Depletion of ER Chaperones and GA Fragmentation in PMD
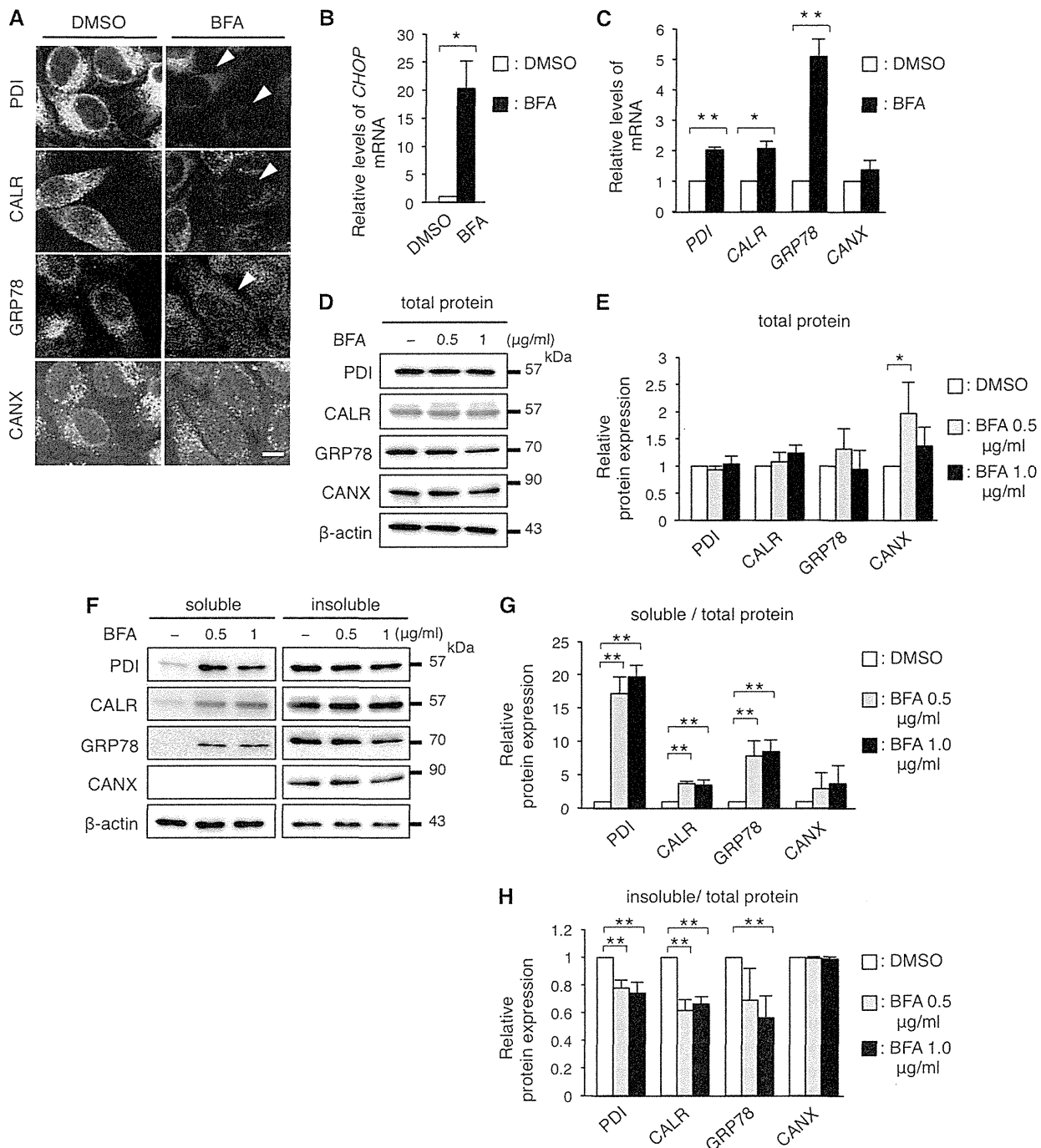

FIGURE 7. **BFA treatment recapitulates the disappearance of PDI, CALR, and GRP78.** *A,* immunocytochemistry of ER chaperones in HeLa cells treated with BFA. HeLa cells were treated with 1 μg/ml of BFA for 8 h were immunostained with the indicated antibodies and observed with a confocal fluorescence 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

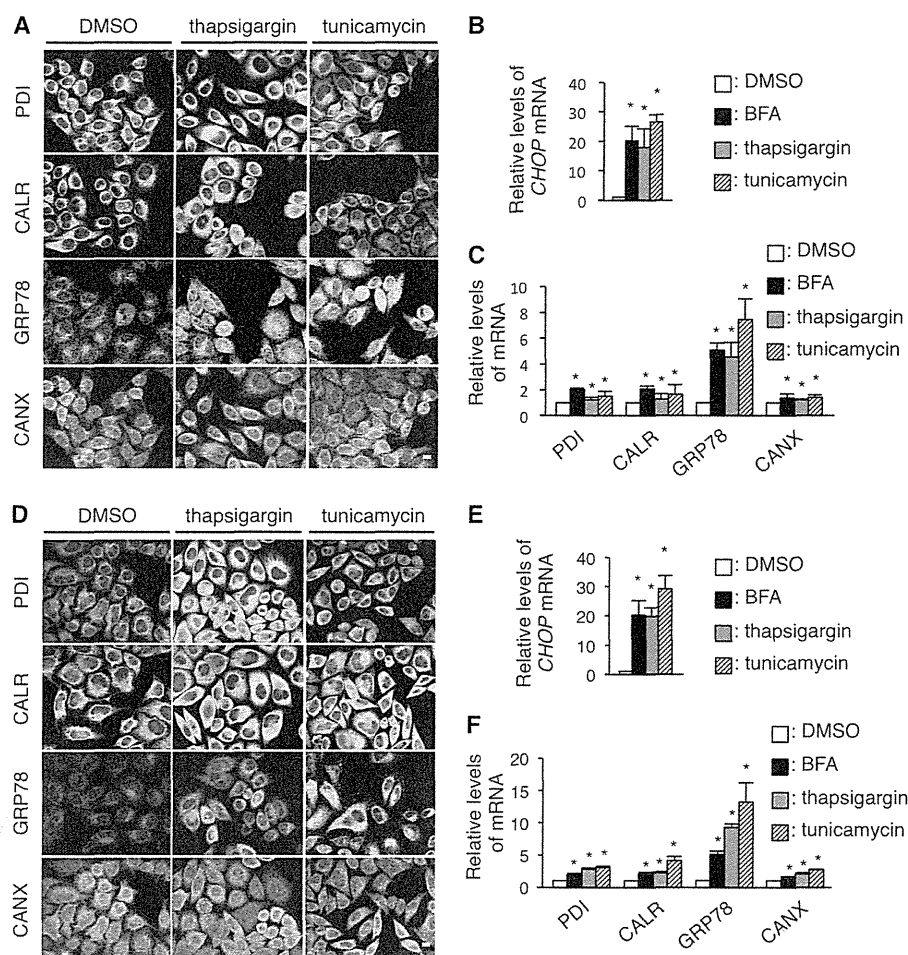## Depletion of ER Chaperones and GA Fragmentation in PMD

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. 9A). 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. 9B). 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. 9C). 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. 9D). 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.
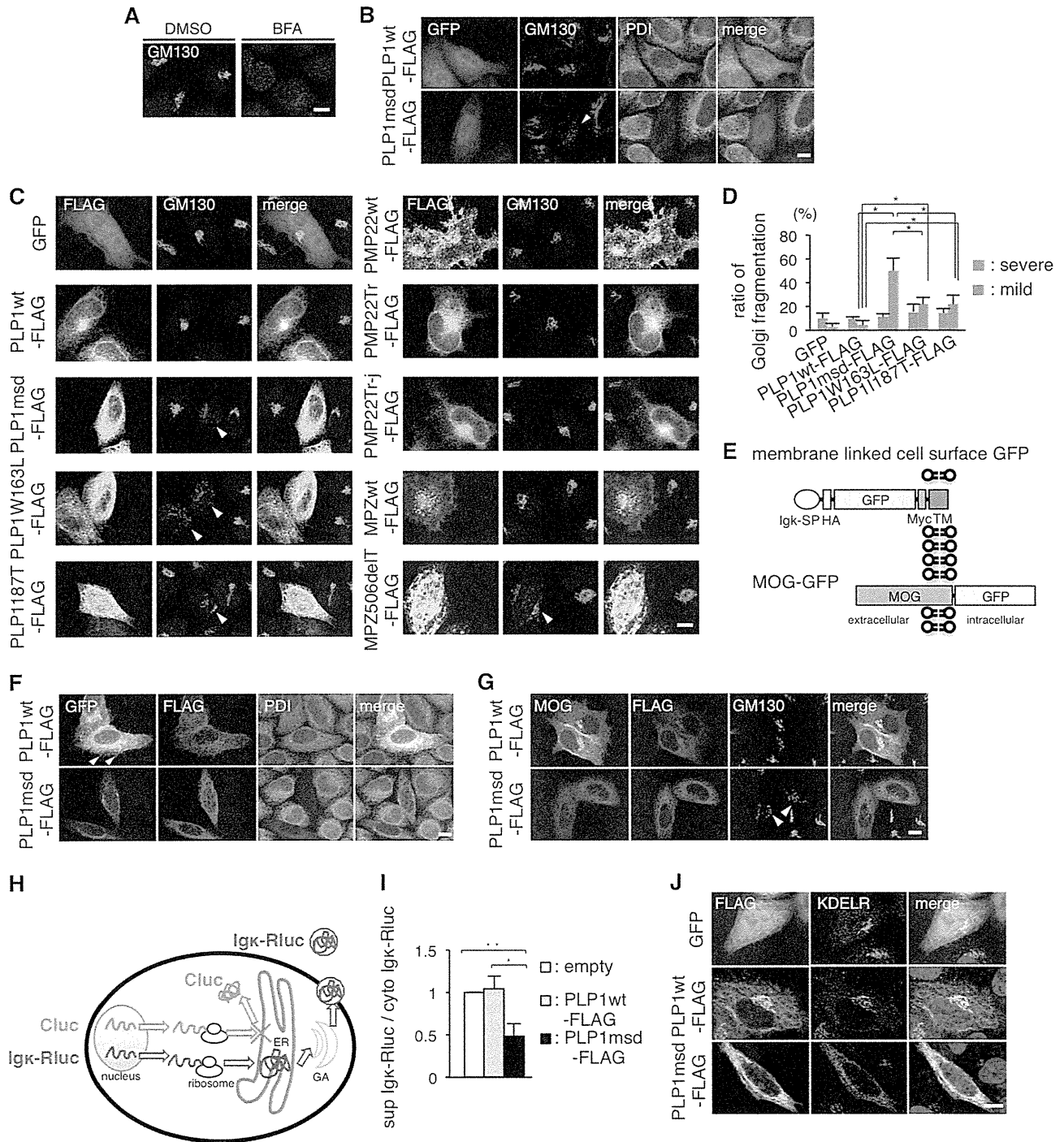
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Obstruction of Membrane and Secretory Protein Transport by PLP1msd—Newly synthesized membrane and secretory proteins are transported from the ER to the GA when they undergo post-translational modification (39). ER chaperone proteins are subsequently 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

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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.

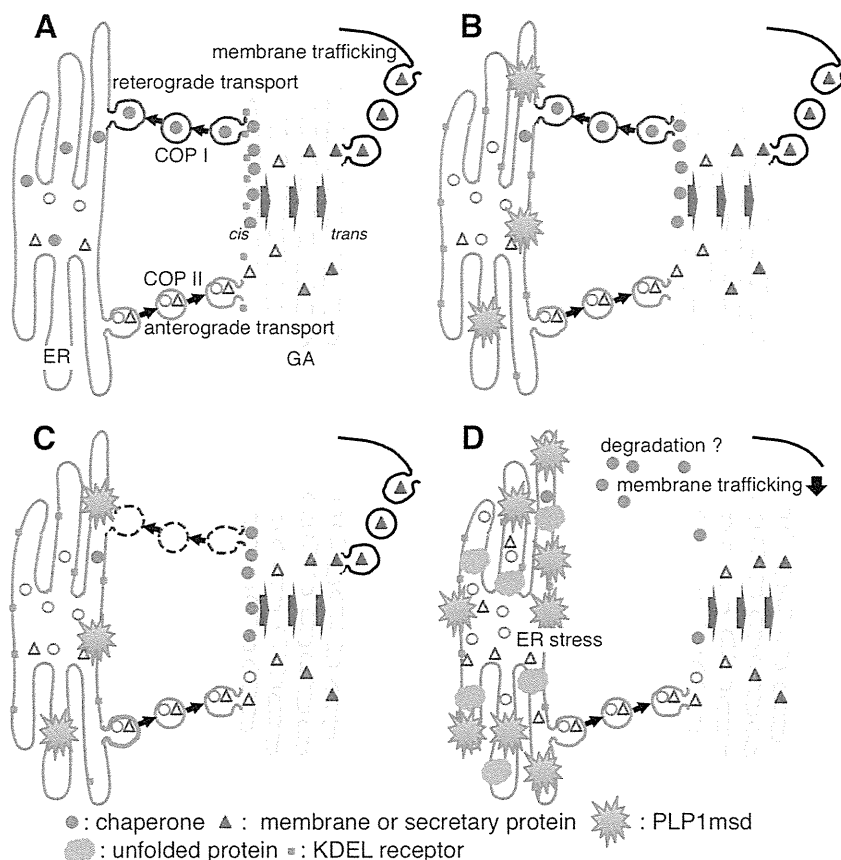## Depletion of ER Chaperones and GA Fragmentation in PMD

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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