The KDEL Receptor Modulates the Endoplasmic Reticulum Stress Response through Mitogen-activated Protein Kinase Signaling Cascades*

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† The abbreviations used are: ER, endoplasmic reticulum; BiP, immunoglobulin-binding protein; UPR, unfolded protein response; ATF, activating transcription factor; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; mAb, monoclonal antibody; JNK, c-Jun amino-terminal kinase; ERK, extracellular signal-regulated kinase; CREB, cAMP-response element binding protein; DTT, dithiothreitol; GTPase-activating protein; ARF, ADP ribosylation factor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling assay; TRAF2, tumor necrosis factor receptor-associated factor 2.

The accumulation of misfolded proteins in the endoplasmic reticulum (ER) evokes the ER stress response. The resultant outcomes are cytoprotective but also pro-apoptotic. ER chaperones and misfolded proteins exit to the secretory pathway and are retrieved to the ER, during which process the KDEL receptor plays a significant role. Using an expression of a mutant KDEL receptor that lacks the ability for ligand recognition, we show that the impairment of retrieval by the KDEL receptor led to a mis-sorting of the immunoglobulin-binding protein BiP, an ER chaperone that has a retrieval signal from the early secretory pathway, which induced intense ER stress response and an increase in susceptibility to ER stress in HeLa cells. Furthermore, we show that the ER stress response accompanied the activation of p38 mitogen-activated protein (MAP) kinases and c-Jun amino-terminal kinases (JNKs) and that the expression of the mutant KDEL receptor suppressed the activation of p38 and JNK1 but not JNK2. The effect of the expression of the mutant KDEL receptor was consistent with the effect of a specific inhibitor for p38 MAP kinases, because the inhibitor sensitized HeLa cells to ER stress. We also found that activation of the KDEL receptor by the ligand induced the phosphorylation of p38 MAP kinases. These results indicate that the KDEL receptor participates in the ER stress response not only by its retrieval ability but also by modulating MAP kinase signaling, which may affect the outcomes of the mammalian ER stress response.

Proteins destined for the secretory pathway are inserted into the endoplasmic reticulum (ER) cotranslationally and subjected to quality control. ER molecular chaperones such as the immunoglobulin-binding protein (BiP) and calnexin facilitate the correct folding or degradation of these newly synthesized proteins as well as that of misfolded proteins (1). An increase in misfolded proteins in the ER caused by deleterious effects upon the folding environments in the ER or by genetic mutations evokes the ER stress response, which includes an induction of the synthesis of ER chaperones called the unfolded protein response (UPR) (2), suppression of general protein synthesis (3, 4), and enhancement of the ER-associated degradation of misfolded proteins (5–7).

Despite these responses, the persistent accumulation of misfolded proteins leads to protein aggregation in the cell as well as in the extracellular tissue, frequently accompanied by cellular dysfunction and cell death. Now a growing amount of evidence indicates that the ER stress response is involved in the pathogenesis of several diverse disorders called conformational diseases (8). The mammalian ER stress response is a complicated process that is coordinately induced by ER transmembrane kinases such as ATF6, IRE1, and PERK (9–11). Further complexity comes from the fact that the activation of these kinases not only enhances the expression of ER chaperones for cytoprotection but also causes apoptosis (12, 13). The underlying molecular mechanism of the transition between these two opposite outcomes during the ER stress response is uncertain.

Yeast genetic analyses have revealed that the UPR affects a wide variety of gene transcriptions regulating not only ER chaperones and protein degradation but also the early secretory pathway (14, 15), suggesting that the UPR may involve the whole secretory pathway rather than just that portion within the ER. In fact, some misfolded proteins in mammalian cells (16–19) as well as in yeast (20–22) have been shown to be transported from the ER but were then retrieved to the ER. The yeast UPR is initiated by the activation of IRE1, which induces the synthesis of ER chaperones, although it does not seem to be accompanied by apoptosis (2). ER luminal chaperones like BiP/Kar2 are localized in the ER partly because of the interaction with the ER matrix and partly because of the retrieval from the secretory pathway to the ER (1). These luminal chaperones have a carboxyl-terminal His-Asp-Glu-Leu (HDEL) amino acid sequence that is recognized by the transmembrane receptor, ERD2, in post-ER compartments when the chaperones are secreted from the ER (23). Interestingly, yeast cells without the IRE1 gene are viable, and yeast cells bearing mutant alleles of the ERD2 gene in which ERD2-mediated retrieval is impaired are also viable; however, both mutations synthetically cause growth arrest. Thus, the secretion of ER chaperones and misfolded proteins from the early secretory
pathway per se does not seem to be critical in yeast if the synthesis of chaperones is compensated for by the function of IRE1 (24).

Mammalian ER luminal chaperones like BiP have a carboxy-terminal Lys-Asp-Glu-Leu (KDEL) amino acid sequence instead of HDEL (25), which is recognized by the KDEL receptor, the mammalian homologue of ERD2, in post-ER compartments including the Golgi intermediate compartment and the Golgi complex when the chaperones are secreted from the ER (26). ER chaperones and the KDEL receptor are sorted into the transport vesicle coated with COP I complex and retrieved to the ER (27).

In this study, we show that the KDEL receptor enhances mitogen-activated protein kinase (MAPK) signaling during the ER stress response, which affects the outcomes of the response. This finding reveals a novel function of the KDEL receptor; it modulates the ER stress response with its signaling function in addition to its retrieval function. Furthermore, it suggests diverse biological scenarios where the ER stress response may be concerned in mammals, considering the pivotal roles of MAP kinase signaling such as development, cell differentiation, cell survival, and cell death (28).

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—HeLa cells were grown in a complete medium that consisted of Dulbecco's modified essential medium (Sigma) with 10% fetal calf serum, 2 mM glutamine, 50 μg/ml streptomycin, and 50 unit/ml penicillin G at 37°C in a 5% CO₂ incubator. The following antibodies were used: a mouse mAb (9E10) against the Myc epitope (American Type Culture Collection); a mouse mAb against Golgi p58; a mouse mAb against γ-tubulin (Sigma); a mouse mAb (KR-10) against the KDEL receptor; a mouse mAb (SPA-827) against BiP (KDEL sequence) (StressGen Biotechnologies); a mouse mAb (AF8) against calnexin (kindly provided by M. Brenner, Boston, MA) (29); a rabbit polyclonal antiserum against phospho-JNK (Cell Signaling); a goat polyclonal antiserum against phospho-p38 (Cell Signaling); a goat polyclonal antiserum against phospho-CREB; a rabbit polyclonal antiserum against phospho-p38; a rabbit polyclonal antiserum against phospho-ERK1/2; a rabbit polyclonal antiserum against phospho-JNK; a rabbit polyclonal antiserum against phospho-MKK3/6 (Cell Signaling); a Cy-2-conjugated donkey antibody against mouse IgG; and a Cy-3-conjugated donkey antibody against goat IgG (Jackson ImmunoResearch Laboratories).

**Northern Blot Analysis**—The total RNA from the cells was isolated with TRIzol reagent (Invitrogen). Twenty micrograms of each RNA were separated on 1% formaldehyde denaturing agarose gels, transferred to a nylon membrane by capillary action, and cross-linked with UV light using a cross-linker (Amersham Biosciences). Hybridization was performed for 24 h at 65°C in a hybridization buffer containing 20× SSC Denhardt's solution and herring sperm DNA with a 32P randomly labeled cDNA fragment. A rat cDNA encoding BiP was used for a probe. The hybridized membrane was washed at 65°C with 0.1× SSC containing 0.1% SDS. The radioactive signals were analyzed by BAS 2500 and Image Gauge software (Fuji Photo Film Co., Ltd.). The blots were stripped and re-probed with a cDNA encoding murine β-actin to ensure the equivalent loading of RNA samples, and the expression level of BiP mRNA was normalized by the signal intensity of β-actin mRNA.

**RESULTS**

**Ligand Recognition by the KDEL Receptor Is Saturable**—Transient transfection of the KDEL receptor results in various expression levels in HeLa cells. Overexpression of the KDEL receptor has been shown to enhance transport from the Golgi complex to the ER, which results in the redistribution of the whole Golgi complex to the ER (30); however, moderate expression of the KDEL receptor does not disturb the Golgi structure, and only those cells with moderate expression become stable transfectants (33). To examine the role of the KDEL receptor in the ER stress response, we made HeLa cells that stably expressed either the wild-type or a mutant human KDEL receptor. This binding defective mutant, which had a single amino acid replacement (R169N), was localized to the Golgi complex as was the wild-type, but did not respond to the KDEL sequence effectively as described previously (32). The transient expression of the lysozyme-KDEL in these stable cells induced the transport of the wild-type KDEL receptor, but not that of the mutant, to the ER (Fig. 1A). The lysozyme-KDEL was localized in the ER even in the mutant KDEL receptor cells, which indicates that minimal retrieval was maintained in these cells by the endogenous KDEL receptor. In fact, metabolic labeling experiments revealed that stably transected
KDEL receptors expressed at levels less than endogenous KDEL receptors in these cells (Fig. 1B).

The expression of ER chaperones is induced extensively upon ER stress, which may saturate KDEL receptor-mediated retrieval because the expression of the KDEL receptor is not inducible in mammals (34). We examined the effect of ER stress on the distribution of BiP, an endogenous ligand of the KDEL receptor, in the secretory pathway by using sucrose gradient analysis in cells stably expressing the wild-type (Fig. 2A) or the mutant (bind.def) KDEL receptor (Fig. 2B). Although most endogenous BiP was found in the ER in the resting state, a significant amount was detected in the post-ER fractions of these cells when the cells were treated with tunicamycin, which prevented protein glycosylation in the ER and induced the UPR. Under these circumstances, we detected more BiP being secreted to the medium in the mutant KDEL receptor cells than in the HeLa cells and the wild-type KDEL receptor cells despite an equivalent expression level of BiP within these cells (Fig. 2C). These results suggested that ER chaperones associating with

**Fig. 1.** Ligand recognition induced the redistribution of the wild-type KDEL receptor to the ER. A, the subcellular localization of the wild-type or the mutant KDEL receptor (bind.def) in the stable cells transiently transfected with the lysozyme-KDEL was evaluated by confocal laser scan microscopy with double labeling using a rabbit anti-lysozyme antiserum (red) and a mouse anti-Myc mAb (green). The scale bar represents 10 μm. B, cells were metabolically labeled with [35S]methionine, lysed, and immunoprecipitated with an anti-native KDEL receptor mAb or an anti-Myc mAb. The immunoprecipitates were separated by SDS-PAGE.

**Fig. 2.** A fraction of the BiP is secreted from the ER under stressed conditions. A and B, sucrose gradient analysis (20–50%; top, fraction 1; bottom, fraction 12) of endogenous BiP in cells stably expressing the wild-type KDEL receptor-Myc (KDELR) (A) or the mutant (bind.def) KDEL receptor-Myc (B) with or without treatment with tunicamycin 2.5 μg ml⁻¹ for 24 h. An aliquot of each fraction was analyzed by SDS-PAGE. The distribution of GRP94, BiP, Myc-tagged KDEL receptors, Golgi p58, and calnexin was determined by Western blotting. C, HeLa cells and the cells stably expressing the wild-type or the mutant (bind.def) KDEL receptor-Myc with or without treatment with tunicamycin 2.5 μg ml⁻¹ for 24 h were collected. The secreted BiP in the medium was immunoprecipitated with an anti-BiP antiserum and analyzed by SDS-PAGE, followed by Western blotting. The expressions of BiP, γ-tubulin, and Myc-tagged KDEL receptors in the cell lysates were also determined by Western blotting.
misfolded proteins might be secreted from the ER and retrieved by the KDEL receptor. When retrieval is limited, they may escape from the early secretory pathway, especially under stressed conditions.

The Loss of BiP from the Early Secretory Pathway Causes Persistent UPR—The accumulation of misfolded proteins in the ER leads to the recruitment of BiP from ER transmembrane kinases such as ATF6 and IRE1, which results in the activation of these kinases and the synthesis of ER chaperones, including BiP (35). The loss of BiP in the early secretory pathway may enhance this process. We examined the induction of BiP gene transcription with Northern blot analysis. Although the BiP mRNA in all cells increased upon tunicamycin treatment, it was prominent and persistent in the mutant KDEL receptor cells (Fig. 3A). ATF6 is localized in the ER in the resting state, and its translocation to the Golgi upon ER stress has been shown to activate ATF6, resulting in the initiation of the UPR (36). We examined the subcellular localization of endogenous ATF6 by immunofluorescence microscopy using a goat anti-ATF6 antiserum. C, HeLa cells and the cells stably expressing the mutant KDEL receptor (bind.def.) were treated with tunicamycin (5 μg/ml) for 0–24 h. The cell lysates were analyzed by SDS-PAGE, and the expressions of GRP94, BiP, IRE1α, and γ-tubulin were examined by Western blotting.

Sensitization to ER Stress—We assessed whether retrieval by the KDEL receptor might contribute to cell survival in the UPR, because intense UPR due to an overexpression of ATF6 or IRE1 causes cell death (12, 13). HeLa cells and the wild-type or the mutant KDEL receptor cells were treated with tunicamycin and DTT. Significantly, the mutant KDEL receptor cells were sensitive to ER stress, whereas cells expressing the wild-type tolerated the stress (Fig. 4A). By TUNEL assay, we detected more apoptotic features in the mutant KDEL receptor cells undergoing tunicamycin treatment (Fig. 4B). Hoechst 33258 staining revealed prominent, condensed chromatins and the fragmentation of nuclei in the mutant tunicamycin-treated KDEL receptor cells (Fig. 4C). These features were consistent with programmed cell death by ER stress (37). Although BiP expressed itself at an equivalent level in these different cells (Fig. 2C), the impairment of the retrieval led to intense UPR that sensitized the mutant KDEL receptor cells to ER stress, suggesting that the retrieval of BiP and misfolded proteins from post-ER compartments by the KDEL receptor plays a significant role in the ER stress response.

The KDEL Receptor Enhances MAP Kinase Signaling—UPR signaling has been shown to be transduced through IRE1 to cytosolic factors such as TRAF2, caspase-12, and JNKs (38, 39). The transcriptional factor CHOP is also induced through IRE1, PERK, and ATF6 activation (9, 12, 13). These signaling cascades have been shown to contribute to cell death during the ER stress response (37, 39–41). We examined these death-related pathways in the HeLa cells after treating them with tunicamycin. We observed an enhanced expression of CHOP in the mutant KDEL receptor stable cells in comparison to the wild-type stable cells (Fig. 5, top panel). As for JNK activation,
we found an increased phosphorylation of JNKs in the wild-type stable cells; however, in the mutant KDEL receptor cells, although JNK2 phosphorylation was enhanced in the resting state that accompanied the phosphorylation of c-Jun, the phosphorylation of JNK1 was suppressed (Fig. 5, bottom panel). This observation prompted us to examine other signaling molecules of MAPK cascades. Interestingly, we found that p38 MAP kinases were phosphorylated in the wild-type stable cells upon tunicamycin treatment; this accompanied the phosphorylation of ATF2, CREB (downstream effectors of p38), and MKK3/6 (kinases for p38), especially at 3–6 h after stimulation, but these phosphorylations were suppressed in the mutant stable cells despite an equivalent expression of BiP in the wild-type stable cells.

Then, we assessed whether these differences in MAPK cascades activity might affect cell survival and death during the ER stress response. We examined the effects of p38 activation upon tunicamycin-induced cell death in HeLa cells by using a specific inhibitor for p38 (SB202190). Although SB202190 treatment alone did not affect cell viability, it significantly promoted tunicamycin-induced cell death in the HeLa cells (Fig. 6, A and B). As a control, treatment with a JNK inhibitor (SP600125) and an ERK inhibitor (PD98059) had no significant effects (Fig. 6A). Next, we wanted to know whether the activation of the KDEL receptor might induce the activation of p38 MAP kinases. We have shown previously that an artificial KDEL-tagged protein as well as endogenous KDEL proteins secreted from the ER were recognized by the KDEL receptor in post-ER compartments and retrieved to the ER. This process accompanied the activation of the KDEL receptor and its downstream signaling events such as the activation of a GTPase-activating protein (GAP) for the ADP-ribosylation factor 1 (ARF1) (17, 31). HeLa cells stably expressing the wild-type KDEL receptor and inducibly expressing the KDEL-tagged lysozyme by glucocorticoid responsive promoter (17) were treated with dexamethasone or tunicamycin. We found that the expression of the lysozyme-KDEL induced the accumulation of phosphorylated p38, although it did not induce the expression of BiP (Fig. 6C).

These results suggest that activation of the KDEL receptor by endogenous ligands (such as BiP), possibly complexed to misfolded proteins (Fig. 5) and an artificial ligand (Fig. 6C), enhances the phosphorylation of p38 MAP kinases and that p38 activation contributes to the resistance of HeLa cells to ER stress. MAPKs have diverse functions in mammals, and those functions are spatially and temporally regulated (28). In fact, the activation of p38 MAP kinases has been shown to induce both cell survival (42) and cell death (43). Thus, the effects of the KDEL receptor on MAPK signaling may cause different outcomes in other cell types. Taken together, these results indicate that the KDEL receptor modulates MAPK signaling during the ER stress response, which affects the outcomes of the response.

**DISCUSSION**

In this study, we have shown that an overload of misfolded proteins and ER chaperones in the lumen of the ER upon ER stress induced their secretion out of the ER in mammalian cells. These proteins did not escape from the early secretory pathway entirely, because the KDEL receptor retrieved them from post-ER compartments and returned them to the ER. When the retrieval was limited during the ER stress response, which is demonstrated by the expression of a mutant KDEL
ceptor led to the suppression of MAPK signaling cascades. HeLa cells stably expressing the wild-type or the mutant KDEL receptor (bind.def.) were treated with tunicamycin (Tm, 5 μg ml⁻¹) for 0–24 h. The cell lysates were analyzed by SDS-PAGE, and the expressions of GRP94, BiP, Myc-tagged KDEL receptors, γ-tubulin, and CHOP (top panel) and phospho-JNKs (p-JNK2 and p-JNK1), JNKs, phospho-p38 (p-p38), p38, phospho-ERKs (p-ERK1 and p-ERK2), phospho-cJun (p-cJun), phospho-ATF2 (p-ATF2), phospho-CREB (p-CREB), and phospho-MKK3/6 (p-MKK3/6) (bottom panel) were examined by Western blotting. The activation of p38 and ATF2 was accessed by the relative density of phospho-p38 to p-p38 and phospho-ATF2 to γ-tubulin. The line graphs represent the mean values and S.E. of the three experiments.

Although yeast ERD2 is an essential protein for cell viability and preserving the Golgi morphology and function (23), its retrieval function has been revealed to be dispensable (44), suggesting that the main function of ERD2 is not retrieval but rather maintaining Golgi membrane trafficking. Consistent with the yeast studies, we have found in mammals that the KDEL receptor mediates Golgi membrane trafficking through the regulation of ARF GAP1 (33). Nevertheless, the KDEL receptor mediating Golgi membrane trafficking is linked to the retrieval function, because stimulation by KDEL proteins, including artificial KDEL-tagged proteins (31, 45) as well as endogenous ER chaperones associating with misfolded proteins (17), can activate the KDEL receptor and ARF GAP1 in post-ER compartments when they exit from the ER. As a result, ARF GAP1 is recruited from the cytosol to the membranes...
to promote the hydrolysis of GTP on ARF1 to GDP (46, 47), which is required for the proper sorting of cargo proteins into COPI vesicles (48, 49). In fact, some misfolded proteins in mammalian cells (16–18) as well as in yeast (20–22) have been shown to leak out from the ER but then be retrieved to the ER. Thus, ER chaperones, accumulated in post-ER compartments and complexed to misfolded proteins, could be input signals that the KDEL receptor senses, and enhancing the COPI transport could be one of the outputs.

The KDEL receptor is a multi-membranous intracellular receptor for ER chaperones with the KDEL sequence. The expectation that activation of the KDEL receptor by ligands may lead to multiple signal transduction events other than its interaction with ARF-GAP1 is quite natural, considering the well characterized signal transduction pathways of cell surface receptors (50). Although precise mechanisms remain uncertain, our study indicates that those signaling events have connections to MAPK signaling cascades. The cross talk between the ER stress response and MAPK cascades is well established in that IRE1 transduces the ER stress signals to ASK1 and JNK, which participates in cell death in neurogenic cells (38, 41). The KDEL receptor may modify such signaling or, alternatively, it may have other independent connections to MAPK cascades. What we found was that the mutant KDEL receptor suppressed the phosphorylation of p38 MAP kinases and JNK1 but not JNK2. The selective suppression of p38 MAP kinases and JNK1, but not JNK2, has been reported in activated macrophages treated with anthrax lethal factor; this treatment results in the apoptosis of those macrophages (42). The activation of the KDEL receptor may stimulate the MKKs for p38 as shown in Fig. 5, or it may also suppress the phosphatase function for p38 (51), because we observed an accumulation of phospho-p38 upon activation of the KDEL receptor by the ligand in Fig. 6C. These possibilities will be examined further.

ER stress causes misfolded proteins and induces an expansion of ER chaperones. Among the chaperones, BiP has been acknowledged to be a sensor molecule in the UPR that initiates both survival and apoptotic responses. The mammalian UPR is acknowledged to be a sensor molecule in the UPR that initiates both survival and apoptotic responses. The mammalian UPR has been suggested to be a self-regulatory process. At rest, BiP associates with IRE1 and PERK kinases and suppresses their activity again (35). On the other hand, if the stress continues or is beyond the capacity of the ER quality control, the overload of misfolded proteins seems to initiate an apoptotic process through the activation of the same kinases. The activation of IRE1 induces the expression of BiP as well as that of CHOP/GADD153, a transcription factor that causes Bel-2 down-regulation and cell death (12, 52). IRE1 also transduces signals to a cytosolic factor, TRAP2, that activates the JNK pathway (38) as well as the caspase-12 dependent apoptotic pathway (39). The loss of BiP from the early secretory pathway may enhance these processes, and, thus, the retrieval function of the KDEL receptor is important. Furthermore, our present study indicates that the signaling function of the KDEL receptor mediating MAPK cascades is also one of the critical factors that determine the fates of the cells during the ER stress response.

MAPKs have been acknowledged to determine the fates of cells in diverse biological situations, including cell differentiation and development in addition to cell survival and cell death (28). One interesting assumption is that the way the KDEL receptor modulates MAPK activity shown in this study may also be involved in other diverse situations, which implies that the ER stress response may participate in many biological processes, physiological as well as pathological.

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