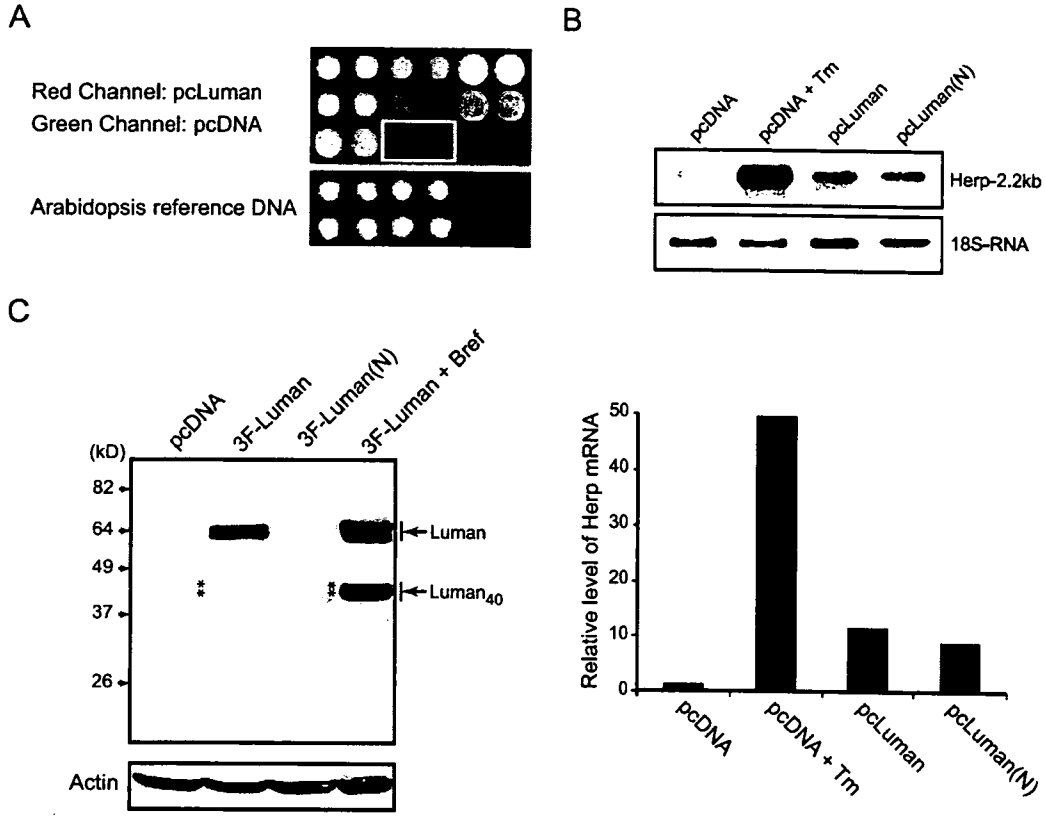

FIG. 1. Induction of cellular Herp expression by Luman. (A) Scanned image of a representative microarray used in the study. 293 cells were either transfected with pcLuman or the vector pcDNA3.1. cDNA samples were labeled with Alexa Fluor 555 or 647 and hybridized to the 1.7K human cDNA microarray. (B) 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 • g of 3F-Luman, 3F-Luman(N), or the parental vector pcDNA3.1. For the positive control, cells were treated with brefeldin A (1 • g/ml) in the presence of MG132 (5 • M) for 5 h. The affinity-purified FLAG monoclonal antibody M2 (Sigma) was used as the primary antibody in the Western blotting. • -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 • l of 20 • M 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 • l 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 • g of protein sample and 100 • l 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 • M 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/• g 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

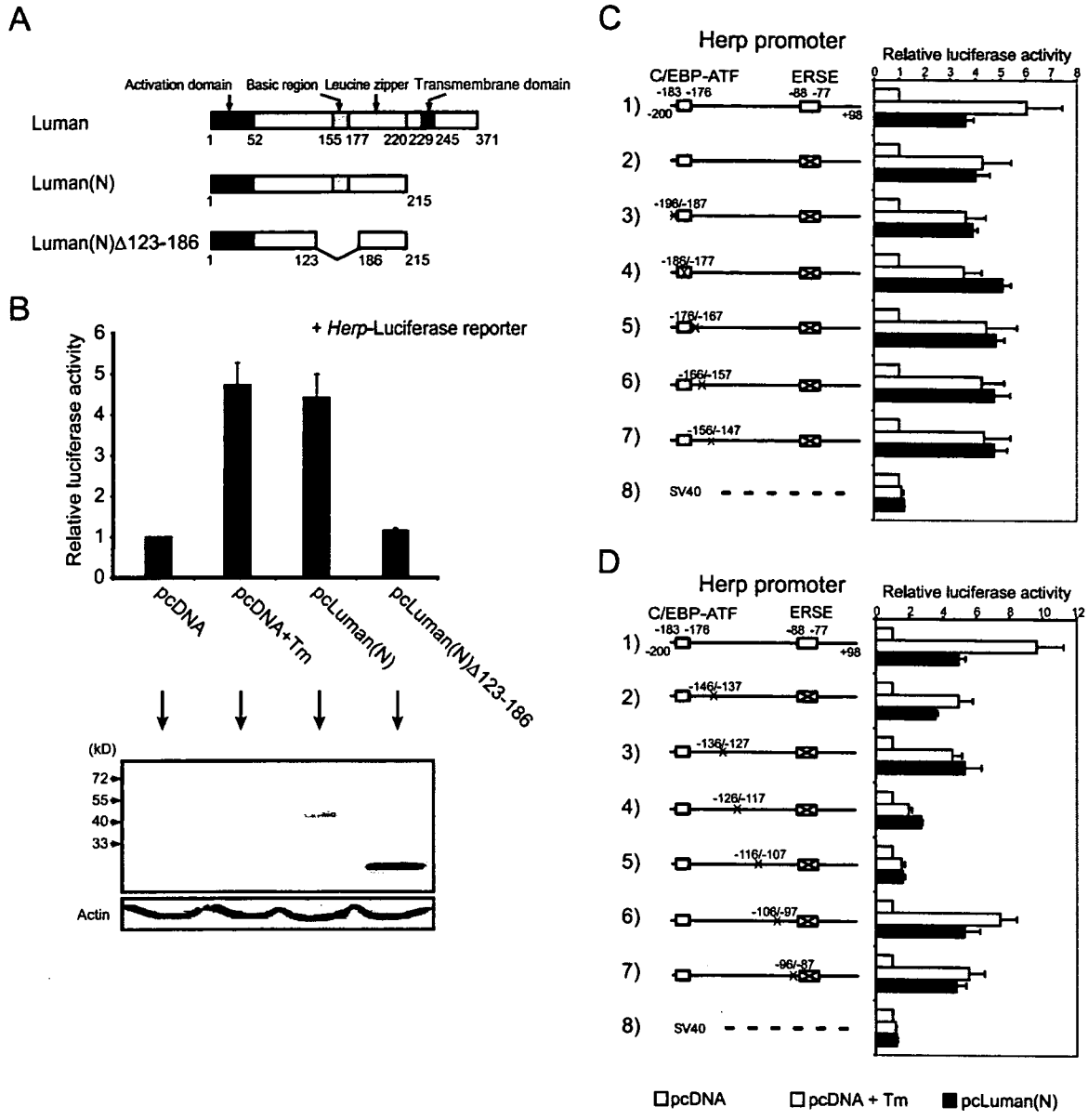

FIG. 2. Luman induces transcription from the Herp promoter. (A) Schematic structure of full-length and N-terminal Luman as well as the • 123-186 mutant lacking the basic region responsible for DNA binding. (B) Activation of a Herp promoter (• 200/• 98) reporter by Luman. 293 cells were transiently transfected with pGL3-Herp-Luciferase reporter together with the reference Renilla luciferase plasmid pRL-SV40 and the effector plasmids encoding Luman(N) and Luman(N)• 123-186. 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 • -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-

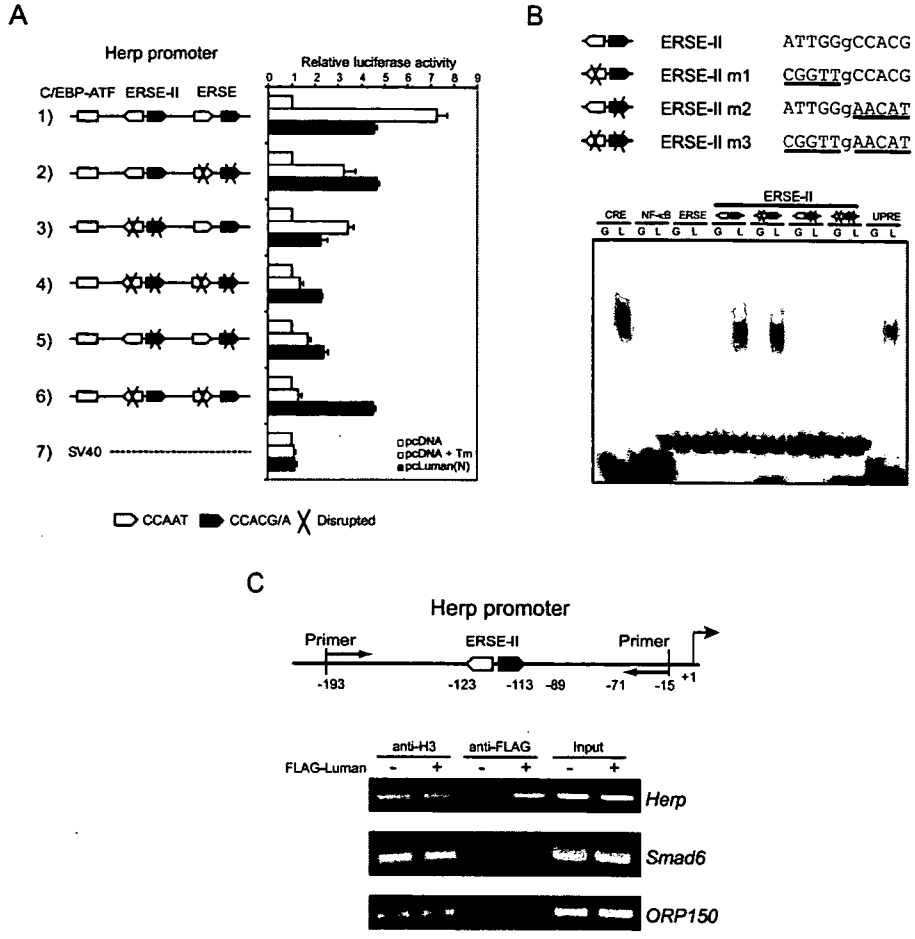

FIG. 3. Luman binds and activates transcription from the second half-site of ERSE-II. (A) Dual luciferase assays were performed as described in the legend for Fig. 2. In all the half-site mutants of ERSE and ERSE-II, the nucleotides A, C, G, and T were substituted for C, A, T, and G, respectively. (B) 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)• 123-186, 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)• 123-186 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-• B-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-• B 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-

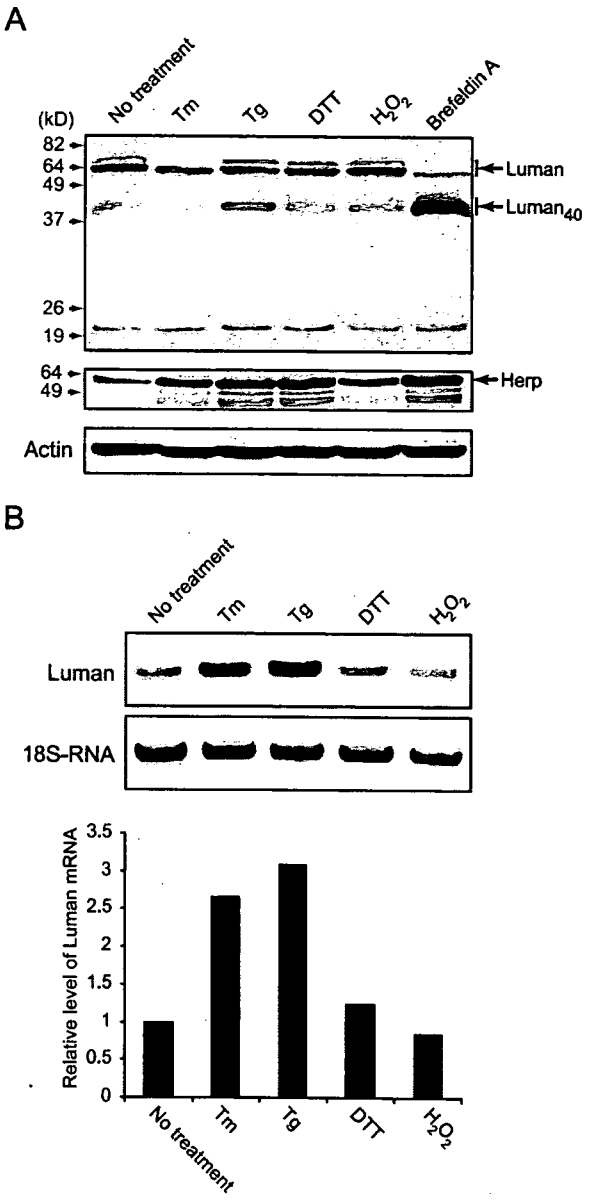

FIG. 4. Activation of Luman by ER stress. (A) Induction of Luman cleavage upon various ER stressor treatments. 293 cells were treated with 2 • g/ml Tm, 300 nM Tg, 1 mM DTT, 300 nM H_2O_2,or 1 • g/ml brefeldin A for 8 h in the presence of 5 • M MG132. Cells were lysed in sample buffer. Affinity-purified polyclonal antibody (Rb5660) against Luman(N) and a Herp antibody (17) were used as primary antibodies in Western blotting. • -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).

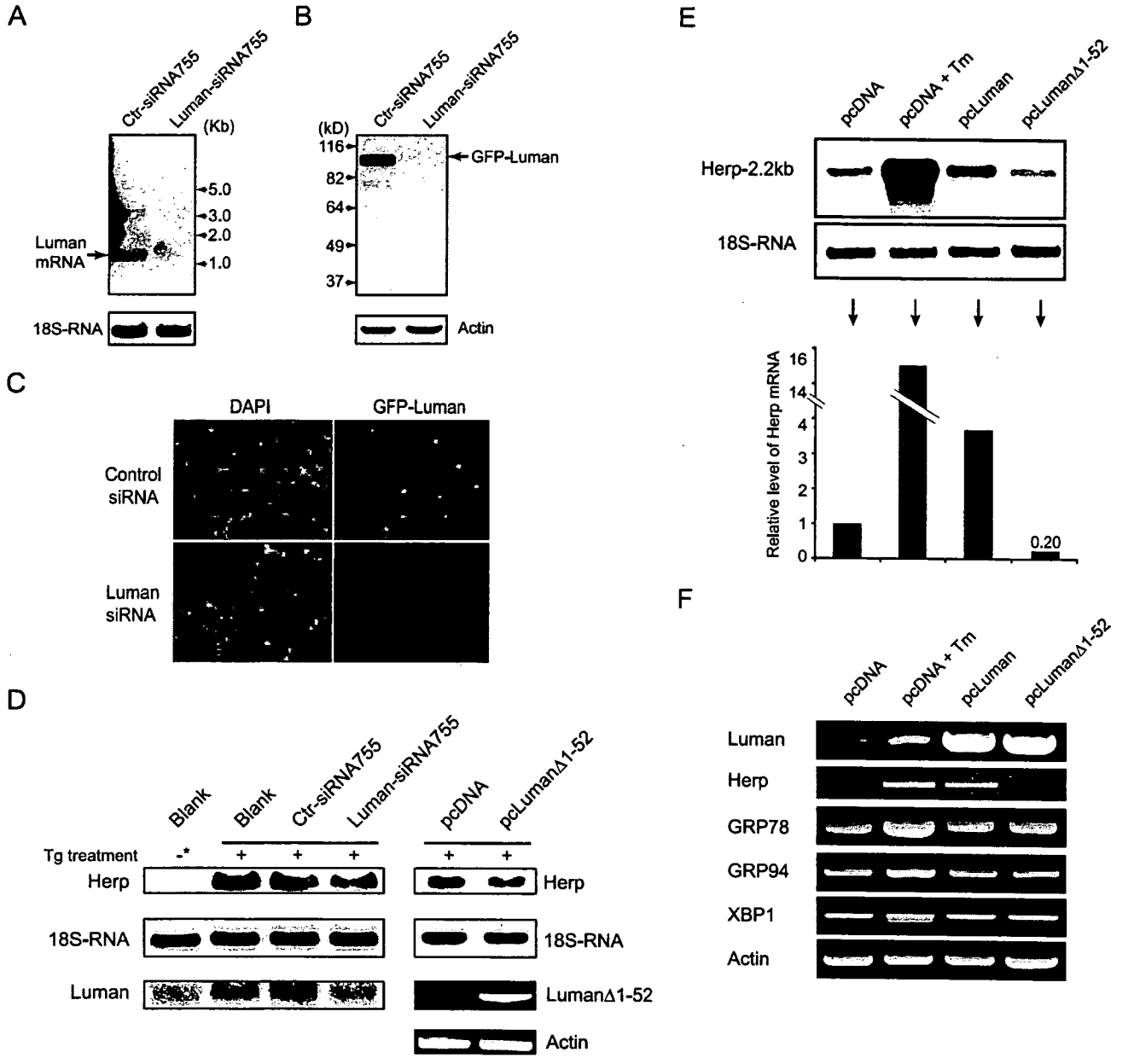

FIG. 5. Luman contributes to the induction of cellular Herp during the ER stress response. (A) Endogenous Luman knockdown by siRNA (Northern blotting). 293 cells were transfected with Luman Stealth siRNA 755 and its corresponding control siRNA (Invitrogen). RNA was extracted with TRIzol (Invitrogen) 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 Luman• 1-52. 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 Luman• 1-52 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 Luman• 1-52. 293 cells were transfected with pcDNA3.1 treated or untreated with 2 • g/ml tunicamycin (Tm) for 8 h, pcLuman, or pcLuman • 1-52. 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, • 1-52, 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, pcLuman• 1-52, or the control pcDNA3.1 with or without Tm treatment. We found that, while overexpression of the wild-type Luman induced Herp, Luman• 1-52 reduced the Herp transcript level by fivefold (Fig. 5E). Luman• 1-52 was also found to be a more effective repressor for cellular Luman than siRNA755 (data not shown). To investigate whether overexpression of Luman or Luman• 1-52 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 pcLuman• 1-52.

It has been reported previously that transcriptional induction from the Herp ERSE-II is affected by the absence of 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•$^{/+}$ and IRE1•$^{/+}$ 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).

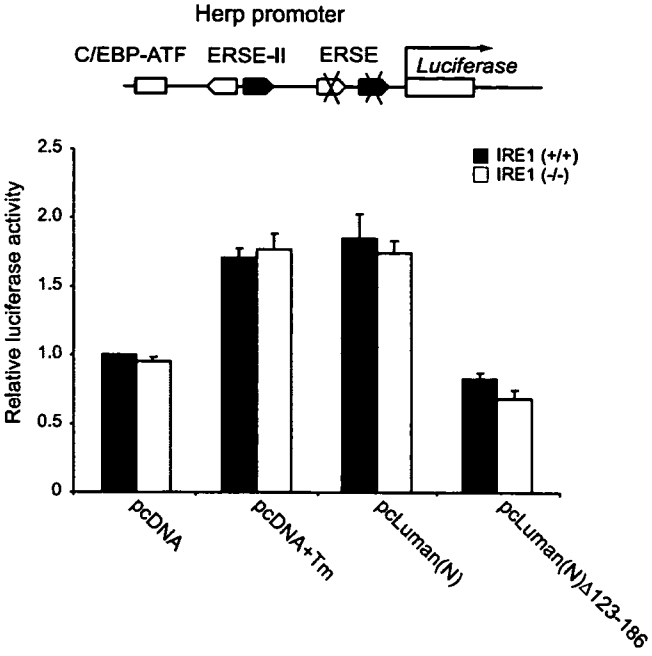

FIG. 6. Induction of Herp transcription by Luman is independent of the IRE1/XBP1 pathway. IRE1•$^{/+}$ and IRE1•$^{/+}$ cells were transfected with pcLuman(N), pcLuman(N)• 123-186, and the vector pcDNA3.1, along with the Herp reporter as shown. Dual luciferase assays were performed as described above.

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

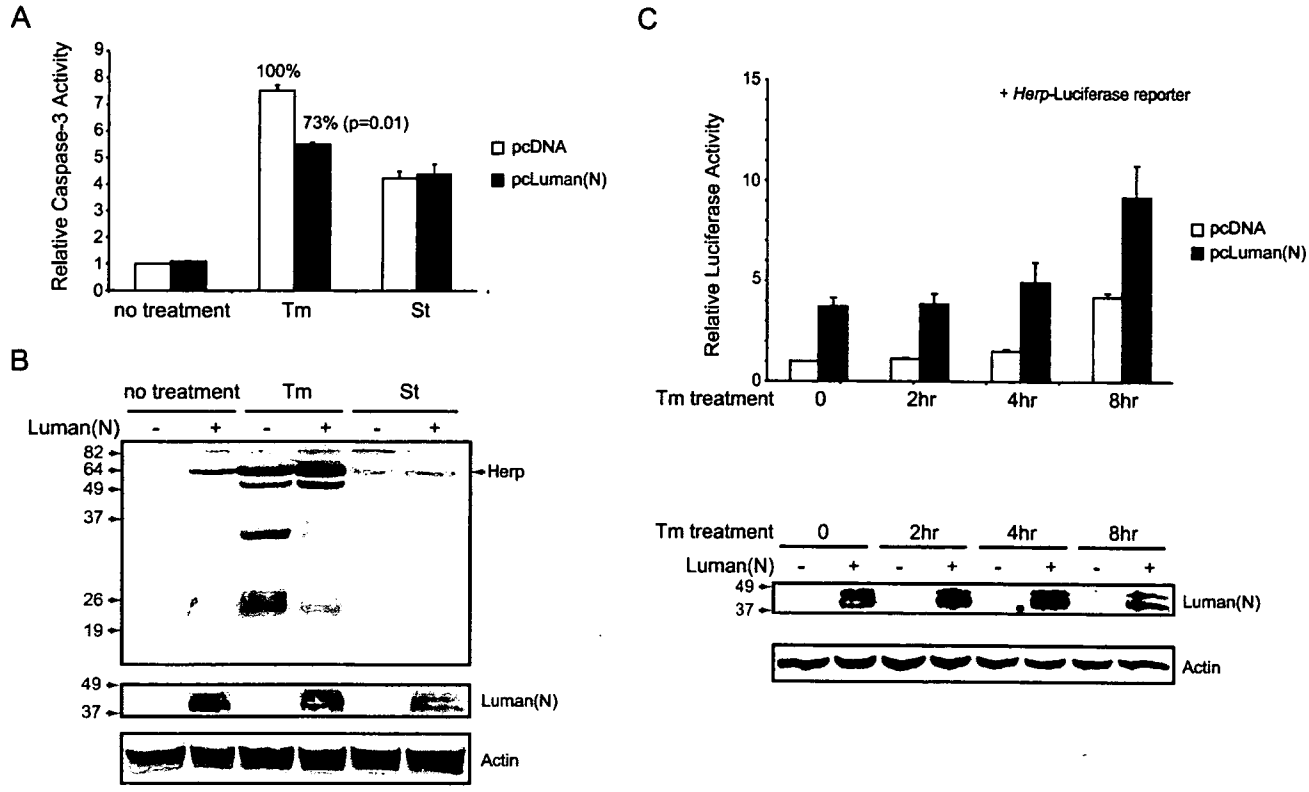

FIG. 7. Luman protects cells from ER stress-induced apoptotic cell death. (A) Luman represses the caspase 3 activity during ER stress. HeLa cells were transiently transfected with pcDNA3.1 or pcLuman(N). Caspase 3 activity was analyzed after cells were treated with Tm (2 • g/ml) for 48 h or staurosporine (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. • -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-• (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 eIF2• 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 IRE1•-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 IRE1•$^{-/-}$ 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

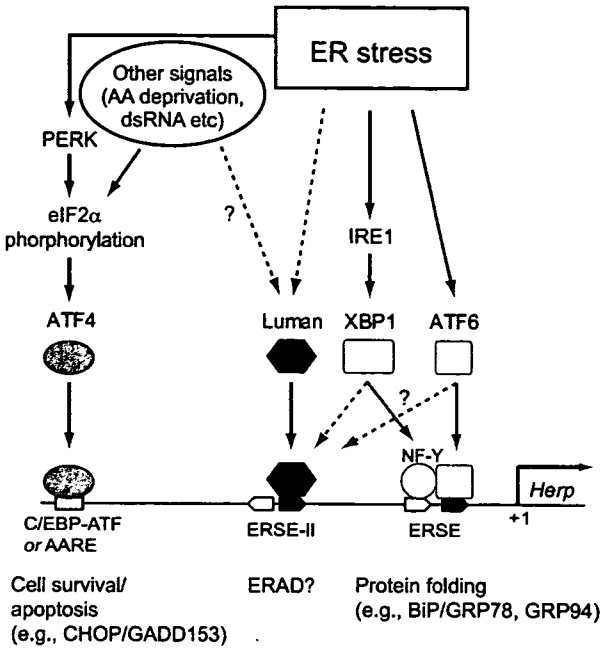

FIG. 8. Summary of cis-acting elements and their regulatory factors in regulation of Herp gene expression during the mammalian UPR. The text at the bottom shows the cellular processes linked to the cis element and the genes in which the element has been found, in addition to Herp. Abbreviation: AA deprivation,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

by Luman• 1-52 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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SHORT ARTICLE

Peripheral ghrelin transmits orexigenic signals through the noradrenergic pathway from the hindbrain to the hypothalamus

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Summary

Ghrelin, a gastrointestinal peptide, stimulates feeding when administered peripherally. Blockade of the vagal afferent pathway abolishes ghrelin-induced feeding, indicating that the vagal afferent pathway may be a route conveying orexigenic ghrelin signals to the brain. Here, we demonstrate that peripheral ghrelin signaling, which travels to the nucleus tractus solitarius (NTS) at least in part via the vagus nerve, increases noradrenaline (NA) in the arcuate nucleus of the hypothalamus, thereby stimulating feeding at least partially through a-$_1$ and b-$_2$ noradrenergic receptors. In addition, bilateral midbrain transections rostral to the NTS, or toxin-induced loss of neurons in the hindbrain that express dopamine b hydroxylase (an NA synthetic enzyme), abolished ghrelin-induced feeding. These findings provide new evidence that the noradrenergic system is necessary in the central control of feeding behavior by peripherally administered ghrelin.

Introduction

Ghrelin, a newly discovered member of the family of gut-brain peptides, functions in feeding control and growth hormone (GH) secretion by binding to the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999; Nakazato et al., 2001; Tschöp et al., 2000; Wren et al., 2000). This peptide, which is produced primarily by endocrine cells of the stomach, is released into the circulation (Date et al., 2000; Dornonville de la Cour et al., 2001). Ghrelin is also produced by neurons of the hypothalamus, where it serves as part of the neural networks (Cowley et al., 2003). GHS-R is extensively distributed throughout the brain, including the hypothalamus and brainstem where are essential for energy homeostasis. Given the GHS-R expression pattern, ghrelin, when given centrally, peripherally, or both, may increase food intake directly via effects on neurons present in the hypothalamus or brainstem. We recently demonstrated, however, that blockade of the gastric vagal afferent pathway abolished peripheral ghrelin-induced feeding (Date et al., 2002). A similar study demonstrated that intraperitoneal injection of ghrelin into vagotomized mice did not stimulate food intake (Asakawa et al., 2001). These findings suggest that the gastric vagal afferent pathway as well as the humoral pathway may have some significant part in conveying ghrelin-mediated orexigenic signals to the brain.

Several gastrointestinal hormones, including ghrelin, cholecystokinin (CCK), peptide YY, and glucagon-like peptide 1, transmit signals of starvation and satiety to the brain at least in part via the vagal afferent system (Date et al., 2002; Smith et al., 1981; Koda et al., 2005; Abbott et al., 2005). Feeding-related information, travels directly to the nucleus tractus solitarius (NTS), where it can be converted to additional signals that transmit a feeling of hunger or fullness to the hypothalamus. In the present study, we focused on the importance of the neural pathways from the NTS to the hypothalamus in transmitting peripheral ghrelin signals.

To investigate the neural pathways involved in the transmission of ghrelin orexigenic signals from the NTS to the hypothalamus, we examined the effects of bilateral midbrain transections on ghrelin-induced feeding. The NTS contains the A2 noradrenergic cell group, which projects to regions of the hypothalamus that include the ARC (Sawchenko and Swanson, 1981). Therefore, we examined the role of the central noradrenaline (NA) system in peripheral ghrelin feeding stimulation. Using real-time PCRs, we quantified the expression of dopamine b hydroxylase (DBH), an enzyme necessary to convert dopamine into NA, within the NTS. We also measured overflow NA within or near the ARC after intravenous administration of ghrelin using in vivo microdialysis. We studied the effects of adrenergic antagonists and the elimination of NA innervation within the ARC on ghrelin-induced food intake. Using immunohistochemical techniques, we demonstrated that the NPY neurons activated following intravenous administration of ghrelin are innervated by DBH-containing fibers.

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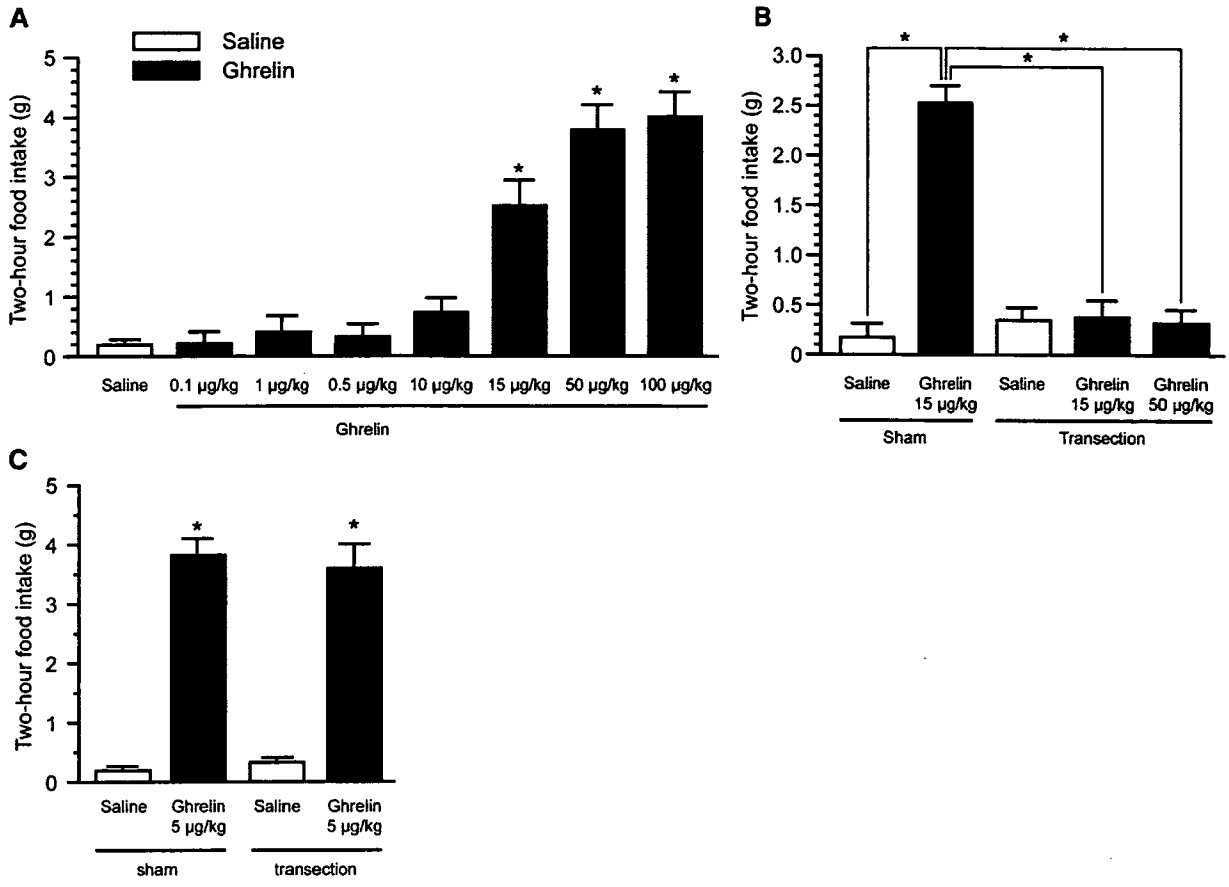

Figure 1. Effect of bilateral midbrain transections on ghrelin-induced feeding behavior

A) Two hour food intake (mean ± SEM) of sham-treated rats after a single intravenous administration of ghrelin (0.1–100 mg/kg). *$p < 0.0001$ versus saline.

B) Food intake of rats with bilateral midbrain transections after a single intravenous administration of ghrelin (15 and 50 mg/kg). *$p < 0.0001$.

C) Food intake of rats with bilateral midbrain transections after single intracerebroventricular administration of ghrelin (5 mg/kg). *$p < 0.0001$.

Error bars represent the SEM.

Results and Discussion

Midbrain transections and peripheral ghrelin-induced feeding

To investigate if intravenous administration of ghrelin stimulates feeding via the ascending efferent fibers of the NTS, we examined ghrelin-induced food intake in rats with bilateral midbrain transections (Crawley et al. 1984). Before this experiment, we confirmed that there were no significant differences in body weight or food intake between control and actual transected groups up to eight days after the surgery (see Supplemental Results and Figure S1 in the Supplemental Data available with this article online). There were also no significant differences in the feeding response after fasting for 12 hr, energy expenditure, locomotor activity, body fat, or food preference between the two groups seven days after surgery (Supplemental Results and Figure S2). Therefore, we performed feeding experiments using rats seven days after the surgery. The lowest effective dose of intravenously (i.v.) administered ghrelin for rats subjected to sham surgery (sham-treated rats) was 15 mg/kg; this value was used as the standard dose in the subsequent experiments (Figure 1A). Intravenous administration of ghrelin (R 15 mg/kg) significantly increased food intake (10:00–12:00 hr) in sham-treated rats, whereas ghrelin-induced feeding was absent in midbrain transected rats (Figure 1B) ($n = 10$ per group). Because bilateral midbrain transections may nonspecifically suppress feeding in response to ghrelin, we tested the orexigenic effect of centrally administered ghrelin in the midbrain transected rats. Intracerebroventricular administration of ghrelin similarly increased food intake in the transected and control groups (Figure 1C) ($n = 7$ per group). This finding demonstrates that bilateral midbrain transections specifically blocked peripherally administered ghrelin-induced feeding, but did not affect centrally administered ghrelin-induced feeding. Centrally and peripherally administered ghrelin may therefore stimulate feeding by distinct mechanisms. Midbrain transections severing the ascending efferent fibers of the NTS block feeding reduction of CCK that transmits satiety signals to the brain via the afferent limb of the vagus nerve (Crawley et al., 1984). In contrast, Grill and Smith showed that CCK-induced feeding reduction is still observed in chronic decerebrate rats (Grill and Smith, 1988). We described some differences in the surgery between midbrain transection and chronic decerebration in Supplementary Methods (Grill and Norgren, 1978) (Supplemental Experimental Procedures).

We have already shown the possibility that peripheral ghrelin signals for starvation are transmitted to the NPY neurons of the

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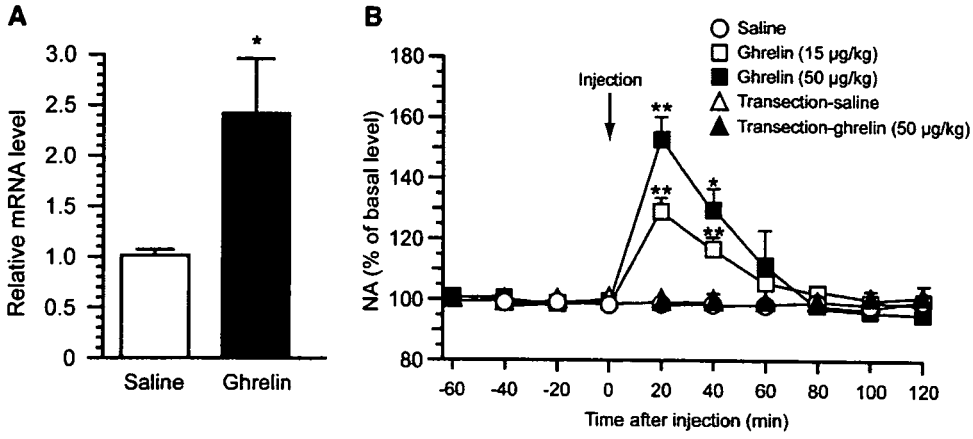

Figure 2. Ghrelin stimulates feeding via the NA system

A) DBH mRNA levels in rats receiving either ghrelin (15 mg/kg, i.v.) or saline. *p < 0.03 versus saline.

B) Effect of intravenous ghrelin on NA levels within the ARC in sham-treated and midbrain-transected rats. NA levels are represented as percentages of the mean concentration of NA in four consecutive dialysate samples taken before ghrelin injection. *p < 0.01, **p < 0.0001 versus sham saline.

Error bars represent the SEM.

ARC at least partially via the vagal afferent pathway (Date et al., 2002). The possibility remains, however, that i.v. administered ghrelin may bind directly to receptors present on neurons in the ARC, as the ARC, situated at the base of the hypothalamus, is incompletely isolated from the general circulation by the blood-brain barrier (Banks and Kastin, 1985; Merchenthaler, 1991). The present study shows that ghrelin-induced feeding was abrogated in transected rats. This result indicates that neural pathways ascending from the NTS may play an important role in the transmission of ghrelin orexigenic signals to the hypothalamus. Conveyed to the NTS, these signals could be relayed to the hypothalamus through other transmitters produced by neurons located in the NTS.

NA system and peripheral ghrelin-induced feeding

Although afferent projections from the NTS to the hypothalamus are not exclusively noradrenergic, the noradrenergic pathway is the major constituent. We here showed that DBH mRNA levels increased significantly in the NTS after ghrelin (15 mg/kg) administration (Figure 2A). Considering that the NTS is the termination area of the vagal afferent fibers that receive vicerosensory information from the gastrointestinal tract, it seems reasonable to expect that peripheral ghrelin induces Fos expression in the NTS. We were not, however, able to detect any increase in the number of Fos-expressing neurons in the NTS (Date et al., 2005). This finding is consistent with previous data from other groups (Wang et al., 2002; Rüter et al., 2003). These results may depend on the fact that peripherally administered ghrelin decreases the firing rate of gastric vagal afferent fibers by binding to its receptor present in the vagal afferent terminals (Asakawa et al., 2001; Date et al., 2002). Thus, these findings suggest that inhibitory signals caused by peripherally administered ghrelin affect DBH expression in the NTS.

Peripherally administered ghrelin and synthetic GHSs primarily activate neurons located in the ARC (Hewson and Dickson, 2000). Most peripheral ghrelin-induced Fos-positive neurons in the ARC express NPY (Wang et al., 2002; Date et al., 2002). NPY and AgRP, which are colocalized in neurons of the ARC, have been implicated in the stimulation of feeding behavior. Pharmacological examinations indicated that centrally administered NPY Y-1 receptor antagonists block the orexigenic effect of ghrelin injected peripherally (Asakawa et al., 2001). Furthermore, Chen et al. demonstrated that peripherally administered ghrelin does not induce food intake in $NPY^{2/2}$, $AgRP^{2/2}$ double-knockout mice (Chen et al., 2004). These findings suggest that the ARC plays a crucial role in regulating peripheral ghrelin signals. In order to examine whether peripherally administered ghrelin affects the release of NA in the ARC, which is not only a noradrenergic terminal area but also a target site of peripheral ghrelin signals, we monitored overflow NA within or near the ARC using a microdialysis system. Overflow NA is thought to include both newly released NA and NA that was not subject to reuptake. Examination of overflow NA within or near the ARC after intravenous administration of 15 and 30 mg/kg ghrelin to sham-treated rats (n = 7 per group) revealed significantly increased NA concentrations within and near the ARC, reaching 129.7 6 4.7% and 152.8 6 7.5% of the control levels, respectively (Figure 2B). Ghrelin administration, however, did not induce NA release in transected rats. Hindbrain noradrenergic neurons innervating the hypothalamus are implicated in mediation of the feeding response to glucose deprivation (Ritter et al., 2001), suggesting that the NA system in the brain contributes significantly to feeding regulation and/or energy homeostasis. The present study demonstrates that ghrelin, an orexigenic signal produced in the periphery, increases DBH mRNA levels in the NTS and increases NA levels within the ARC. These results suggest that noradrenergic inputs, projecting from the hindbrain to the ARC, are critical for the feeding behavior induced by peripheral ghrelin. This study, however, has yet to elucidate whether peripheral ghrelin signals transmitted via the vagal afferent pathway affect the NA system in the ARC or whether ghrelin bound to the receptor present in the area postrema or NTS stimulates it. To clarify this issue, further examinations to evaluate NA overflow in the ARC of vagotomized animals are needed.

CCK, an anorectic peptide produced by the gastrointestinal tract, increases the firing rate of the vagal afferent fibers, and thereby transmits satiety information to the NTS. Recently, Sutton et al. showed that the CCK-induced reduction in feeding is modulated by a melanocortinergic pathway through extracellular signal-regulated kinase signaling in the NTS (Sutton

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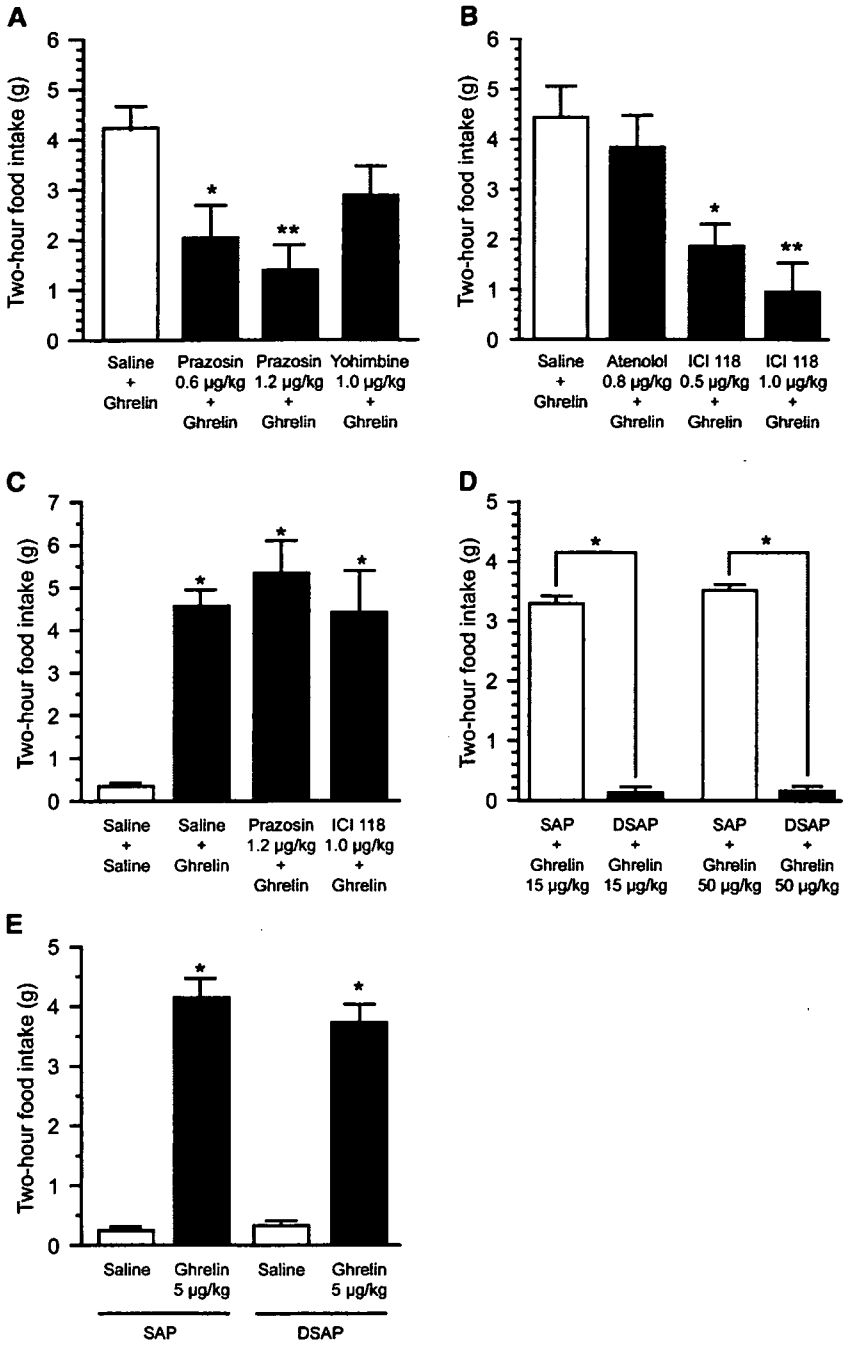

Figure 3. The effects of either pretreatment with adrenoceptor antagonists or disruption of DBH-containing neurons on ghrelin-induced feeding

A) Effect of i.c.v.-administered a_1 or a_2 antagonists on feeding induced by ghrelin (15 mg/kg). *$p < 0.05$, **$p < 0.005$ versus rats given saline plus ghrelin.

B) Effect of i.c.v.-administered b_1 or b_2 antagonists on feeding induced by ghrelin (15 mg/kg). *$p < 0.005$, **$p < 0.001$ versus rats given saline plus ghrelin.

C) Food intake of rats treated with an a_1 or a b_2 antagonist after a single intracerebroventricular administration of ghrelin (5 mg/kg). *$p < 0.0005$ versus saline.

D) Effect of DSAP treatment on ghrelin-induced feeding. *$p < 0.0001$.

E) Food intake of DSAP-treated rats after a single intracerebroventricular administration of ghrelin (5 mg/kg). *$p < 0.0001$.

Error bars represent the SEM.

et al., 2005). There is also a report that fourth ventricular administration of the MC4-melanocortin receptor antagonist SHU9119 completely blocked the peripherally administered CCK-induced decrease in feeding (Fan et al., 2004). These findings indicated that the NTS is crucial for the integration of peripheral ascending signals with descending signals from the hypothalamus that relate to feeding. The precise molecular mechanisms that underlie the effect of peripheral ghrelin signals on NTS function remain to be elucidated. To fully understand the noradrenergic pathway from the NTS to the hypothalamus, further investigations into the identities of the intracellular signaling systems in the NTS that are mobilized by peripheral ghrelin, and the signals from the forebrain that modulate peripheral ghrelin signaling in the NTS are required.

NA can utilize at least four distinct receptor subtypes: a_1, a_2, b_1, and b_2 (O'Dowd et al., 1989). We examined which of these receptors was involved in ghrelin-induced feeding by treatment with an antagonist for each adrenoceptor. Ghrelin-induced feeding was attenuated in rats pretreated with either the specific a_1 antagonist prazosin or the specific b_2 antagonist ICI 118, but not the a_2 antagonist yohimbine or the b_1 antagonist atenolol (Figures 3A and 3B) (n = 7 per group). After injection of these adrenergic antagonists intracerebroventricularly (i.c.v.), rats were observed for behavioral signs of nausea (elongation of the body, gaping, raising the tail, and lowering the belly to the floor), ataxia, sedation, and anxiety (locomotion within the cage and avoidance of the front of the cage). The rats did not exhibit any of these signs during the testing period. We also tested

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the orexigenic effect of centrally administered ghrelin in rats treated with prazosin or ICI 118, as these antagonists may nonspecifically suppress feeding in response to ghrelin. Centrally administered ghrelin increased feeding similarly in the prazosin- and ICI 118-treated groups and the control group (Figure 3C). This result suggests that although NA antagonists specifically suppressed feeding induced by peripherally administered ghrelin, centrally administered ghrelin induces feeding by a mechanism that is independent of the noradrenergic system. Considering that NA excites approximately 50% of the neurons in the ARC, probably due to a direct postsynaptic response through a_1- or b-adrenoceptors (Kang et al., 2000), peripherally administered ghrelin may activate NPY/AgRP neurons in the ARC through the NA system. A recent study also suggested the possibility that the GABAergic system is involved in ghrelin-induced feeding. Cowley et al. showed that ghrelin induced depolarization of ARC NPY neurons and hyperpolarization of ARC POMC neurons using hypothalamic slices (Cowley et al., 2003). Given that NPY/AgRP neurons expressing are GABAergic, central ghrelin may induce the release of GABA from NPY axonal terminals and thereby modulate the activity of postsynaptic POMC neurons.

NA exerts a variety of responses that depend on the type of neurons and the expression of different adrenoceptor subtypes (Nicoll et al., 1990). Infusion of exogenous NA can cause either increases or decreases in food intake (references in Wellman, 2000), which may depend on the site of application or changes in the numbers of adrenoceptors according to the circadian cycle. For example, NA injection into the hypothalamic paraventricular nucleus (PVN) increases feeding through PVN a_2-adrenoceptors, whereas it decreases feeding through PVN a_1-adrennoceptors (Goldman et al., 1985; Wellman et al., 1993). The circadian pattern in the number of a_2-adrenoceptors within the PVN exhibits a sharp increase in a_2-adrenoceptors at the onset of the dark phase, a time when feeding is greatly enhanced. Taken together, it may be difficult to determine whether microinjection of an NA agonist or antagonist into the hypothalamic nuclei results in a physiologically significant effect. Therefore, in the present study, we focused on the role of endogenous NA induced by peripherally administered ghrelin in the control of food intake. We demonstrated that a_1- and b_2-receptor antagonists attenuated feeding induced by ghrelin. This result indicates that a_1- and/or b_2-adrenoceptors in the ARC play an important role in peripheral ghrelin-induced feeding.

To eliminate NA innervation of the ARC, we used DSAP, a monoclonal antibody specific for DBH, the enzyme that converts dopamine into NA, conjugated to saporin (SAP) (Fraley and Ritter, 2003). DSAP, an immunotoxin that allows an antibody against the NA synthetic enzyme DBH to selectively deliver the saporin toxin, can successfully destroy hindbrain neurons that contain DBH (Rinaman, 2003). Bilateral DSAP injections into the ARC induced an approximately 70% reduction in DBH-positive neurons in the NTS in comparison to the number of DBH neurons present in rats treated with an SAP control solution (data not shown). DSAP injections also completely disrupted peripherally administered ghrelin-induced feeding (Figure 3D) (n = 7 per group). We also tested the orexigenic effect of centrally administered ghrelin in the DSAP-treated rats. Centrally administered ghrelin increased feeding similarly in the DSAP-treated group and the control group (Figure 3E) (n = 7 per group). This finding suggests that the noradrenergic system in the ARC is not involved in centrally administered ghrelin-induced feeding.

There are several catecholaminergic neuronal cell groups in the hindbrain. DBH-positive neurons projecting to the hypothalamus are found within the A2 cell group located in the caudal medial and commissural NTS and the A1/C1 cell group located in the ventrolateral medulla (VML). Most NA neurons within the A2 group directly project to the hypothalamus, central nucleus of the amygdala, and bed nucleus of the stria terminalis, whereas the A2 NA neurons also project to these forebrain areas in part via the A1/C1 group. As viscerosensory signals from the gastrointestinal tract are carried to the caudal medial and commissural NTS via the vagal afferent pathway, NA neurons in A2 may be an integral component of the brainstem circuits that mediate ghrelin-induced feeding. Given the projection from the A2 group to the A1/C1 group, these integrative circuits would include a role for NA neurons in the VML. Our findings suggest that NA neurons in the hindbrain are necessary to convey ghrelin-related orexigenic signals to the hypothalamus.

Innervation of NPY neurons by DBH-containing fibers

To examine the effect of peripheral ghrelin signals ascending from the NTS on neurons in the ARC, we investigated DBH innervation and ghrelin-induced Fos expression using unilateral midbrain-transected rats as described previously (Ericsson et al., 1994; Sawchenko, 1988). We compared DBH innervation and Fos expression in the ARC ipsilateral and contralateral to the lesion. Midbrain transections significantly decreased the DBH-imunoreactive innervation ipsilateral to the lesion (Figures 4C and 4D). This finding is consistent with the fact that the ascending catecholamine input to the hypothalamus is largely unilateral. In lesioned rats, peripherally administered ghrelin resulted in a significant increase in Fos expression in the ARC that was contralateral to the lesion (ipsilateral side, 24.3 6 1.8 neurons; contralateral side, 50.6 6 1.9 neurons; $p < 0.001$) (Figures 4A and 4B). When saline was injected i.v. to lesioned rats, Fos expression did not differ significantly on the two sides of the brain (ipsilateral side, 11.6 6 1.3 neurons; contralateral side, 11.9 6 1.0 neurons; $p > 0.1$) (data not shown). These results suggest that the midbrain transections that were effective in reducing DBH-positive innervation blocked the response of neurons in the ARC to peripherally administered ghrelin.

Electron microscope immunohistochemistry demonstrated that NPY-immunoreactive perikaryon and dendritic process often received synapses from DBH-containing axon terminals (Figures 4E–4G). Approximately 40%–50% of hypothalamic NPY neuron innervation arises from catecholaminergic neurons in the hindbrain (Everitt and Hokfelt, 1989). NPY, a potent orexigenic peptide, is thought to be the final mediator of ghrelin feeding signals. To examine the anatomical linkage of NPY neurons, which are activated by ghrelin, with DBH-immunoreactive fibers, we performed immunohistochemistry. Intravenous ghrelin injection significantly increased Fos expression in 53% of the NPY neurons in the ARC (Figure 4H), in accordance with previous studies (Date et al., 2002). Triple labeling immunofluorescence demonstrated that 54% of these NPY neurons in the ARC induced to express Fos by ghrelin treatment were innervated by DBH-immunoreactive fibers (Figure 4I). These results suggest that ghrelin signals activate NPY neurons via the noradrenergic pathway ascending from the NTS to the ARC, resulting in increased feeding.

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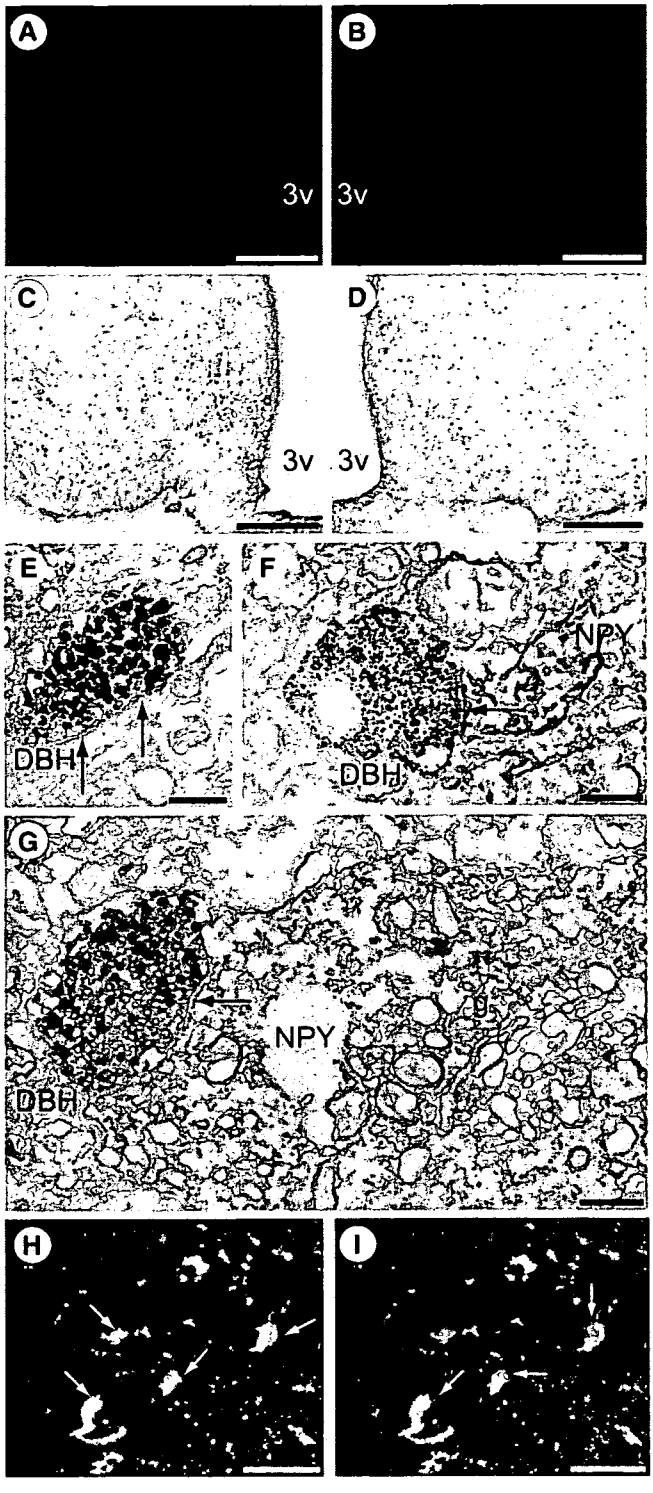

Figure 4. The effect of unilateral midbrain transections on ghrelin-induced Fos expression and activation of NPY neurons by ghrelin via the catecholaminergic pathways

A) DBH-immunoreactive fibers project to the ARC contralateral to the lesion.

B) DBH-immunoreactive innervation ipsilateral to the lesion decreases as compared to that on the contralateral side.

C) Peripherally administered ghrelin (15 mg/kg) induces Fos protein expression contralateral to the lesion.

D) Ghrelin-induced Fos expression ipsilateral to the lesion decreases as compared to that on the contralateral side.

E) DBH-immunoreactive axon terminal making synapses with immunonegative dendritic process (arrow, synapse).

F) DBH-immunoreactive axon terminal making synapses with NPY-immunoreactive dendritic process (arrow, synapse).

G) DBH-immunoreactive axon terminal making synapses with NPY-immunoreactive perikaryon (arrow, synapse).

H) Intravenous administration of ghrelin (15 mg/kg) upregulates Fos expression in NPY neurons of the ARC (arrows) (blue, Fos; green, NPY).

I) Fifty-four percent of ghrelin-activated NPY neurons receive projections from DBH-immunoreactive fibers (arrows) (blue, Fos; green, NPY; red, DBH). g; Golgi apparatus; 3v, third ventricle. The scale bar represents, respectively, 100 mm (A and B), 200 mm (C and D), 400 nm (E–G), and 50 mm (H and I).

The present study focused on the hypothesis that the neural pathway from the brainstem to the ARC plays a crucial role in transmitting peripheral ghrelin signals and peripheral ghrelin regulates feeding at least partially via NA-mediated neuronal transmission. Although the central circuits for feeding may have been altered in response to bilateral midbrain transections or DSAP treatment, the results shown here are consistent with the hypothesis. The hypothesis, if correct, is a counterpoint to the most widely accepted model for neuroendocrine energy balance regulation. We have shown previously that peripheral ghrelin-induced feeding is absent in either vagotomized or capsaicin-treated rats. We showed here that ghrelin-induced feeding is also canceled in midbrain transected rats. Thus, it may seem that peripheral ghrelin signals for starvation are transmitted to the hypothalamus only via the vagal afferent pathway and neural pathways from the NTS. However, we have to consider the possibility that vagotomy and/or midbrain transections affect several peripheral substances as well as central circuits relative to feeding. In addition, the present study has yet to address the direct relationship between peripheral ghrelin signals via the vagal afferent pathway and the NA system in the ARC. Taken together, it may be difficult to assert that peripheral ghrelin signals are transmitted only via the neural pathways. We, therefore, think that the humoral pathway and the neural pathway are important routes to convey peripheral energy balance information to the brain. Very recently, we found that peripherally administered leptin decreased 2 hr and 4 hr food intake in vagotomized, midbrain transected, and sham-operated rats, and the leptin-induced reduction in feeding was less pronounced in vagotomized and transected rats than in the sham-operated rats (unpublished data). These findings suggest that the vagal afferent pathway and/or the ascending efferent pathway from the brainstem to the hypothalamus are necessary elements for the effectual action of leptin on feeding and energy homeostasis. Feeding is regulated by a complicated interaction of many orexigenic and anorectic signals; sophisticated interactions between humoral pathways and neural pathways may be necessary to maintain energy homeostasis. We have shown that the central noradrenergic system is a candidate to mediate peripheral ghrelin signals. Although the pathways linking peripheral ghrelin to NA transmission are likely to be more complicated given the remarkable number of signals that provide input to the NTS and ARC, we believe that this study provides an important clue to understanding the feedback loops linking the brain and peripheral tissues in the control of feeding and energy homeostasis.

Experimental procedures

Experimental animals

We maintained male Wistar rats (Charles River Japan, Inc.), weighing 255.9 ± 2.0 g, under controlled temperature and light conditions (0800–2000 hr light).

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For feeding and microdialysis experiments, an intravenous cannula was implanted into the right jugular vein of each rat under anesthesia. We performed unilateral or bilateral midbrain transections 5 days after implantation, as described details in the Supplemental Experimental Procedures. To confirm that the transection surgeries were successful, the brains were immunostained using an anti-DBH antiserum diluted 1:1000 (Chemicon International, Inc.) by the avidin-biotin complex method (Date et al., 1999) after the feeding tests were completed (Figure S3A). To facilitate the penetration of a microdialysis probe, a guide cannula (500 mm outside diameter; AG-12, Eicom) was stereotaxically implanted 1.0 mm above the ARC (0.2 mm lateral to the midline, 2.4 mm caudal to the bregma, and 9.0 mm ventral to the dura), fixed to the skull with acrylic dental cement, and sealed with a dummy cannula (350 mm external diameter; AD-12, Eicom). To inject adrenergic receptor antagonists into the rats, we implanted intracerebroventricular cannulae into the lateral cerebral ventricle. To block noradrenergic innervation of the ARC, we microinjected either a SAP-conjugated DBH-specific mouse monoclonal antibodies (DSAP; Advanced Targeting Systems; 42 ng/0.2 ml in phosphate buffer [pH 7.4], n = 6) or SAP-conjugated normal mouse IgG (SAP control solution) (Advanced Targeting Systems; 8.82 ng/0.2 ml, n = 6) bilaterally into the ARC (Ritter et al., 2001). Only animals exhibiting progressive weight gain after these surgeries were used in subsequent experiments. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Food intake

First, rat ghrelin (Peptide Institute, Inc.) at 0.1–100 mg/kg (100 ml), or saline alone (100 ml) was administered i.v. at 1000 hr to ad libitum-fed rats that had undergone a sham operation (n = 7 per group). Second, rat ghrelin (15 or 50 mg/kg [100 ml]) was administered i.v. to rats that had undergone bilateral midbrain transection. Third, rat ghrelin (15 or 50 mg/kg [100 ml]) was administered i.v. to rats that had been treated with either DSAP or SAP control solution. Fourth, ghrelin (5 mg/kg [10 ml]) was injected i.c.v. at 1000 hr into rats that had undergone bilateral midbrain transections or sham operations, or into rats that had been treated with either DSAP or SAP control solution. The dose of centrally administered ghrelin (5 mg/kg) is often used as a standard while investigating the effect of i.c.v.-administered ghrelin on food intake under various conditions (Nakazato et al., 2001; Kamegai et al., 2000; Toshinai et al., 2003). Thus, this dosage is recognized as the most appropriate in constantly inducing food intake when administered i.c.v. Therefore, this dosage was also selected as a standard to evaluate i.c.v. administered ghrelin induced feeding. After ghrelin injection, rats were immediately returned to their cages. Two hour food intake was then measured.

Quantitative RT-PCR

Two hours after intravenous administration of ghrelin (15 mg/kg) or saline to rats, total RNA was extracted from the NTS using TRIZOL Reagent (Invitrogen Corp.). Quantitative RT-PCR for DBH was conducted with a LightCycler system (Roche Diagnostics) using a LightCycler-Fast Start DNA Master SYBR Green I kit (Roche) and the following primer set for rat DBH: 5'-CTAGGGCCCTGGGCGCCAAGGCATT-3' and 5'-GCCAGAGGAGTCGCG CCGGCCTT-3'. Known amounts of DBH cDNA were used to obtain a standard curve. Rat rRNA levels were also measured as an internal control.

Microdialysis

One week after midbrain transection, the rats were lightly anesthetized with isoflurane, and the dummy cannula was replaced with a microdialysis probe. The tip of the microdialysis probe, covered with hollow fibers (1.0 mm in length, 220 mm external diameter, regenerated cellulose membrane with a molecular weight cutoff of 48 kDa; Eicom), was set to extend 1 mm beyond the guide cannula to reach the ARC. Microdialysis was performed under free-moving conditions. A microinfusion pump was used to continually perfuse the probe with modified physiological Ringer's solution (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$ [pH 6.5]) at a constant flow rate of 1 ml/min. To measure NA, chromatographic analysis of dialysates was carried out by HPLC with electrochemical detection as described previously (Ishizuka et al., 2000). The perfusate from the ARC was automatically injected into the HPLC every 20 min. After a 3 hr stabilization period, baseline NA levels were assessed in four consecutive dialysate samples. At the end of each experiment, rats were sacrificed with an overdose of pentobarbital sodium; the brains were then fixed in 10% neutral buffered formalin. Placement of the microdialysis probe was verified histologically in 40-mm cresyl violet-stained coronal sections (Figure S3B).

Effect of adrenoceptor blockers on ghrelin-induced feeding

At 0930 hr, rats were i.c.v. administered either vehicle alone (saline, n = 6) or one of the specified adrenergic receptor antagonists: prazosin (selective a_1 antagonist: 0.6 or 1.2 mg/kg, n = 6 each) (Sigma Chemical Co.), yohimbine (selective a_2 antagonist: 1.0 mg/kg, n = 6) (Sigma), atenolol (selective b_1 antagonist: 0.8 mg/kg, n = 10) (Sigma), or ICI 118 (selective b_2 antagonist: 0.5 or 1.0 mg/kg, n = 6 each) (Sigma). Thirty minutes after adrenergic receptor antagonist injection, ghrelin (50 mg/kg) was administered intraperitoneally to rats; 2 hr food intake was measured. We also tested the orexigenic effect of centrally administered ghrelin in rats that had been injected with prazosin or ICI 118. Thirty minutes after prazosin (1.2 mg/kg, n = 6) or ICI 118 (1.0 mg/kg, n = 6) injection, ghrelin (5 mg/kg) was administered i.c.v. to rats; 2 hr food intake was measured. The rats fasted between the two injections.

Immunohistochemistry

Ghrelin (15 mg/kg) or saline was injected i.v. into rats 90 min before transcardial perfusion with fixative containing 4% paraformaldehyde (n = 5 per group). The brains of animals were then cut into 20-mm thick sections. The sections were first incubated with anti-c-Fos antiserum (1:500, Santa Cruz Biotechnology), and then with Alexa Flour 350-conjugated donkey anti-goat IgG (Molecular Probes, Inc.). Next, samples were incubated with anti-NPY antiserum (1:500, ImmunoStar, Inc.), then with Alexa Flour 488-conjugated chicken anti-rabbit IgG (Molecular Probes, Inc.). Finally, the samples were incubated with anti-DBH antiserum (1:1,000, Chemicon International, Inc.), then with Alexa Flour 568-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). Samples were then observed under a BH2-RFC microscope (Olympus Corp.). We counted the number of Fos-immunoreactive cells in the bilateral ARCs (bregma: 22.30 to 23.30 from Paxinos and Watson's rat brain atlas). Sections from unilaterally transected rats were also incubated with anti-DBH antiserum, and then with Alexa Flour 568-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). A significant (>60%) depletion of DBH-immunoreactive fibers was determined by semi-quantitative comparison of the strength of the DBH-positive innervation of the ARC ipsilateral and contralateral to the lesion by two independent observers (Sawchenko, 1988). Sections from unilaterally transected rats were incubated with anti-c-Fos antiserum (Santa Cruz Biotechnology), and then stained by the avidin-biotin complex method (Date et al., 1999). The number of Fos-immunoreactive cells was compared in the ARC ipsilateral and contralateral to the letion. Fos-expressing cells of the ARC in a 0.7-mm right triangle (0.245 mm^2) were counted in every fifth section (ten tissue sections per rat) using a cell-counting program written for NIH Image (v1.62; NIH).

Electron microscope immunohistochemistry

Three Wistar rats were perfused as described above. The brain was cut into 30–40 mm thick sections using an Oxford vibratome (Oxford Instruments). Electron microscope immunohistochemistry was performed using anti-NPY antiserum and anti-DBH antiserum as described previously (Toshinai et al., 2003).

Statistical analysis

We analyzed groups of data (means ± SEM) using analysis of variance (ANOVA) and post hoc Fisher tests. p values less than 0.05 were considered to be significant (two-tailed tests).

Supplemental data

Supplemental Results, Supplemental Experimental Procedures, Supplemental References, and three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/4/ - - - /DC1/.

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Homocysteine-induced endoplasmic reticulum protein (Herp) is up-regulated in sporadic inclusion-body myositis and in endoplasmic reticulum stress-induced cultured human muscle fibers

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Abstract

Herp is a stress-response protein localized in the endoplasmic reticulum (ER) membrane. Herp was proposed to improve ER-folding, decrease ER protein load, and participate in ER-associated degradation (ERAD). Intra-muscle-fiber ubiquitinated multiprotein-aggregates containing, among other proteins, either amyloid-b (Ab) or phosphorylated tau are characteristic of sporadic inclusion-body myositis (s-IBM). ER stress and proteasome inhibition appear to play a role in s-IBM pathogenesis. We have now studied Herp in s-IBM muscle fibers and in ER-stress-induced or proteasome-inhibited cultured human muscle fibers. In s-IBM muscle fibers: (i) Herp was strongly immunoreactive in the form of aggregates, which co-localized with Ab, GRP78, and b2 proteasome subunit; (ii) Herp mRNA and protein were increased. In ER-stress-induced cultured human muscle fibers: (i) Herp immunoreactivity was diffusely increased; (ii) Herp mRNA and protein were increased. In proteasome-inhibited cultured human muscle fibers: (i) Herp immunoreactivity was in the form of aggregates; (ii) Herp protein was increased, but its mRNA was not. Accordingly, in s-IBM muscle fibers: (i) increase of Herp might be due to both ER-stress and proteasome inhibition; (ii) co-localization of Herp with Ab, proteasome, and ER-chaperone GRP78 could reflect its possible role in processing and degradation of cytotoxic proteins in ER.

Keywords: inclusion-body myositis; homocysteine-induced endoplasmic reticulum protein; endoplasmic reticulum stress; unfolded/ misfolded proteins; proteasome inhibition; amyloid-b.
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The endoplasmic reticulum (ER) functions include processing, folding, and exporting of newly synthesized proteins into the secretory pathway (Ellgaard and Helenius 2001). An efficient system of molecular chaperones in the ER is required to assure proper folding of misfolded abnormal and native proteins (Kaufman 1999). Unfolded proteins accumulating in the ER lead to ER-stress (Rutkowski and Kaufman 2004; Shen et al. 2004). This elicits the unfolded protein response (UPR), a functional mechanism by which cells attempt to protect themselves against ER-stress (Mori 2000; Rutkowski and Kaufman 2004). The UPR involves: (i) attenuating translation to reduce protein overload and subsequent accumulation of unfolded proteins (Mori 2000; Liu and Kaufman 2003); (ii) transcriptional induction of ER

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Abbreviations used: Ab, amyloid-b; AbPP, amyloid-b precursor protein; AD, Alzheimer disease; ATF4, activating transcription factor 4; BiP/GRP78, immunoglobulin heavy chain-binding protein/glucose-regulated protein 78; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); Herp, homocysteine-induced ER protein; s-IBM, sporadic inclusion-body myositis; ULD, ubiquitin-like domain; UPR, unfolded protein response; XBP-1, X-box-binding protein 1.

