Luman is capable of binding and activating transcription from the unfolded protein response element

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Abstract

Luman (or LZIP, CREB3) is a transcription factor with an endoplasmic reticulum (ER)-transmembrane domain. Due to its structural similarities with ATF6, it is thought that Luman might also be involved in cellular stress responses. Here we report that Luman can bind and activate transcription from the consensus unfolded protein response element (UPRE). Mutations that disrupted the binding of Luman to the UPREs impaired its ability to activate transcription from these sites. Overexpression of Luman stimulated transcription of EDEM, a downstream effector of the mammalian unfolded protein response involved in ER-associated degradation (ERAD). Unlike ATF6, however, Luman was not activated by proteolytic cleavage in response to endoplasmic reticulum stressors such as tunicamycin and thapsigargin. These results suggest that the activation of ERAD by Luman is likely through a pathway different from the common ER stress response, and that additional factor(s) are required for the activation of this Luman-mediated pathway.
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Keywords: Unfolded protein response; Endoplasmic reticulum stress response; Luman/LZIP/CREB3; ER degradation enhancing α-mannosidase-like protein; ER-associated degradation

Luman (also called LZIP or CREB3) [1–3] is a cellular transcription factor that was identified through its interaction with herpes simplex virus (HSV)-related host cellular factor (HCF)-1 (or C1) [4,5]. It has subsequently been shown that VP16 mimics Luman [1–3,6–9] as well as another cellular protein Zhangfei [10] in its ability to bind HCF-1, leading to the hypothesis that Luman may be involved in cellular signaling events associated with HSV-1 latency/re-activation [6].

Luman is a basic domain leucine zipper (bZIP) transcription factor that is able to bind consensus DNA elements such as CRE, AP-1, and C/EBP [1]. Although

Luman mRNA is ubiquitous, endogenous Luman protein has only been found in the trigeminal ganglional neurons. In these cells and in transfected cell lines, Luman is found primarily associated with the endoplasmic reticulum (ER) membrane [6]. Extensive similarities between Luman and ATF6 have led us to propose that Luman undergoes a proteolysis event similar to the regulated intramembrane proteolysis (RIP) process of SREBPs and ATF6, two well-characterized ER-associated bZIP transcription factors (reviewed in [11–14]). As a type II transmembrane protein like ATF6, all known functional domains of Luman, including the activation domain, HCF-1-binding motif, and bZIP region, are located in the N-terminal region and hence, on the cytoplasmic side of the ER membrane. In response to yet unknown signal cues, the N-terminus of

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Luman may be liberated from the ER by proteolytic cleavage and translocates to the nucleus, leading to the activation of its target genes [6,15].

ATF6, a bZIP transcription factor with structural similarities to Luman, is a key player in the mammalian unfolded protein response (UPR) [16–18]. The UPR is a cellular stress response triggered by an excess of mis-folded/unfolded proteins within the ER. Accumulation of improperly folded proteins activates three UPR pathways; ATF6, PERK-ATF4, and IRE1-XBP1 (reviewed in [13,14]). UPR-related transcription factors are activated and bind to specific DNA elements, such as the ER stress response element (ERSE) [16,19] and the unfolded protein response element (UPRE) [20,21], leading to the expression of target genes. Although cross-talk occurs across these pathways, XBP1 is currently the only known transcription factor to activate UPRE sequences [22,23].

This report identifies a second protein, Luman, which can bind and activate transcription from UPRE consensus sequences. In transient transfection experiments Luman stimulated transcription of ER degradation enhancing α-mannosidase-like protein (EDEM), a component of the ER-associated degradation (ERAD) machinery of the mammalian UPR [21,24–26]. However, unlike ATF6, Luman was not activated by proteolytic cleavage in response to ER stressors such as tunicamycin and thapsigargin. These results suggest that the potential activation of ERAD by Luman is different from the currently known ER stress response.

Materials and methods

Plasmid constructs. The plasmids pcLuman, pcLuman1–215, and pcLuman53–371 were previously described [3]. LumanΔ123–186 was created using the QuikChange II Mutagenesis Kit (Stratagene) from the construct pcLuman using primers 1 and 2 (in Table 1).

The p3× ERSE-Luc plasmid was constructed by inserting a fragment of the GRP78 promoter (−378 to +13 bp) containing three ERSE sequences [16] into pGL3-basic (Promega) by PCR using HEK-293 genomic DNA as template and oligonucleotides 3 and 4 (in Table 1) as primers.

Oligonucleotides 5–7 (in Table 1) were used to construct UPRE mutant reporters based on the p5× UPRE-Luciferase plasmid [20] (gift from Ron Prywes, Columbia University), which contains the human *c-fos* minimal promoter linked to the firefly luciferase gene, to create p1× UPRE wild-type and mutant luciferase reporters.

Cell culture, transfection, and luciferase assays. HEK 293 cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum. Cells were transfected by the calcium phosphate precipitation method as previously described [6,10]. At 28 h post-transfection, cells were treated with 2 μg/mL tunicamycin (Sigma) or 100 nM thapsigargin (Sigma) for 16 h. Dual luciferase assays were performed according to the manufacturer's instructions (Promega) using a Turner TD-20e Luminometer and relative luciferase activity (firefly luciferase/*Renilla* luciferase) was calculated. Assays were independently repeated at least three times. Data are shown with standard error.

Electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides 8–14 (in Table 1) were end-labeled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase. Bacterially expressed GST and GST 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 poly(dI/dC) in a buffer containing 20 mM Hepes (pH 7.9), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 5% glycerol, and 1.5 mg/mL BSA. DNA-binding proteins were resolved on a 4% non-denaturing polyacrylamide gel and visualized with a Typhoon 9400 PhosphorImager (Amersham Biosciences). Images were analyzed using IMAGEQUANT TLv2003.01 (Molecular Dynamics).

RNA isolation, cDNA preparation, and real-time PCR analysis. Cells were transfected and treated with tunicamycin or thapsigargin as described above. Total RNA was extracted using the RNeasy kit (Qiagen); cDNA was made using the Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen).

Real-time PCR analysis was conducted using the gene-specific Taqman Assay-On-Demand system (Applied BioSciences) according to the manufacturer's instructions. Samples were analyzed during the last minute of the primer annealing/elongation step of every cycle. Data were analyzed using ABI 7300 System Sequence Detection Software v1.2.2 (Applied BioSciences). The presented data are an average of three independent trials performed in duplicate.

Table 1
Oligonucleotides used in this study

No.	Purpose	Used in construct	Sequence
1	PCR	pcLumanΔ123–186	5′-GCTAGGCTAGTACTGGCCCAGAATATGGGG
2	PCR	pcLumanΔ123–186	5′-CCCCATATTCTGGGCCAGTACTAGCCTAGC
3	PCR	p3× ERSE-Luc	5′-CGGGGTACCGGTCACTCCTGCTGGACCTA
4	PCR	p3× ERSE-Luc	5′-CCGCTCGAGACCTCACCGTCGCCTACTC
5	Linker	p1× UPRE	5′-TCGAGACAGGTGC**TGACGTGG**CGATTCC
6	Linker	p1× UPRE mutant 1	5′-TCGAGACAGGTGC**TGT<u>C</u>GTGG**CGATTCC
7	Linker	p1× UPRE mutant 2	5′-TCGAGACAGGTGC**TGT<u>T</u>GTGG**CGATTCC
8	EMSA	CRE	5′-CTAGCCCGGTGACGTCATCGCA
9	EMSA	NF-κB	5′-CTAGCTATGGGGAMTTTCCGCTA
10	EMSA	CHOP-C/EBP	5′-CTAGCRRRTGCAATMCCCGGCA
11	EMSA	ERSE	5′-CTAGCCCTTCA**CCAAT**CGGCGGCCT**CCACG**ACGGA
12	EMSA	UPRE	5′-CTAGCACAGGTGC**TGACGTGG**CGATTCA
13	EMSA	UPRE mutant 1	5′-CTAGCACAGGTGC**TG<u>T</u>CGTGG**CGATTCA
14	EMSA	UPRE mutant 2	5′-CTAGCACAGGTGC**TG<u>TT</u>GTGG**CGATTCA

Bold letters indicate consensus core sequence of the element; underlined letters denote mutated nucleotides.

Western blotting. Cells were harvested and lysed in 2× SDS-sample buffer and subjected to SDS–PAGE followed by Western blotting. A Luman-specific antibody (M13) [6] 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). Data are representative of two independent experiments.

Results

Luman activates transcription from UPRE consensus sequences

Because of the structural similarities between Luman and ATF6, and their common transmembrane localization on the ER membrane, we hypothesized that Luman might also be involved in the UPR. Genes regulated by the UPR contain within their promoters either of the two novel enhancer sequences, ERSE and UPRE (Fig. 1A). We sought to investigate whether Luman could activate transcription from these elements in reporter assays.

Luman constructs (pcLuman or pcLuman1–215) were cotransfected into 293 cells with the 5× UPRE-Luciferase reporter. As Fig. 1B shows, full-length Luman was found to activate the UPRE reporter 10 times above the background level, while the activated nuclear form of Luman, Luman1–215, activated 30 times over background. Luman did not demonstrate transactivation activity on the p3× ERSE-Luc reporter plasmid (Fig. 1C).

We verified that reporter activity was dependent upon the transactivation potential of Luman by showing that transfection of cells with a construct lacking the activation domain of Luman (Luman53–371) failed to produce significant UPRE reporter activity (Fig. 2A). Western blotting assays with the cellular lysates showed that equivalent levels of Luman1–215 and Luman53–371 were expressed in the transfected cells (Fig. 2B). Although full-length Luman was expressed five times higher than the activated nuclear form of Luman (Luman1–215), it was three times less effective in activating the 5× UPRE reporter. As well, the normalized transactivation activity of the full-length Luman was not significantly higher than that of the Luman protein without activation domain, Luman53–371. We therefore conclude that Luman is capable of transactivating the promoters containing the UPRE and that this requires untethering of Luman from its ER membrane-anchoring site.

Luman1–215 cannot activate transcription from mutant UPRE sequences

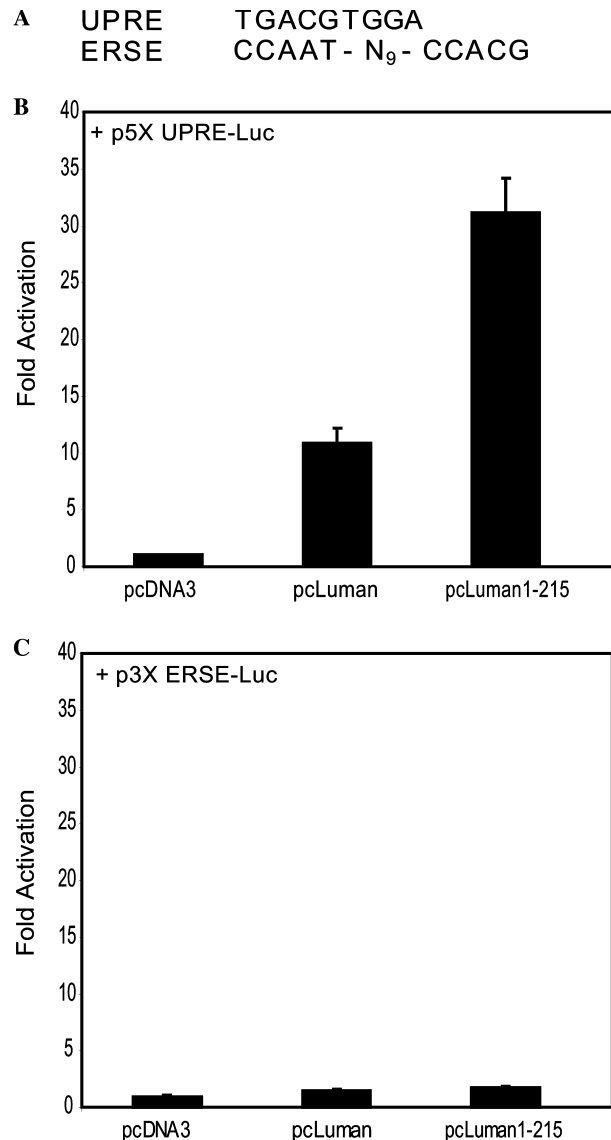To ensure that the observed activation of the UPRE-luciferase reporter was mediated specifically through the

Fig. 1. Luman specifically activates transcription from UPRE but not ERSE consensus sequences. 293 cells were cotransfected with 1 μg of Luman plasmid and 1 μg the reporter plasmids, (A) Consensus sequences of UPRE and ERSE. (B) p5× UPRE-luciferase or (C) p3× ERSE-Luc, and pRL-SV40 (30 ng). The parental vector of the Luman plasmids, pcDNA3, was used as a control. Dual luciferase activities were measured 44 h post-transfection. Values are normalized to *Renilla* luciferase before being referenced to the control.

UPRE sequence, we introduced single or double point mutations in the UPRE site and tested the ability of Luman to activate transcription from the mutated sequences (Fig. 3A). 293 cells were transfected with Luman1–215, and either a wild type or a mutant 1× UPRE-Luciferase reporter plasmid. As shown in Fig. 3B, while Luman1–215 was able to activate the wild-type UPRE reporter approximately 20-fold over the background level, its transactivation of UPRE mutant reporters was severely reduced.
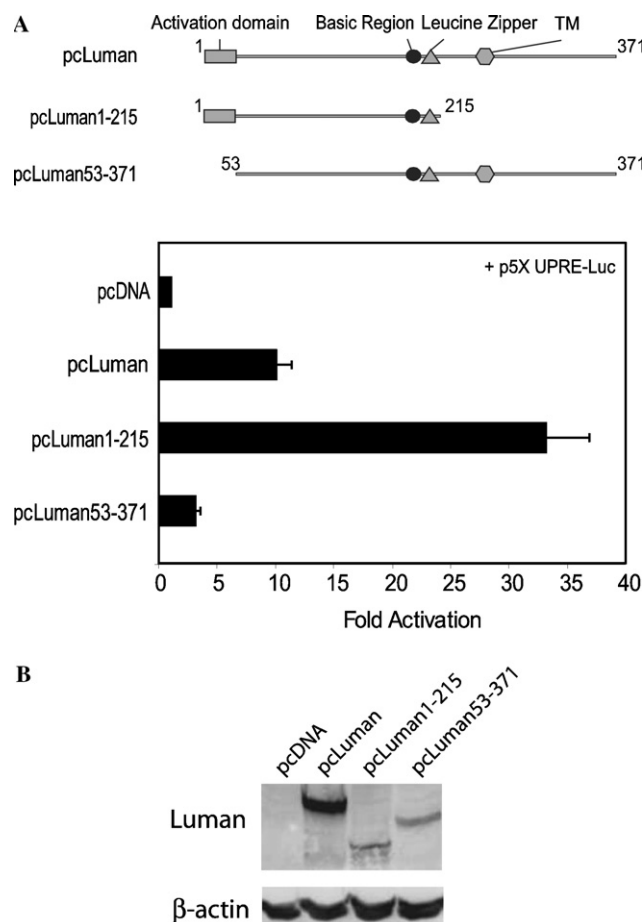
Fig. 2. Activation of the UPRE by Luman1–215 requires the Luman activation domain. 293 cells were transfected with 1 μg of each Luman plasmid along with the reporter plasmids, p5× UPRE-luciferase (1 μg) and pRL-SV40 (30 ng). The parental vector, pcDNA3, was used as a control. (A) Dual luciferase activities were measured 44 h post-transfection. Values are normalized to *Renilla* luciferase before being referenced to the control. TM, transmembrane domain. (B) Cells were lysed 44 h post-transfection and subjected to SDS–PAGE and Western blotting. Primary antibody was a 1:1000 dilution of either a Luman antibody M13 or a β-actin antibody (AC-15, Sigma); secondary antibody was a 1:30,000 dilution of HRP-anti-rabbit or HRP-anti-mouse, respectively.

Luman specifically binds the UPRE consensus sequence in vitro

To examine if the loss of activation of the mutant UPRE reporters was due to the disruption of Luman binding to the UPRE sites, we conducted an electrophoretic mobility shift assay (EMSA) using recombinant Luman protein and the UPRE oligonucleotides (Fig. 3A), along with CRE, C/EBPβ, NF-κB, and ERSE as controls. As expected we found that Luman bound both the CRE and C/EBPβ oligonucleotides but not the NF-κB (Fig. 4). Luman was able to bind the UPRE oligonucleotide, supporting the hypothesis that the transcriptional activation seen in Fig. 1B was due to a physical interaction between Luman and the UPRE site.
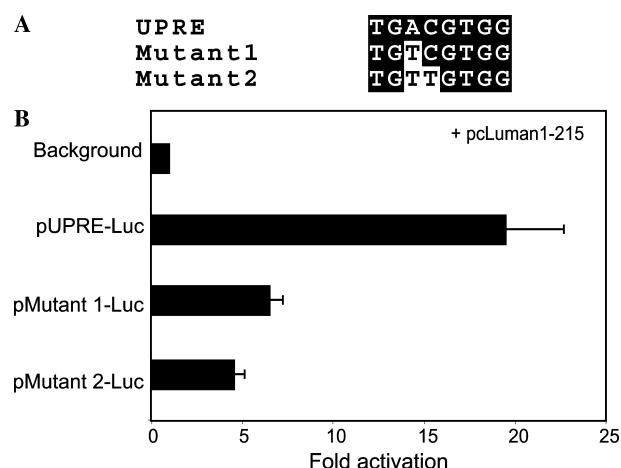

Fig. 3. Luman1–215 was unable to activate transcription from mutant UPRE sequences. 293 cells were transfected with 1 μg Luman1–215, 1 μg of the reporter plasmids, pUPRE-Luc, pMutant1-Luc or pMutant2-Luc, and 30 ng pRL-SV40. Cotransfection of pUPRE-Luc and pcDNA3 was used as a background control. Values are normalized to *Renilla* luciferase before being referenced to the control.
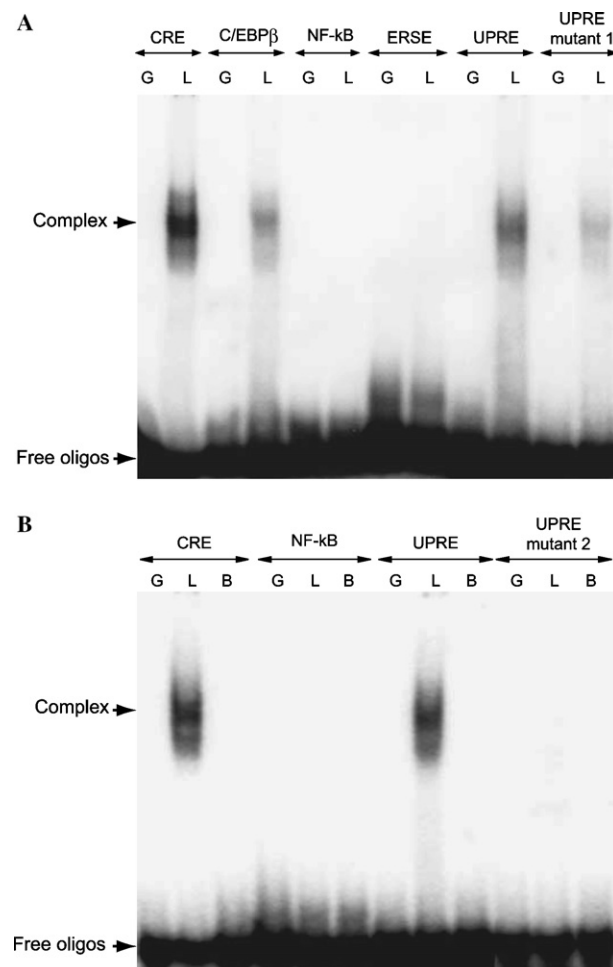

Fig. 4. Luman specifically binds the consensus UPRE. Equal amounts of purified GST (G), GST-Luman (L), and GST-Luman Δ123–186 (B) proteins were incubated with the indicated double-stranded probes in EMSAs for 25 min and separated on 4% non-denaturing PAGE.

Consistent with the results of report assays (Fig. 3B), Luman either did not bind the UPRE mutant oligonucleotides (mutant 2) or bound very poorly (mutant 1). Luman was not found to bind ERSE as we expected (Fig. 3A), since Luman did not activate the ERSE promoter in the reporter assay (Fig. 1C) and it would require NF-Y proteins to form a DNA-binding complex like ATF6 and XBP1 [23]. Luman Δ123–186, which lacks the bZIP DNA-binding domain, did not bind any of the DNA elements (Fig. 4B).

Expression of Luman1–215 induced EDEM mRNA levels

Since Luman was capable of activating transcription from UPRE sites in reporter assays, we were interested to see if expression of Luman could trigger the unfolded protein response in the cell. Four target genes (EDEM, GRP78, XBP1, and CHOP) known to be involved in the UPR [13,14] were chosen for investigation. Tunicamycin and thapsigargin, two UPR-inducing reagents, were used as positive controls. Real-time PCR quantitation assays were performed using cell samples treated with these drugs or transfected with the activated N-terminal form of Luman (Fig. 5). We found that Luman induced transcription of EDEM by ∼2-fold, and to approximately 50% of the level observed with tunicamycin treatment. No effect was observed for the other UPR target genes.

Luman is apparently not activated by ER stress inducers

The data described above suggested to us that like ATF6 Luman might be involved in ER stress response. We were therefore interested to know whether induction of ER stress could trigger the proteolytic processing of Luman as in the case with ATF6, thereby enhancing the activation potential of Luman. Full-length and the activated N-terminal form of Luman were transfected into 293 cells, along with the 5× UPRE-Luciferase reporter. As Fig. 6A shows, three ER stressors, tunicamycin, thapsigargin, and DTT, induced over 30-fold activation of the UPRE reporter (blank columns, Fig. 6A). However, these treatments did not appear to have synergistically enhanced the reporter activation by the full-length Luman or Luman1–215. Instead they only produced an additive effect on UPRE activation. The difference between pcLuman and pcDNA-transfected samples within each treatment was not significantly greater than that of the untreated sample (e.g., Fig. 6A, compare the difference between the gray and blank columns in tunicamycin group to the untreated group). To investigate whether ER stress treatment triggered the proteolytic processing of Luman, we performed Western blotting analysis of the transfected cell lysates (Fig. 6B). No apparent processing of Luman was observed in the ER stressor-treated samples.
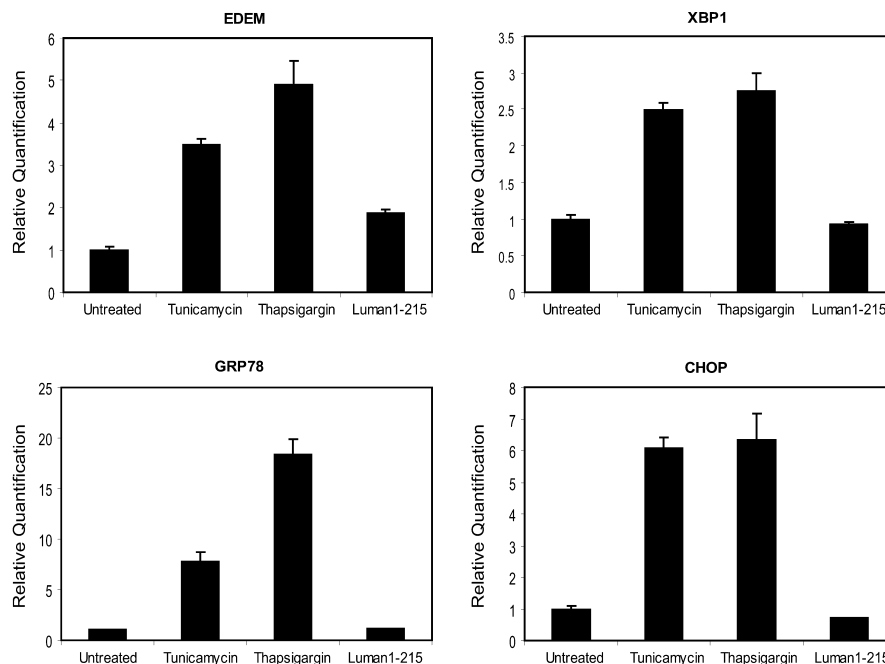

Fig. 5. Luman induces transcription of EDEM. 293 cells were transfected with 2 μg pcLuman1–215 or pcDNA3 (in triplicate). One replicate served as an untreated sample while the others were treated with 2 μg/mL tunicamycin or 100 nM thapsigargin for 16 h to activate the UPR. Data were normalized with GAPDH as an endogenous control. The relative quantification (RQ) values were calculated using the ABI 7300 System Sequence Detection Software v1.2.2 (Applied BioSciences) with the untreated sample as the calibrator (RQ = 1).
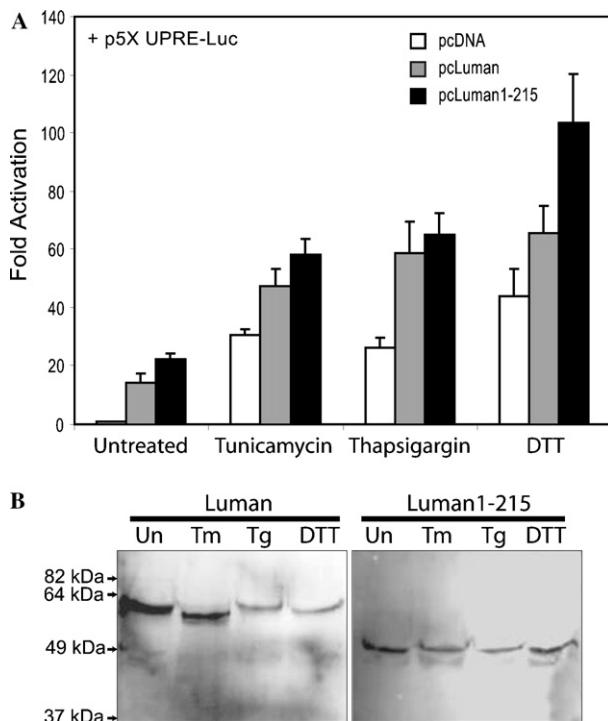
Fig. 6. Common ER stressor treatments have no effect on Luman's transactivation potential or cleavage. (A) 293 cells were transfected with 1 μg of each Luman plasmid along with the reporter plasmids, p5× UPRE-luciferase (1 μg) and pRL-SV40 (30 ng). The parental vector, pcDNA3, was used as a blank control. Cells were treated with 2 μg/mL tunicamycin, 100 nM thapsigargin or 3 mM DTT for 16 h prior to lysis. Dual luciferase activities were measured 44 h post-transfection. Values are normalized to *Renilla* luciferase before being referenced to the control. (B) The cell lysates from the above transfections were analyzed by Western blotting using 1:5000 dilution of an N-terminal Luman antibody as the primary and 1:30,000 dilution of HRP-anti-rabbit antibody as the secondary antibody. Lysates from pcDNA3-transfected cells did not produce any bands on the Western blot (result not shown). *Abbreviations:* Un, untreated; Tm, tunicamycin; and Tg, thapsigargin. *Note.* The change of the apparent molecular weight of Luman in Tm-treated sample is believed to be caused by deglycosylation by Tm [15].

Discussion

Luman has been implicated in cellular signaling processes related to herpesvirus re-activation from latency because of the mimicry by HSV protein VP16 [2,3,6,7]; however, its downstream target genes have not yet been identified. Here we report that Luman can specifically bind and activate transcription from UPREs but not ESREs, and that expression of Luman induced transcription of endogenous EDEM, a protein required for ER-associated degradation of luminal glycoproteins and a key effector protein in the UPR [24].

Until now XBP1 was the only transcription factor known to directly induce transcription from UPRE-containing promoters [21–23,27]. XBP-1 itself is activated by post-transcriptional processing by IRE1 and can sub-

sequently induce the expression of EDEM [22]. Although Luman can potentially activate ERAD, it is unclear whether Luman is related to the known UPR pathways, since its transactivation activity on UPRE reporters and the presumed proteolytic processing were not seemingly affected by ER stressors (Fig. 6). The inactivity of Luman on ERSE-containing promoters (Fig. 1) also separates its role from ATF6. It may be argued that the activation of Luman by ER stress requires the involvement of other cellular factors specific to certain cell types. To date, the induction of Luman processing by ER stress is not observed in several cell lines that have been examined. It is worth noting that in a recent report, Oasis, a protein structurally similar to ATF6 and Luman, only mediates ER stress responses in a cell type-specific manner [28]. Nonetheless, the proteolytic cleavage of transfected Oasis protein can be induced by ER stress, independent of cell type. Another possible explanation for Luman's unresponsiveness to ER stress inducers is that activation of UPRE or possible ERAD by Luman is independent of the known ER stress response. An unknown signaling pathway and the XBP1-mediated ER stress response may converge on the ERAD machinery. As we have not yet found cell types that express endogenous Luman protein, except for a fraction of neurons in the trigeminal ganglia, future studies using the IRE1$^{-/-}$ ES cell line [27] could help delineate Luman's role in ERAD in relation to the IRE1/XBP1 pathway.

It is known that viruses often establish ways to interfere with the UPR, thereby ensuring successful production of large amounts of viral protein [29]. HSV-1 has been shown to manipulate the UPR for its glycoprotein processing [30]. Recent studies have also suggested that VP16 may influence the UPR through mimicry of ATF6 [31]. Thus, it would be reasonable to speculate that through mimicry of Luman by VP16, HSV-1 may have yet another mechanism to usurp the UPR for viral replication.

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