ERdj5 Sensitizes Neuroblastoma Cells to Endoplasmic Reticulum Stress-induced Apoptosis*

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Down-regulation of the unfolded protein response (UPR) can be therapeutically valuable in cancer treatment, and endoplasmic reticulum (ER)-resident chaperone proteins may thus be targets for developing novel chemotherapeutic strategies. ERdj5 is a novel ER chaperone that regulates the ER-associated degradation of misfolded proteins through its associations with EDEM and the ER stress sensor BiP. To investigate whether ERdj5 can regulate ER stress signaling pathways, we exposed neuroblastoma cells overexpressing ERdj5 to ER stress inducers. ERdj5 promoted apoptosis in tunicamycin, thapsigargin, and bortezomib-treated cells. To provide further evidence that ERdj5 induces ER stress-regulated apoptosis, we targeted Bcl-2 and the ER stress sensor BiP. To investigate whether ERdj5 promotes a stress-induced gene expression program. During the integrated stress response (ISR), specific mRNAs escape the translational repression, and the synthesis of transcription factors, such as ATF4, activates genes (CHOP and BiP) that promote stress resistance and cell survival (4–6). ATF4 controls also the expression of GADD34, a subunit of the PP1-holophosphate complex that dephosphorylates eIF2α promoting recovery from the translational inhibition during the UPR (7). Activation of the UPR can result in restoration of ER homeostasis; however, both persistent ER stress and failure of the adaptive response initiate ER-dependent apoptotic cascades (8).

Although the UPR protects the cells from normal variations that occur in the cellular environment, pathological conditions, including cancer, can also activate the response (9–11). Tumors are known to depend on an intact UPR for growth. The ER stress chaperones GRP78/BiP and GRP94 are induced at different stages of tumor progression, and the X box-binding protein (XBP1), the major transcriptional regulator of the IRE1 arm of the UPR, is essential for tumor growth under hypoxic conditions (8, 12–14). Convinced evidence that the UPR is required for tumorigenesis comes from studies where BiP+/−, PERK−/−, and XBP1-knockdown cells formed tumors in mice that were smaller and grew significantly more slowly compared with those from wild type cells, directly implicating BiP, PERK, and XBP1 in tumor growth in vivo (13–16). Several groups are now screening for drugs that target the UPR. Both PERK and IRE1 are obvious candidates, because as kinases they are viable drug targets (17). Drugs such as bortezomib (Velcade) kill tumor cells in vitro by causing ER stress while inhibiting part of the UPR (18). Activation of the UPR alters chemosensitivity, conferring cellular resistance, or conversely works synergistically with anticancer agents, such as etoposide, doxorubicin, cisplatin, and melphalan (19).

The ER contains several proteins endowed with oxidoreductase activity that catalyze formation, isomerization, and reduction of disulfide bonds in proteins that are undergoing folding and quality control (20). Among these proteins, ERdj5 is a recently identified, ER-resident molecule that features a unique combination of domains, including a dnap, a protein-disulfide isomerase (PDI)-like, and a thioredoxin domain. Either full-
length ERdj5 or its dnaj domain alone have been shown to bind BiP in vitro in an ATP-dependent manner (21). ERdj5 has recently been reported to have reductase activity, cleave the disulfide bonds of misfolded proteins, and accelerate their ER-associated degradation through its physical and functional associations with EDEM (ER degradation-enhancing mannosi-dase-like protein) and BiP (22). As in the case of the heat shock proteins Hsp70, Hsp27, and BiP as well as in the other members of the thioredoxin system that are overexpressed in many cancers, ERdj5 exhibits elevated expression levels in tumor tissue from human hepatocellular carcinoma (HCC) patients, suggesting a role for this protein in the ER stress response in tumors (23–26). The aim of this study was to examine whether ERdj5, through regulating the UPR, could alter cancer cell resistance to ER stress. We show that ERdj5 decreases neuroblastoma cell survival by inducing ER stress-regulated apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Generation of Stable Cell Lines—Neuroblastoma SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. A stable SH-SY5Y cell line overexpressing ERdj5 was established after stably transfecting SH-SY5Y cells with the recombinant pIREShneo-ERdj5 expression vector. In addition, a double-transfected SH-SY5Y cell line was established after stably transfecting the ERdj5-overexpressing SH-SY5Y cells with the recombinant pIRESpuro-Bcl-2c6 expression vector. The ER-targeted Bcl-2 expression plasmid (pIRESpuro-Bcl-2c6) contains the full-length human Bcl-2 cDNA with a deletion at the C-terminal 658–720 base pairs (corre-sponding to amino acids 210–239) ligated with the ER-tar-geting signal sequence of human cytochrome b5. Furthermore, the full-length human ERdj5 cDNA as well as the cDNA encoding the fragment of ERdj5 that corresponds to the dnaj domain together with the ER targeting signal and the ER retention sequence KDEL were subcloned into pLenti6/V5 vector. Stable SH-SY5Y cell lines expressing the full-length ERdj5 as well as the dnaj domain of ERdj5 were established after transfecting the cells with pLenti6/V5 recombinant plasmids and selecting the transfected cells with blasticidin.

Treatments and Quantification of Apoptosis by FACS Analysis—Cells were grown in medium with or without the ER stress inducers tunicamycin and thapsigargin, the proteasome inhibitor bortezomib that induces ER stress, the translational stress inducers tunicamycin and thapsigargin, the proteasome inhibitor cycloheximide, and the genotoxic agents doxorubicin and etoposide for 24 and 48 h. Cells were collected, washed with PBS, fixed with ethanol, and incubated with propidium iodide for 24 and 48 h. Cells were collected, washed with PBS, and incubated with 1 μM biotinylated annexin V and 1 μM blue dye (Dojindo Laboratories). The cells were then washed, resuspended in PBS, and analyzed with a BD Biosciences FACSCalibur.

Measurement of Intracellular Ca2+ Levels—Cells were left untreated or were treated with 250 nM thapsigargin for 3 h. Cells were collected, washed in PBS, and incubated with 1 μM calcium green-1 (Molecular Probes) for 30 min at 37 °C. Intracellular calcium was quantified using flow cytometry.

Survival Assays—Cells were plated at a density of 7.5 × 10^3 cells/well/6-well plate. 24 h later, the culture medium was replaced with a medium containing thapsigargin. 30 h later, cells were imaged in the same dishes containing PBS using a ×10 objective in a Zeiss Axiovert S 100 microscope equipped with Zeiss AxioCam MRC camera. For the colony outgrowth assay, cells were plated at a density of 1 × 10^3 cells/well/6-well plate and treated with 150 nM thapsigargin for 24 h. Cells were switched to regular medium and after 12 days were fixed with methanol/acetic acid (3:1, v/v) prior to staining with Giemsa (Sigma).

Immunoblotting and Translation Assays—For analysis of ATF4, GADD34, CHOP, BiP, eIF2α, and phosphorylated eIF2α by immunoblotting, 7.5 × 10^3 cells were plated onto 6-well plates 24 h prior to treatment. Cells were then washed with PBS, scraped in ice-cold PBS, and lysed in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, and 1% Triton X-100) including protease inhibitors (1 mM EDTA, Roche Applied Science protease inhibitor mixture, 2 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (50 mM NaF, 1 mM Na3VO4, and 10 μM/ml phosphatase inhibitor mixture 1; Sigma). The lysates were subjected to SDS-PAGE, transferred onto nitrocellulose membrane, and probed with the following antibodies: anti-eIF2α and phosphorylated eIF2α (Cell Signaling); anti-PERK, anti-phosphorylated PERK, anti-CADD34, CHOP/GADD153, and ATF4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-BiP/GRP78 (Transduction Laboratories); and anti-actin-β (Sigma). The ERdj5 antibody used in this study was produced as described previously (23). In summary, two peptides sequences of 14 amino acids were selected from within the ERdj5 human sequence (positions 102–116 (APVKYHGD) and 640–654, FYERAKRFQEEQIN) for peptide synthesis and immunization. Serum was affinity-puri-fied against both peptides. The proteins were visualized using the ECL Western blotting detection reagents (Amer-sham Biosciences).

To measure protein synthesis, cells were plated on 6-well plates, allowed to attach for 24 h, washed with PBS, and placed in cysteine- and methionine-free medium containing 5% dia-lyzed fetal bovine serum. Cells were treated with thapsigargin for the indicated times and labeled for 15 min with 50 μCi/ml [35S]cysteine and [35S]methionine in the same medium. Cells were washed with PBS, harvested, and lysed in 1% Triton X-100-containing buffer. Proteins were separated in a 9% SDS-polyacrylamide gel followed by autoradiography to visualize radiolabeled proteins.

Immunohistochemistry—Cells were placed on 12-mm glass coverslips 24 h prior to transfection. 48 h after transfection, cells were washed with PBS, fixed in 4% paraformaldehyde/PBS, and permeabilized in PBST (0.1% Tween 20). Coverslips were blocked in PBST containing 5% goat serum for 1 h and then exposed to mouse anti-Bcl-2 antibody (Santa Cruz Biotechnology). After washing with PBST, cells were incubated with Alexa Fluor 488 anti-mouse (Molecular Probes, Inc., Eugene, OR). Slides were mounted in FluorSave (Calbiochem) and analyzed with a Leica ASMDW microscope equipped with a Leica DM IRE2 camera.
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RESULTS

ERdj5 Induces ER Stress-mediated Apoptosis—We previously demonstrated that ERdj5 exhibits ATP-dependent binding to the master regulator of the UPR BiP and that ERdj5 mRNA levels are induced upon ER stress (21). Therefore, we wanted to investigate whether ERdj5 could affect ER stress signaling pathways. For this purpose, we tested whether ERdj5 could induce apoptosis in response to ER stress in neuroblastoma cells. We stably overexpressed ERdj5 in SH-SY5Y neuroblastoma cells by using the pIRESneo vector system (Fig. 1A). These cells as well as cells carrying the empty pIRESneo vector were treated with the glycosylation inhibitor tunicamycin and the Ca\(^{2+}\)-ATPase inhibitor thapsigargin, two pharmacological activators of ER stress. ERdj5 overexpression induced apoptosis in response to ER stress, as determined by sub-G\(_1\), fraction detected by FACS analysis (Fig. 1B). Apoptosis was also induced by ERdj5 overexpression when the cells were treated with the selective and potent inhibitor of the proteasome and the clinically used agent bortezomib (Velcade) (Fig. 1C) (27). Inhibition of translation with cycloheximide reduced the tunicamycin-induced apoptotic cell death observed in ERdj5-overexpressing SH-SY5Y cells, indicating that ERdj5 may induce apoptosis by regulating protein synthesis (Fig. 1D). Under persistent ER stress, the proapoptotic proteins Bak/Bax undergo conformational alteration in the ER membrane, permitting Ca\(^{2+}\) efflux into the cytosol (28). Increased cytosolic Ca\(^{2+}\) levels activate the calcium-dependent protease m-Calpain, which subsequently induces procaspase-12-regulated apoptosis (8). The increase in the intracellular Ca\(^{2+}\) concentration observed in thapsigargin-treated ERdj5-overexpressing cells (Fig. 2) correlated with the ERdj5-induced apoptosis under ER stress (Fig. 1B). To provide further evidence that ERdj5 induces ER stress-regulated apoptosis, we targeted Bcl-2 to ER in ERdj5-overexpressing neuroblastoma cells by stably overexpressing Bcl-2 containing the ER-targeting signal sequence of cytochrome b5 (Bcl-2cb5). These cells as well as ERdj5-overexpressing cells carrying an empty pIRESpuro vector were treated with tunicamycin and thapsigargin and the non-ER genotoxic stressors doxorubicin and etoposide. In SH-SY5Y cells doubly transfected with Bcl-2cb5 and ERdj5, Bcl-2cb5 showed ER localization, as determined by a subcellular localization pattern similar to that of the DsRed fluorescent protein containing the ER targeting sequence of calreticulin and the ER retention sequence KDEL (Fig. 3, A and B). Apoptosis was induced to a similar extent in both Bcl-2cb5-overexpressing cells and the cells carrying the empty vector after treatment with the non-ER stressors doxorubicin and etoposide (Fig. 4, A and B). In contrast, expression of Bcl-2cb5 reduced the levels of apoptosis induced by the ER stress inducers tunicamycin and thapsigargin in the ERdj5-overexpressing cells (Fig. 4, A and B). Taken together, these results suggest that overexpression of ERdj5 induces ER stress-regulated apoptosis.

ERdj5 Inhibits the ER Stress-induced eIF2\(\alpha\) Phosphorylation and Translation Repression—Upon ER stress, inhibition of translation initiation is mediated by increased phosphorylation of eIF2\(\alpha\) (3). Based on our cycloheximide results, which suggested that ERdj5 can regulate protein synthesis, we hypothesized that ERdj5 promotes apoptosis by inhibiting the ER stress-induced eIF2\(\alpha\) phosphorylation and the subsequent translational repression. To test this hypothesis, ERdj5-overexpressing and control SH-SY5Y cells were continuously exposed to 250 \(\text{nm}\) thapsigargin. At various time points thereafter, the rate of protein synthesis was measured by incorporation of radiolabeled methionine and cysteine into newly synthesized proteins. eIF2\(\alpha\) phosphorylation was analyzed by immunoblotting with an monoclonal antibody specific for the phosphorylated form of the protein. In control cells, thapsigargin treatment induced a profound but transient suppression of new protein synthesis and eIF2\(\alpha\) phosphorylation that was maximal between 0.5 and 1 h. Protein synthesis was derepressed, and eIF2\(\alpha\) phosphorylation was partially decreased later in the course of the stress response (Fig. 5, A and B). At this later phase during the translational recovery, translation of the major ER stress-inducible chaperones GRP78 and GRP94 was induced. These observations are consistent with previous studies showing transient eIF2\(\alpha\) phosphorylation and translation repression in ER-stressed cells (6). In contrast, in thapsigargin-treated ERdj5-overexpressing cells, protein synthesis was slightly repressed during the first 1 h while at the same time eIF2\(\alpha\) phosphorylation increased to a lesser extent compared with the control cells (Fig. 5, A and B). Translation of the ER chaperones GRP78 and GRP94 followed a similar time-dependent pattern.

ERdj5 Inhibits the ER Stress-induced PERK Phosphorylation—PERK is the endoplasmic reticulum kinase that phosphorylates eIF2\(\alpha\) in response to various stimuli that induce ER stress, including treatment of cells with thapsigargin (42). We hypothesized that ERdj5 abolishes the ER stress-induced eIF2\(\alpha\) phosphorylation through inactivating PERK. Activation of this kinase involves autophosphorylation, which results in a shift in the apparent molecular weight on a polyacrylamide gel (3, 42). We continuously exposed ERdj5-overexpressing and control SH-SY5Y cells to 250 \(\text{nm}\) thapsigargin. At different times after the addition of thapsigargin, we analyzed PERK phosphorylation by immunoblotting. In particular, the shift in the molecular weight was monitored using an anti-PERK antibody. The levels of the phosphorylated PERK were additionally detected with an antibody specific for the phosphorylated form of the protein. As shown in Fig. 5C, under thapsigargin treatment, PERK exhibits partially slightly slower electrophoretic mobility due to phosphorylation in control cells but not in ERdj5-overexpressing cells. In agreement with the retarded electrophoretic mobility in control cells, the levels of phosphorylated PERK detected with the specific anti-phospho-PERK antibody at 0.5 and 1 h were higher in thapsigargin-treated control (pIRESpuro) compared with ERdj5-overexpressing cells (Fig. 5C). This is consistent with the increased eIF2\(\alpha\) phosphorylation levels at the same time points in the thapsigargin-treated control compared with ERdj5-overexpressing cells (Fig. 5, A and B). These results suggest that ERdj5 is highly likely to attenuate the ER stress-induced eIF2\(\alpha\) phosphorylation and translational repression through retaining PERK in a dephosphorylated, inactive form.

ERdj5 Down-regulates the Integrated Stress Response—eIF2\(\alpha\) phosphorylation at Ser-51 triggers expression of stress-induced genes, such as transcription factors ATF4 and CHOP as well as GADD34, GADD34, by regulating the dephosphorylation of
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FIGURE 1. Apoptosis induced by ERdj5 in response to ER stress. A, immunoblot of ERdj5 in SH-SY5Y cells stably transfected with the recombinant plRESneo-ERdj5 vector and SH-SY5Y cells stably transfected with the empty plRESneo vector. B, SH-SY5Y stably overexpressing ERdj5 (ERdj5) and cells transfected with the empty plRES vector (plRES) were left untreated (UT) or were treated with 2 μg/ml tunicamycin (Tm) and 250 nM thapsigargin (Tg) for 24 h. Cells were fixed, stained with propidium iodide (PI), and analyzed for apoptosis by FACS. The M1 bar in the flow cytometric histograms indicates the sub-G1 cell population that represents the apoptotic cells. The number beside each histogram shows the percentage of apoptotic cells indicated by the M1 bar. C, ERdj5 and plRES SH-SY5Y cells were left untreated or were treated with the indicated concentrations of bortezomib (BZ) for 24 h. D, ERdj5 and plRES SH-SY5Y cells were left untreated or were treated with 2 μg/ml tunicamycin in the presence or absence of 10 μM cycloheximide (CHX) for 48 h. The graphs show the percentage of apoptotic cells in each condition. Each value represents the mean ± S.E. for three independent experiments. The asterisks show a significant difference from the plRES cells at the same treatment. **, p < 0.05.
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FIGURE 2. ERdj5 increases intracellular \( \text{Ca}^{2+} \) levels in response to ER stress. ERdj5-overexpressing SH-SY5Y cells (ERdj5) and cells carrying the empty pIRES vector (\( \text{pIRES} \)) were left untreated (UT) or were treated with 250 \( \text{nM} \) thapsigargin (Tg) for 3 h. Cells were stained with calcium green-1 and analyzed by FACS analysis.

FIGURE 3. Expression of Bcl-2 that targets the ER in ERdj5-overexpressing cells. A, SH-SY5Y cells overexpressing ERdj5 were stably transfected with Bcl-2 containing the ER-targeted signal sequence of cytochrome \( \delta_6 \). Cells were transiently transfected with DsRed fluorescent protein containing the ER targeting sequence of calreticulin and the ER retention sequence KDEL. Cells were then fixed, stained with anti-Bcl-2 antibody, and imaged on a Leica ASMDW microscope. The lower panel shows amplified the part of the cell indicated by the white frame shown in the picture of the upper panel. B, immunoblot of Bcl-2 from ERdj5-overexpressing SH-SY5Y cells expressing the Bcl-2cb5.

eIF2\( \alpha \), is responsible for the recovery from the translational repression at a later stage of the stress response (6). Since overexpression of ERdj5 impaired the eIF2\( \alpha \) phosphorylation in stressed cells, we examined whether increased ERdj5 levels also impacted on the expression of downstream UPR target genes. Western blot analysis showed a block in the accumulation of the ER stress-inducible proteins ATF4, CHOP, BiP, and GADD34 in thapsigargin-treated ERdj5-overexpressing cells (Fig. 6A). Since ATF4, BIP, and GADD34 have been shown to impart stress resistance (4–6), we compared the ability of control and ERdj5-overexpressing cells to survive exposure to the ER stress inducer thapsigargin in both colony and cell growth assays (Fig. 6, B and C). Thapsigargin-treated ERdj5-overexpressing cells exhibited decreased survival compared with the cells carrying the empty vector, consistent with the data from the FACS analysis, where ERdj5 overexpression was shown to induce apoptosis in response to ER stress. These results indicate that ERdj5 may induce neuroblastoma cell death by downregulating basic ISR components.

ERdj5 Inhibits ER Stress-induced eIF2\( \alpha \) Phosphorylation and Increases Apoptosis by Utilizing Its \( \text{dnaj} \) Domain—ERdj5 has been shown to feature a \( \text{dnaj} \), a PDI-like, and a thioredoxin domain. The \( \text{dnaj} \) domain was reported to interact \( \text{in vitro} \) with BiP (21). BiP dissociates from PERK upon ER stress, resulting from activation of PERK in eIF2\( \alpha \) phosphorylation (3). The \( \text{dnaj} \) domain was hypothesized as the BiP-interacting domain responsible for the ERdj5-mediated abolishment of the ER stress-induced eIF2\( \alpha \) phosphorylation. To test this hypothesis, we generated SH-SY5Y cells stably expressing the \( \text{dnaj} \) domain of ERdj5 containing the ER targeting signal sequence and the ER retention motif KDEL by using the pLenti system (Fig. 7C). For control purposes, SH-SY5Y cells were stably transfected with the recombinant pLenti full-length ERdj5 vector (Fig. 7C). The \( \text{dnaj} \)-expressing, ERdj5-overexpressing, and control cells were treated with 200 \( \text{nM} \) thapsigargin and collected at different times after the addition of the ER stressor. Treatment of the cells transfected with the empty pLenti vector with thapsigargin increased the eIF2\( \alpha \) phosphorylation at 0.5 and 1 h, as in the case of control SH-SY5Y cells stably transfected with the empty pIRES vector (Fig. 7, A and B). In contrast, in thapsigargin-treated cells stably transfected with the pLenti full-length ERdj5, eIF2\( \alpha \) phosphorylation was slightly increased only at 0.5 h. The \( \text{dnaj} \) domain of ERdj5 inhibited the eIF2\( \alpha \) phosphorylation in thapsigargin-treated cells in a similar fashion as the full-length ERdj5 (Fig. 7, A and B). To examine whether the \( \text{dnaj} \) domain of ERdj5, by inhibiting the ER stress-induced eIF2\( \alpha \) phosphorylation, increases apoptosis, we treated \( \text{dnaj} \)-expressing SH-SY5Y cells as well as full-length ERdj5-overexpressing cells and cells transfected with the empty pLenti vector with thapsigargin for 24 h and analyzed them for apoptosis by FACS. As shown in Fig. 7D, both the expression of the \( \text{dnaj} \) domain of ERdj5 and the overexpression of full-length ERdj5 induced apoptosis in thapsigargin-treated SH-SY5Y cells to the same extent. These results indicate that the \( \text{dnaj} \) property is likely to be responsible for the ERdj5-mediated induction of apoptosis and dephosphorylation of eIF2\( \alpha \) observed under ER stress.

DISCUSSION

Cancer cells activate survival mechanisms to circumvent death imposed by different types of insults. Recent evidence shows that the tumor microenvironment represents physiologic ER stress and that the UPR is crucial for survival of tumor cells subjected to persistent hypoxia (15). Accumulation of unfolded proteins within the ER results in the induction of proteins to facilitate an increase in the rate at which such proteins can be correctly folded. Induction of BiP has been widely used as a marker for ER stress and the onset of UPR. Overexpression of BiP has been reported in many types of cancer cell lines and tumor biopsies. Recent studies showed that BiP conferred resistance against doxorubicin and etoposide-mediated apoptosis in cancer cells, at least in part, through inhibition of Bax and caspase-7 activation (12, 29).

Interestingly, although the molecular chaperone calnexin and the multifunctional protein calreticulin are induced in can-
cer cell lines, their overexpression in cancer cells has been shown to enhance apoptosis in response to ER stress (30–33). Like other ER-resident proteins, thiol-oxidoreductase chaperones of the PDI family are up-regulated in cancer cells as a part of the response to alleviate stresses induced by ER stressors and chemotherapeutic drugs. For instance, PDI expression increases in response to hypoxia in glial cells and is strongly expressed on invasive glioma cells in both xenografts and at the invasive front of human glioblastomas (34, 35).

ERp57 is another ER-resident chaperone with similarity to the PDI polypeptide backbone characterized by the presence of two thioredoxin domains. Down-regulation of ERp57 has been reported to increase neuroblastoma cell death induced by fenretinide or velcade (36). Down-regulation of ER stress responses seems to be therapeutically valuable, and ER-resident proteins may thus be targets for developing novel chemotherapeutic strategies (37).

ERdj5 is a novel, recently identified ER-resident molecule. It features dnaj, PDI-like, and thioredoxin domains. This unique combination of domains suggests that ERdj5 can function as an assistant in protein folding and quality control in endoplasmic reticulum. This is in agreement with the observation that ERdj5 overexpression in SH-SY5Y cells stably transfected with the empty pIRESpuro vector (ERdj5 puro) as well as ERdj5-overexpressing cells carrying the empty pIRESpuro vector (ERdj5 puro) were left untreated (UT) or were treated with 2 µg/ml tunicamycin (Tm), 250 nM thapsigargin (Tg), 50 µM etoposide (ET), and 0.5 µM doxorubicin (Dox) for 24 h. Cells were stained with propidium iodide and analyzed for apoptosis by FACS. The M1 bar in the flow cytometric histograms indicates the sub-G1 cell population representing the apoptotic cells. The number in each histogram shows the percentage of apoptotic cells indicated by the M1 bar. B, the graph shows the percentage of apoptotic cells. Values represent the mean ± S.E. for three independent experiments. The asterisks show a significant difference from the control ERdj5-overexpressing cells carrying the empty pIRESpuro vector (ERdj5 puro) with the same treatment. **, p < 0.05.

FIGURE 4. ER-targeted Bcl-2 inhibits the apoptosis induced by ERdj5 under ER stress. A, ERdj5-overexpressing SH-SY5Y cells stably transfected with Bcl-2cb5 (ERdj5 Bcl-2cb5) as well as ERdj5-overexpressing cells carrying the empty pIRESpuro vector (ERdj5 puro) were left untreated (UT) or were treated with 2 µg/ml tunicamycin (Tm), 250 nM thapsigargin (Tg), 50 µM etoposide (ET), and 0.5 µM doxorubicin (Dox) for 24 h. Cells were stained with propidium iodide and analyzed for apoptosis by FACS. The M1 bar in the flow cytometric histograms indicates the sub-G1 cell population representing the apoptotic cells. The number in each histogram shows the percentage of apoptotic cells indicated by the M1 bar. B, the graph shows the percentage of apoptotic cells. Values represent the mean ± S.E. for three independent experiments. The asterisks show a significant difference from the control ERdj5-overexpressing cells carrying the empty pIRESpuro vector (ERdj5 puro) with the same treatment. **, p < 0.05.

FIGURE 5. ERdj5 abolishes the ER stress-induced eIF2α phosphorylation and translation repression. A, upper panel, autoradiogram of an SDS-polyacrylamide gel showing [35S]methionine/cysteine incorporation into newly synthesized proteins in untreated (UT) and thapsigargin (Tg)-treated pIRESpuro (stably transfected with the empty vector) and ERdj5-overexpressing cells. The arrowheads to the right of the autoradiogram indicate the ER stress-inducible chaperones GRP94 and GRP78. Lower panels, immunoblots of phosphorylated eIF2α (eIF2α-p) and total eIF2α from different cells under the same treatments. B, graphic presentation of [35S]methionine/cysteine incorporation into newly synthesized proteins (upper graph) and -fold induction of phosphorylated eIF2α (lower graph) in untreated and thapsigargin-treated pIRESpuro and ERdj5-overexpressing cells. The level of [35S] is set at 100%, whereas the signal of phosphorylated eIF2α from untreated pIRESpuro cells is set as 1. Shown are mean ± S.E. of experiments performed in triplicate. C, immunoblots of phosphorylated PERK (PERK-p) and total PERK from untreated and thapsigargin-treated pIRESpuro (cells carrying the empty vector) and ERdj5-overexpressing cells.
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How does ERdj5 induce apoptosis in response to ER stress? The UPR relieves the ER stress by inducing protein folding and degradation pathways and inhibiting protein synthesis (1). However, when the ER stress is prolonged or the adaptive response fails, apoptotic cell death ensues (8). We showed that ERdj5 interferes with the phosphorylation of eIF2α, suggesting that the translational branch of the UPR is affected. If ERdj5 promotes apoptosis by abolishing translational repression, treatment with the translational inhibitor cycloheximide was expected to inhibit the apoptosis observed in ERdj5-overexpressing cells under ER stress. Indeed, this apoptosis was inhibited by cycloheximide, suggesting that ERdj5 can regulate the mechanisms that control protein synthesis. The endoplasmic reticulum kinase PERK is activated by ER stress through autophosphorylation. Active PERK phosphorylates eIF2α, attenuating protein synthesis (4). Our cell culture experiments showed that the apoptosis observed in ERdj5-overexpressing cells under ER stress correlated with attenuation of both the translational repression and eIF2α and PERK phosphorylation, strengthening our initial notion that the increased levels of ERdj5 under ER stress may down-regulate the translational arm of the UPR. Inhibition of ER stress-induced eIF2α phosphorylation followed by increased apoptosis was observed when the dnaj domain of ERdj5 was expressed in SH-SYSY cells. This suggests that ERdj5 is likely, by utilizing its dnaj domain and regulating BiP-PERK interaction under ER stress, to modulate PERK and eIF2α phosphorylation. eIF2α phosphorylation is known to activate the cytoprotective ISR. Accumulation of the ATF4 transcription factor and its ISR target genes (GADD34, CHOP, and BiP) is strictly dependent on stress-induced eIF2α phosphorylation (3, 4, 5, 38, 39). Since ERdj5 inhibited the stress-induced eIF2α phosphorylation, we consider the possibility that ERdj5, by inhibiting the activation of the ISR pathway, could have an impact on the survival of ER-stressed cells. Our results show that the ER-stressed ERdj5-overexpressing cells having a compromised ISR, as reflected in the decreased expression of the ISR target genes, ATF4, GADD34, CHOP, and BiP, exhibit decreased survival. This is consistent with the ERdj5-mediated induction of apoptosis observed in ER-stressed cells.

Our results support the hypothesis that ERdj5 down-regulates the translational branch of the UPR and sensitizes neuroblastoma cells to ER stress-regulated apoptosis. This does not exclude the possibility of ERdj5 inducing neuroblastoma cell death through affecting an additional UPR pathway, given its role in assisting the retrotranslocation and ER-associated degradation of misfolded proteins (22). In addition, ERdj5 is likely to induce apoptosis through a UPR-independent pathway. BiP is known to confer growth advantage to tumor cells by enhancing tumor cell proliferation, protection against apoptosis, and induction of tumor angiogenesis. In addition to the BiP role in controlling UPR signaling, it may facilitate, as a major ER chaperone, the processing and trafficking of critical growth factors and their receptors (40). BiP has also been detected on the surface of tumor cells to mediate the Akt signal transduction pathway that induces tumor cell proliferation. In addition, as an interactive partner of caspase-7, BiP can block its activation under ER stress (41). Therefore, the ERdj5-mediated induction
of apoptosis observed in stressed cells might be explained by activation of caspase-7 through ERdj5-BiP interaction (21).

One interesting question not addressed by this study is how ERdj5 modulates the phosphorylation of PERK in stressed cells. Activation of PERK and eIF2α phosphorylation is negatively regulated by BiP. Upon ER stress, BiP dissociates from PERK, leading to its activation (3, 42, 43). ERdj5 is likely to regulate PERK activity through direct interaction with PERK. As an alternative hypothesis, ERdj5 can modulate the BiP-PERK complex and consequently PERK activity through interaction with BiP. Further validation of these models requires careful examination of the interactions between BiP and PERK in control and ERdj5-overexpressing untreated and ER-stressed cells.

Based on the findings presented here, we propose a particular role for this novel with unique properties chaperone molecule, ERdj5, in regulating the UPR and the UPR-related apoptosis. In contrast to the cell growth advantage that confers up-regulation of the ER-resident chaperones and protein members of the PDI family, overexpression of ERdj5 induces ER stress-regulated apoptosis and decreases neuroblastoma cell survival. ER stress responses represent homeostatic mechanisms allowing cells to survive the consequences of perturbations in protein folding in the endoplasmic reticulum; however, it is not clear how these mechanisms interact with signaling pathways controlling apoptosis. The point at which homeostatic mechanisms cannot cope and apoptosis is induced is critical to understanding the relationships between ER stress and apoptosis and how ER stress can be used to increase chemotherapeutic drug targeting to tumor cells. This study by elucidating the role of a novel ER-resident chaperone in UPR is likely to shed new light on the control of the mechanisms that regulate ER stress-mediated apoptosis. Our results provide evidence that ERdj5 can regulate UPR pathways that control apoptosis. As such, it might represent a novel molecule to enhance the

FIGURE 7. ERdj5 inhibits the ER stress-induced eIF2α phosphorylation and induces apoptosis through utilizing its dnaj domain. A, immunoblots of phosphorylated eIF2α (eIF2α-p) and total eIF2α from untreated (UT) and thapsigargin (Tg)-treated SH-SYSY cells carrying the empty vector (pLenti), ERdj5-overexpressing cells (ERdj5), and cells expressing the dnaj domain of ERdj5 (dnaj). B, graphic presentation of fold induction of phosphorylated eIF2α in untreated and thapsigargin-treated pLenti, ERdj5-overexpressing, and dnaj-expressing cells. The signal of phosphorylated eIF2α from untreated pLenti cells is set as 1. Shown are the means of experiments performed in triplicate. C, immunoblot of full-length ERdj5 and dnaj domain of ERdj5 in lysates of SH-SYSY cells stably transfected with the empty pLenti vector, the full-length ERdj5 (ERdj5), and the dnaj domain of ERdj5 (dnaj). D, SH-SYSY stably transfected with the full-length ERdj5 (ERdj5), the dnaj domain of ERdj5 (dnaj), and the empty pLenti vector (pLenti) were left untreated (UT) or treated with 200 nM thapsigargin (Tg) for 24 h. Cells after staining with propidium iodide were analyzed for apoptosis by FACS.
anti-tumor effectiveness and selectivity of drugs for cancer cells.

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