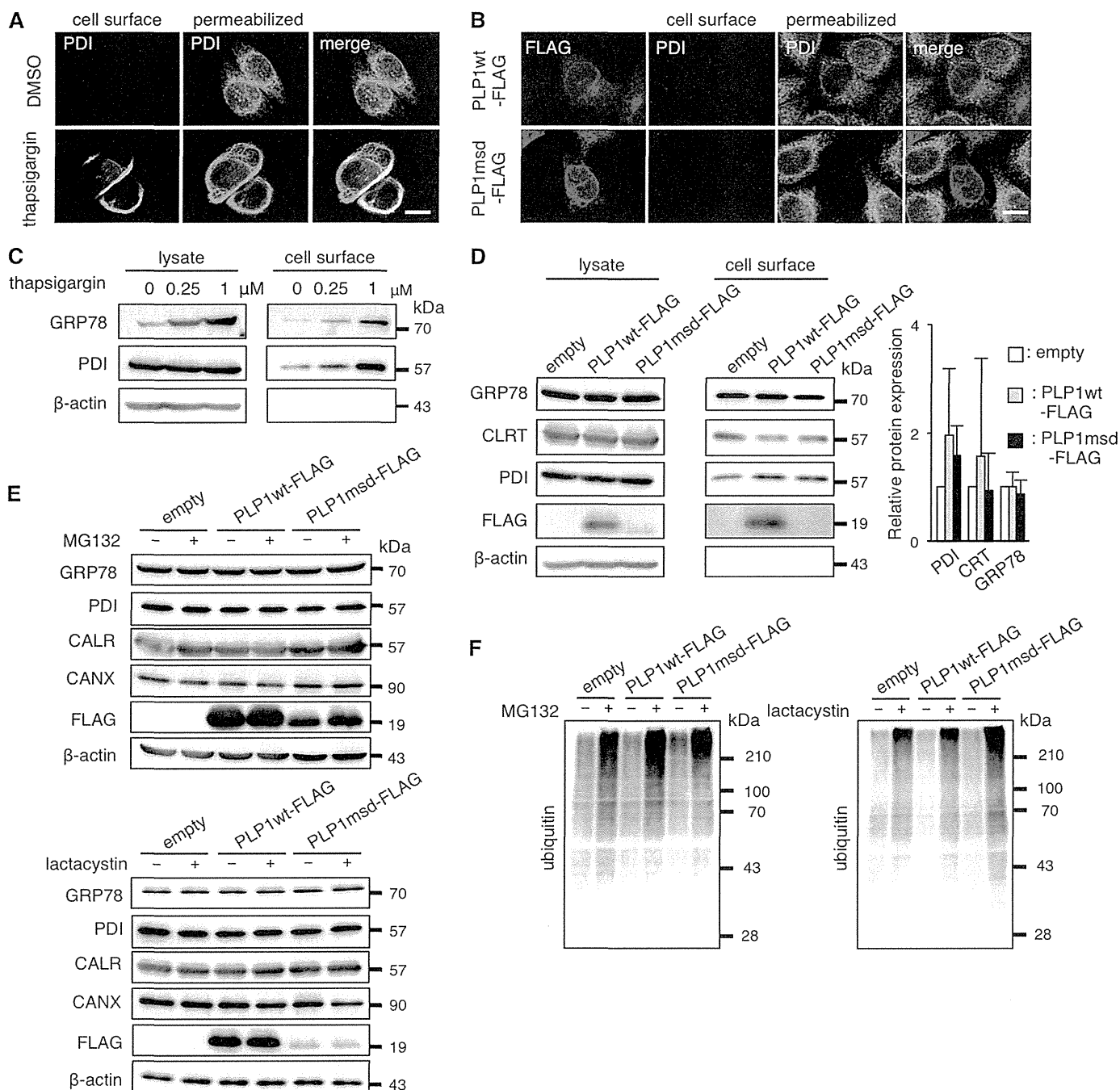FIGURE 3. **PLP1msd does not increase cell surface expression of the ER chaperones.** *A* and *B,* immunocytochemical analysis of cell surface PDI on HeLa cells treated with 1 μM thapsigargin (*A*) or transfected with the PLP1msd gene (*B*). Cell surface PDI (*green*) were stained with the anti-PDI antibody without permeabilization followed by intracellular staining with the same antibody (*magenta*). *Scale bar,* 10 μm. *C* and *D,* biochemical analysis of cell surface expression of the ER chaperones. HeLa cells were treated with thapsigargin for 16 h (*C*). Transfection was performed before 24 h of cell surface biotinylation (*D*). Cell surface proteins were labeled with biotin, precipitated with streptavidin beads followed by immunoblotting with anti-PDI, anti-CALR, and anti-GRP78 antibodies. *E* and *F,* protease inhibitors do not increase total amounts of the ER chaperones in HeLa cells expressing PLP1msd. Transfected cells were treated with 5 μM MG132 for 16 h or 1 μM lactacystin for 8 h followed by immunoblotting with the anti-PDI, anti-CALR, anti-GRP78, anti-CANX (*E*) and anti-ubiquitin antibodies (*F*). Protein amounts were measured by densitometry. The results are represented as fold-induction against the control experiment using the empty vector. Values are represented as the mean ± S.E. from three independent experiments (*D*).

Down-regulation of Pdi in the SC and Primary Culture of msd Mice—In our *in vitro* analyses, expression of the PDI, CALR, and GRP78 proteins did not increase in cells expressing PLP1msd, despite the significant increase in their transcripts (Fig. 2*A*). To determine whether this also occurs *in vivo*, we further investigated the mRNA and protein expression of these ER chaperones in the SCs isolated from male *msd* mice, which

carry the *Plp1A243V* allele, on P14, when the *Plp1* gene is most strongly expressed in the SCs of mutant mice (8). Expression of the *Chop* transcript was significantly higher in *msd* mice than in wild-type mice, suggesting that cells in the SCs of *msd* mice were under ER stress (Fig. 6*A*). We then analyzed the expression of these ER chaperone mRNA by quantitative RT-PCR (Fig. 6*B*). The expression of *Grp78* mRNA was significantly

A

B

C
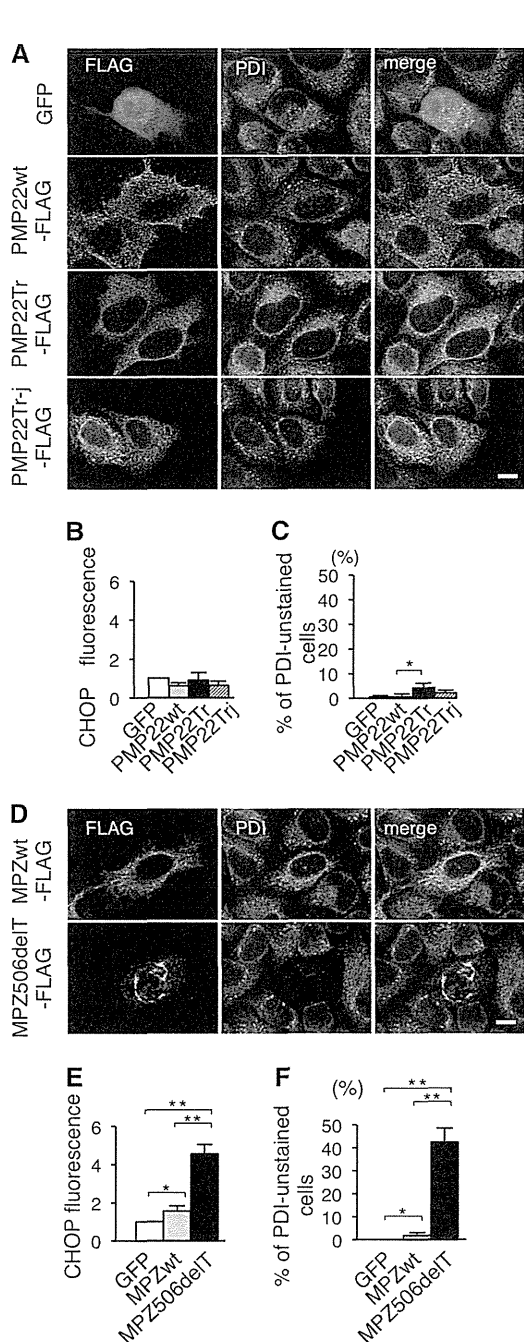

D

E

F

FIGURE 4. **Effect of PMP22 and MPZ mutations on ER chaperones.** *A* and *D,* immunocytochemistry of PDI in HeLa cells transfected with the PMP22wt and mutant PMP22 genes (*A*) or the MPZwt and MPZ506delT genes (*D*). HeLa cells transfected with the indicated vectors were immunostained with the anti-FLAG (*green*) and anti-PDI (*magenta*) antibodies followed by observation with a confocal fluorescence microscope. Note that cells expressing MPZ506delT showed a extremely faint staining pattern for PDI (*arrowhead*). *Scale bar,* 10 μm. *B* and *E,* relative expression of CHOP in HeLa cells transfected with the PMP22wt and mutant PMP22 genes (*B*) or the MPZwt and MPZ506delT genes (*E*). HeLa cells transfected with the indicated vectors were stained with the anti-FLAG and anti-CHOP antibodies together with DAPI to visualize the nuclei. The relative fluorescence intensity of CHOP in the nuclei was analyzed by densitometry. *C* and *F,* proportion of unstained cells with anti-PDI antibody in HeLa cells transfected with the PMP22wt and mutant PMP22 genes (*C*) or the MPZwt and MPZ506delT genes (*F*). Bar graphs are represented as fold-induction ± S.E. against the mean of control experiment from three independent experiments with >100 cells counted in each experiment (*, $p \leq 0.05$; **, $p \leq 0.005$).
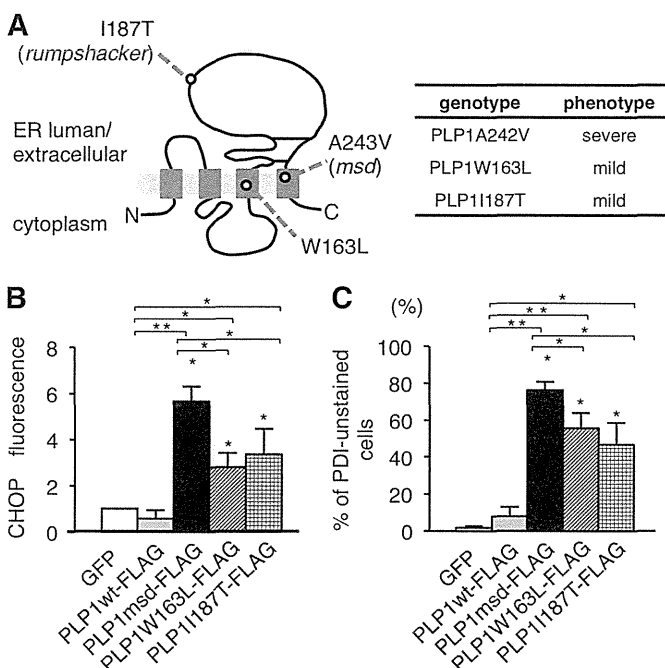
A

genotype	phenotype
PLP1A242V	severe
PLP1W163L	mild
PLP1I187T	mild

B

C

FIGURE 5. **Effect of different PLP1 mutations on ER chaperones.** *A,* schematic diagram of PLP1 with the positions of mutations examined in this study (*left*) and their associated phenotypes (*right*). *B,* expression of CHOP in HeLa cells transfected with the PLP1wt and mutant PLP1 genes. The fluorescence intensity of CHOP in the nuclei was analyzed by densitometry as described in the legend to Fig. 4*B. C,* proportion of unstained cells with anti-PDI antibody in HeLa cells transfected with PLP1wt and the indicated PLP1 mutant genes as described in the legend to Fig. 4*C.* Bar graphs are represented as fold-induction ± S.E. against the mean of control experiment from three independent experiments with >100 cells counted in each experiment (*, $p \leq 0.05$; **, $p \leq 0.005$).

increased (2-fold) in *msd* mice. *Pdi* and *Calr* were also up-regulated, but to a lesser extent. However, at protein levels, we observed discordance (Fig. 6, *C* and *D*). Pdi protein expression in *msd* mice was significantly decreased. The expression of Calr showed a similar tendency, although these results did not reach significance ($p = 0.06$). In contrast, Grp78 and Canx protein expression did not differ significantly when compared with wild-type mice.

We also immunocytochemically examined primary MGCs isolated from the brains of embryonic day (E) 14.5 wild-type or *msd* mice. On the 4th day after induction of oligodendrocyte differentiation, profound maturation with increased MBP immunoreactivity was evident in the wild-type MGCs (Fig. 6*E*). In contrast, rapid regression of Oligo2-positive cells and decreased MBP immunoreactivity were observed in the *msd* MGCs, suggesting that oligodendrocyte maturation induced apoptosis in *msd* on the 4th day after induction. Pdi was detected in the cell body of Plp1-positive mature oligodendrocytes in wild-type MGCs, whereas, it was only faintly stained in Plp1-positive oligodendrocytes in the *msd* MGCs. (Fig. 6*F*). These results suggest that endogenous PLP1msd depletes Pdi from the ER of the oligodendrocytes.

Inhibition of GA to ER Transport Is Associated with the Disappearance of PDI, CALR, and GRP78—To determine the underlying mechanism for PDI, CALR, and GRP78 depletion from the ER in cells expressing PLP1 mutants, we further analyzed the effects of the following chemical ER stressors on the
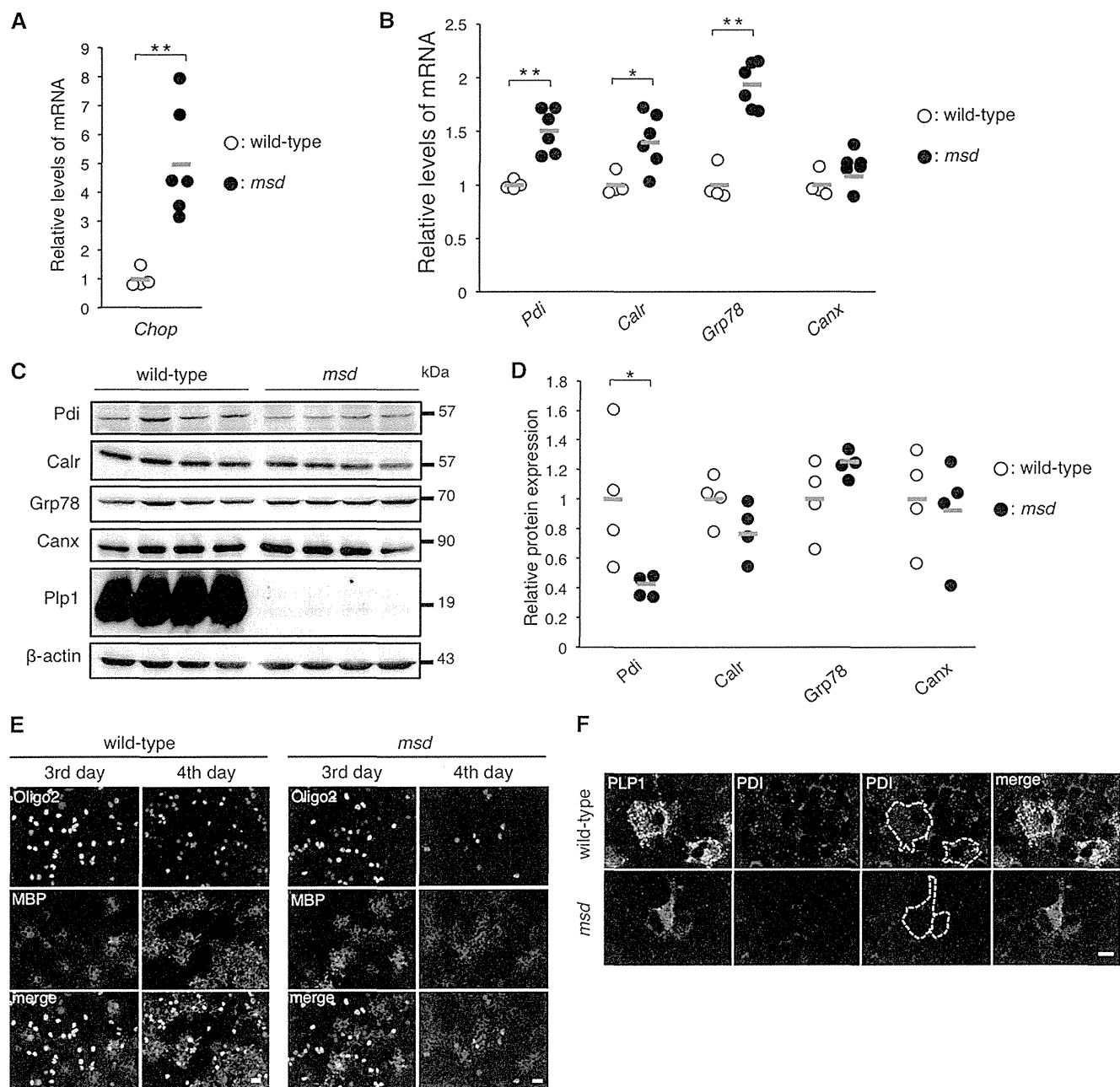
FIGURE 6. **PDI is down-regulated in the SCs and primary oligodendrocytes of** *msd* **mice.** *A* and *B,* quantitative analysis of *Chop* (*A*), *Pdi, Calr, Grp78,* and *Canx* genes (*B*) in the SCs of *msd* mice. Quantitative RT-PCR was performed to analyze the expression levels of *Pdi, Calr, Grp78,* and *Canx* in the SCs of wild-type (*open circle, n* = 4) and *msd* (*filled circle, n* = 6) mice at P14. *GAPDH* was used as an internal control. The results are represented as fold-induction against the means of wild-type mice. *Red horizontal bars* indicate the mean. *C* and *D,* relative amounts of ER chaperones in the SCs of *msd* mice. The SCs of P14 wild-type (*open circle, n* = 4) and *msd* (*filled circle, n* = 4) mice were subjected to immunoblotting with the indicated antibodies (*C*). The amounts of the proteins were measured by densitometry and normalized to β-actin (*D*). *E,* expression of MBP and Olig2 in primary oligodendrocytes of *msd* mice. Immunocytochemistry of MBP and Olig2 in primary oligodendrocytes of *msd* mice. Primary mixed glial cultures were prepared from the forebrains of E14.5 wild-type or *msd* mice. On the 3rd and 4th days after induction of oligodendrocyte differentiation, the oligodendrocytes were immunostained with anti-MBP (*magenta*) and anti-Olig2 (*green*) antibodies and observed with a confocal fluorescence microscope. *Scale bar,* 5 μm. *F,* immunocytochemistry of PDI in primary oligodendrocytes of *msd* mice. Primary oligodendrocytes prepared from the forebrains of wild-type or *msd* mice at E14.5 and immunostained with anti-PLP1 (*green*) and anti-PDI (*magenta*) antibodies and observed with a confocal microscope. *Scale bar,* 5 μm. *A, B,* and *D,* *, $p \leq 0.05$; **, $p \leq 0.005$.

expression of ER chaperones: thapsigargin, a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor; tunicamycin, an *N*-glycosylation inhibitor; and brefeldin A (BFA), a GA-ER transport inhibitor. These three compounds greatly up-regulated the transcripts of *CHOP* and *GRP78,* confirming that they work as ER stressors (Figs. 7, *B* and *C,* and 8, *B, C, E,* and *F*). These compounds also slightly but significantly increased the expression of *PDI, CALR,* and *CANX* transcripts.

Next, HeLa cells were treated with these compounds for 8 h, followed by immunocytochemistry with anti-PDI, anti-CALR, anti-GRP78, or anti-CANX antibodies. Thapsigargin and tunicamycin did not alter the expression of the chaperones (Fig. 8*A*), even after an extended incubation (Fig. 8*D*). In contrast, BFA treatment clearly diminished PDI, CALR, and GRP78 from the ER; however, CANX expression remained unchanged (Fig. 7*A*). In BFA-treated HeLa cells, total amounts of the chaperone

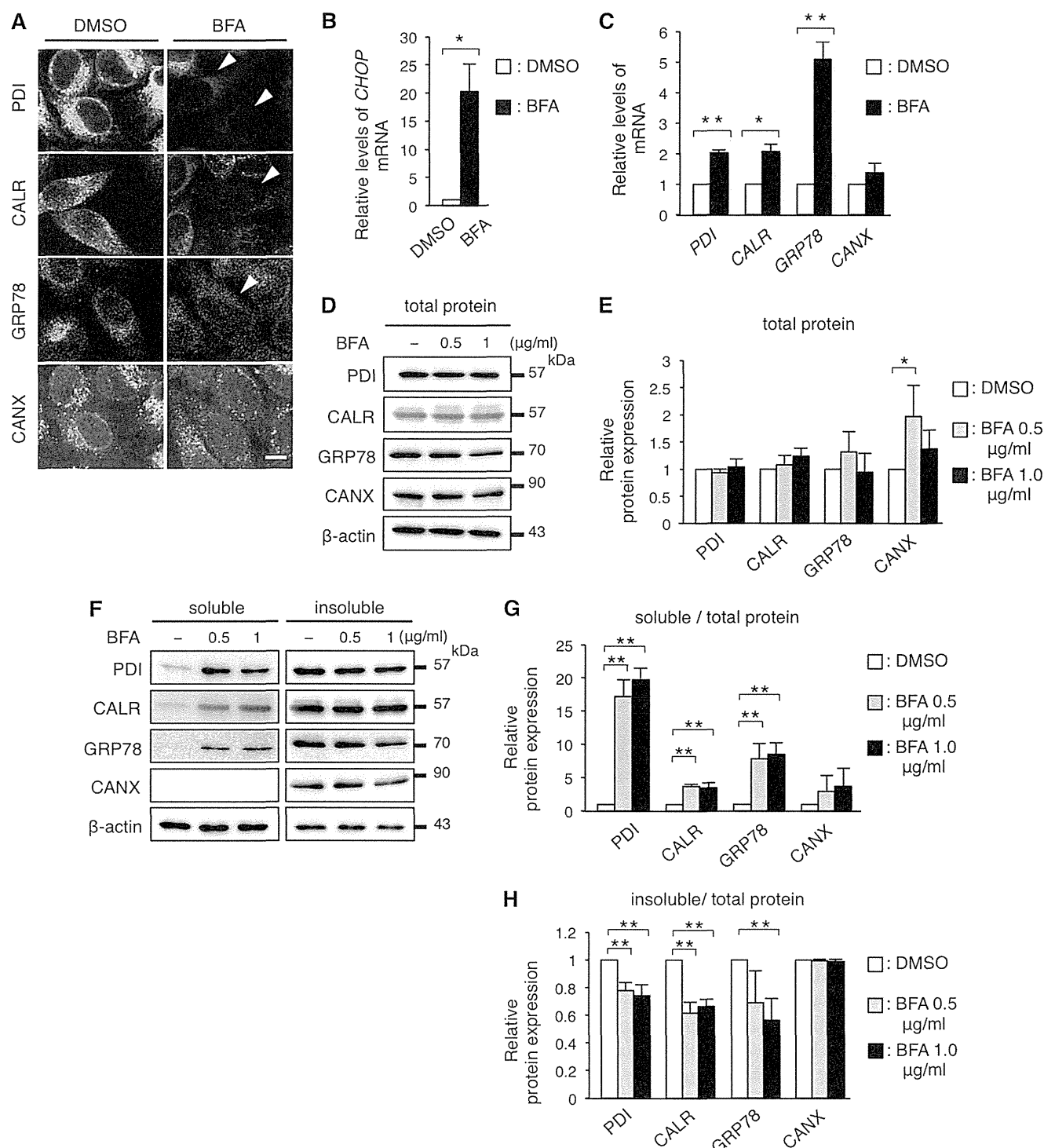## Depletion of ER Chaperones and GA Fragmentation in PMD

FIGURE 7. **BFA treatment recapitulates the disappearance of PDI, CALR, and GRP78.** *A*, immunocytochemistry of ER chaperones in HeLa cells treated with BFA. HeLa cells were treated with 1 µg/ml of BFA for 8 h were immunostained with the indicated antibodies and observed with a confocal fluorescence microscope. *Scale bar*, 10 µm. Note that cells treated with BFA showed extremely faint staining (*arrowheads*) for PDI, CALR, and GRP78. *B* and *C*, relative expression of the transcripts of the ER chaperones in HeLa cells treated with BFA. Expression levels of the transcripts of *CHOP* (B), *PDI, CALR, GRP78*, and *CANX* mRNA (*C*) in HeLa cells treated with BFA were analyzed by qRT-PCR and normalized to *GAPDH*. *D* and *E*, total amounts of PDI, CALR, GRP78, and CANX in HeLa cells treated with BFA. HeLa cells were treated with BFA as in *A* and subjected to immunoblotting with the indicated antibodies (*D*). The amounts of the proteins were measured by densitometry and normalized to β-actin (*E*). *F-H*, digitonin fractionation of HeLa cells treated with BFA. Digitonin fractionation was performed as described in the legend to Fig. 2*D* and the extracts were subjected to immunoblotting with the indicated antibodies (*F*) followed by quantitative analysis (*G* and *H*) as in Fig. 2, *E* and *F*. Results are represented as fold-induction compared with DMSO control experiment. Values are represented as the mean ± S.E. from three independent experiments (*, $p \leq 0.05$, **, $p \leq 0.005$).

proteins (except for CANX at 0.5 µg/ml of BFA treatment) were mostly unaffected despite their increased transcripts (Fig. 7, *D* and *E*), as was observed in HeLa cells transfected with the

PLP1msd gene (Fig. 2, *B* and *C*). We performed fractionation experiments with 0.01% digitonin as described in Fig. 2*D*. In the BFA-treated HeLa cells, the proportion of PDI, CALR, and
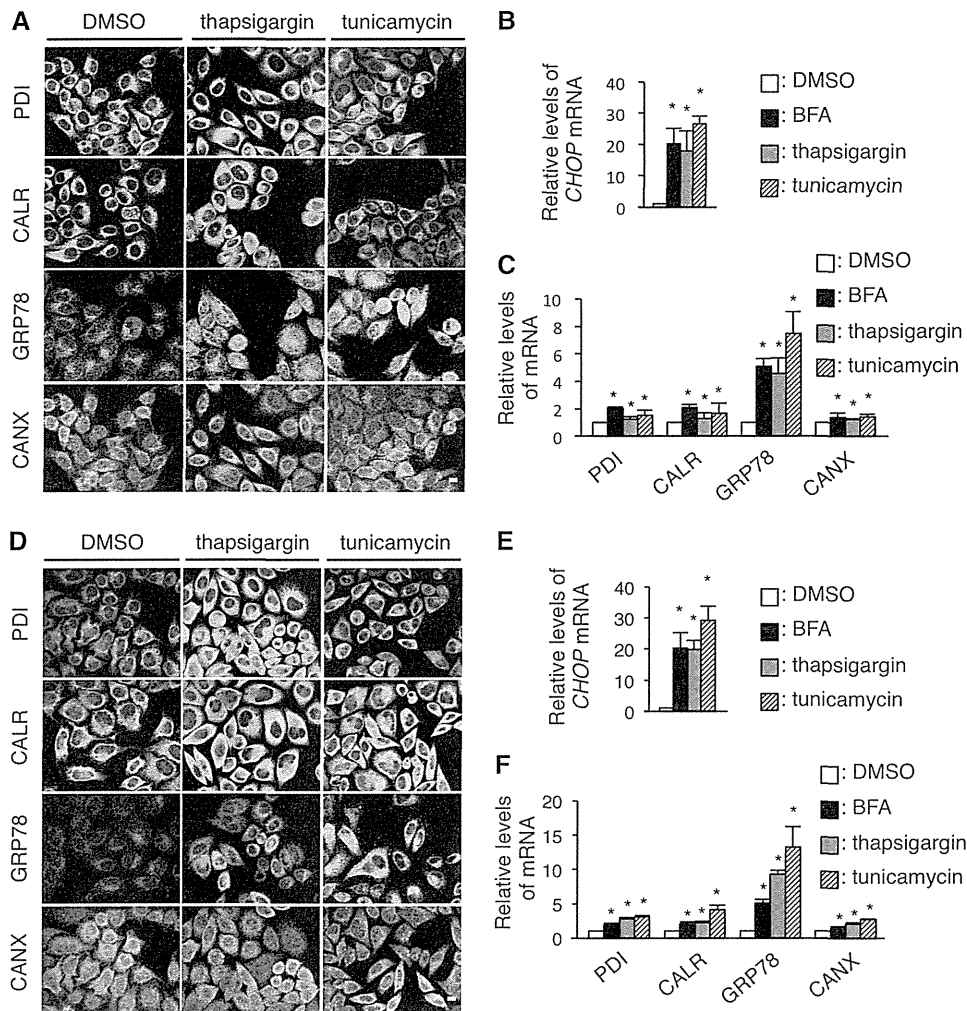
FIGURE 8. **Thapsigargin and tunicamycin treatments do not cause the depletion of PDI, CALR, and GRP78.** *A* and *D,* immunocytochemistry of ER chaperones in HeLa cells treated with thapsigargin and tunicamycin. HeLa cells were treated with 1 μM thapsigargin or 2 μM tunicamycin for 8 (*A*) or 24 h (*D*), immunostained with the indicated antibodies and observed with a confocal fluorescence microscope. *Scale bar,* 5 μm. *B, C, E,* and *F,* quantitative RT-PCR for *CHOP* (*B* and *E*) *PDI, CALR, GRP78,* and *CANX* (*C* and *F*) genes in HeLa cells treated with 1 μM thapsigargin or 2 μM tunicamycin for 8 h (*B* and *C*) or 24 h (*E* and *F*). The *GAPDH* gene was used as an internal control. Results are represented as fold-induction compared with DMSO control experiment. Values are represented as the mean ± S.E. from three independent experiments (*, $p \leq 0.05$; **, $p \leq 0.005$).

GRP78 in the digitonin-soluble fraction containing the cytosol and plasma membrane was significantly higher, whereas the proportion of these proteins in the insoluble fraction containing ER proteins was lower than in untreated cells (Fig. 7, *F–H*); similar to that observed in cells expressing the mutant PLP1 (Fig. 2, *D–F*). This multitude of common evidence between BFA-treated and PLP1msd-transfected cells suggests that these may have a common mechanism underlying depletion of PDI, CALR, and GRP78.

The Fragmentation of GA in Cells Expressing PLP1msd and Phenotypically Milder PLP1 Mutants—As previously reported (37), BFA induces fragmentation of the GA (Fig. 9*A*). To further examine whether the mutant proteins affect the structure of the GA, we co-transfected GFP vector and the PLP1msd gene in HeLa cells and immunostained cells with antibodies against GM130, a GA marker, and PDI. We found that the GA was fragmented in GFP⁺ cells, which are expressing PLP1msd (Fig. 9*B*). These cells were not stained with anti-PDI antibody. This GA fragmentation was not observed in cells expressing PLP1wt. GM130 co-localized with the dense signal of PLP1wt at the peri-
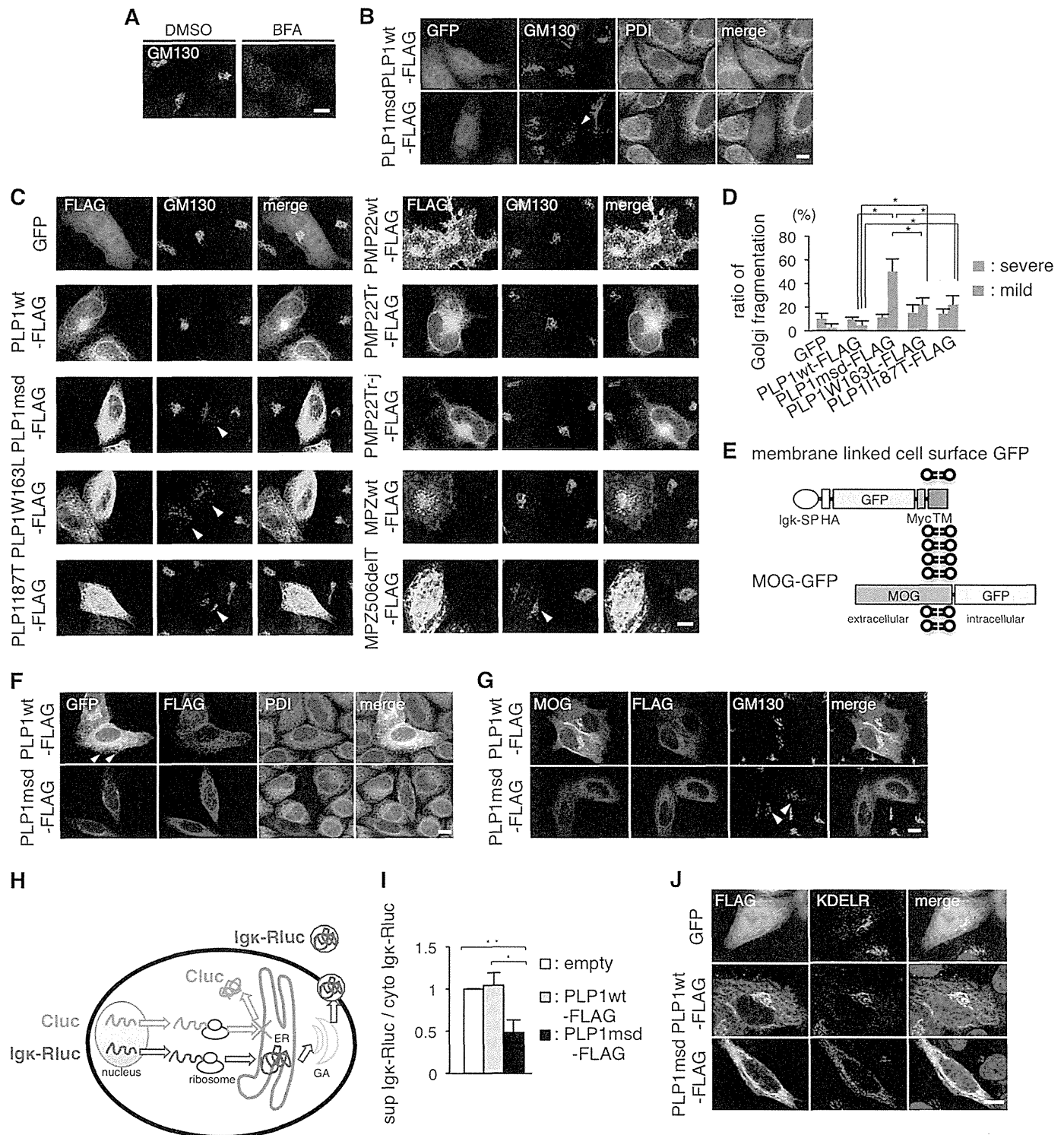
nuclear structure, presumably staining normal GA. We also found that expression of the *MPZ506delT* mutation also induced fragmentation of the GA in HeLa cells, whereas MPZwt, PMP22wt, and both Tr and Tr-j PMP22 mutants did not induce any morphological changes of the GA (Fig. 9*C*). These findings suggest that ER stressor proteins also induce GA fragmentation.

Next, to determine whether fragmentation of the GA is associated with the phenotypic variation in PMD patients, we evaluated the GA structure in HeLa cells transfected with the PLP1msd and two milder PLP1 mutants and classified the GA morphology into three categories, "normal," "mild fragmentation," and "severe fragmentation," as previously reported elsewhere (38). Cells expressing each mild allele showed a higher proportion of "severe" GA fragmentation than those expressing PLP1wt; however, this proportion was lower than in cells expressing PLP1msd (Fig. 9*D*). These results suggest that GA fragmentation is involved in pathogenesis of disease-causing PLP1 mutations, and that the degree of GA fragmentation is associated with the severity of PMD, in conjunction with depletion of the ER chaperones.

Obstruction of Membrane and Secretory Protein Transport by PLP1msd—Newly synthesized membrane and secretory proteins are transported from the ER to the GA when they undergo post-translational modification (39). ER chaperone proteins are subsequently transported back to the ER from the *cis*-Golgi. In contrast, other membrane and secretory proteins that reach the *trans*-Golgi are sorted to carriers for further transport to various cellular destinations (40) (Fig. 10A). We hypothesized that

PLP1msd interferes with the maturation of these proteins because it depletes the ER chaperones, which assist with protein folding in the ER, and induces morphological changes in the GA. To test this hypothesis, we created an expression vector encoding membrane-linked cell surface GFP, summarized in Fig. 9E. When HeLa cells were co-transfected with either PLP1wt-FLAG or PLP1msd-FLAG along with the cell surface GFP, we found that expression of the reporter protein was mis-

localized in cells co-transfected with the PLP1msd gene (Fig. 9F). On the other hand, co-transfection with the PLP1wt gene showed GFP fluorescence in the cell surface. Such phenomenon was also observed in HeLa cells co-transfected with the MOG gene (41), which is an oligodendrocyte-specific membrane protein, fused with the GFP gene (MOG-GFP, summarized in Fig. 9, E and G). These findings suggested the possibility that PLP1msd impairs the transport of membrane proteins from the ER to the cell surface through the GA.

Furthermore, we analyzed whether intracellular transport of the secretory proteins was also affected by PLP1msd. We created a reporter secretory protein, in which *Renilla* luciferase (Rluc) was fused at the N terminus with the signal sequence of Igκ light chain (Igκ-Rluc). This fusion protein penetrates into the ER and is secreted to the extracellular space through the GA. HeLa cells were co-transfected with the regular firefly luciferase (cytoplasmic luciferase, Cluc) gene and the Igκ-Rluc gene along with an empty vector, PLP1wt-FLAG or PLP1msd-FLAG gene (Fig. 9H). We then simultaneously measured Cluc and Igκ-Rluc in cell lysate and culture supernatants with a luminometer. Total Rluc activity normalized to Cluc activity did not differ among cells expressing empty vector, PLP1wt, and PLP1msd, confirming a stable translation ratio between ER-mediated and non-ER-mediated processes (data not shown). Of note, we found that expression of the Igκ-Rluc protein in the culture supernatant normalized to intracellular Igκ-Rluc was significantly lower in cells transfected with the PLP1msd gene (Fig. 9I). Immunocytochemistry revealed that the Igκ-Rluc reporter protein is faintly localized in the GA in cells expressing PLP1wt; whereas, the same protein is clearly accumulated in the ER of cells expressing PLP1msd (data not shown). Together, these results suggest that PLP1msd induces obstruction of membrane and secretory protein transport.

PLP1msd Disturbs the Localization of KDEL Receptor in the GA—PDI, CALR, and GRP78 contain a carboxyl-terminal retrieval signal KDEL (Lys-Asp-Glu-Leu) motif (42). The KDEL motif is recognized by the KDEL receptor in the GA after releasing the chaperones from the ER, and then the chaperones are retrogradely transported back to the ER by the receptor in a coatomer protein I-dependent manner (43) (Fig. 10A). In contrast, CANX, which lacks a KDEL motif, was not depleted by PLP1msd transfection. These results promote us to investigate subcellular localization of the KDEL receptor in cells expressing PLP1msd. The KDEL receptor mainly localizes in the GA (44). Surprisingly, in the PLP1msd-transfected cells, the KDEL receptor was displayed as a mesh-like distribution through the cells and co-localized well with PLP1msd (Fig. 9J). In the cells expressing PLP1wt, we observed that the KDEL receptor co-localized with PLP1wt at the perinuclear structure, which was probably localized in the GA as shown in Fig. 9B. These results suggest that PLP1msd induced mislocalization of the KDEL receptor.

DISCUSSION

Involvement of ER stress and the subsequent UPR has been implicated in pathogenesis of multiple human inherited diseases, including cystic fibrosis (45), retinitis pigmentosa (46), CMT (22), and PMD (8, 11). Although there is wide phenotypic variation in each of these diseases, even among the mutations in same genes, little is known about the factors that determine the difference in ER stress and the severity of disease. In this study, we investigated the organelle changes in cells expressing different *PLP1* missense mutations associated with a wide-range of clinical severities in PMD. We demonstrated that accumulation of the ER stress-associated mutant PLP1 leads to depletion of some important ER chaperones and GA fragmentation, both of which are more profound in cells expressing mutants associated with more severe phenotypes. We also found that an ER stress-related MPZ mutant also induces these cellular phenotypes; however, two PMP22 mutants, which cannot induce ER stress despite their ER retention, do not induce them. Based on these findings, we suggest that the cellular phenotypes of ER chaperone depletion and GA fragmentation may be involved in

FIGURE 9. **PLP1msd overexpression induces GA fragmentation, retention of MOG in the ER, and reduction of protein secretion.** *A* and *B*, immunocytochemistry of GM130 in HeLa cells treated with BFA (*A*) and HeLa cells expressing PLP1wt or PLP1msd (*B*). HeLa cells were treated with DMSO (as a control) or BFA as described in the legend to Fig. 7A. HeLa cells co-transfected with GFP (to visualize transfected cells) along with PLP1wt-FLAG or PLP1msd-FLAG were immunostained using anti-GM130 (*blue*) and anti-PDI (*magenta*) antibodies and were observed with a confocal fluorescence microscope. Cells expressing PLP1msd showed the fragmentation of the GA (*arrowhead*). Scale bar, 10 μm. *C*, immunocytochemistry of GM130 in HeLa cells expressing PLP1, PMP22, and MPZ mutants. HeLa cells transfected with the indicated vectors were immunostained with the anti-FLAG (*green*) and anti-GM130 (*magenta*) antibodies. Note that cells expressing PLP1 mutants and MPZ506delT showed fragmentation of GA detected by GM130 staining (*arrowheads*). Scale bar, 10 μm. *D*, the proportion of cells showing GA fragmentation in HeLa cells transfected with PLP1wt or mutant PLP1 genes, as shown in *B* and *C*. FLAG-positive cells were classified into 3 categories based on GA morphology, as normal, mild fragmentation, and severe fragmentation and the number of cells in each class was counted. Data for normal are not shown. The results are represented as the mean ± S.E. from three independent experiments with >100 cells counted in each experiment (*, $p \leq 0.05$; **, $p \leq 0.005$). *E*, scheme for the construction of membrane-linked cell surface GFP and MOG-GFP. *SP*, signal peptide; *Igκ*, immunoglobulin κ light chain; *HA*, hemagglutinin; *TM*, transmembrane of platelet-derived growth factor receptor. *F* and *G*, subcellular localization of membrane-linked cell surface GFP (*F*) or MOG-GFP (*G*) in HeLa cells expressing PLP1wt-FLAG or PLP1msd-FLAG. HeLa cells co-transfected with membrane-linked cell surface GFP or MOG-GFP along with PLP1wt-FLAG or PLP1msd-FLAG were immunostained using anti-FLAG (*blue*) and anti-PDI (*magenta*) antibodies. Cells expressing PLP1wt showed the cell surface GFP expression (*arrowhead*). Scale bar, 10 μm. *H*, scheme for the luciferase reporter assay. Secretary *Renilla* luciferase (Igκ-Rluc, *blue*), which is fused with Igκ signal peptide, penetrates into the ER and is secreted to the extracellular space through the GA. Firefly luciferase (Cluc, *red*), which is a cytosolic protein, served as an internal control. This system enables the measurement of secretion of the secretory reporter protein in live cells by comparing the total and supernatant activities of Igκ-Rluc. Co-transfection with the Cluc gene not only detects leakage of these reporter proteins to the supernatant from dead cells, but also compares translation efficacies between cytoplasmic and secretary proteins. *I*, luciferase reporter assay to evaluate the effect of PLP1msd on the secretory protein transport. HeLa cells were co-transfected with the firefly luciferase (Cluc) and Igκ-Rluc genes along with an empty vector, PLP1wt-FLAG, or PLP1msd-FLAG. Each of Cluc and Igκ-Rluc in the cell lysate and supernatant was simultaneously measured by a luminometer. Efficient of secretion of Igκ-Rluc was calculated as follows: *Renilla* luciferase activity of supernatant/total (supernatant plus cytosol) *Renilla* luciferase activity. Results are represented as fold-induction compared with empty vector control experiment. Values are represented as the mean ± S.E. from three independent experiments (*, $p \leq 0.05$; **, $p \leq 0.005$). *J*, immunocytochemistry of KDEL receptor in HeLa cells expressing PLP1wt or PLP1msd. HeLa cells transfected with the indicated vectors were immunostained with the anti-FLAG (*green*) and anti-KDEL receptor (*magenta*) antibodies and observed with a confocal fluorescence microscope. Scale bar, 5 μm.
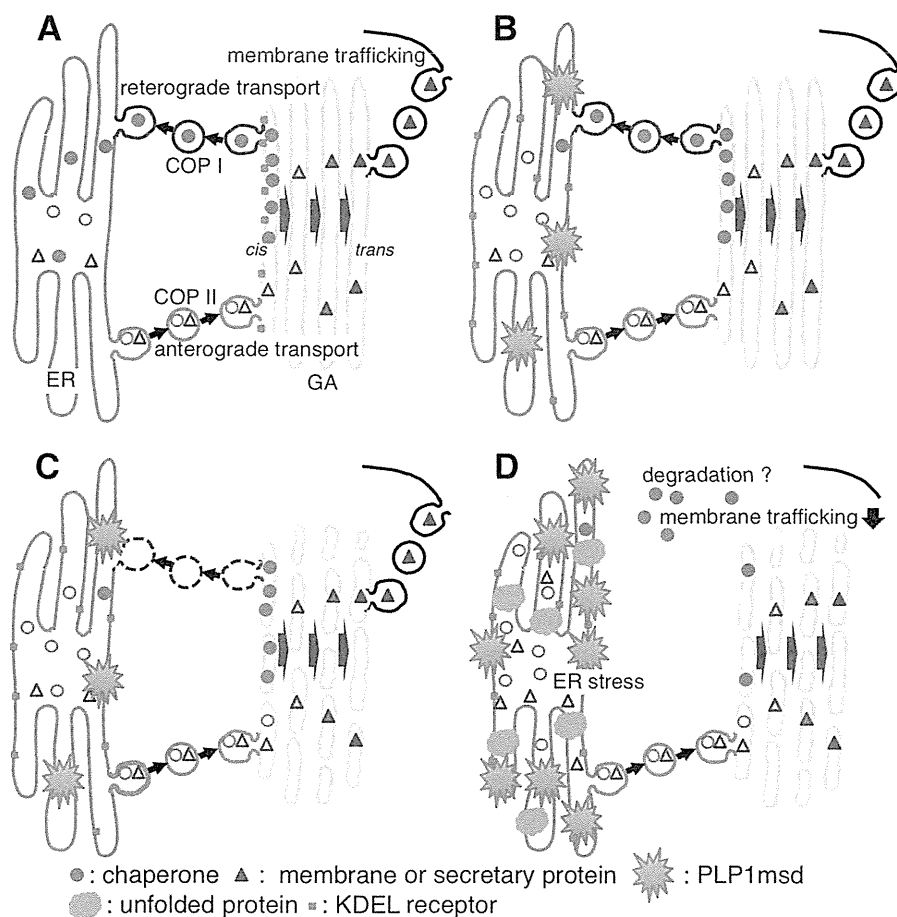
FIGURE 10. **Scheme for the mechanism of ER chaperone depletion and GA fragmentation.** *A*, under physiological conditions, efficient amounts of mature ER chaperones (*filled circles*) can fold unfolded proteins into their correct conformation. Immature ER chaperones with KDEL motifs (*open circles*), along with membrane and secretory proteins (*triangles*), are first transported from ER exit sites to the entry (*cis*) side of the GA to undergo post-translational modifications (coatomer protein (*COPII*) II-mediated anterograde transport). The ER chaperones are recycled back to the ER through the interaction with the KDEL receptor (*red square*) in the GA (coatomer protein I-mediated retrograde transport). In contrast, mature membrane and secretory proteins that reach to the *trans*-Golgi are further transported to various cellular destinations. *B*, ER stress-related mutant protein (*red spines*) induces dysfunction of retrograde transport from the GA to the ER by KDEL receptor mis-localized in the ER, resulting in a reduced supply of ER chaperones. *C*, this inhibition of retrograde transport, and probably the ER stress itself, may lead to the fragmentation of the GA. *D*, dysfunction of the ER to GA transport may jam membrane and secretory protein trafficking, leading to the further accumulation of misfolded proteins (*orange*). These changes in cellular homeostasis triggered by misfolded mutant proteins may further accelerate ER stress.

the pathogenesis of particular mutations in certain genes in ER stress-related diseases.

We observed that PDI, CALR, and GRP78 were depleted in the ER of HeLa cells transfected with the PLP1msd gene, whereas CANX remained unaffected. Similar phenomenon was also observed in endogenous Pdi and Calr in the SCs of *msd* mice (Fig. 6, *C* and *D*). By contrast, we could not find an obvious decrease of endogenous Grp78 in the mutant SCs, possibly due to the large enhancement of mRNA up-regulation. We considered the features that were either functionally or structurally common among the depleted chaperones. Functionally, each of these chaperones has a distinct role in protein folding and maintenance of ER homeostasis. For example, PDI catalyzes the formation and rearrangement of molecular disulfide bonds for protein folding (47). CALR and CANX are calcium-binding proteins implicated in the trimming of *N*-glycosylation and storage of calcium in the ER (48). GRP78 controls activation of the UPR, acting as a sensor for misfolded proteins in the ER (49). Based on this evidence, it is unlikely that the depletion is linked to a particular function of these chaperones.

Structurally, PDI, CALR, and GRP78 contain a carboxyl-terminal retrieval signal KDEL motif (42), which is recognized by the KDEL receptor to transport them back to the ER (43). This retrieval mechanism by the KDEL receptor contributes to quality control at the ER (50). We observed that the KDEL receptor was localized in the ER in cells expressing PLP1msd, whereas the same protein was localized in the GA in PLP1wt and control cells (Fig. 9*J*). Moreover, depletion of PDI, CALR, and GRP78 was also observed in HeLa cells treated with the chemical ER stressor, BFA, which inhibits retrograde transport from the GA to the ER (51, 52). Interestingly, the other chemical ER stressors tested, thapsigargin and tunicamycin, did not recapitulate the findings. These results suggest that misfolded mutant proteins may induce ER chaperone depletion by inhibition of their KDEL receptor-mediated retrograde transport of these chaperones by mis-localizing KDEL receptor.

Our results suggested that PLP1 and MPZ mutants, and possibly other mutant proteins that evoke ER stress, specifically deplete chaperones containing a KDEL motif from the ER. These proteins were unlikely degraded by the ERAD-protea-

some system (Figs. 2, *B* and *C*, and 3, *E* and *F*). We further demonstrated that the proportion of these chaperone proteins in the digitonin-soluble fraction, which contains the plasma membrane and cytosolic proteins, increased in cells expressing PLP1msd (Fig. 2, *D* and *E*). However, we found no change in the amounts of these chaperone proteins on the cell surface (Fig. 3, *B* and *D*). These results suggest that KDEL-containing ER chaperones mainly translocate from the ER to the cytosol in cells expressing ER stress proteins. However, we could not rule out a possibility that small populations may translocate to the plasma membrane, as described previously (29).

In contrast, the GA fragmentation observed in cells treated with BFA was also observed in cells treated with thapsigargin (data not shown). GA fragmentation has been reported in another ER stress-related disorder, ALS (53). These findings suggest that GA fragmentation may be a common pathology in ER stress-related diseases.

An association between cellular pathology and clinical severity for PLP1 mutations has been reported. Gow and Lazzarini (10) reported a cellular mechanism that the amount of mutant *PLP1* gene product accumulated in the ER accounts for disease severity in PMD. Recent studies showed that differences in the UPR (9) and ER quality control (11) have the potential to modulate disease severity. These reports suggest that retention of PLP1 mutants determines the severity of ER stress and clinical outcome. Consistent with these findings, the depletion of ER chaperones and GA fragmentation are closely linked to clinical severity (Figs. 5*C* and 9*D*), indicating that these cellular phenotypes are associated with disease pathology. In addition, we also demonstrated that PLP1msd not only induces ER stress, but also inhibits secretion and cell surface expression of proteins, probably due to impairment of ER chaperone transport from the GA to the ER and/or GA fragmentation. These trafficking defects may also contribute to the pathogenesis of disease by preventing cell-to-cell and cell-to-environment communications. Additional studies are required to elucidate how these mutants affect the maturation and trafficking of other membrane and secretory proteins.

Based on our findings, we propose a novel model for mechanisms to explain how mutant misfolded proteins affect intracellular homeostasis, as summarized in Fig. 10. When misfolded proteins accumulate in the ER, they inhibit GA to ER retrograde transport by KDEL receptor mis-localization in the ER (Fig. 10*B*). Ultimately, inhibition of retrograde transport results in depletion of KDEL-containing ER chaperones from the ER. Blockage of GA to ER retrograde transport also contributes to abnormal accumulation of ER chaperones in the *cis*-Golgi, which may, in part, contribute to GA fragmentation (Fig. 10*C*). As a consequence, the ER to GA transport of membrane/secretory proteins is disturbed, and misfolded proteins and other membrane/secretory proteins accumulate in the ER, resulting in a trafficking defect and further acceleration of ER stress (Fig. 10*D*). This unexpected discovery and new model for the disease mechanism may promote our understanding of how different mutations in the same gene differently evoke ER stress and affect disease phenotype. Our findings may have further implications for ER stress-related diseases in which the UPR modulates pathology. Because practically no effective treat-

ment is available for these diseases, ER chaperone and GA may serve as potential targets for therapeutic intervention.

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ORIGINAL COMMUNICATION

Epidemiological, clinical, and genetic landscapes of hypomyelinating leukodystrophies

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Abstract To determine the epidemiological, clinical, and genetic characteristics of congenital hypomyelinating leukodystrophies, including Pelizaeus–Merzbacher disease (PMD), we conducted a nationwide epidemiological survey in Japan. A two-step survey targeting all medical institutions specializing in pediatric neurology and childhood disability (919 institutes) in Japan was performed. Detailed information was collected for 101 patients (86 males and 15 females) with congenital hypomyelinating leukodystrophies. The prevalence of congenital hypomyelinating disorders was 0.78 per 100,000 people (0–19 years old), and the incidence was 1.40 per 100,000 live births. Molecular testing was performed in 75 % of patients, and *PLP1* gene abnormalities were observed in 62 %. The incidence of PMD with *PLP1* mutations was estimated to be 1.45 per 100,000 male live births and that for congenital hypomyelinating disorders with unknown cause to be 0.41 per 100,000 live births. Patients with *PLP1* mutations showed a higher proportion of nystagmus and hypotonia, both of which tend to disappear over time. Our results constitute the first nationwide survey of congenital hypomyelinating disorders, and provide the epidemiological, clinical, and genetic landscapes of these disorders.

Keywords Hypomyelinating leukodystrophy · Epidemiology · Pelizaeus–Merzbacher disease

Introduction

Congenital hypomyelinating leukodystrophies are a heterogeneous group of inherited diseases characterized by a substantial deficit in myelin deposition in the brain. These disorders are distinguished from classic demyelinating leukodystrophies characterized by myelin degeneration. Therefore, clinical manifestations, pathogenesis, and potential treatment approaches can be distinct for these two groups of disorders. Recently, at least 11 congenital

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hypomyelinating leukodystrophies, of which Pelizaeus–Merzbacher disease (PMD) is the best characterized prototype, have been clinically and genetically recognized and differentiated [7], including Pelizaeus–Merzbacher like disease (PMLD) [19], hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) [20], chromosome 18q-deletion syndrome (18qdel) [10], Allan–Herndon–Dudley syndrome [4], mitochondrial Hsp60 chaperonopathy (MitChap60) [11], Salla disease [22], diffuse cerebral hypomyelination with cerebellar atrophy and hypoplasia of the corpus callosum [15], hypomyelination and congenital cataract [24], ataxia, delayed dentition, and hypomyelination [23], peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and Hirschsprung disease [9]. However, epidemiological and clinical diagnostic data specific to this group of disorders are lacking.

In the diagnostic evaluation, the recognition of hypomyelination has been revolutionized by magnetic resonance imaging (MRI) technology [1, 16]. In addition, molecular detection of disease-causing mutations, especially proteolipid protein 1 (PLP1) gene testing for PMD, has further promoted the diagnostic clarification of hypomyelinating disorders. However, despite the availability of such neuroimaging and molecular tools, the cause of the disease remains unknown in many patients. Therefore, substantial information regarding the epidemiological and clinical picture of congenital hypomyelinating disorders remains undetermined.

Here, we conducted a nationwide epidemiological study in Japan to determine the prevalence, incidence, clinical characteristics and manifestations, and frequency of disease-causing mutations in patients with congenital hypomyelinating leukodystrophies.

Methods

Study design

This study was conducted as a part of the Rare and Intractable Disease Research Program, directed by the Ministry of Health, Labour and Welfare, Japan, and was performed in collaboration with the Japanese Society of Pediatric Neurology. The Institutional Review Board at National Center of Neurology and Psychiatry, Japan approved this study in accordance with the ethical standards. We selected all medical hospitals with one or more board-certified pediatric neurologists regardless of the size of the institution (747 institutes), all pediatric departments in medical university hospitals (48 institutes, not including the above), and institutions for children with severe physical and intellectual disabilities (124 institutes), a total of 919 institutes in

Japan. All patients who visited pediatric neurologists in these institutes from 1 April 2008 to 31 March 2009 were approached. This approach enabled sufficient coverage of neuropediatric medical institutes, where most patients with hypomyelinating leukodystrophies visit for diagnosis, follow-up, treatment, and rehabilitation, and thus achieving practically a 100 % sampling rate without performing a large-scale investigation. Incidence (the rate of occurrence per birth) and prevalence (the number of patients in a population) were determined using demographic statistics obtained from the Vital Statistics of Japan yearly survey carried out by the Ministry of Health, Labour and Welfare, Japan (http://www.mhlw.go.jp/toukei/list/81-1.html). The population of Japan is calculated to be roughly 128 million.

Inclusion criteria

Diagnosis of congenital hypomyelinating leukodystrophies was defined by MRI findings and clinical features [16]. MRI diagnosis should include the observation of diffuse hyperintensity of the cerebral white matter in T2-weighted images, suggesting a persistent and substantial deficit in myelin deposition in the brain. Clinical features include developmental retardation in childhood with no or slow clinical deterioration. Demyelinating leukodystrophies were strictly excluded from this survey at the time of enrollment.

Primary and secondary surveys

In the primary survey, we sent a simple questionnaire to the 919 selected medical institutes to determine if patients with congenital hypomyelinating disorders were present in each institute by requesting the number of such patients. In the secondary survey, we sent a survey sheet to the responders of the primary survey to collect detailed clinical information. The questionnaires included questions on age, sex, family history, clinical diagnosis, clinical symptoms, changes of clinical symptoms, findings in the neuroimaging study, implementation and results of genetic testing, and availability for genetic counseling and carrier testing.

Statistical analyses

Two-tailed p values for odds ratios were calculated using Fisher's exact test.

Results

Prevalence and incidence

The primary survey (70 % recovery rate) identified 164 patients with possible congenital hypomyelinating

leukodystrophies from 95 institutes. We obtained completed and returned secondary survey questionnaire sheets from 108 patients, with a 65.5 % recovery rate. Ultimately, 101 patients (86 males and 15 females) met our diagnosis criteria for congenital hypomyelinating leukodystrophies, after exclusion of seven patients with a diagnosis of demyelinating leukodystrophies, such as Alexander disease, and duplicated registrations (Table 1). Based on these numbers, an estimation of 220 patients with congenital hypomyelinating leukodystrophies present in Japan was obtained.

We estimated the prevalence of congenital hypomyelinating leukodystrophies in Japan as 0.78/100,000 people (0–19 years old) and the incidence as 1.40/100,000 live births. More than 80 % of the patients were under 20 years old (supplementary Figure 1A). A family history of congenital hypomyelinating leukodystrophies was present in 22 patients, including 11 patients with *PLP1* gene abnormalities.

Clinical diagnosis

The most common clinical diagnosis was PMD (Table 1). Other diagnoses included PMLD, H-ABC, and 18qdel; more than 20 % of patients had no specific diagnosis and remained unclassified. These diagnoses were made primarily on the basis of MRI findings and clinical features,

Table 1 Characteristics of the patients

Characteristics	Total $n = 101$
Range of ages (mean, median)	0–48 (12.1, 9)
Sex, n (%)	
Male	86 (85)
Female	15 (15)
Family history, n (%)	22 (22)
Clinical diagnosis, n (%)	Total $n = 101$
PMD	66 (65)
PMLD	6 (6)
H-ABC	4 (4)
18q deletion syndrome	1 (1)
Others	1 (1)
Unknown	23 (23)
Molecular diagnosis, n (%)	Total $n = 76$
PLP1 abnormalities	47 (62)
Duplication	26 (63)
Point mutation	15 (37)
Others	2 (3)
Unknown	27 (35)

PMD Pelizaeus–Merzbacher disease, *PMLD* Pelizaeus–Merzbacher like disease, *H-ABC* hypomyelination with atrophy of the basal ganglia and cerebellum

and the results of molecular testing were not necessarily required in the diagnostic decision, although in nearly half of the patients, conclusive diagnostic information via molecular testing was obtained (49/101 patients).

Molecular diagnosis

Almost 75 % of the patients received molecular testing for one or more genes; the majority were tested for *PLP1*, which is responsible for PMD. More than 60 % of these patients had *PLP1* abnormalities. Information about the mutation types was available in 41 patients; 26 patients had genomic duplications and 15 patients had point mutations. Besides *PLP1* mutations, chromosomal abnormality was reported in one patient with 18qdel. In 36 % of the patients, no molecular diagnosis was achieved. Based on these data, we estimated the incidence of PMD with *PLP1* mutations to be 1.45 per 100,000 male live births. *GJC2* was examined in 11 patients negative for *PLP1* testing. In all patients but one, in which a potential alteration in one allele was seen, no mutation could be found. The incidence for congenital hypomyelinating leukodystrophies with unknown cause was estimated to be 0.41/100,000 live births.

Clinical characteristics

All patients experienced their initial symptoms before 1 year of age, and 91 % showed symptoms within 6 months of birth. The most common initial symptoms were nystagmus and developmental delay (Table 2). Patients with *PLP1* mutations showed nystagmus as their initial symptom more commonly than the patients without the mutations did. In contrast, patients having no *PLP1* mutations were more likely to show developmental delay as their initial symptom. Nystagmus was a more common initial symptom in patients with earlier disease onset, whereas developmental delay was more frequent in patients with later onset (supplementary Figure 1B). Of note, five patients had respiratory problems as their initial symptom; all five patients had *PLP1* mutations. In addition, all of the patients with *PLP1* mutations showed their initial symptoms before the 5th month of life (supplementary Figure 1C). The median age for achieving clinical diagnosis was 1 year and 8 months. Almost 70 % of patients were diagnosed at <3 years of age (supplementary Figure 1D). Meanwhile, eight patients were diagnosed at age 10 years or older, including two individuals in their 40s. These individuals might have had no opportunity for *PLP1* molecular testing at their initial diagnostic evaluation before, and therefore, might have had to wait for a recent re-evaluation opportunity.

The most common symptoms over the entire clinical course were nystagmus (74 %), hypotonia (71 %), and the

Table 2 Initial symptoms

Initial symptoms	All No. (%) n = 101	PLP1+ No. (%) n = 47	PLP1− No. (%) n = 22	P value PLP1+ vs. PLP1−
Nystagmus	57 (56)	33 (70)	9 (41)	0.0332
Developmental delay	34 (34)	8 (17)	14 (64)	0.0002
Others	14 (14)	7 (15)	2 (9)	NS

Others include respiratory failure (n = 5)

PLP1+ patients with PLP1 mutations, PLP1− patients without PLP1 mutations

symptoms associated with spastic diplegia/quadriplegia (increased deep tendon reflex, spasticity, quadriplegia, and pathological reflex) (Table 3). A comparison between patients with PLP1 mutations and those without revealed that nystagmus and hypotonia were more common in patients with PLP1 mutations (Table 3). In contrast, rigidity and dystonia were less common in those with PLP1 mutations. No difference in the frequency of other symptoms was observed.

To determine the changes in clinical manifestations over time, we selected patients aged 7 years and older and investigated the frequency of each symptom prior to and at the time of the survey (supplementary Table 1). In patients with PLP1 mutations, the frequencies of nystagmus and hypotonia decreased over time (Fig. 1). No such tendency was observed in patients without PLP1 mutations, indicating that the disappearance of these symptoms is characteristic to PLP1 mutations. Changes in the frequency of other symptoms, including quadriplegia, rigidity, dystonia, and cerebellar signs, were not observed.

Imaging findings

In accordance with the inclusion criteria, bilateral diffuse hyperintensity of cerebral white matter in T2-weighted MRI images was recognized in all patients. Other MRI findings are summarized in supplementary Table 2. Minor findings, including diffuse cerebral atrophy, cerebellar atrophy, and caudate nucleus lesions, were observed less frequently in patients with PLP1 mutations than those without.

Clinical examination

The auditory brainstem response (ABR) was measured in more than 80 % of patients (Table 4). Compared to 83 % of patients not carrying PLP1 mutations, all patients with PLP1 mutations had an abnormal ABR. A nerve conduction study was performed in 37 % of patients, three of whom, including two with PLP1 mutations, demonstrated

Table 3 Clinical features

Symptoms	All No. (%) n = 101	PLP1+ No. (%) n = 47	PLP1− No. (%) n = 22	P value PLP1+ vs. PLP1−
Nystagmus	75 (74)	43 (91)	12 (55)	0.0008
Hypotonia	72 (71)	37 (79)	12 (55)	0.0049
Quadriplegia	56 (55)	24 (51)	15 (68)	NS
Spasticity	59 (58)	31 (66)	11 (59)	NS
Increased DTR	62 (61)	28 (60)	13 (59)	NS
Swallowing difficulty	29 (29)	11 (23)	8 (36)	NS
Hearing loss	24 (24)	11 (23)	8 (36)	NS
Pathological reflex	36 (35)	13 (28)	9 (41)	NS
Rigidity	20 (20)	5 (11)	7 (32)	0.04
Dystonia	14 (14)	3 (6)	6 (27)	0.02
Cerebellar signs	21 (21)	9 (19)	3 (14)	NS
Athetosis	11 (11)	4 (9)	4 (18)	NS
Seizure	26 (26)	9 (19)	8 (36)	NS

DTR deep tendon reflexes, PLP1+ patients with PLP1 mutations, PLP1− patients without PLP1 mutations

an abnormality. Chromosomal examinations and a thyroid hormone study were performed in approximately half of the patients.

Availability for genetic counseling and carrier diagnosis

Approximately 30 % of the families of patients having conclusive molecular diagnoses proceeded to carrier testing. Genetic counseling was provided to all families. Of the institutes included in this survey, 48 % have an on-site genetic counseling service available, and 14.5 % have access to affiliated medical institutes with a genetic counseling service. However, 37.5 % have no access to genetic counseling as a part of their medical service; most of these are institutes for severely retarded children, and were responsible for the daily support and rehabilitation of children after completion of the diagnostic study.

Supportive care and treatment

Seventeen percent of patients underwent tracheotomies that included laryngotracheal separation, another 8 % required mechanical ventilation, and 27 % of patients, including those receiving a gastrotomy, required ingestion of food via a feeding tube. Rehabilitation was performed in 57 % of patients. Nearly 30 % of the patients use antiepileptic drugs and/or muscle relaxants. No difference in the frequency of care and treatment for patients with and without PLP1 mutations was observed.
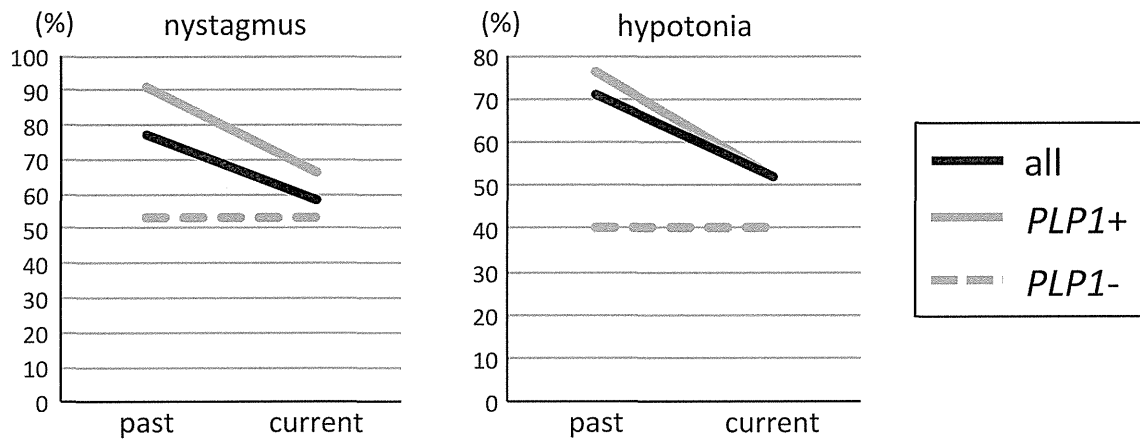
Fig. 1 Change in the clinical symptoms of patients aged 7 years and older. Patientsaged 7 years and older (*n* = 66) were selected from the cohort (*black line*). Of these, patients with genetic testing information were sub-grouped into those with *PLP1* abnormality (*grey line*; *n* = 33) and without (*dotted line*; *n* = 15). Frequencies of the symptoms present in the past and current presentation were shown for nystagmus (*left*) and hypotonia (*right*)

Table 4 Clinical examinations

Clinical examinations	All (*n* = 101)		PLP1+ (*n* = 47)		PLP1− (n = 22)		*P* value *PLP1*+ vs. *PLP1*−
	No. performed (%)	No. with abnormality (%)	No. performed (%)	No. with abnormality (%)	No. performed (%)	No. with abnormality (%)	
ABR	83 (82)	72 (87)	43 (92)	43 (100)	18 (82)	15 (83)	NS
SEP	22 (22)	10 (46)	5 (11)	2 (40)	9 (41)	7 (78)	0.008
NCV	37 (37)	3 (8)	15 (32)	2 (13)	12 (55)	1 (8)	NS
Chromosomal analysis	53 (53)	3 (6)	20 (43)	1 (5)	18 (82)	0 (0)	0.0038
Thyroid hormone	50 (50)	3 (6)	18 (38)	3 (17)	15 (68)	0 (0)	0.0374
LH, FSH	9 (9)	0 (0)	2 (4)	1 (50)	3 (14)	0 (0)	NS

ABR auditory brainstem response, *SEP* somatosensory evoked potentials, *NCV* nerve conduction velocities, *PLP1*+ patients with *PLP1* mutations, *PLP1*− patients without *PLP1* mutations, *LH* luteinizing hormone, *FSH* follicle stimulation hormone

Discussion

By using this nationwide questionnaire-based study, we sought to determine an epidemiological and molecular diagnostic view of congenital hypomyelinating leukodystrophies, and to reveal the clinical characteristics of this group of disorders in Japan. By targeting this specific group of diseases, we identified a number of findings. First, we estimated the prevalence of the congenital hypomyelinating leukodystrophies as 0.78/100,000 people (0–19 years old) and the incidence as 1.40/100,000 live births. In combination with molecular diagnostic information, we further estimated the incidence of genetically confirmed PMD as 1.45 per 100,000 male live births. Second, 62 % of patients with congenital hypomyelinating leukodystrophies have *PLP1* mutations. Third, patients with *PLP1* mutations show a higher propensity of nystagmus and hypotonia, both of which may disappear over time.

Only a few epidemiological studies have been conducted on leukodystrophies, including hypomyelinating disorders. One nationwide survey conducted in Germany determined the incidence of major forms of leukodystrophies as 2.0/100,000 live births [5]. Based on the proportion of PMD identified (via genetic testing) in this study (6.5 %), the incidence of PMD can be estimated as approximately 0.26/100,000 male live births. However, this is possibly an underestimation because molecular confirmation of *PLP1* abnormalities was required as part of the inclusion criteria; however, at the time of this study (1996), molecular testing for *PLP1* duplication was not clinically available. In another recent study conducted in Utah, USA, the incidence of leukodystrophies was 13.0/100,000 live births [3]. In this chart-based study, almost half of the patients had no known diagnosis, possibly enhancing the overall incidence. Of note, PMD was the second most frequent leukodystrophy in the Utah study, representing

7.4 % of all patients. Based on these data, an incidence of 1.9/100,000 male live births can be estimated for PMD that is consistent with the rate of 1.45/100,000 obtained in our study of a Japanese population. Neither of the aforementioned studies determined the frequency of hypomyelinating leukodystrophies other than PMD. In contrast, our study has specifically targeted congenital hypomyelinating leukodystrophies for the first time to unveil their epidemiological features. We found that 36 % of the patients with hypomyelinating leukodystrophies received no specific diagnosis, leading to an estimated incidence of 0.41/100,000 live births for congenital hypomyelinating leukodystrophies with unknown cause.

We confirmed that PMD is the most common congenital hypomyelinating leukodystrophies. Notably, PLP1 mutations were found in 62 % of patients who underwent molecular diagnostic testing. Therefore, PLP1 molecular testing should be recommended as a first line of examination if hypomyelinating leukodystrophies are considered a potential clinical diagnosis. Of the PMD patients, 63 % carried duplications and 37 % had point mutations. A similar proportional distribution of mutations has been estimated at the diagnostic laboratory level [8, 12, 18], but has not been determined by epidemiological studies. Meanwhile, 36 % of patients may have congenital hypomyelinating leukodystrophies caused by other genes. We found that patients possessing and lacking PLP1 mutations showed largely overlapping clinical and MRI findings. Mutations in SLC16A2 and GJC2 genes were reported to be responsible for approximately 10 % of patients who did not have PLP1 mutations [6, 21], while SOX10 mutations were infrequent [13]. Nonetheless, comprehensive and systematic screening of the human genome is required to fully delineate the molecular diagnostics of these patients with unknown cause of the disease. In fact, exome analyses have recently identified three new genes for congenital hypomyelinating leukodystrophies [2, 14, 17].

In our comparison between patients with and without PLP1 mutations, we found that most symptoms were present in both groups at similar frequencies (Table 3). However, nystagmus and hypotonia were both more common in patients with PLP1 mutations than in those without. In addition, these two symptoms tend to disappear over time only in patients with PLP1 mutations (Fig. 1). Therefore, the presence of nystagmus and hypotonia at earlier stages of the disease and the disappearance of these symptoms in later stages is the unique feature of PMD caused by PLP1 mutations. Hypotonia is a relatively common neurological symptom in children with motor and/or intellectual disabilities, as seen in Down syndrome. However, nystagmus is not as common in other pediatric neurological disorders, and PLP1 molecular testing is therefore recommended particularly for children exhibiting nystagmus.

Of the other diagnostic examinations, an abnormal ABR was commonly observed in patients with hypomyelinating leukodystrophies, regardless of the presence or absence of PLP1 mutations (Table 4). Although an abnormal ABR is not linked to specific gene abnormalities, it is an easily measurable response for infants and toddlers, and has even been used for the screening of hearing in neonates. In combination with early onset nystagmus, an ABR may serve as a useful and convenient tool to detect congenital hypomyelinating leukodystrophies before an MRI reveals confirmatory evidence for hypomyelination, usually at an age of 1 year and later.

There are some limitations to this study. First, we recruited the patients mainly through pediatric neurologists; therefore, we might have excluded adult patients. The lower frequencies of enrolled adults may also be associated with the mortality of these disorders. Because our study did not determine the longitudinal outcomes of patients, we were unable to assess the mortality rate of the congenital hypomyelinating leukodystrophies. Considering these factors, we calculated the prevalence using a population aged 19 years and under; our epidemiological values should therefore be only minimally influenced by these potential biases. Second, mutational analyses of known genes other than PLP1, such as GJC2, SLC16A2, HSPD1, and SLC17A5, were only performed when clinically suspected as contributing to the disease. In most patients, PLP1 was the only gene examined, because only PLP1 could be assessed by commercial laboratory testing. Scanning of other genes requires a research-based setting, which is available for only a limited number of patients. Third, conventional PLP1 sequencing may not detect alterations deep in introns; thus, it is possible that the proportion of PMD in our cohort may have been underestimated. Fourth, the prevalence of hypomyelinating leukodystrophies was lower than their incidence, probably because of the low number of patients older than 8 years (supplementary Figure 1A). The reason for this discrepancy is unknown, but it may be associated with the mortality of these conditions, dropout from long-term follow-up at specialized hospitals, and lower performance rate of genetic testing in older patients. It should also be noted that hypomyelinating leukodystrophies are often misdiagnosed as cerebral palsy, especially in elderly patients. This may also contribute to the underestimation of the prevalence of hypomyelinating leukodystrophies.

In conclusion, we report the results of the first nationwide epidemiological survey of congenital hypomyelinating leukodystrophies conducted in Japan. By targeting hypomyelinating leukodystrophies, we could distinguish this group of disorders from demyelinating leukodystrophies. As a result, we have provided, for the first time, data on the epidemiological, clinical, and genetic landscapes of congenital hypomyelinating leukodystrophies.

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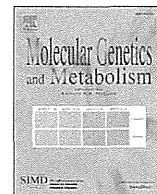
GJC2 promoter mutations causing Pelizaeus–Merzbacher-like disease

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ABSTRACT

Objective: Pelizaeus–Merzbacher-like disease is a rare hypomyelinating leukodystrophy caused by autosomal recessive mutations in GJC2, encoding a gap junction protein essential for production of a mature myelin sheath. A previously identified GJC2 mutation (c.-167G > A) in the promoter region is hypothesized to disrupt a putative SOX10 binding site; however, the lack of additional mutations in this region and contradictory functional data have limited the interpretation of this variant.
Methods: We describe two independent Pelizaeus–Merzbacher-like disease families with a novel promoter region mutation and updated in vitro functional assays.
Results: A novel GJC2 mutation (c.-170G > A) in the promoter region was identified in Pelizaeus–Merzbacher-like disease patients. In vitro functional assays using human GJC2 promoter constructs demonstrated that this mutation and the previously described c.-167G > A mutation similarly diminished the transcriptional activity driven by SOX10 and the binding affinity for SOX10.
Interpretation: These findings support the role of GJC2 promoter mutations in Pelizaeus–Merzbacher-like disease. GJC2 promoter region mutation screening should be included in the evaluation of patients with unexplained hypomyelinating leukodystrophies.

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1. Introduction

Hypomyelinating leukodystrophies are a rare cause of disease of the central nervous system (CNS) characterized by abnormal myelin formation [1]. The prototype condition for hypomyelinating leukodystrophies is Pelizaeus–Merzbacher disease (PMD) (OMIM 312080), an X-linked condition [2] that is due to a mutation in the proteolipid protein 1 gene (*PLP1*) (OMIM 300401). Pelizaeus–Merzbacher-like disease (PMLD) (OMIM 608804) is a clinically similar disease without detectable abnormalities within the *PLP1* gene. PMLD is instead an autosomal recessive hypomyelinating leukodystrophy that was shown to be caused by mutations in the gap junction protein gamma-2 gene (*GJC2*) (OMIM 608803) that encodes the connexin 47 protein (Cx47), a connexin family member and gap junction protein important in astrocytes and oligodendrocytes

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[3,4]. Mutation of GJC2 does not allow Cx47 to reach the membrane, resulting in loss of function [5]. Additionally, the GJC2 promoter region contains SOX10 transcriptional factor binding sites, which allow for SOX10 to play a role in myelin formation [5].

More than twenty different coding mutations have so far been identified in the GJC2 coding region [2,4,6–11]. An additional mutation, c.-167G > G, was identified in the putative promoter region in individuals with the phenotype of PMLD [3,5,12,13]. This promoter mutation was first identified in the homozygous state, has now been reported in 15 individuals from 5 families [3,5,12,13], and has additionally been found in two patients [12] in the heterozygous state with another previously published mutation [7] within the coding sequence of GJC2. There is evidence suggesting that some c.-167A > G cases arose from a single founder [3,13] and this mutation is thought to account for nearly a third of GJC2-PMLD phenotypes [13]. GJC2 mutations account overall for only 10% of unsolved cases of hypomyelination, suggesting that mutations in GJC2 and its promoter region at the SOX10 binding site are a rare cause of this phenotype [6]. Mutation c.-167A > G was demonstrated to result in decreased SOX10 dependent transcription of the luciferase reporter gene in constructs containing mouse Gjc2 promoter region [5]. However,

Original Research

Attenuation of endoplasmic reticulum stress in Pelizaeus-Merzbacher disease by an anti-malaria drug, chloroquine

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Abstract

Pelizaeus-Merzbacher disease (PMD) is a hypomyelinating disorder caused by the duplication and missense mutations of the proteolipid protein 1 (*PLP1*) gene. PLP1 missense proteins accumulate in the endoplasmic reticulum (ER) of premature oligodendrocytes and induce severe ER stress followed by apoptosis of the cells. Here, we demonstrate that an anti-malaria drug, chloroquine, decreases the amount of an ER-resident mutant PLP1 containing an alanine-243 to valine (A243V) substitution, which induces severe PMD in human. By preventing mutant PLP1 translation through enhancing the phosphorylation of eukaryotic initiation factor 2 alpha, chloroquine ameliorated the ER stress induced by the mutant protein in HeLa cells. Chroloquine also attenuated ER stress in the primary oligodendrocytes obtained from myelin synthesis deficit (*msd*) mice, which carry the same *PLP1* mutation. In the spinal cords of *msd* mice, chloroquine inhibited ER stress and upregulated the expression of marker genes of mature oligodendrocytes. Chloroquine-mediated attenuation of ER stress was observed in HeLa cells treated with tunicamycin, an N-glycosylation inhibitor, but not with thapsigargin, a sarco/ER Ca^{2+}ATPase inhibitor, which confirms its efficacy against ER stress caused by nascent proteins. These findings indicate that chloroquine is an ER stress attenuator with potential use in treating PMD and possibly other ER stress-related diseases.

Keywords: PMD, PLP, ER stress, UPR, chloroquine, treatment

Introduction

In the central nervous system, oligodendrocytes form the myelin sheath by wrapping axons with multiple layers of their plasma membrane; this enables rapid impulse conduction along the axons and prevents neuronal death.[1-3] Compact myelin, the lipid-rich major component of the myelin sheath, has a unique composition with more than 70% lipid by dry weight, and 80% of its protein mass consists of just two proteins, myelin basic protein (MBP), and proteolipid protein 1 (PLP1).[1] Although *shiverer*[4] (*Mbp*-deficient) mice develop a severe hypomyelinating disorder in the central and peripheral nervous systems and a

corresponding shortened lifespan, the phenotypes of *Plp1*-knockout mice[5] and *PLP1*-null humans[6,7] display extremely mild myelin defects with almost normal lifespan.

While complete deficiency of *PLP1* only causes modest neurological symptoms, duplication and missense mutations of the *PLP1* gene result in a severe hypomyelinating disorder, Pelizaeus-Merzbacher disease (PMD). PMD is an X-linked recessive leukodystrophy characterized by failure of myelination in the central nervous system.[8,9] Duplication of the *PLP1* gene accounts for 60–70% of PMD cases,[10,11] but it remains unclear why an excess amount of PLP1 induces severe hypomyelination. In contrast, missense mutations of
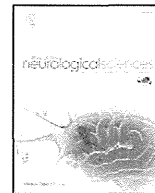
Short communication

A novel homozygous mutation of GJC2 derived from maternal uniparental disomy in a female patient with Pelizaeus–Merzbacher-like disease

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ABSTRACT

Pelizaeus–Merzbacher-like disease (PMLD) is an autosomal recessive hypomyelinating disorder of the central nervous system characterized by nystagmus, motor developmental delay, ataxia, and progressive spasticity. The gap junction protein gamma-2 gene (GJC2), encoding the gap junction protein connexin 47, is one of the genes responsible for this condition. In this study, a novel homozygous mutation in GJC2 (c.746C>G; p.P249R) was identified in a 21-year-old female patient with PMLD. Although her mother was a carrier of this mutation, the Mendelian inheritance pattern could not be determined because the paternal sample was unavailable. Alternatively, chromosomal microarray testing together with single nucleotide polymorphism typing (CGH + SNP) was performed to determine the gene copy number and analyze the haplotype in the 1q42.13 region in which GJC2 is located. The result showed no deletion, but the GJC2 region was involved in the loss-of-heterozygosity region. Furthermore, haplotype of chromosome 1, in which GJC2 is located, revealed that both copies of chromosome 1 were derived from the patient's mother, indicating maternal uniparental disomy of chromosome 1. This study showed the advantage of the SNP genotyping microarray for detecting the origin of the mutation.

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1. Introduction

Hypomyelination, which can be easily revealed by brain magnetic resonance imaging (MRI), is a consequence of the immature condition of oligodendrocytes in the brain white matter, manifesting as pyramidal signs and neurodevelopmental delay [1]. Although many genetic backgrounds are related to this condition, Pelizaeus–Merzbacher disease (PMD) is the most frequently seen in the X-linked inheritance mode of this condition as the genetic background and most of the patients are males. Patients with PMD often show nystagmus, stridor, and hypotonia in early infancy and later develop spasticity, ataxia, tremor and deterioration of psychomotor development [2]. Abnormalities of the proteolipid protein 1 gene (PLP1), including nucleotide alterations and gene copy number aberrations, are the cause of PMD. However, there are many related conditions associated with hypomyelination other than PMD [1].

Among them, PMD and Pelizaeus–Merzbacher-like disease (PMLD) are almost indistinguishable based on the basis of clinical and neuroradiological features at the onset of symptoms [3]. If a patient's genotype is inconsistent with an X-linked recessive inheritance pattern, genes other than PLP1 may be related including the gap junction protein ganna-2 gene (GJC2/GJA12 #608803) [3,4], the aminoacyl tRNA synthetase complex-interacting multifunctional protein 1gene (AIMP1/p43; MIM#603605) [5], and the heat shock 60 kDa protein 1 gene (HSPD1; MIM#118190) [6]. Because approximately 8% of all patients and 16% of female patients with PMLD showed mutations in GJC2, a reasonable approach for the genetic testing for female patients who have clinical symptoms of PMD/PMLD is to begin with a sequence analysis of GJC2 [7,8]. GJC2, located on 1q42.13, encodes connexin 47, which is a member of a large family of homologous connexins. It has been identified in a board range of mammalian tissues. It has 4 transmembrane regions with 2 extracellular and 3 intracellular domains. It is highly expressed in oligodendrocytes and predominantly present at oligodendrocyte-astrocyte gap junctions and participates in linking these 2 types of glial cells [7,9–11].

In this study, we identified a novel homozygous mutation in GJC2 in a female patient with PMLD. Chromosomal microarray testing revealed maternal uniparental disomy (UPD) as the cause of this homozygous

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