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Luman/CREB3 Induces Transcription of the Endoplasmic Reticulum (ER) Stress Response Protein Herp through an ER Stress Response Element▽

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Luman/CREB3 (also called LZIP) is an endoplasmic reticulum (ER) membrane-bound transcription factor which is believed to undergo regulated intramembrane proteolysis in response to cellular cues. We previously found that Luman activates transcription from the unfolded protein response element. Here we report the identification of Herp, a gene involved in ER stress-associated protein degradation (ERAD), as a direct target of Luman. We found that Luman was transcriptionally induced and proteolytically activated by the ER stress inducer thaspsigargin. Overexpression of Luman activated transcription of cellular Herp via ER stress response element II (ERSE-II; ATTGG-N-CCACG) in the promoter region. Mutagenesis studies and chromatin immunoprecipitation assays showed that Luman physically associates with the Herp promoter, specifically the second half-site (CCACG) of ERSE-II. Luman was also necessary for the full activation of Herp during the ER stress response, since Luman small interfering RNA knockdown or functional repression by a dominant negative mutant attenuated Herp gene expression. Like Herp, overexpression of Luman protected cells against ER stress-induced apoptosis. With Luman structurally similar to ATF6 but resembling XBP1 in DNA-binding specificities, we propose that Luman is a novel factor that plays a role in ERAD and a converging point for various signaling pathways channeling through the ER.

Unfolded and misfolded proteins accumulate under endoplasmic reticulum (ER) stress and constitute a fundamental threat to all living cells. The cellular response to such stress is necessary to restore homeostasis in the ER. During the ER stress response or unfolded protein response (UPR), ER-resident molecular chaperones and foldases are induced to augment the folding capacity of the ER, and translation is attenuated to reduce the biosynthetic load of the ER (for reviews, see references 31, 39, and 44). Another mechanism for organisms to reduce the unfolded protein burden in the ER is to retrotranslocate proteins to the cytoplasm, where they are ubiquitinated and degraded by the proteasome; this mechanism is currently termed ER-associated degradation (ERAD) (20, 35). When these mechanisms are not able to remedy the stress situation, apoptosis is initiated in eukaryotic organisms (9, 29, 37, 38).

Current studies of the UPR mechanism in mammalian cells have identified three branches of the signaling pathway, represented by three types of ER transmembrane proteins: pancreatic eukaryotic initiation factor subunit 2α (eIF2α) kinase (PERK) (10) (also called PKR-like ER kinase) (52), activating transcription factor 6 (ATF6), and inositol requiring 1 (IRE1). The activation of PERK by ER stress leads to phosphorylation of eIF2α, which causes translational repression but selective translational activation of the basic leucine zipper (bZIP) factor ATF4 (8, 15, 24, 56). ATF6 is an ER membrane-bound bZIP transcription factor that is expressed ubiquitously and activated by the regulated intramembrane proteolysis mechanism (2, 11, 12, 47, 60) that was first identified in SREBPs (1). In response to ER stress, ATF6 is cleaved in a two-step process by site 1 and site 2 proteases (S1P and S2P) (5, 48, 60). The released N terminus, which encodes the transcription activation domain and the bZIP region, translocates to the nucleus to activate ER chaperone genes, such as BiP/GRP78 and GRP94, through the ER stress response element (ERSE). ERSE, with a consensus sequence of CCAAT-N9-CCACG, is a *cis*-acting element that is necessary and sufficient for transcriptional induction of ER chaperone genes (40, 61, 64, 65). IRE1 is a kinase endoribonuclease which upon the ER stress response initiates spliceosome-independent splicing of XBP1 mRNA (3, 22, 34, 50, 63), resulting in an alternative splicing product of a potent bZIP transcription factor (63). Like ATF6, XBP1 activates ER chaperone genes via ERSE (63), but it also activates transcription through the unfolded protein response element (UPRE), another ER stress-responsive *cis*-acting element with the consensus sequence TGACGTGG/A (57, 62). The known candidate genes regulated by this enhancer element include HRD1 (16), Derlins (33), and EDEM (ER degradation-enhancing α-mannosidase-like protein) (30, 62), all of which are believed to play a role in the ERAD. It has been demonstrated that induction of EDEM transcription is specifically mediated by the IRE1/XBP1 pathway (62).

Luman/CREB3 (27) (also called LZIP [7]) is identified through its association with herpes simplex virus-related host

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cell factor 1 (HCF-1) (21, 58). It is a type II ER transmembrane bZIP transcription factor and shares similar domain structure with ATF6 and another ER stress response protein, OASIS (19). The mode of interaction between Luman and HCF-1 is mimicked by the herpes simplex virus 1 protein VP16, which has led to the hypothesis that Luman may play a role in the viral reaction from latency (7, 25, 28). The cellular function of Luman, however, is not well defined. It has been shown that Luman can be cleaved by the S1P protease that also processes ATF6 (36), but whether Luman is indeed controlled by the same regulated intramembrane proteolysis machinery has not been established, nor have the triggering signals been identified. Recently, we have shown that Luman is the only other known transcription factor in addition to XBP1 that can bind and activate transcription from UPRE-containing promoters and that overexpression of Luman also induces transcription of the cellular EDEM protein (6). Here we report the identification of ERAD-related Herp (homocysteine-induced ER protein) (17) or Mif1 (55) as a direct downstream target of Luman. We show that Luman induces cellular Herp expression during the UPR via transactivation of the ER stress response element II (ERSE-II) (18) enhancer element in its promoter. We propose that Luman is a novel factor that plays a role in ERAD and a cross talk point between different pathways that go through the ER.

MATERIALS AND METHODS

DNA constructs. The reporter plasmids pGL3-Herp (−200/+98)–luciferase and the Herp promoter mutant variants (18), as well as the constructs pcLuman, pcFLAG-Luman (encoding Luman with an amino-terminal FLAG epitope), pcLuman(N), and pcLumanΔ1-52 (6, 25, 27, 28), were described previously. The Luman(N)Δ123-186 fragment was amplified by PCR and cloned into the EcoRI/XhoI site of pcDNA3.1 (Invitrogen) to create pcLuman(N)Δ123-186. Similarly, 3F-Luman and 3F-Luman(N), which contain three FLAG epitope tags at the N-terminal end of Luman, were made by replacing ATF6 in the 3×FLAG-ATF6 plasmid (5) kindly provided by Ron Prywes (Columbia University).

Cell culture, transfection, and luciferase assays. HeLa, 293, and IRE1α (+/+) and (−/−) mouse embryonic fibroblast (MEF) cells (a gift from Randal Kaufman, University of Michigan) (22) were cultured in Dulbecco's modified Eagle's medium (high glucose; Sigma) containing 10% (vol/vol) fetal bovine serum and 1% penicillin and streptomycin at 37°C in a 5% CO_2 atmosphere. Cell cultures were grown to approximately 70% confluence prior to transfection using the calcium phosphate method (26) or Lipofectamine 2000 (Invitrogen) for MEF cells or small interfering RNA (siRNA) experiments. The cells were cotransfected with 1.0 μg/35-mm dish of pcDNA3.1, pcLuman(N), or Luman(N)Δ123-186 and 1.0 μg/dish of the indicated pGL3-Herp-luciferase reporter plasmids or pGL3-Basic (Promega) with a simian virus 40 (SV40) promoter, together with 0.040 μg/well of the *Renilla* luciferase plasmid pRL-SV40 (Promega) as an internal control. At 20 h posttransfection, the medium was replaced to allow the cells to recover for 8 h. Tunicamycin was then added and incubated for 16 h. The cells were harvested, and dual luciferase assays were carried out according to the manufacturer's instruction (Promega). Reporter activity was calculated as relative luciferase activity (firefly luciferase/*Renilla* luciferase) to correct for transfection efficiency. Assays were independently repeated at least three times, and results are shown with standard errors.

Total RNA isolation and Northern blotting. Cells were transfected with 5 μg/10-cm plate plasmid DNA and treated with ER stress inducers as indicated. Total RNA was extracted using the RNeasy kit (QIAGEN); cDNA was synthesized using the Superscript II RNase H- reverse transcriptase (Invitrogen) and oligo(dT) primers. An 837-bp Herp and a 404-bp *Luman* cDNA fragment were labeled by random priming with [α-^{32}P]dCTP and used as probes. The blots were visualized using a Typhoon 9400 PhosphorImager (Amersham).

RT-PCR. Total RNA was harvested as described above. cDNA was derived from the RNA using oligo(dT) and the Superscript II RNase H- reverse transcriptase (Invitrogen). The primers utilized in the reverse transcription-PCR (RT-PCR) were the following: GRP78, 5′-TTGCTTATGGCCTGGATAAGAGGG and 5′-TGTACCCTTGTCTTCAGCTGTCAC; GRP94, 5′-CCCGCTGATCAGAGACATGC and 5′-TTCCTGTGACCCATAATCCCA; Luman, 5′-AAGAGGGGACCCAGATGACT and 5′-AGGAGGAGGCAGAAGGAGAC; Herp, 5′-CTTGGAGCTGAGTGGCGAC and 5′-CAATGTCCAGGAGAGGCAATC; β-actin, 5′-GAGAAAATCTGGCACCACACC and 5′-CAGGAAGGAAGGCTGGAAGAG. Splicing of XBP1 mRNA was analyzed using RT-PCR as previously reported (46). Primers 5′-CGAGCTCGGATCCACTAGTAA (targeting pcDNA3.1) and 5′-CTGCTGCTGGTTTTGTTTGA (targeting Luman) were designed to selectively amplify LumanΔ1-52 transcripts encoded by the pcDNA3.1 vector, not the cellular Luman mRNA.

Microarray analysis. Total RNA and cDNA from 10 μg of total RNA was prepared from 293 cells transfected with pcDNA3.1 (as control) or pcLuman as described above. cDNA was labeled with either Alexa Fluor 555 or 647 carboxylic acid (Invitrogen), purified using the QIAQuick purification kit (QIAGEN), and hybridized to 1.7K human arrays (University Health Network Microarray Facility, Toronto, Canada). Microarray images were acquired using a GenePix 4000A scanner (Molecular Devices). Microarray data from three independent experiments were quantified using GenePix Pro 3.0 software (Molecular Devices) and normalized using a lowess algorithm in GeneTraffic (Stratagene). Statistical analysis of microarray (54) was used to perform a one-class response, and a list of genes was produced.

Western blotting. To detect the N-terminal proteolysis product of Luman, a polyclonal antibody (Rb5660) was raised against the N-terminal region of Luman (amino acids 1 to 215), which was purified by affinity chromatography by sequentially passing through glutathione *S*-transferase (GST) and GST–N-Luman columns. In addition, another Luman polyclonal antibody against the full-length protein (M13) (25), a polyclonal Herp antibody (17), a FLAG monoclonal antibody (M2; Sigma), and β-actin monoclonal antibody (clone AC-15; Sigma) were used as primary antibodies. Blots were visualized using ECL Plus (Amersham) on a Typhoon 9400 PhosphorImager (Amersham).

For brefeldin A (Sigma) treatment, used as the positive control, cells were treated for 5 to 8 h with 1 μg of brefeldin A per ml of medium and 5 mM MG132. Cells were washed with 1× phosphate-buffered saline prior to lysis in sample buffer and subsequent Western blot analyses.

EMSA. The oligonucleotides CRE (for the cyclin AMP response element) (5′-CTAGCCCGG**TGACGTCA**TCGCA), NF-κB (5′-CTAGCTATGGGGAMTTTCCGCTA), ERSE (5′-TCGACCTCCGCCTCAG**CCAAT**GGCGGCAG**CCACA**GAGCGTT), ERSEII (5′-TCGAGGATCCGGACGCCG**ATTGGGCCACG**TTGGGAGAGTGCCT), ERSEII mutant 1 (5′-TCGAGGATCCGGACGCCG**CGGTTGCCACG**TTGGGAGAGTGCCT), ERSEII mutant 2 (5′-TCGAGGATCCGGACGCCG**ATTGGGAACAT**TTGGGAGAGTGCCT), ERSEII mutant 3 (5′-TCGAGGATCCGGACGCCG**CGGTTGAACAT**TTGGGAGAGTGCCT), and UPRE (5′-CTAGCACAGGTGC**TGACGTGG**CGATTCA) were synthesized as probes for the electrophoretic mobility shift assay (EMSA) (bold portions of sequences indicate the consensus core, and the portions in italics indicate mutated sequences). The double-stranded oligonucleotides of these probes were end labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase. Bacterially expressed GST and GST-Luman fusion proteins were quantified using the Bradford assay (Bio-Rad). Recombinant proteins (50 ng) were incubated for 25 min at room temperature with labeled probe and 1 μg of poly(dI-dC) in a buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 5% glycerol, and 1.5 mg/ml bovine serum albumin. DNA-binding proteins were resolved on a 4% nondenaturing polyacrylamide gel and visualized with a Typhoon 9400 PhosphorImager (Amersham). Images were analyzed using ImageQuant software (Molecular Dynamics).

ChIP. 293 cells cultured in 10-cm plates were transfected with 5 μg/plate of pcFLAG-Luman or pcDNA3.1 as described above. The chromatin immunoprecipitation (ChIP) assay was performed by following a protocol reported elsewhere (66) with minor modifications. Briefly, after cross-linking in 1% formaldehyde, the cells were lysed and sonicated. The supernatant was precleared with protein A beads (Amersham). Equal amounts of samples were used in the immunoprecipitation. A 5% aliquot of the precleared chromatin was taken as input, and the rest was incubated with 1 μg of M2 anti-FLAG monoclonal antibody (Sigma) or 1 μg of H3 polyclonal antibody (FL-136; Santa Cruz Biotechnology). After reversing the formaldehyde-induced cross-linking, the chromatin DNA was used in a PCR to produce a 179-bp Herp product with the primers 5′-CAGACGCGGCGGGTTGCA and 5′-GCTTCGGGCGCCTTTTATAGA for the endogenous Herp promoter. Similarly, primers 5′-GGGGAGGAAGGAGTGGAG and 5′-TTAGCCACCAACCTCTCG for the ORP150 promoter and primers 5′-GCGAGGTCCCTCATGTTGTT and 5′-GTTGGGGACCACACGACTTC for the Smad6 promoter were used in control ChIP assays. The gel electrophoresis images were acquired on a Bio-Rad Gel Doc 2000 gel documentation system after ethidium bromide staining.

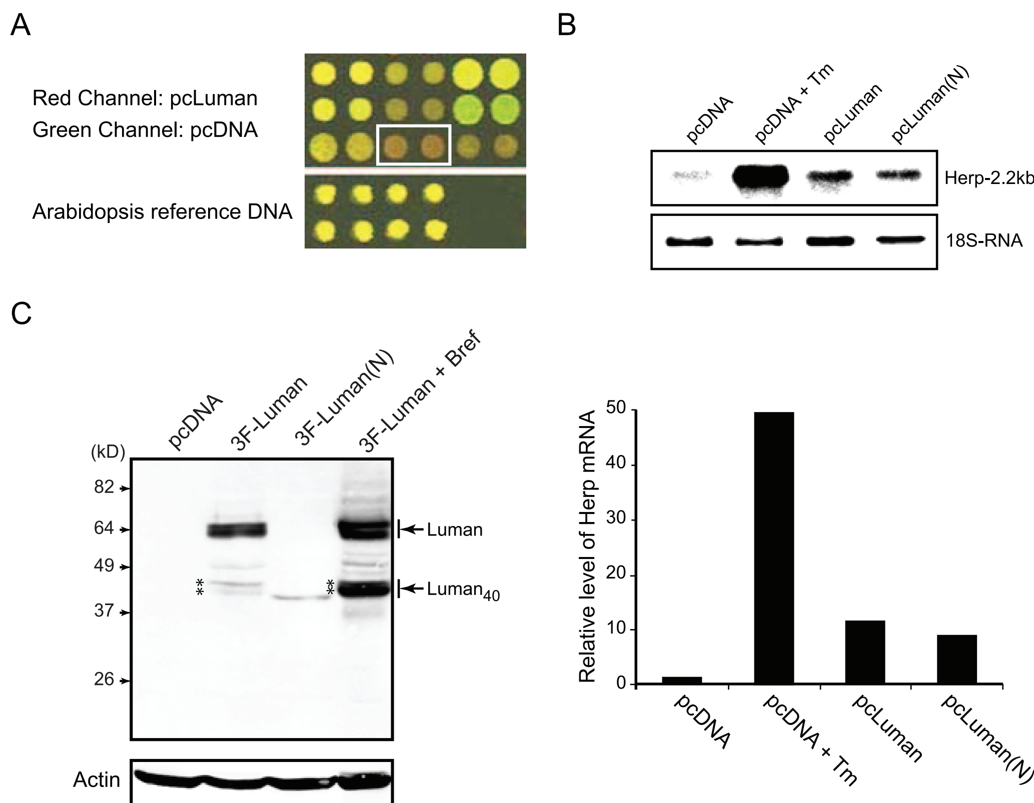

FIG. 1. Induction of cellular Herp expression by Luman. (A) Scanned image of a representative microarray used in the study. 293 cells were either transfected with pcLuman or the vector pcDNA3.1. cDNA samples were labeled with Alexa Fluor 555 or 647 and hybridized to the 1.7K human cDNA microarray. (B) Induction of Herp mRNA expression by Luman. Cells were transfected with pcLuman, pcLuman(N), or the vector only. Treatment with the ER stressor Tm was used as a positive control. Total RNA was extracted and subjected to Northern blot analysis using a DNA probe specific for Herp. The relative intensities of the bands were normalized against 18S rRNA, shown at the bottom. (C) Overexpression of Luman triggers its proteolytic cleavage. HeLa cells in 35-mm dishes were transfected with 1 μg of 3F-Luman, 3F-Luman(N), or the parental vector pcDNA3.1. For the positive control, cells were treated with brefeldin A (1 μg/ml) in the presence of MG132 (5 μM) for 5 h. The affinity-purified FLAG monoclonal antibody M2 (Sigma) was used as the primary antibody in the Western blotting. β-Actin was used as a loading control. Bands with an asterisk on the left are proteolysis products of full-length Luman and are labeled $Luman_{40}$.

Knockdown of Luman gene expression by siRNA. For initial testing, 25 μl of 20 μM Luman Stealth siRNA755 (5′-GGACCCAGAUGACUCCACAGCA UAU) or its specific control siRNA (5′-GGAGACGUAUCAACCGACACCC UAU) (Invitrogen) was used to transfect 293 cells using Lipofectamine 2000 reagent (Invitrogen) by following the manufacturer's instructions. In the knockdown experiment with thapsigargin (Tg)-treated 293 cells, two rounds of siRNA transfection were performed at a 24-h interval. The efficiencies of the siRNA knockdown were assessed by Northern/Western blotting and fluorescence microscopy.

Measurement of caspase 3 activity. Cell extracts were prepared by incubating 2×10^6 cells in 200 μl of cell lysis buffer {50 mM HEPES [pH 7.4], 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 0.1 mM EDTA) for 10 min followed by centrifugation at 10,000 × *g* for 10 min at 4°C. The protein assay was carried out to determine the sample concentration using the BCA protein assay kit (Pierce). Caspase 3 activity was measured by mixing 35 μg of protein sample and 100 μl of reaction buffer (50 mM HEPES [pH 7.4], 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA, 10 mM DTT, 10% glycerol) containing 30 μM of Ac-DEVD-AMC [*N*-acetyl-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin); Biomol Research Laboratories, Plymouth Meeting, PA). The continuous liberation of AMC was examined at 37°C using a Bio-Tek FLx800 microplate fluorescence reader (Bio-Tek, Winooski, VT) with an excitation wavelength of 380 nm and emission at 460 nm. The fluorescence units of AMC released/min/μg protein were calculated for all the samples.

Digital images in this study were processed using Adobe Photoshop and Illustrator software.

RESULTS

Identification of Herp as a potential downstream target of Luman. In an effort to uncover the cellular processes in which Luman is involved, we performed gene expression profiling using human cDNA microarrays to identify potential downstream targets of Luman. After statistical analysis of the microarray data, we generated a list of 108 genes that were significantly upregulated and 11 genes that were downregulated by comparing 293 cells that were transfected with Luman versus those with the vector DNA. One of the most strongly upregulated genes was Herp (Fig. 1A). Herp (or Mif1) is a ubiquitin-like integral ER membrane protein that is highly induced during the UPR and has been implicated in ERAD (17, 18, 32, 45, 55). Since we have previously found that Luman may be linked to the UPR (6), we asked whether Herp was a direct downstream target of Luman. To confirm the microarray results, we first carried out Northern blot analysis of 293 cells transfected with Luman. Tunicamycin (Tm), a strong ER stress inducer which inhibits protein N-glycosylation, was used as a positive control (17). We found that Herp mRNA was induced approximately 10-fold in the cells transfected with full-length

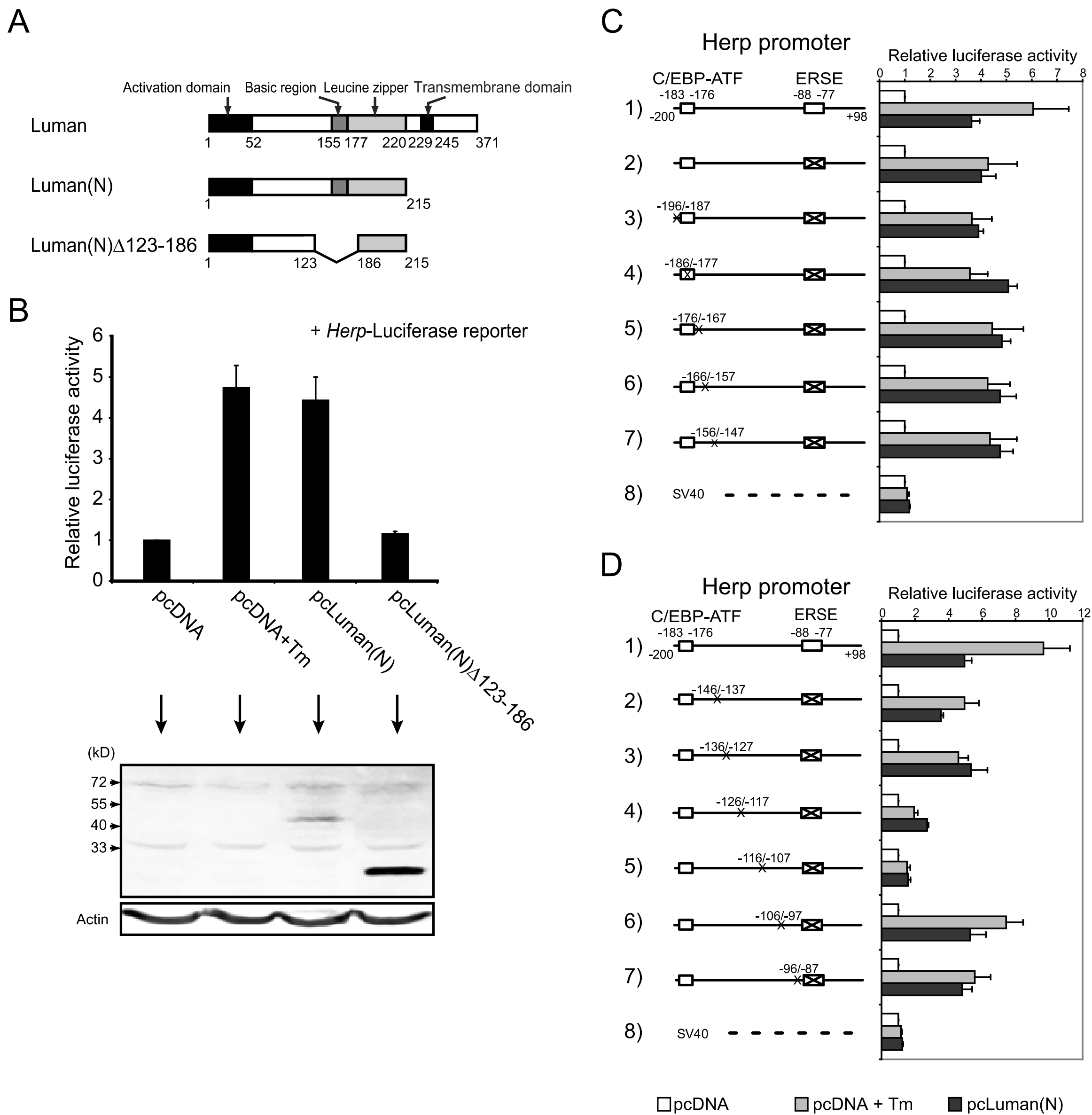

FIG. 2. Luman induces transcription from the Herp promoter. (A) Schematic structure of full-length and N-terminal Luman as well as the Δ123-186 mutant lacking the basic region responsible for DNA binding. (B) Activation of a Herp promoter (−200/+98) reporter by Luman. 293 cells were transiently transfected with pGL3-Herp-Luciferase reporter together with the reference *Renilla* luciferase plasmid pRL-SV40 and the effector plasmids encoding Luman(N) and Luman(N)Δ123-186. The vector pcDNA3.1 and treatment with Tm were used as negative and positive controls, respectively. Luciferase values from three independent experiments were normalized to *Renilla* luciferase activity before being referenced to the control. Cell lysates from the luciferase assays in panel B were subjected to Western blot analysis using Luman antibody M13, shown at the bottom, with β-actin as a loading control. (C and D) Mapping of the Luman-responsive element in the Herp promoter. In both panels C and D, the pGL3-Herp-Luciferase or 10-bp scanning mutation reporter plasmids were cotransfected in 293 cells along with pcDNA3.1 or pcLuman(N). In all scanning mutations of the Herp promoter, nucleotides A, C, G, and T were substituted for C, A, T, and G, respectively. The vector pcDNA3.1 and treatment with Tm were used as negative and positive controls, respectively. The relative luciferase activity was determined by averaging triplicates in three independent experiments, shown with standard errors.

Luman or the activated N-terminal form of Luman, pcLuman(N) (Fig. 1B). These results indicate that Luman can induce transcription of the cellular Herp.

Similar to ATF6 (12, 23, 57, 60), we have observed that in transient-transfection assays full-length Luman exhibits a strong activation potential similar to that of the presumed proteolytically activated form, Luman(N) (Fig. 1B). To investigate the level of aberrant proteolytic cleavage caused by Luman overexpression, HeLa cells were transfected with the same amount of 3×FLAG-Luman and 3×FLAG-Luman(N) plasmid DNA, and Western blot analysis was conducted using an affinity-purified FLAG monoclonal antibody (M2; Sigma) (Fig. 1C). We found that transfection by the full-length plasmid, 3F-Luman, produced the same banding pattern as the brefeldin A-treated positive control, specifically, the full-length Luman and its glycosylated form at ~64 kDa and the pro-

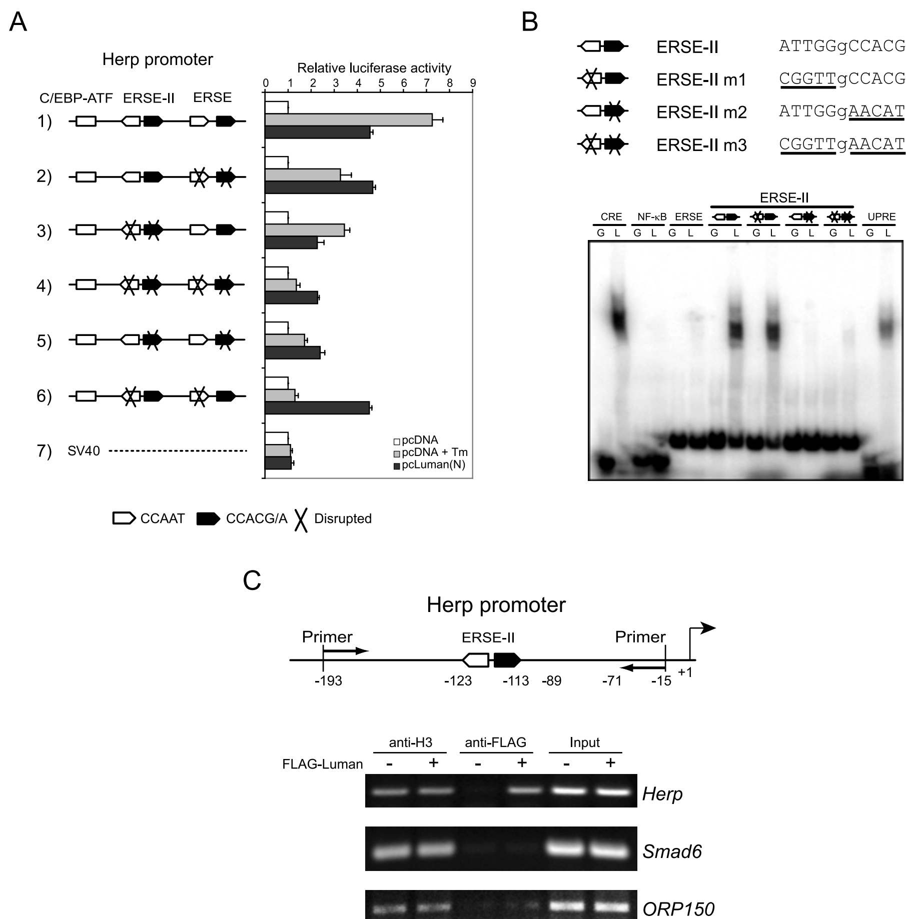

FIG. 3. Luman binds and activates transcription from the second half-site of ERSE-II. (A) Dual luciferase assays were performed as described in the legend for Fig. 2. In all the half-site mutants of ERSE and ERSE-II, the nucleotides A, C, G, and T were substituted for C, A, T, and G, respectively. (B) In vitro binding of Luman to the second half-site of ERSE-II by EMSA. Mutant sequences of the two half-sites of ERSE-II are underlined. Equal amounts of purified GST (G) and GST-Luman (L) proteins were incubated with the indicated double-stranded probes labeled with ^{32}P and separated on a 4% nondenaturing polyacrylamide gel electrophoresis gel. (C) Direct binding of Luman to the Herp promoter as demonstrated by ChIP assay. Top, schematic diagram of the human Herp promoter, with positions of the primer pair used in this ChIP assay indicated. Bottom, 293 cells were transfected with plasmid pcDNA3.1 or pcFLAG-Luman and then cross-linked by formaldehyde. Chromatin was immunoprecipitated with the indicated antibodies. Purified precipitates or input DNA was analyzed by PCR using primers specific for Herp (−193/−15) or the control OPR150 (−311/−28) and Smad6 (−186/+63) promoters. PCR products were subjected to gel electrophoresis and visualized by ethidium bromide staining.

cessed N-terminal form(s) at ~40 kDa (36). Interestingly, even in the presence of the proteasome inhibitor MG132, the transfected Luman(N) protein did not accumulate to the same level as full-length Luman. In fact, its level was similar to the ~40-kDa processed product of the full-length Luman. This is in agreement with the observed similar transactivation potentials of Luman and Luman(N) (Fig. 1B). We noticed that the transfected Luman(N) (amino acids 1 to 215) migrated slightly faster than the proteolysis products, indicating that the actual cleavage site is likely several amino acid residues C-terminal to codon 215.

Luman activates transcription from the Herp promoter. To substantiate the hypothesis that Luman regulates Herp gene expression at the transcription level, we sought to investigate whether Luman is able to activate transcription from the Herp promoter. To this end, we carried out luciferase reporter assays in which cells were cotransfected with the reporter plasmid containing a −200/+98 fragment of *Herp* (18) along with the vector pcDNA3.1 or plasmids encoding N-terminal Luman as well as Luman(N)Δ123-186, which lacks the basic DNA-binding domain (Fig. 2A). Consistent with previous results (18), Tm induced transcription from the Herp promoter (Fig. 2B, compare column 2 with column 1). Luman(N) also activated the Herp reporter ~4.5-fold above the background. In contrast, the mutant Luman(N)Δ123-186 lost the ability to induce the Herp reporter. Immunoblot analysis of the transfected cell lysates indicated that the loss of transactivation potential was not the result of different expression levels of the proteins (Fig. 2B, bottom); rather, Luman activation of the Herp promoter is dependent upon its basic DNA-binding domain.

To map the Luman-responsive element in the Herp promoter that mediates the transcriptional activation by Luman, we utilized a scanning mutagenesis strategy in our reporter assays in which mutations in 10-bp consecutive segments covered the entire Herp promoter region from −200 to −88, just

upstream of a known ERSE site (Fig. 2C and D) (18). In these reporter assays, the pcLuman(N) plasmid was cotransfected into 293 cells along with various scanning mutation reporter plasmids. Since Luman does not bind or transactivate ESRE (6) (Fig. 2C, compare row 2 with row 1), the ERSE at −88/−77 was mutated in all scanning mutants to reduce potential interference by background activation via this site. Of all the mutants, mutation of the −116/−107 (5′-CCACGTTGGG) segment resulted in the most significant loss in reporter activation by Luman(N) and Tm (Fig. 2D). Notably, this is the same region where CCACG, the second half-site of ERSE-II, was previously identified (18). Mutation of the C/EBP-ATF composite site at −183/−178 (29) did not affect the activity of Luman (Fig. 2C, compare row 4 with row 2). The SV40 promoter reporter did not respond to Luman transfection (Fig. 2C and D, bottom rows), indicating that Luman activates the Herp promoter specifically.

ERSE-II is the responsive element of Luman in the Herp promoter. Notably, the promoter of Herp contains not only an ERSE but also an ERSE-II (ATTGG-N-CCACG) site that mediates induction of Herp upon ER stress (18). ERSE-II has the same CCAAT and CCACG consensus sequences as ERSE (CCAAT-N9-CCACG). They are, however, separated by a space of only one nucleotide and placed in the opposite orientation compared with ERSE. Recent studies of the UPR mechanism indicate that ERSE and ERSE-II are regulated differentially (59).

To demonstrate that Luman directly induces Herp transcription through the ERSE-II, we generated specific mutations in ERSE and ERSE-II in the Herp promoter (Fig. 3A). As seen previously (Fig. 2C), the mutation of ERSE did not affect the induction of luciferase activity by Luman(N), although it markedly reduced the activation by Tm (Fig. 3A, compare rows 1 and 2). Of all the mutations that disrupt different half-sites of the two enhancer elements, only the ones that affected the second half-site (CCACG) of ERSE-II significantly reduced the reporter activity induced by Luman, while mutations of the first half-site (ATTGG) showed no effect (Fig. 3A, compare rows 3, 4, and 5 with the rest). These results suggest that the CCACG half-site of ERSE-II is essential for Herp promoter activation by Luman.

To determine if transcriptional activation of ERSE-II is due to direct binding of Luman to the element, we performed EMSAs to examine whether the recombinant Luman protein can physically bind ERSE-II DNA. Oligonucleotides representing ERSE-II and its mutants (m1, m2, and m3) were used, including CRE, NF-κB-binding site, ERSE, and UPRE as controls (Fig. 3B). Consistent with the reporter assay results (Fig. 3A), while Luman formed a complex with wild-type ERSE-II and mutant 1, in which the second half-site was preserved, it failed to complex with ERSE-II mutants 2 and 3 (Fig. 3B). Luman could also bind CRE and UPRE but not NF-κB or ERSE, as reported previously (6, 7, 25, 27).

Next, we carried out ChIP assays to test if Luman binds to the endogenous Herp promoter in vivo (Fig. 3C). 293 cells were transiently transfected with plasmid pcFLAG-Luman expressing FLAG epitope-tagged Luman protein or pcDNA3.1 (mock). After cross-linking and immunoprecipitation, PCR was performed to detect the presence of the Herp promoter DNA using primers flanking the ERSE-II site. Smad6 was arbitrarily chosen as a negative control. ORP150 (14), another ER stress-related gene that is believed to also have an ERSE-II element in its promoter (18), was included. We found that, in FLAG-Luman-transfected cells, FLAG antibody readily pre-

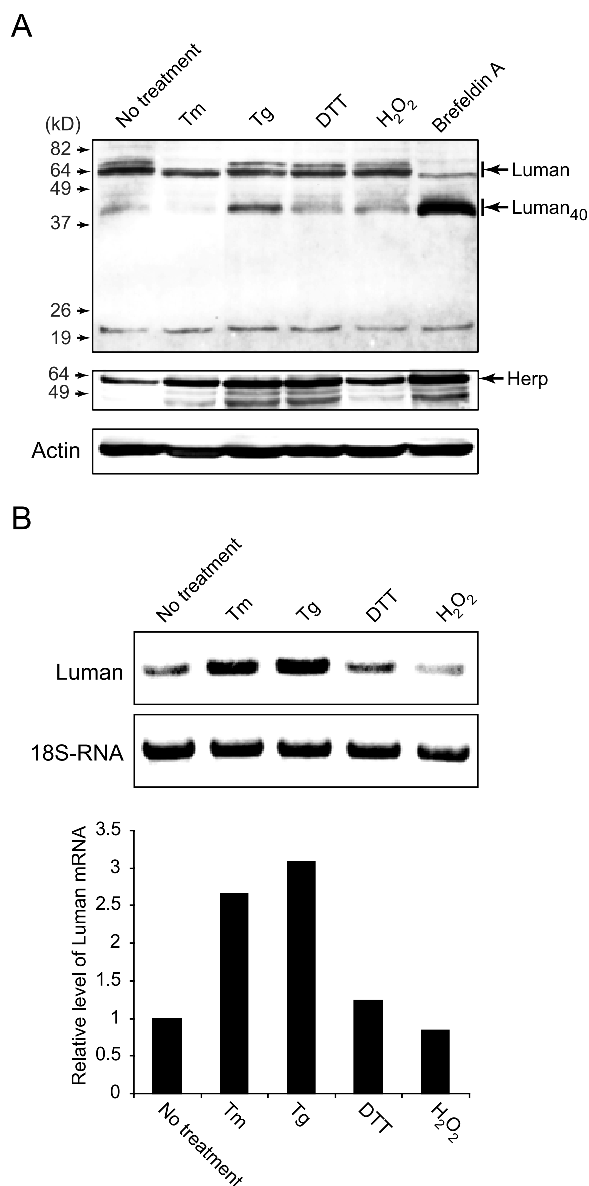

FIG. 4. Activation of Luman by ER stress. (A) Induction of Luman cleavage upon various ER stressor treatments. 293 cells were treated with 2 μg/ml Tm, 300 nM Tg, 1 mM DTT, 300 nM H_2O_2,or 1 μg/ml brefeldin A for 8 h in the presence of 5 μM MG132. Cells were lysed in sample buffer. Affinity-purified polyclonal antibody (Rb5660) against Luman(N) and a Herp antibody (17) were used as primary antibodies in Western blotting. β-Actin was used as a loading control. (B) Induction of Luman transcription by Tm, Tg, DTT, and H_2O_2. After the same ER stressor treatments as for panel A, Northern blot analysis was carried out using Luman cDNA as a probe. Equal loading of RNA was confirmed by staining of 18S rRNA. Relative levels of Luman mRNA (bottom) were calculated by normalization to 18S RNA. Note: the glycosylated form of full-length Luman (A, top band of the Luman doublet) is absent in the treated with Tm (an N-glycosylation inhibitor) or brefeldin A (ER-to-Golgi transport inhibitor), as reported previously (36).

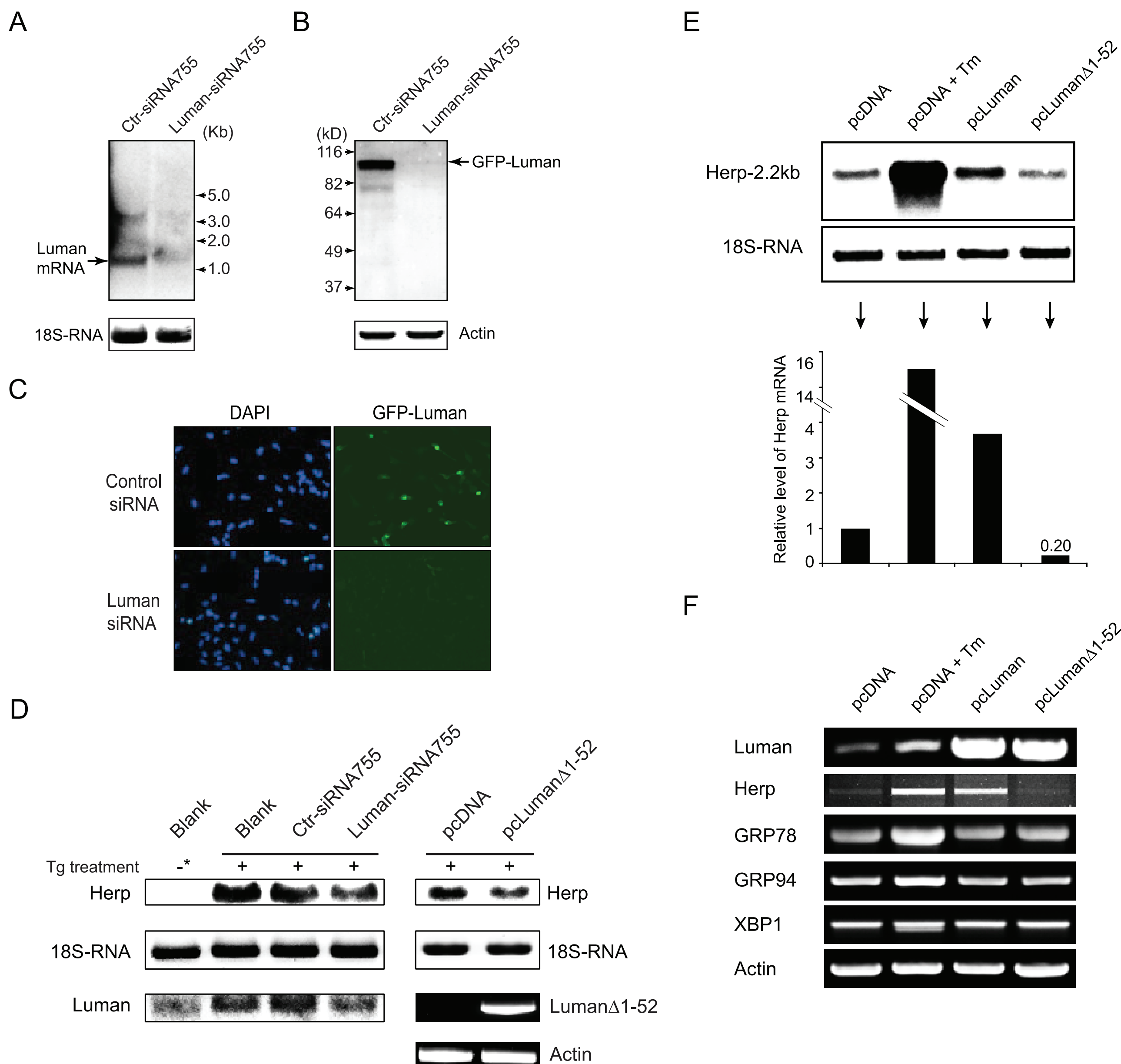

FIG. 5. Luman contributes to the induction of cellular Herp during the ER stress response. (A) Endogenous Luman knockdown by siRNA (Northern blotting). 293 cells were transfected with Luman Stealth siRNA 755 and its corresponding control siRNA (Invitrogen). RNA was extracted with TRIzol (Invitrogen) 24 h posttransfection and subjected to electrophoresis and Northern blot analysis using Luman cDNA as a probe. (B and C) siRNA knockdown of transfected GFP-Luman shown by Western blotting (B) and microscopy (C). At 48 h posttransfection of GFP-Luman and siRNA, 293 cells were lysed in sample buffer and subjected to Western blotting using Luman antibody M13 (B), or cells growing on coverslips were photographed under a Leica DMRA2 microscope using a 63× objective lens (C). (D) Repression of Herp expression through siRNA knockdown of Luman. 293 cells were transfected twice successively with Luman siRNA755 at a ~24-h interval (left panel) or transfected with the Luman dominant negative mutant LumanΔ1-52. At 36 h posttransfection, cells were treated with Tg at 300 nM for 12 h and total RNA was extracted. Northern blot analysis was performed for Herp and Luman, while an RT-PCR specific for LumanΔ1-52 mutant or the actin control was used. *, a faint Herp band can been seen in the Tg-untreated sample under longer exposure. (E) Inhibition of uninduced Herp transcription by dominant negative mutant LumanΔ1-52. 293 cells were transfected with pcDNA3.1 treated or untreated with 2 μg/ml tunicamycin (Tm) for 8 h, pcLuman, or pcLuman Δ1-52. Herp mRNA was detected by Northern blot analysis. Normalized transcript levels are shown at the bottom in the same order. (F) Semiquantitative analysis of cellular Luman, Herp, GRP78, GRP94 transcription, and the alternative splicing of XBP1 mRNA by RT-PCR.

cipitated chromatin containing the Herp promoter and possibly ORP150 but not the Smad6 promoter (Fig. 3C). These results indicate that Luman binds to the Herp promoter specifically in vivo.

Luman contributes to the induction of cellular Herp during the ER stress response. Since Herp is believed to be involved in ERAD (17, 18, 55), we were interested in investigating the potential role of Luman in the induction of Herp during the

mammalian UPR. First we sought to examine whether Luman is proteolytically activated by ER stress. 293 cells were treated with ER stress inducers, including Tm, Tg, DTT, H_2O_2, and brefeldin A, and Western blot analyses were performed. Besides brefeldin A, which strongly induces Luman cleavage by promoting reflux of Golgi-resident protease to the ER, Tg was the only reagent that efficiently triggered Luman cleavage (Fig. 4A). Herp, on the other hand, was strongly induced by all reagents (although that with H_2O_2 was at a lesser level). In addition, we also carried out Northern blot analysis to investigate whether Luman expression could be induced by ER stress (Fig. 4B). We found that the Luman mRNA level was increased by approximately threefold by Tm or Tg treatment, while DTT and H_2O_2 had no effect (Fig. 4B).

To investigate the contribution of Luman to the activation of Herp gene expression during the UPR, we sought to use siRNAs to specifically knock down Luman gene expression. By Northern blot analysis, we found that one RNA duplex, siRNA755, reduced the cellular Luman mRNA by over 75% (Fig. 5A). In 293 cells transiently transfected with GFP-Luman, over 95% knockdown at the protein level was observed by Western blotting (Fig. 5B) and fluorescence microscopy (Fig. 5C). We subsequently used siRNA755 to examine whether knockdown of Luman would affect Herp gene expression during ER stress triggered by Tg. To ensure efficient knockdown of cellular Luman, 293 cells were successively transfected twice with siRNA755, since Tg was known to induce Luman transcription (Fig. 4B). Despite the strong induction of both Luman and Herp by Tg treatment, we found that siRNA755 apparently repressed Luman expression (by ~29% compared to the control siRNA), which also led to a similar level (~31%) of Herp mRNA reduction (Fig. 5D, left). The Luman repression by siRNA755 in Tg-treated cells was not as efficient in the untreated cells; nonetheless, it was reproducible (data not shown). We reason that such low repression efficiency upon Tg treatment might be due to the offsetting effect of strong Luman induction by Tg and, potentially, its interference with the general RNA interference pathway. To confirm the finding that knockdown of Luman gene expression affects Herp induction during the UPR, we transfected cells with a dominant negative mutant of Luman, Δ1-52, in which the activation domain is deleted. A similar level of repression of Herp transcription was also observed (Fig. 5D, right).

To examine whether Luman plays a role in maintaining the Herp expression level without ER stress, we transfected 293 cells with pcLuman, pcLumanΔ1-52, or the control pcDNA3.1 with or without Tm treatment. We found that, while overexpression of the wild-type Luman induced Herp, LumanΔ1-52 reduced the Herp transcript level by fivefold (Fig. 5E). LumanΔ1-52 was also found to be a more effective repressor for cellular Luman than siRNA755 (data not shown). To investigate whether overexpression of Luman or LumanΔ1-52 had an effect on other known UPR pathways, reverse transcription-PCR was performed for GRP78, GRP94, and XBP1 as well as Luman, Herp, and the actin control. Luman was found to only activate Herp but not chaperone production or XBP1 splicing (Fig. 5F). It was noted that the Herp RT-PCR also confirmed the repression effect of pcLumanΔ1-52.

It has been reported previously that transcriptional induction from the Herp ERSE-II is affected by the absence of

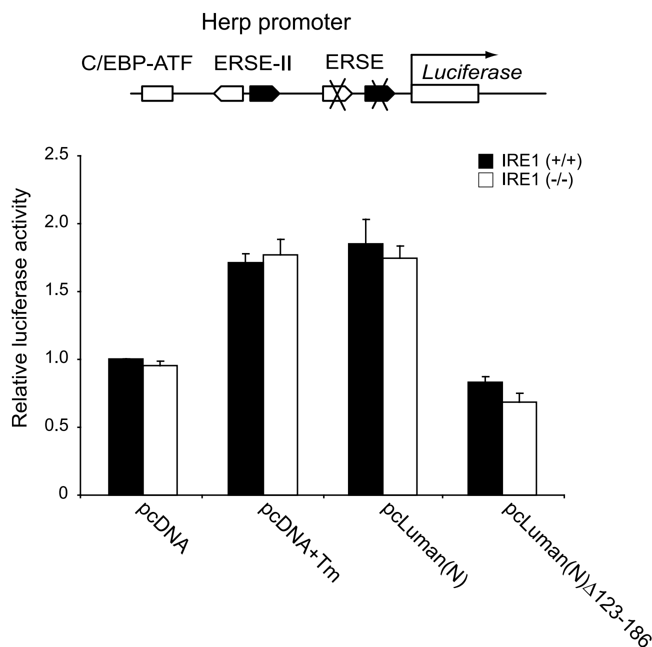

FIG. 6. Induction of Herp transcription by Luman is independent of the IRE1/XBP1 pathway. IRE1$\alpha^{+/+}$ and IRE1$\alpha^{-/-}$ cells were transfected with pcLuman(N), pcLuman(N)Δ123-186, and the vector pcDNA3.1, along with the Herp reporter as shown. Dual luciferase assays were performed as described above.

XBP1 (59). To investigate whether the activation of Herp by Luman also requires an intact IRE1/XBP1 pathway, we assessed the transactivation activity of Luman on the Herp promoter in IRE1$\alpha^{+/+}$ and IRE1$\alpha^{-/-}$ MEF cells. The overall relative luciferase activities in these MEF cells by Tm treatment or by Luman(N) transfection were lower than those seen in 293 cells (Fig. 2C and 3A). It is likely that the ERSE site, which is removed in the reporter plasmid, plays a relatively more important role in the MEF cells. Also, the transfection efficiency of the MEF cells was much lower than that of 293 cells. Nonetheless, we did not notice any difference of the Luman activity between IRE1 +/+ and −/− cells (Fig. 6).

Luman promotes cell survival against ER stress-induced apoptosis, possibly through Herp induction. Herp plays a crucial role in enhancing the cellular tolerance to ER stress and protecting cells from ER stress-induced cell death (4, 13). This prompted us to examine if Luman could also promote cell survival during the UPR. We transfected HeLa cells with pcLuman(N) or vector pcDNA3.1, followed by Tm or staurosporine treatment. Caspase 3 activities were measured and used as an indicator for apoptosis (Fig. 7). While both Tm and staurosporine treatments increased caspase 3 activities in the cells, overexpression of Luman(N) reduced Tm-induced apoptosis by 27% but not the apoptosis caused by staurosporine-induced mitochondria damage. This finding suggests that Luman increased cellular tolerance to ER stress and protected cells from ER stress-induced apoptotic cell death. The level of cell protection by Luman is consistent with the findings obtained through overexpression of Herp (4, 13).

To confirm that Luman indeed induced Herp expression under Tm-triggered ER stress, Western blot analysis of the

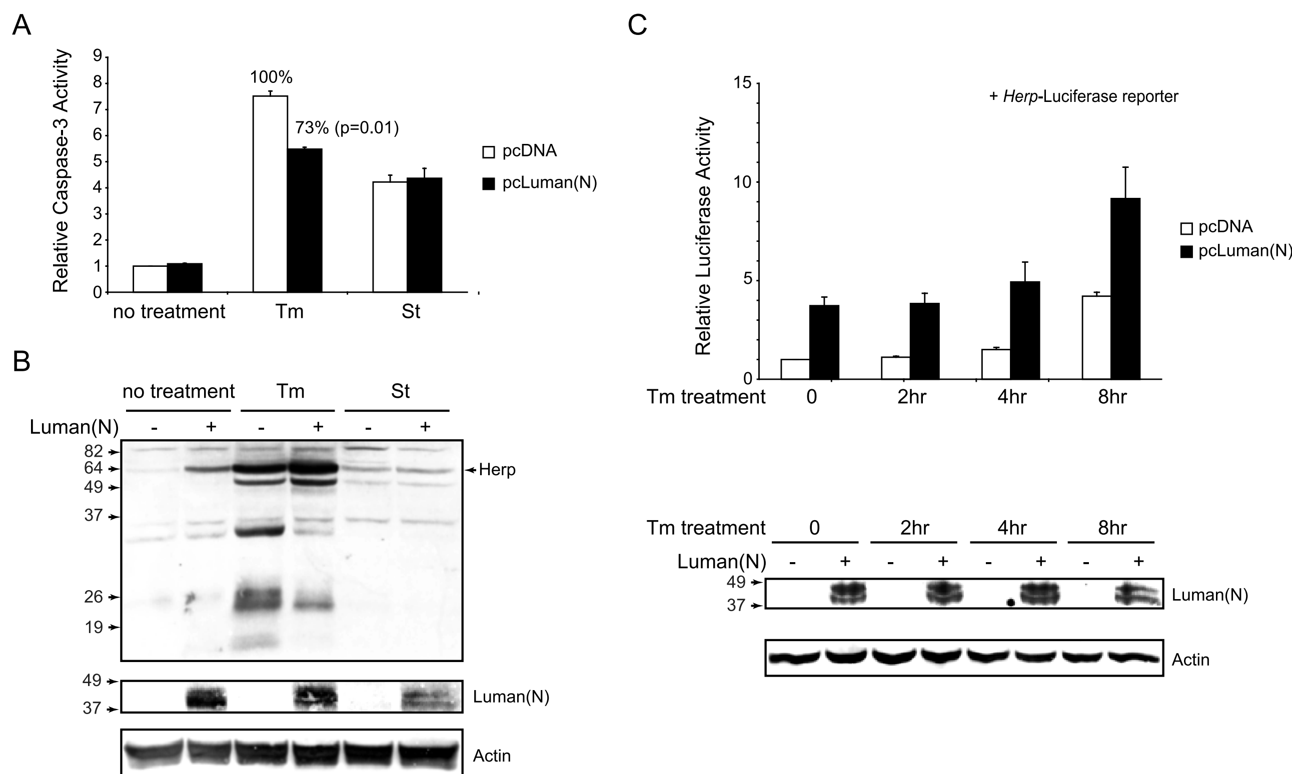

FIG. 7. Luman protects cells from ER stress-induced apoptotic cell death. (A) Luman represses the caspase 3 activity during ER stress. HeLa cells were transiently transfected with pcDNA3.1 or pcLuman(N). Caspase 3 activity was analyzed after cells were treated with Tm (2 μg/ml) for 48 h or staurosporine (St) for 24 h. The activity was normalized to the cells transfected with pcDNA3.1 with no treatment. The averages of the relative values from three independent experiments are shown with standard errors. The *P* value from Student's *t* test is shown for the pcLuman(N) with Tm treatment sample. (B) Luman increases cellular Herp expression in addition to ER stress stimulation. Cell lysates from the caspase 3 assay (shown in panel A) were subjected to Western blot analysis using affinity-purified Luman antibody (Rb5660) and a Herp antibody (17) as primary antibodies. β-Actin was used as a loading control. (C) Luman activates the Herp promoter in addition to Tm-induced ER stress. 293 cells were transiently transfected with the pGL3-Herp-Luciferase reporter together with the reference plasmid pRL-SV40 and pcLuman(N). The vector pcDNA3.1 was used as a negative control. Cells were treated with Tm for the indicated time before cellular lysates were harvested, and dual luciferase assays (top) and Western blotting (bottom) were conducted as described previously.

caspase assay lysates was conducted. Upon Luman(N) transfection, the level of Herp protein was significantly increased in both Tm-treated and untreated cells (Fig. 7B). It is also worth noting that overexpression of Luman(N) changed the banding pattern of the Tm-treated sample. Using the Herp promoter (−200/+98) reporter plasmid, we found that transfection of Luman(N) steadily increased transcription from the Herp promoter in addition to the Tm induction (Fig. 7C). These results suggest that Luman can increase the level of Herp expression over Tm stimulation.

DISCUSSION

In this report we have presented evidence that the ERAD-related protein Herp is a downstream target of Luman. We have shown that Luman can be transcriptionally and proteolytically activated by the ER stress inducer Tg and that Luman activates transcription from the Herp promoter through direct binding of an ERSE-II element, specifically via its second half-site (CCACG). Our results indicate that Luman contributes to the induction of Herp during the UPR. Expression of Luman produces the same effect in the cells as Herp, i.e., enhancement of cellular tolerance to ER stress and protection of cells from ER stress-induced apoptotic cell death. Previously, we found that Luman can bind and activate transcription from the UPRE (6) and that overexpression of Luman induces cellular EDEM, another ERAD-related protein (30, 62). Together with the data presented here, we propose that Luman is a transcription factor that plays a role in the ERAD signaling.

Herp is an ER integral membrane protein and reportedly the most highly induced protein during the UPR (17, 55, 59). The primary suggested cellular role of Herp is ERAD (13, 42, 45, 59). Herp is known to associate with the components of the ERAD pathway (45) and prevents ER stress-induced apoptotic cell death (4, 13). Interestingly the cellular function of Herp, especially its antiapoptotic role, has been linked to the neuronal system (4, 13). During the ER stress response, Herp helps to stabilize ER Ca^{2+} homeostasis (4) and also increases ER folding capacity through ERAD (13). Herp also interacts with presenilins and increases the production of amyloid-β (42, 43). Since the Luman protein has been evidently found in the neurons of mammalian trigeminal ganglia (25), there may be a functional link between Luman and Herp in the ERAD of neurons.

Although Luman is believed to undergo the same regulated

intramembrane proteolysis by the S1P and S2P proteases as OASIS and ATF6 (5, 19, 36, 49, 53), this is the first report to demonstrate that Luman can be proteolytically activated by Tg. We have noted that not all ER stressors activate Luman (Fig. 4). Although Tm enhanced Luman transcription, only Tg induced both Luman transcription and proteolytic cleavage. Compared to brefeldin A, which induced over 90% Luman cleavage, Tg (~40% Luman cleavage induction) is likely not the optimal reagent in triggering the proteolysis of Luman. Therefore, it is likely that Luman is an unconventional ER stress response protein, and the signal(s) that optimally activates Luman has not yet been found. It is known that a diverse array of environmental cues and biological processes can trigger the ER stress response, including lipid metabolism, differentiation of secretory cells, viral infection, DNA damage, and chemical insult (41, 51). Like eIF2α in the PERK/ATF4 pathway, Luman may be preferentially activated by signals that do not originate from but are channeled through the ER (Fig. 8).

It is also of interest that DTT did not induce Luman transcriptionally or proteolytically, albeit it activated Herp efficiently (Fig. 4A). Luman, therefore, is not the only factor that regulates Herp expression during the UPR. In fact, all three known branches of the mammalian UPR are represented in the transcriptional regulation of Herp, namely, ATF6, IRE1/XBP1, and PERK/ATF4 (Fig. 8). Previous mapping studies have identified three enhancer elements, C/EBP-ATF, ERSE, and ERSE-II, in the Herp promoter that can mediate Herp induction in response to ER stress (18, 29, 59). As reported previously (18, 29), we also found that the C/EBP-ATF site is not necessary but is required for optimal activation of Herp expression (Fig. 2C, row 4). The contributions of ERSE and ERSE-II appear to be similar; mutation of either element significantly impaired Herp induction by Tm (Fig. 3A, rows 2 and 3) (18). When both elements were mutated, in either half-site of the element, Herp induction by Tm was diminished (Fig. 3A, rows 4 to 6).

Both XBP1 (63) and ATF6 (64) can bind to ERSE in the presence of NF-Y, and overexpression of both proteins induces the expression of Herp (18, 59). XBP1, however, is not necessary for the transactivation of ERSE (22, 62); the loss of XBP1 activity can be fully compensated by ATF6 in IRE1α-deficient cells (62). At the ERSE-II site in the Herp promoter, all three proteins, Luman, XBP1, and ATF6, can potentially regulate Herp transcription (Fig. 8). In the presence of NF-Y, ATF6 binds to ERSE preferentially but also binds to ERSE-II at a substantially lower efficiency (59). The fact that overexpression of ATF6 only moderately activated transcription from ERSE-II (18) suggests that it plays a lesser role in inducing Herp expression from this site. In contrast to ATF6, both Luman and XBP1 (59) bind to ERSE-II independently from NF-Y. We also found that an intact IRE1/XBP1 pathway does not appear to be essential for activation of ERSE-II in the Herp promoter (Fig. 6). Consistent with our finding, Yamamoto et al. (59) showed that, in the same IRE1α$^{-/-}$ MEF cells, the transcriptional activity from a reporter containing three ERSE-II elements was not affected by the absence of XBP1 splicing. As with ERSE, it is possible that the absence of XBP1 may be compensated by Luman in terms of transcriptional activation through the ERSE-II element (Fig. 6). Conversely, Luman knockdown by siRNA or functional repression

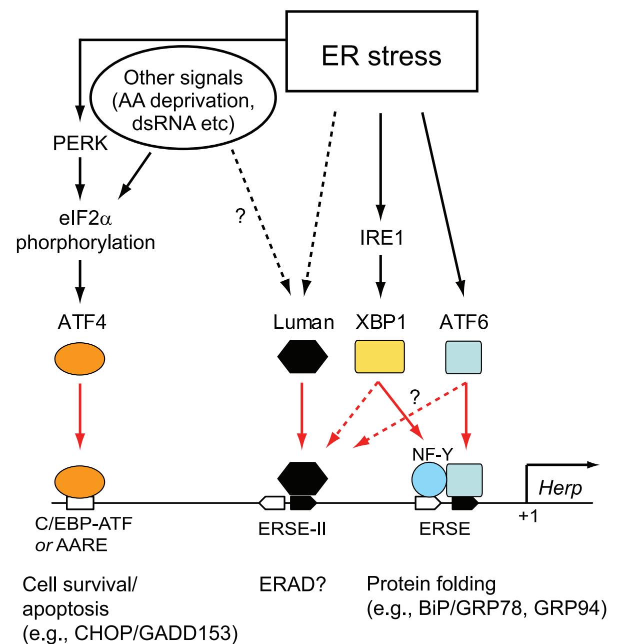

FIG. 8. Summary of *cis*-acting elements and their regulatory factors in regulation of Herp gene expression during the mammalian UPR. The text at the bottom shows the cellular processes linked to the *cis* element and the genes in which the element has been found, in addition to Herp. Abbreviation: AA deprivation, amino acid deprivation.

by LumanΔ1-52 attenuated Herp gene expression (Fig. 5). We therefore believe that Luman is a key factor in the transcriptional regulation of Herp via the ERSE-II site during the ER stress response (Fig. 8).

Among the transcription factors involved in the UPR, Luman has similar domain structures to ATF6 and OASIS (19) but appears to have similar DNA-binding specificity to XBP1. ATF6 binds ERSE and ERSE-II only in the presence of NF-Y (59) and does not bind UPRE. Luman, similar to XBP1, binds ERSE-II and UPRE in an NF-Y-independent manner. Luman is also the only known transcription factor other than XBP1 that binds and activates transcription from both UPRE and ESRE-II. Although we have not examined whether Luman can bind to ERSE in the presence of NF-Y by EMSA, our reporter assay results (Fig. 2C and D and 3A and also reference 6) suggest that, unlike ATF6, Luman does not activate ERSE. While ERSE is mostly found in the promoters of chaperones, such as BiP/GRP78 and GRP94, UPRE and ERSE-II have only been identified in ERAD-related genes, three of which, i.e., EDEM, Herp, and possibly ORP150, are known to be potentially regulated by Luman.

Differences between Luman and ATF6 or XBP1 seem to argue for a unique function of Luman in the mammalian UPR. In terms of transcriptional regulation of Herp, it seems plausible to postulate that induction mediated by the ERSE site is an early regulatory time point in which Herp is controlled in concert with the increase of protein folding capacity. The C/EBP-ATF composite element, also called the amino acid response element, is an ATF4-binding site which has been linked to the regulation of apoptosis (29). Hence, the C/EBP-

ATF composite site may represent a regulatory point late in the stress response when the cell fate will be decided. The induction of ERSE-II, on the other hand, may be a transitional phase between the two, coinciding with activation of the ERAD machinery associated with Luman. With a structure similar to ATF6 and DNA-binding specificities resembling XBP1, Luman might represent a cross talk point of the IRE1/XBP1 and ATF6 pathways and might also be a converging point between the unfolded protein stress and other signals that are channeled through the ER (Fig. 8). Future studies are required to delineate the role of Luman in this important cellular process.

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