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Hepatitis E Virus Replication Requires an Active Ubiquitin-Proteasome System

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The mechanism of hepatitis E virus (HEV) replication remains largely unknown. Here we demonstrate that HEV replication requires an active ubiquitin-proteasome system and that proteasome inhibitors affect HEV replication, possibly by inhibition of viral transcription or/and translation without a significant effect on cellular translation. Overexpression of ubiquitin in inhibitor-treated cells partially reverses the inhibitor effect on HEV replication. The results suggest that HEV replication requires interactions with proteasome machinery, which could be a potential therapeutic target against HEV.

The cellular ubiquitin-proteasome system (UPS) is important for intracellular protein degradation in eukaryotic cells (40, 46). The UPS is composed of ubiquitination and substrate-degrading machinery. Ubiquitination is the conjugation of proteins with ubiquitin and occurs through the sequential enzymatic reactions of an E1 activating enzyme, E2 conjugation enzyme, and E3 ligase (17). Viruses manipulate the infrastructure and metabolism of their host cell to effect their own survival. UPS has been implicated in the infection cycle and virus-host interplay of several viruses (3, 9, 28, 36, 51). Bortezomib is an FDA-approved proteasome inhibitor that has demonstrated clinical efficacy in the treatment of multiple myeloma (7, 10, 27). Therefore, in this study we examined the role of UPS in the replication of hepatitis E virus (HEV) and evaluated the potential use of UPS inhibitors as therapeutic agents against HEV infection.

HEV, a nonenveloped single-strand positive-sense RNA virus in the family *Hepeviridae* (30, 32), is an important but understudied human pathogen (2, 11, 31, 33). The genome of HEV is ~7.2 kb and contains a 5′-m7G cap (20) and three open reading frames (ORFs) (39). The ORF1 protein possesses domains for replicase enzymes (25) and among these, functional activities of RdRp (1), Hel (21, 22), and MetT (29) have been experimentally verified. ORF2 encodes the viral capsid protein (16, 50). ORF3 encodes a small multifunctional protein that interacts with various signaling pathways (5, 6, 13, 24, 26, 33–35, 43–45, 48, 49). The ORF2 and ORF3 proteins are translated from a 2.2-kb subgenomic RNA (15, 18). The expression of the ORF3 protein is not required for virus replication, virion assembly, or infection *in vitro* (12, 14).

It has been reported that proteasome inhibitors affect the replication of herpesviruses (9), vaccinia virus (36), influenza virus (47), human immunodeficiency virus (38), and cytomegaloviruses (42). Many viruses encode proteins that can modify the host's ubiquitin machinery, (19). Recently, a papain-like cysteine protease has been described as a deubiquitinating enzyme in HEV (23), indicating that a ubiquitin system may influence the life cycle of HEV.

For all experiments, a subclone of a human hepatocellular carcinoma cell line, Huh7-S10-3, which is permissive for HEV replication, was used, and the cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum under a 5% CO_2 atmosphere at 37°C. Transfected cells were maintained under the same conditions except at 34.5°C.

First, to determine whether proteasome activity is required for HEV replication, we tested the effects of proteasome inhibitors MG132, lactacystin, and epoxomicin (Sigma) on HEV replication. The toxicities of the inhibitors were tested by the alamarBlue assay (Invitrogen), and we showed that there was >80% cell survival when concentrations of inhibitors were less than or equal to 1 μM (Fig. 1A). Thus, for all further experiments in this study, the concentration of inhibitors we used was 1 μM or less.

HEV replicon expressing the *Renilla* luciferase (Rluc) gene system (designated pSK-HEV-2RLuc) was developed previously using the genotype 1 human HEV infectious clone pSK-HEV-2 (4). The capped RNA transcript of the pSK-HEV-2RLuc clone was transfected into Huh7-S10-3 cells by using the DMRIE-C reagent (Invitrogen). UPS inhibitors were added to culture medium at 24 h posttransfection. The luciferase activities were measured with a dual luciferase reporter assay system (Promega) at 5 days posttransfection. Firefly luciferase RNA was cotransfected with HEV Rluc replicon RNA to normalize the *Renilla* luciferase signal. All the inhibitors tested in this study caused a significant reduction in the level of virus replication, suggesting that the UPS is important for HEV replication. The MG132 inhibitor had a more pronounced effect on virus replication than other inhibitors (Fig. 1B). Furthermore, we found that this inhibition of HEV replication was concentration dependent (Fig. 1C).

To investigate which specific step(s) of the HEV replication cycle might be affected by lack of proteasome activity, we performed an immunofluorescence assay (IFA) to detect viral capsid protein synthesis, and we performed negative-strand-specific reverse transcription-PCR (RT-PCR) to detect replicative negative-strand viral RNA. Briefly, Huh7 cells were transfected with the full-length capped RNA transcripts of the pSK-HEV2 infectious clone, and capsid protein synthesis was monitored by IFA (8). When 1 μM MG132 inhibitor was added to culture medium at 24 h posttransfection, no capsid protein synthesis was detected by IFA (Fig. 2A), further confirming that HEV replication requires an active ubiquitin proteasome system.

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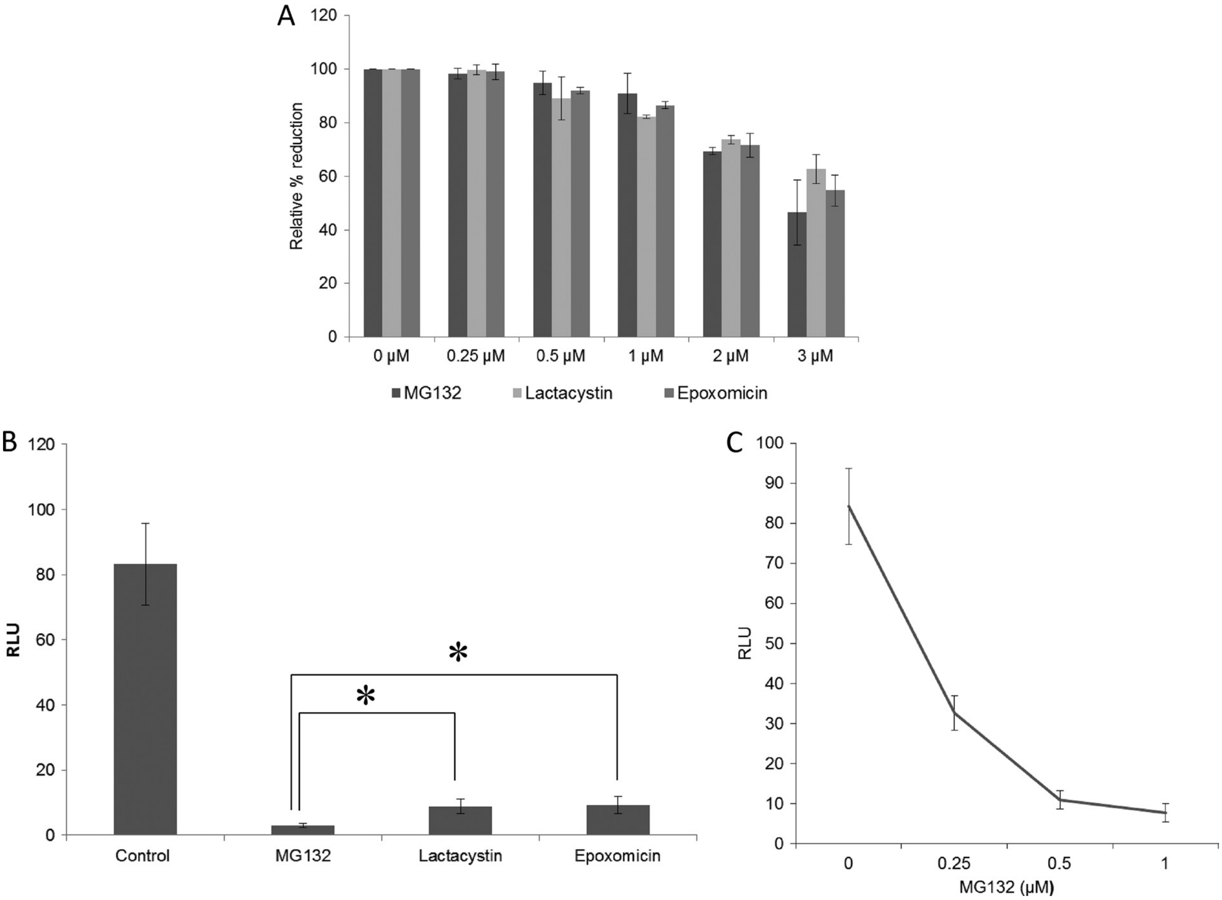

FIG 1 The proteasome inhibitors significantly reduced the levels of HEV replication. (A) Toxicity of inhibitors to Huh7 S10 cells. The results shown are from an alamarBlue assay for Huh7 S10 cells treated with the inhibitors MG132, lactacystin, and epoxomicin. The assay was performed on the fourth day after treatment. The concentrations of inhibitors are indicated. Mean values from three independent experiments are plotted. (B) HEV replication is reduced by treatment with proteasome inhibitors. Relative luciferase activities are shown for Huh7 S10 cells transfected with capped RNA transcript of the pSK-HEV2RLuc clone. Inhibitor treatment started 1 day posttransfection, and the concentration of inhibitors was 1 μM. A luciferase assay was performed on the fifth day posttransfection. Mean values from six independent experiments are plotted. Statistical analysis was performed using analysis of variance followed by Dunnett's procedure, and significance was set at a *P* level of <0.05 (indicated with an asterisk). Data analysis was performed using JMP9 software. (C) Effect of MG132 on HEV replication. Relative luciferase activities are shown for Huh7 S10 cells transfected with capped RNA transcript of the pSK-HEV2RLuc clone. Inhibitor treatment started 1 day posttransfection, and the concentration of MG132 used is indicated. A luciferase assay was performed on the fifth day posttransfection. Mean values from three independent experiments are plotted.

For the detection of negative-strand replicative viral RNA, a strand-specific anchored RT-PCR was carried out essentially as described previously (41). RNA was reverse transcribed with a forward primer (5′-GGGGGGGGGGGGCCGCGCCCATACTT TCGATGA-3′), and both the first and second amplifications were carried out using the forward poly(G) primer (5′-GGGGGGGGG GGGGGGGG-3′) and reverse primer (5′-CAGGGAGCGCGGAA CGGAACGCAG-3′). As for a positive control, a negative-strand HEV RNA was prepared by *in vitro* transcription of a PCR DNA template amplified with a forward primer (5′-CCAGCAGTATTC AAAGACC-3′) and a reverse primer (5′-GATCATCTCCCTA TAGTGAGTCGTATTATTTCAGGGAGCGCGAAACGC-3′; T7 polymerase promoter sequence, underlined). Huh7 cells were transfected with the capped full-length RNA transcripts of the pSK-HEV2 infectious clone, and cells were treated with 1 μM MG132 inhibitor at 1 day posttransfection and harvested on the fifth day posttransfection. No negative-strand viral RNA was detected when cells were treated with MG132 inhibitor (Fig. 2B), suggesting that the proteasome activity is needed for the replication of the HEV genome, possibly by inhibition of viral transcription or translation or both. We believe that inhibition of early or multiple stages of virus replication will result in little or no synthesis of negative-strand RNA, thus explaining our observation of the absolute negative result on the detection of the negative-strand RNA.

Due to the long duration of treatment, we tested the cytotoxicities of the inhibitors in cell culture to determine the concentration ranges of inhibitors. It is possible that inhibitors may impair cellular translation and thus could attribute to the inhibition of viral replication. Therefore, we subsequently tested the effects of the inhibitor drug treatments on green fluorescent protein (GFP) synthesis. In addition, in another set of experiments we expressed part of the viral capsid protein that is known to form virus-like particles (VLPs) in the presence and absence of MG132. Huh7 cells were transfected, inhibitor treatment was the same as described above, and pAcGFP N1 and pTrix-neo-ORF2 (with deletion of amino acids 1 to 111 [Δ1-111])

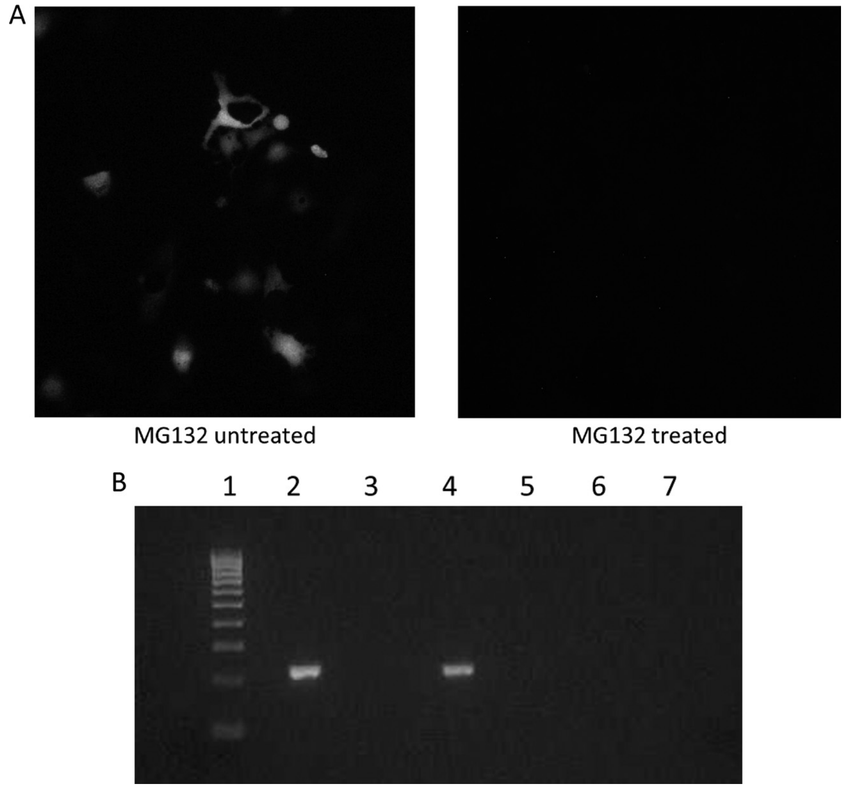

FIG 2 MG132 inhibits viral transcription and/or translation. (A) Immunofluorescent staining of a subclone of Huh7 cells transfected with similar amounts of capped full-length RNA transcripts. (Left) MG132 untreated; (right) MG132 treated. Inhibitor treatment started 1 day posttransfection, and cells were stained for HEV ORF2 protein by using chimpanzee 1313 anti-HEV immune serum. (B) Detection of HEV replication by strand-specific anchored RT-PCR. A subclone of Huh7 cells was transfected with similar amounts of capped full-length RNA transcript, 1 μM MG132 treatment started 1 day posttransfection, and cells were harvested on the fifth day posttransfection. For detection of replicative negative-sense viral RNA, a strand-specific anchored RT-PCR was carried out. Lane 1, 100-bp marker; lane 2, RT-PCR results for positive-control negative-strand RNA transcript generated by *in vitro* transcription; lane 3, PCR performed with positive-control negative-strand RNA transcript generated by *in vitro* transcription (reaction without RT); lane 4, RT-PCR performed with RNA isolated from full-length capped RNA transfected cells; lane 5, PCR performed with RNA isolated from full-length capped RNA transfected cells (reaction without RT); lane 6, RT-PCR performed with RNA isolated from full-length capped RNA-transfected cells and treated with 1 μM MG132; lane 7, RT-PCR performed with mock-transfected cells.

were transfected to Huh7 cells. Immunoblotting was performed with anti-GFP rabbit polyclonal antibody (1:500), anti-HEV chimpanzee polyclonal serum (1:200), and anti-actin goat polyclonal antibody (1:200) (all from Santa Cruz Biotechnology) and with appropriate secondary antibody. Comparable levels of GFP, capsid protein, and actin were observed in drug-treated and untreated cells (Fig. 3A and B). Also, when the full-length RNA genome of HEV was transfected into Huh7 cells in the presence of MG123, no capsid protein was detected. These results strongly suggest that there is inhibition of viral replication without a significant effect on cellular translation.

It has been shown that MG132 reduces the pool of free ubiquitin in cells (28). MG132 inhibits budding of human parainfluenza virus 5 by depletion of free ubiquitin in cells by blocking the 26S proteasomal degradation of polyubiquitinated proteins (37). Therefore, to determine whether the observed inhibition of HEV replication by MG132 was due to depletion of free ubiquitin, we cotransfected plasmid pRK5-HA-ubiquitin (kindly provided by Ted Dawson [Addgene plasmid]) with capped viral RNA transcript, and the effect on viral replication was monitored. An increase in viral replication was observed when the cells were cotransfected with capped HEV RNA transcript with pRK5-HA-ubiquitin compared to cells cotransfected with capped HEV RNA transcript with the pTrix neo plasmid (Fig. 4A). Immunoblotting

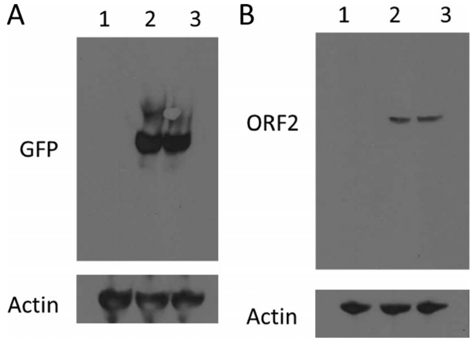

FIG 3 The inhibitory effect of MG132 on HEV replication does not result from the inhibition of translation. (A) Effect of MG132 treatment on GFP synthesis. A subclone of Huh7 cells was transfected with a similar amount of pAcGFP N1 plasmid in six-well plates. Inhibitor treatment started 1 day posttransfection, cells were harvested on the fifth day posttransfection, and immunoblotting was performed with anti-GFP polyclonal serum produced in a rabbit. Lane 1, mock-transfected cells; lane 2, pAcGFP N1-transfected cells; lane 3, pAcGFP N1-transfected cells with 1 μM MG132 treatment. (B) Effect of MG132 treatment on ORF2 protein synthesis. A subclone of Huh7 cells were transfected with a similar amount of pTrix-neo-ORF2 (Δ1–111) plasmid in six-well plates. Inhibitor treatment started 1 day posttransfection, cells were harvested on the fifth day posttransfection, and immunoblotting was performed with chimpanzee 1313 anti-HEV immune serum. Lane 1, mock-transfected cells; lane 2, pTrix-neo-ORF2 (Δ1–111)-transfected cells; lane 3, pTrix-neo-ORF2 (Δ1–111)-transfected cells treated with 1 μM MG132.

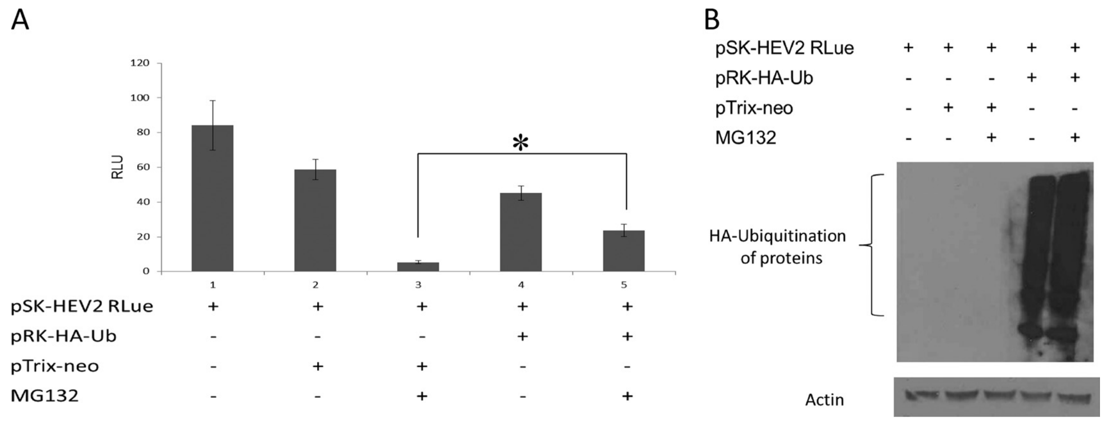

FIG 4 Overexpression of HA-ubiquitin partially restores virus replication. (A) Effect of HA-ubiquitin overexpression on HEV replication in the context of MG132 treatment. In six-well plates, cotransfection of capped RNA transcripts of the pSK-HEV2RLuc clone and pRK-HA-Ub/pTrix-neo was carried out. MG132 treatment started 1 day posttransfection. A luciferase assay was performed on the fifth day posttransfection. Mean relative light unit (RLU) values from three independent experiments are plotted. Statistical analysis was performed using analysis of variance followed by contrast procedure, and significance was set at a P level of <0.05 (indicated with an asterisk). Data analysis was performed using JMP9 software. (B) Representative results from an immunoblot assay performed with the anti-HA tag monoclonal antibody of the experiment shown in panel A.

was performed to detect the expression of hemagglutinin (HA)-ubiquitin in the transfected cells by using an anti-HA tag monoclonal antibody produced in mice (Sigma) (Fig. 4B). In the case of overexpression of HA-ubiquitin and MG132 treatment, recycling of ubiquitin molecules may be affected; in this case, the pool of free HA-ubiquitin must be large enough to cause depletion of free HA-ubiquitin but sufficient to restore virus replication. Therefore, we believe that the reason that there was no difference between lanes 4 (without MG132) and 5 (with MG132) is likely due to the overexpression of HA-ubiquitin. Viral replication was not completely restored, and this may have been due to cotransfection efficiency. Nevertheless, the results suggest that depletion of free ubiquitin may be important for inhibition of viral replication.

In summary, in this study we demonstrated an important role of UPS in the life cycle of HEV. Proteasome inhibitors affected viral replication, possibly by inhibition of viral transcription or/and translation. The results strongly suggested that an active proteasome system is essential for HEV replication, and therefore proteasome inhibitors could be useful as therapeutics against HEV infection.

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