Characterization of inclusion bodies with cytoprotective properties formed by seipinopathy-linked mutant seipin

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Gain-of-toxic mutations in the N-glycosylation motif of the seipin/BSCL2 gene (namely, the N88S and S90L mutations) cause autosomal dominant motor neuron diseases, termed ‘seipinopathy’. Expressed mutant seipin is improperly folded and accumulates in the endoplasmic reticulum (ER), leading to an unfolded protein response (UPR). Furthermore, cells expressing mutant seipin contain unique cytoplasmic inclusion bodies (IB) that form via a different mechanism from that of ubiquitinated inclusions, or aggresomes. Whether the formation of these IB is pathogenic or protective in neurodegenerative diseases remains unclear. Here, we determined that mutant seipin IB are negative for two well-established ER markers, immunoglobulin-heavy-chain-binding protein and calnexin, indicating a distinct compartmentalization from the main ER, and that mutant seipin IB are formed via a mechanism that is independent of major UPR transducers and ER chaperons. Electron microscopy and coexpression study with variant α1-antitrypsin cDNA showed that seipin IB are compatible with unique cytoplasmic vesicles known as ER-derived protective organelles (ERPO). We also obtained evidence that seipin IB exhibit a cytoprotective property via the attenuation of ER stress. These findings suggest that ERPO, such as seipin IB, are a novel adaptation machinery against the accumulation of unfolded proteins in the ER.

INTRODUCTION

The seipin/BSCL2 gene, the function of which remains unknown, is a loss-of-function gene for congenital generalized lipodystrophy type 2, which is characterized by severe lipoatrophy, insulin resistance, hypertriglyceridemia and mental retardation (1–5). Meanwhile, gain-of-toxic mutations in the N-glycosylation motif of the seipin/BSCL2 gene (namely, the N88S and S90L mutations) cause autosomal dominant motor neuron diseases, or seipinopathy, with a wide clinical spectrum, in which upper and lower motor neurons are variously affected (6–8). Both the N88S and S90L mutations disrupt the N-glycosylation motif and result in improper protein folding and accumulation in the endoplasmic reticulum (ER). Expressed mutant seipin activates the unfolded protein response (UPR) pathway and induces cellular dysfunction, suggesting that seipinopathy is strongly associated with ER stress (8–10). Recently, we also demonstrated that N88S seipin transgenic mice develop progressive motor deficits, recapitulating the symptomatic and pathological phenotypes of seipinopathy, as well as the upregulation of ER stress markers (11).

Another important point is that cells and brain tissue expressing N88S and S90L mutant seipin form unique cytoplasmic inclusion bodies (IB) (8–11). Interestingly, our recent studies have revealed that seipin inclusions exhibit a distinct feature of aggresomes, which are well-established IB formed by many proteins associated with neurodegeneration, such as huntingtin, α synuclein and Parkin (12,13); these results suggest that seipin IB are formed via a different novel pathway.

Recent evidence has revealed that IB reduce the intracellular levels of toxic proteins linked with neurodegeneration, suggesting that they may serve a neuroprotective function (14,15). In this study, we characterized the IB of seipinopathy-linked mutated seipin to assess whether these structures may be a novel adaptive mechanism against the toxic accumulation of misfolded proteins in the ER. Seipin IB are formed via a
unique pathway that is independent of major UPR transducers and ER chaperons. We also demonstrated that these IB exhibit the cytoprotective features of ER-derived protective organelles (ERPO), which have been recently implicated in polymeric mutant α1-antitrypsin (16,17). These findings provide important knowledge regarding a novel protein quality control system, ERPO, against the accumulation of unfolded proteins in the ER.

RESULTS

Seipinopathy-linked mutant seipin impairs the secretory pathway

Previous (9,10) and supplementary studies (Supplementary Material, Fig. S1) have shown that mutant seipin is improperly folded in the ER, leading to the formation of IB and ER stress in expressed cultured neuronal cells. The accumulation of mutant seipin in the ER may impair ER function and the trafficking of proteins through the secretory pathway. To address this issue, HeLa cells were co-transfected with wild-type or mutant seipin and progranulin, which is a well-established secretory protein linked to neurodegeneration (18). As shown in Figure 1, the rate of progranulin secretion in mutant seipin-expressing cells was significantly reduced, compared with that in empty vector or wild-type seipin-expressing cells. These findings indicate that the expression of mutant seipin leads not only to ER stress, but also to the impairment of the secretory machinery.

Mutant seipin in IB is located in a distinct compartment from the main ER

A previous paper demonstrated that wild-type seipin is localized in ER labeled using a well-established ER chaperone, calnexin (CNX), but that mutant seipin IB are not colocalized with CNX, suggesting that mutant seipin in IB is located in a distinct compartment from the main ER (9,10). To further characterize the components of IB and their relation to the ER, we stained IB with another ER marker, anti-KDEL antibody. As shown in Figure 2, KDEL immunoreactivity is colocalized with wild-type seipin and the majority of mutant seipin in cells, but the IB are negative for KDEL immunoreactivity in HeLa cells expressing N88S mutant seipin. In the differentiated motor neuronal cell line NSC-34D expressing seipin, we also found that IB were not colocalized with either of the ER markers, KDEL or CNX (Supplementary Material, Fig. S2). These findings indicate that seipin IB are sequestered from the main ER.

Characterization of mutant seipin IB in transgenic mice

To explore the characterization of seipin IB in vivo, we histologically examined N88S seipin transgenic mice, which do not exhibit neuronal death but do exhibit a disruption in axonal transport, resulting in a severe motor phenotype (11). In these mice, the cortical neurons in the frontal cortex and the motor neurons in the spinal cord exhibit mutant seipin IB, as described in our previous paper. Overall, 60.7% (206/339) and 19.2% (35/182) of the neurons expressing the transgene in the cortex and anterior horn in the spinal cord, respectively, harbored IB (Fig. 3). Predictably, IB in the cortical neurons and motor neurons in the spinal cord were not colocalized with two well-established ER markers, immunoglobulin-heavy-chain-binding protein (BiP) and CNX. Furthermore, these IB also did not stain positive for a poly-ubiquitin antibody, in agreement with in vitro findings (9,10) (Supplementary Material, Fig. S3), indicating that mutant seipin IB exhibit unique features even in an in vivo mouse model.

Seipin IB are negative for autophagosome and lysosome markers

Autophagy is a major pathway for the degradation of long-lived proteins and cellular organelles as a homeostatic mechanism in all eukaryotic cells. To determine the relation between seipin IB and autophagy, we co-transfected HeLa cells with the autophagosomal marker LC3-GFP and mutant seipin. Upon cell starvation, LC3-GFP forms ‘dot-like’ autophagosomes in the cytoplasm, as described by others (16,19,20), but is not colocalized with seipin IB (Supplementary Material, Fig. S4A – F). We
also examined the distribution of the lysosome marker, Lamp2, in mutant seipin-expressing cells. As shown in Supplementary Material, Figure S4G–L, mutant seipin IB were separate from areas of Lamp2 staining. These findings indicated that the formation of seipin inclusions is not associated with autophagosomes or lysosomes.

Ultrastructural analysis of mutant seipin IB

To determine the ultrastructure of IB, we performed an electron microscopy analysis of seipin-expressing cells. N2a cells stably transfected with wild-type or mutant seipin were observed using transmission electron microscopy (TEM). Mutant seipin-expressing cells exhibited numerous electron-light vesicles with a single membrane and no apparent connection with the ER (Fig. 4A–C). Using immunoelectron microscopy, labeling for mutant seipin was detected in the membrane of vesicles (Fig. 4D). These observations suggest that the ultrastructure of mutant seipin IB is a vesicular organelle that is separate from the main ER compartment.

Mutant seipin IB are colocalized with the expressed variant α1-antitrypsin in ERPO

Based on the currently available information, these biochemical and morphological characteristics are very similar to unique IB induced by variant α1-antitrypsin Z with an E342K mutation (ATZ), which is associated with infantile

Figure 2. Subcellular localization of seipin IB in HeLa cells. HeLa cells transfected with myc-wild-type (A–C) or N88S seipin (D–L) were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and double-labeled with 9E10 (green) and anti-KDEL antibody (red). (J–L) High magnifications of (G)–(I). Scale bar = 10 μm. Arrowheads indicate seipin IB.
liver disease and adult-onset emphysema, as described by Granell et al. (16,17). ATZ IB are electron-light vesicles that contain several ER components, but not CNX, and are ER-derived and yet separate from the main ER. Granell et al. proposed that ATZ IB, termed ‘ERPO’, are an ER quality control mechanism with cytoprotective properties.

Figure 3. Immunohistological analysis of brain and spinal cord tissues from 32-week-old N88S Tg mice. (A and B) Immunohistochemistry with anti-myc (red) and BiP (green) or CNX (green) antibody in cortex and spinal cord tissues from N88S Tg mice shows that mutant seipin IB is not colocalized with the ER markers BiP and CNX. Sections were counterstained with DAPI (blue). Scale bar = 20 μm.
To directly compare the formation of mutant seipin IB and ATZ IB, we next co-transfected cells so that they would express both mutant seipin and ATZ. As shown in Figure 5, mutant seipin IB coincided with ATZ IB. We concluded that the formation of mutant seipin IB occurs via the same mechanism as that of ATZ IB, indicating that mutant seipin IB are the same as ERPO.

Formation of mutant seipin IB is independent of UPR transducer and critical ER chaperons

Three major ER-resident stress sensors, IRE1, PERK and ATF6, are activated during the induction of the UPR. The signals from these transducers coordinately mediate UPR signaling, the transcription of UPR target genes and the attenuation of translation (21,22). To reveal whether major UPR transducers mediate seipin IB, we examined mouse embryonic fibroblasts (MEF) with a homozygous deletion of PERK (23), the other UPR-mediated transcription factor, X-box-binding protein 1 (XBP-1) alleles (24), or site-2 protease (S2P)-deficient Chinese hamster ovary (CHO) cells (25), in which ATF6 cleavage was not detected. The formation of mutant seipin IB was not affected in these mutant cells transiently transfected with N88S seipin (Fig. 6A–F). To test the requirement for IRE1 in IB formation, we next studied HeLa cells overexpressing the dominant-negative K536A kinase mutant mIre1b. As shown in Figure 6G and H, mutant mIre1β also does not interfere with IB formation. Together, these results demonstrated that mutant seipin IB are formed via UPR transducer-independent machinery.

To test the requirement for critical ER chaperons in IB formation, we next utilized MEF from genetically modified CNX (26) and BiP mice (27). Supplementary Material, Figure S5 shows that both CNX knock-out (KO) MEF and mutant BiP knock-in MEF, in which the mutant BiP lacked the ER retrieval sequence KDEL, do not affect the formation of seipin IB. Therefore, these findings indicated that the major ER chaperons, BiP and CNX, are also not essential for the formation of mutant seipin IB.

Cellular compartment analysis of mutant seipin in cells with/without IB

Recent evidence has revealed that IB reduce the intracellular levels of toxic proteins linked with neurodegeneration, suggesting that they may serve a neuroprotective function (14,28,29). To address this issue, we transfected HeLa cells with mutant N88S seipin for 48 h, and cells with or without IB were distinguished using immunostaining (Fig. 7). The levels of mutant seipin expression in randomly chosen cells were then determined based on their fluorescence intensities using confocal microscopy. No significant difference in the whole cellular levels of p35f and mutant FUS in cells with/without IB was seen [whole cellular intensity: IB−, 3.31 ± 1.53 × 10^6 (n = 30); IB+, 3.30 ± 1.90 × 10^6 (n = 30)]. As shown in Figure 3, the mean levels of the cytoplasmic reticular distribution of mutant seipin in cells with IB were significantly lower, suggesting that IB formation was associated with a decrease in mutant FUS in normal ER [mean cytoplasmic intensity: IB−, 168 ± 33 (n = 30); IB+, 109 ± 54 (n = 30)].

Mutant seipin IB exhibit a cytoprotective property through the attenuation of ER stress

Since Granell et al. (16,17) proposed that the sequestration of mutated ATZ into IB is a cell-protective mechanism that...
maintains ER function, we wished to determine whether seipin IB contribute to cytoprotection or facilitate toxicity. To address this issue, we transfected HeLa cells with mutant seipin. After 48 h, cells with or without IB were distinguished using immunostaining among cells with mutant seipin expression, and the extent of cell death was determined using Hoechst 33342 or TUNEL staining. Approximately 30% of the cells expressing mutant proteins contained IB, as described in a previous paper (10). The levels of seipin expression in 100 cells from three independent experiments were determined based on the immunofluorescence intensity using fluorescent microscopy and the Image J software (Fig. 8A–C). No significant difference in the total levels of seipin expression in cells with or without IB was observed [intensities: IB−/Tunnel−, 34 713 ± 17 679 (n = 26); IB−/Tunnel+, 32 610 ± 13 868 (n = 74); IB+/Tunnel−, 36 400 ± 19 129 (n = 86); IB+/Tunnel+, 35 152 ± 15 633 (n = 14)]. As shown in Figure 7D and E, 73.0 ± 4.9% of the HeLa cells without IB expressed CHOP, whereas only 13.5 ± 4.6% of the cells with IB were CHOP-positive in three independent experiments, indicating that significantly more cells without IB were CHOP-positive (P = 0.0001). Taken together, these results suggest that the formation of seipin IB exerts a cytoprotective property via the attenuation of ER stress.

DISCUSSION

This study presents several important findings relevant to the formation and cellular properties of mutant seipin-containing IB. Wild-type and the majority of mutant seipin are localized in the ER, whereas the present immunocytochemistry and electron microscopy studies demonstrated that seipin IB are aberrantly sequestered from the main ER (Figs 2–4 and Supplementary Material, Fig. S2) and that the mechanism of IB formation is independent of major UPR transducers and ER chaperons (Fig. 6 and Supplementary Material, Fig. S5). A coexpression study showed that mutant seipin is colocalized in IB with accumulated AZT, indicating that seipin IB and ERPO are formed via the same pathway (Fig. 5). Furthermore, we also provided evidence that seipin IB exert a cytoprotective property via the attenuation of ER stress.

A point mutation (E342K) in α1-antitrypsin (ATZ) is the most common genetic cause of liver disease in children. In the hepatocytes of individuals carrying this mutation, α1-antitrypsin forms membrane-surrounded IB. Recently, Granell et al. (16,17) reported that the sequestration of
mutated α1-antitrypsin (ATZ) into IB is a cell-protective mechanism that maintains ER function and named these structures ‘ERPO’. They also found that the IB are ER-derived and yet separate from the main ER and do not have markers for autophagosomes and lysosomes. In this study, we demonstrated that seipin IB share properties similar to ERPO, including the accumulation of misfolded ER-membrane proteins, ER-derived membrane-surrounded IB and a cytoprotective property. Furthermore, both expressed mutant seipin and ATZ were completely colocalized in IB (Fig. 5). We concluded, therefore, that seipin IB are ERPO. We also show that the ER chaperons CNX and BiP did not affect the assembly of seipin IB in CNX KO and mutant BiP knock-in MEF (Supplementary Material, Fig. S5). Interestingly, Granell et al. (16) demonstrated that increased levels of CNX led to the retention of mutated α1-antitrypsin in the ER and inhibited the formation of IB. Therefore, in CNX KO MEF, other ER chaperons, such as calreticulin, may compensate for CNX and lead to the retention of mutant seipin within the ER.

Whether the formation of IB is pathogenic or protective in neurodegenerative diseases is controversial. Recent evidence shows that IB reduce the intracellular levels of diffuse toxic proteins and prolong cell survival, suggesting that IB might protect neurons. Arrasate et al. (14) devised a real-time technique for following the IB formation of Huntingtin and cell survival in the same cultured neuron. They concluded that IB formation leads to decreased levels of toxic Huntingtin and improves cell survival. We also demonstrated that the reticular distributions of mutant seipin in cells with IB were significantly lower than those in cells without IB, suggesting that IB formation potentially decreases mutant seipin in the normal ER (Fig. 7).

When interpreting the results of the present study, several points should be kept in mind. First, the final fate of seipin inclusions, especially the degradation process, is unclear. Cytoprotective inclusions may be retained in cells evading rapid degradation and may function as long-lived organelles. Although Supplementary Material, Figure S4 shows that transiently expressed seipin IB were not colocalized with Lamp2 and LC3, we cannot exclude the possibility that lysosomes and autophagy ultimately remove seipin IB as aberrant organelles. In a future study, long-term observation under undivided conditions is needed to determine the fate of seipin IB.

Second, the molecular machinery responsible for the formation of seipin IB remains unknown. The present study using expressed dominant-negative and KO cells (Fig. 6 and Supplementary Material, Fig. S5) showed that the formation of seipin IB is independent of the three major ER-resident stress sensors and translation factors: IRE1, PERK, and ATF6. Some specific molecules in the ER are thought to organize aberrant ER vesicles, ERPO. Further comprehensive analyses to identify molecules that may interact with ERPO are needed to better understand the machinery of ERPO formation.

Figure 6. Seipin IB formation is independent of major UPR transducers. (A–F) Wild-type and PERK−/− (A and B), XBP-1−/− (C and D) MEF and S2P-deficient CHO cells (E and F) were transfected with myc-wild-type or N88S seipin. The cells were then stained with anti-myc antibody. (G–I) N2a cells stably expressing N88S seipin were transfected with K536A myc epitope-tagged IRE1β plasmids and double-stained with rabbit polyclonal human seipin antiserum (SCT14) (green) and anti-myc antibody (red). Mutant cells of UPR transducers and dominant-negative IRE did not affect the formation of seipin IB. Scale bar = 20 μm.
The possible mechanisms of ERPO formation are outlined in Figure 9. Wild-type ER membrane protein is correctly folded in the ER, whereas mutant proteins are improperly folded, leading to their accumulation in ER and, consequently, ER stress. Unidentified molecule(s) assemble and shut misfolded proteins into a vesicle, ERPO, that then buds from the main ER. Consequently, the removal of misfolded proteins from the main ER attenuates ER stress and ameliorates ER function.

Finally, the data presented here indicate that ERPO are a novel ER quality control system for adaptation against ER stress. Although further studies are needed to elucidate the molecular machinery of ERPO, we hope that an understating of ERPO may lead to important new insights into ER stress-associated diseases, including neurodegenerative diseases.

MATERIALS AND METHODS

Cell cultures and reagents

Wild-type (K1) and S2P-deficient (clone M19) CHO cells, which were provided by T.Y. Chang (Dartmouth Medical School, Hanover, NH, USA), were cultured as described (30). All other cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum. Transfections were performed with Lipofectamine or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. To detect autophagy, Earle’s balanced salt solution (Sigma) was used for starvation. Stable transfectants were selected in a medium containing 0.4 mg/ml of G418 (Gibco) for 4 weeks.

To transfect differentiated NSC-34 (NSC-34D) cells, the cells were grown to 80% confluence on a cover glass and the growth medium was exchanged for a medium comprising 1:1 DMEM plus Ham’s F12, 1% FCS and 1% modified Eagle’s medium non-essential amino acids (31), followed by transfection using Lipofectamine 2000 (Invitrogen). After 48 h in this medium, the differentiated cells (NSC-34D) were fixed for immunofluorescence staining as described below.

MEF from CNX KO mice were cultured as described (26), and MEF from mutant BiP knock-in mice (27) were kindly provided by Dr Tomohiko Aoe. PERK−/− (32) and XBP-1−/− fibroblasts (24) were kindly provided by David Ron (Skirball Institute, New York University, New York, NY, USA) and Laurie H. Glimcher, respectively.

cDNAs

The human seipin expression plasmids were previously described (9,10). Plasmids supplying progranulin (33), myc epitope-tagged IRE1β expression (wild-type and K536A) plasmids, GFP-LC3 (20) and alpha1-antitrypsin with E342K
mutation (ATZ)-GFP plasmids (16) were kindly provided by Dr Andrew Bateman (McGill University of Medicine, Canada), Dr David Ron (Skirball Institute), Dr Mamoru Shibata (Keio University, Japan) and Dr Giulia Baldini (University of Arkansas for Medical Sciences, AR, USA), respectively.

**Antibodies**

Mouse monoclonal anti-KDEL and rabbit polyclonal anti-CNX antibodies were purchased from Stressgen (San Diego, CA, USA). The 9E10 (anti-myc) and mouse monoclonal anti-GADD153 and Lamp2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

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**Figure 8.** IB formation exerts a cytoprotective property. HeLa cells grown on glass cover slips were transfected with myc-N88S seipin plasmids. After 48 h, cover slips were washed in PBS and stained with TUNEL (green) or Hoechst 33342 (blue) and 9E10 (red). Images were acquired with a fluorescence microscope, and the cell viability/death of seipin-positive cells with/without IB was determined. (A and B) The total pixel intensities of expressed mutant seipin were counted in 100 transfected cells with/without IB in three independent experiments, and the results were plotted in (A) and (B). Cells undergoing apoptosis were considered to be those with condensed nuclei or TUNEL-positive (A) or Hoechst 33342 staining (B). (C) Representative images of transfected cells stained with anti-myc antibody, Hoechst 33342 and TUNEL. (D and E) IB formation inhibited CHOP expression. HeLa cells transfected with myc-N88S seipin plasmids were stained with antibody 9E10 (green) and anti-CHOP antibody (red). The total intensities of expressed mutant seipin were counted in 100 transfected cells in three independent experiments, and CHOP positivity/negativity was determined. (E) Representative images of transfected cells stained with anti-myc antibody, anti-CHOP and Hoechst 33342. The statistical analysis was performed using a Student t-test.
Mouse monoclonal anti-α-tubulin and anti-V5 were purchased from Invitrogen. Mouse monoclonal anti-neurofilament (SMI-312) was purchased from Abcam (Cambridge, UK). A rabbit polyclonal antiserum that recognizes human seipin (SCT14) was described previously (10).

**Immunoblot analysis**

Cells were briefly sonicated in cold lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.25% sodium dodecyl sulfate (SDS), 5 mM ethylenediaminetetraacetic acid and protease inhibitor cocktail from Sigma]. For the condition medium, the media were collected and centrifuged at 1000 r.p.m. for 5 min and the supernatant was collected. The total protein concentration in the supernatant was determined using a Bio-Rad protein assay kit. Then, the proteins were analyzed using immunoblotting as follows.

To measure progranulin secretion in the medium, HeLa cells were co-transfected with wild-type or N88S mutant seipin and progranulin-tagged V5 plasmids. After 48 h, the transfected HeLa cells were washed with the growth medium and incubated at 37°C for the indicated times in the same medium. The medium was then collected and centrifuged at 500g for 3 min to remove cell debris.

Protein samples were separated by reducing SDS–polyacrylamide gel (PAGE) on a 4–20% Tris–glycine gradient gel (Invitrogen), after which the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was incubated with the primary antibodies (anti-KDEL, 1:2500; anti-GADD 153, 1:1000; anti-α-tubulin, 1:1000; anti-V5, 1:1000). This step was then followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detection using enhanced chemiluminescence reagents, according to the method described by the supplier (PerkinElmer Life Sciences, Boston, MA, USA). The protein levels in Figure 1 were determined using densitometry and an Epson ES-2000 scanner (Tokyo, Japan) and the Image J software (National Institutes of Health, Bethesda, MD, USA).

**Immunofluorescence detection**

Cells grown on coated cover slips were transfected with the appropriate expression plasmids. After 48 h, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min and then permeabilized in 0.2% Triton X-100 for 5 min. After the blocking of non-specific binding, the cover slips were incubated with the primary antibody (1:500, polyclonal anti-myc antibody; 1:1000, anti-V5 antibody; 1:1000, SMI-312; 1:1000, anti-KDEL; 1:1000, anti-GADD 153 antibody; 1:1000, anti-CNX antibody; or 1:1000, anti-Lamp2 antibody) diluted in phosphate buffered saline (PBS), 0.2% Tween-20 and 3% bovine serum albumin. After three washes, the cover slips were incubated with fluorescein isothiocyanate-conjugated anti-rabbit and Texas Red-conjugated anti-mouse secondary antibodies and mounted. Immunofluorescent staining was examined using a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) and the intensities were quantified using the Leica Application Suite Advanced Fluorescence software.

**N88S mutant seipin transgenic mice**

The generation and breeding of N88S mutant seipin transgenic mice has been previously described (11). Genotyping from tail DNA was performed using the following primer pair: 5′-ACAAGCTACTTGTTTTTTGCAG-3′ and 5′-CTAGCTGCTCTGCCACCTG-3′. All animal experiments were approved by the Ethics Committee of Keio University and were conducted according to the Animal Experimentation Guidelines of Keio University School of Medicine.

**Immunohistochemical staining**

Brain and spinal cord tissues were fixed by dipping in 10% formaldehyde in PBS and embedded in paraffin. Sections of formalin-fixed, paraffin-embedded tissues were dipped in xylene to remove the paraffin, followed by dehydration in an ethanol series. The sections were then incubated with 0.05% saponin for 30 min at room temperature in preparation for immunohistochemistry. Immunohistochemistry was performed essentially as described previously (34,35).

**Electron microscopy**

Cells were grown on cover slips under the same conditions as for optical microscopy. After a brief rinse in PBS, cells attached to a glass surface were fixed with 2% PFA and 2% glutaraldehyde in 0.1 m phosphate buffer, pH 7.4, for 10 min...
at room temperature and then for 20 min at 4°C. The cells were then postfixed with 2% osmium tetroxide in the same buffer for 1 h at 4°C. After embedding in Araldite (Electron Microscopy Sciences, Ft Washington, PA, USA), ultrathin sections of the cell monolayers were cut using an ultramicrotome (LKB Ulrotome V Bromma 2088; LKB, Bromma, Sweden). The sections were stained with uranyl and lead citrate and then examined using TEM (JEM-1200EX; JEOL, Japan).

For immunoelectron microscopy, post-embedding immunogold labeling was performed. Cells were fixed with 2% PFA and 0.05% glutaraldehyde in a 0.1M 4-(2-hydroxyethyl)-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) for 1 h and then with the same fixative in 0.1M HEPES buffer (pH 8.5) overnight at room temperature. The specimens were dehydrated with ethanol and embedded in LR-White resin. UltraThin sections were incubated with polyclonal anti-seipin antibody (SCT14) followed by anti-rabbit IgG conjugated with colloidal gold (British BioCell International, UK). Consequently, the sections were treated with 2% glutaraldehyde in a 0.1M phosphate buffer and were double-stained with uranyl acetate and lead citrate and then examined using TEM.

Cell death assay

HeLa cells plated on cover slips were transfected as described above. After 48 h, the cells were stained with 1.5 μg/ml of Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 15 min at room temperature or in TUNEL solution (Roche) for 30 min at 37°C, followed by immunofluorescent staining with antibody 9E10, as described above (36). Fluorescent images of three to four random fields were acquired using a Nikon (Skirball Institute, New York University, New York, NY, USA) microscope (Eclipse TE300 fluorescence microscope (Nikon, Kanagawa, Japan) with a 20× or 40× objective lens, and the seipin-positive cells (approximately 300 cells/experiment) and apoptotic cells (Hoechst- or TUNEL-positive condensed nuclei) were counted. Data from independent experiments were expressed as the mean ± standard deviation and were statistically examined using a one-way analysis of variance.

Statistical analysis

The statistical analysis of the data was performed using the Student t-test and the Statview 5.0 system (Statview, Berkley, CA, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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