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West Nile virus genome amplification requires the functional activities of the proteasome

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ABSTRACT

The lifecycle of intracellular pathogens, especially viruses, is intimately tied to the macromolecular synthetic processes of their host cell. In the case of positive-stranded RNA viruses, the ability to translate and, thus, replicate their infecting genome is dependent upon hijacking host proteins. To identify proteins that participate in West Nile virus (WNV) replication, we tested the ability of siRNAs designed to knock-down the expression of a large subset of human genes to interfere with replication of WNV replicons. Here we report that multiple siRNAs for proteasome subunits interfered with WNV genome amplification. Specificity of the interference was shown by demonstrating that silencing proteasome subunits did not interfere with Venezuelan equine encephalitis virus replicons. Drugs that blocked proteasome activity were potent inhibitors of WNV genome amplification even if cells were treated 12 h after infection, indicating that the proteasome is required at a post-entry stage(s) of the WNV infection cycle.

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Introduction

The genus *Flavivirus*, within the family *Flaviviridae*, consists of multiple viruses of particular importance to public health, including yellow fever virus, Japanese encephalitis virus, dengue virus and West Nile virus (WNV). These viruses are responsible for significant morbidity and mortality worldwide. WNV first became a public health concern in the United States in 1999, when it was introduced into New York City. Since then, there have been more than 27,000 reported cases of disease associated with WNV infection with over 11,000 cases presenting with severe neurological pathologies, resulting in nearly 1200 deaths (Centers for Disease Control and Prevention, 2008). Unfortunately, as with many other flaviviruses, there are no approved antiviral therapies to control WNV disease and, as of yet, no approved vaccine for protection against WNV diseases.

Similar to other members of the genus, the WNV genome consists of a positive-sense, single-stranded RNA approximately 11 kilobases in length. The genome encodes a mere 11 proteins, 3 nonstructural (C, prM/M and E) and seven nonstructural (NS1–NS5) (Brinton, 2002), all of which has been demonstrated to play some role in replication of the viral genome (Lindenbach et al., 2007). Due to the limited amount of genetic material it carries, WNV, like other viruses, is expected to be

dependent upon many components of the macromolecular machinery of the host cell. As a consequence, it is expected that WNV proteins and the viral genome must interact with a multitude of host proteins to infect cells and efficiently reproduce itself. In fact, several such interactions at various stages of WNV infection have been identified.

One of the first virus–host interactions that can occur is the interaction between the virus particle and a receptor(s) on the host cell surface to promote viral entry. A recent report suggests that WNV entry may be mediated, in part, by $\alpha v \beta_3$ integrin (Chu and Ng, 2004b) or DC-SIGN (Davis et al., 2006) resulting in internalization of the virus via a clathrin-dependent endocytic pathway (Chu and Ng, 2004a). Further, infection of the virus can also be facilitated by binding to cell-surface heparan sulfate (Gilfoy and Mason, 2007; Lee and Lobigs, 2000). Three different host proteins, translation elongation factor-1 α , T cell intracellular antigen-1 (TIA-1) and TIA-1-related protein (TIAR) have been shown to interact with the 3'-terminal stem loop of the WNV genome (Blackwell and Brinton, 1995; Blackwell and Brinton, 1997; Li et al., 2002). The interaction between TIA-1 and TIAR and WNV is reported to interfere with WNV-induced stress granule formation and processing body assembly (Emara and Brinton, 2007), which are sites of translational control and mRNA aggregation and degradation under times of stress. Additionally, the Src kinase c-Yes was demonstrated to be an important host protein involved in WNV particle release from infected cells, an activity that appears to manifest itself at the level of particle trafficking from the endoplasmic reticulum to the extracellular space (Hirsch et al., 2005).

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The ubiquitin–proteasome system is a ubiquitous extralysosomal protein degradation system that clears unneeded proteins from both the cytosol and the nucleus. It plays a key role in a vast number of normal cellular processes including the removal of misfolded or abnormal proteins, the cellular stress response, the removal of unneeded proteins during cell differentiation, cell cycle regulation and the cellular immune response (Ciechanover, 1994; Glickman and Ciechanover, 2002). Proteins targeted for degradation are tagged with ubiquitin by a covalent bond that is produced by three enzymatic entities: an activating enzyme, E1, a carrier enzyme, E2, and a ligase, E3, which ultimately binds ubiquitin onto the protein (Glickman and Ciechanover, 2002; Nandi et al., 2006). Mono- or polyubiquitinated proteins are then trafficked to the 26S proteasome, where they are degraded.

The eukaryotic 26S proteasome is a ubiquitous, highly conserved multi-protease complex consisting of a barrel-shaped, proteolytic core complex (the 20S proteasome) with a 19S regulatory complex, or cap, at one or both ends (Groll et al., 1997; Perkins et al., 1994; Tanaka et al., 1988; Voges et al., 1999). The 20S proteasome is composed of two copies of seven alpha subunits ($\alpha 1$ – $\alpha 7$) and seven beta subunits ($\beta 1$ – $\beta 7$) arranged into 4 stacked rings. The two outer rings contain the alpha subunits, which are important for controlling entry and exit of proteins as well as binding to specific regulatory proteins. The two inner rings, on the other hand, are composed of the beta subunits, which represent the catalytic active sites of the proteasome (Nandi et al., 2006). Up to three primary proteolytic activities have been attributed to the proteasome: trypsin-like activity (cleavage after hydrophobic amino acids), chymotrypsin-like activity (cleavage after the basic amino acids) and caspase-like (cleavage after the acidic amino acids). In eukaryotic proteasomes, each of these protease activities is associated with a specific beta subunit, $\beta 2$, $\beta 5$ and $\beta 1$, respectively (Dick et al., 1998; Kisselev et al., 2003; Nussbaum et al., 1998). Interestingly, in mammals a specialized form of the proteasome has evolved in which the three catalytic beta subunits are replaced by different catalytic subunits, $\beta 2i$, $\beta 5i$ and $\beta 1i$ (Demartino and Gillette, 2007). This proteasome, referred to as the immunoproteasome, replaces the constitutive proteasome following immune stimulation and is responsible for generating antigenic peptides that are displayed by the class I major histocompatibility complex {MHC I (Baumeister et al., 1998; Goldberg et al., 2002; Rock et al., 1994)}.

The life cycle of viruses is intimately tied to the life cycle of the cells; therefore, it is unsurprising that viruses have evolved mechanisms to both hinder the host's response and parasitize host functions in their own replication. Members of multiple families of both RNA and DNA viruses have been shown to modulate the ubiquitin–proteasome system to their advantage for a variety of reasons, including immune evasion, viral entry or release, transcriptional regulation and apoptosis inhibition (Banks et al., 2003; Gao and Luo, 2006; Shackelford and Pagano, 2005). Many viruses, including human cytomegalovirus (Kikkert et al., 2001; Shamu et al., 2001), Epstein Barr virus (Levitskaya et al., 1997) and several other herpes viruses (Boname and Stevenson, 2001; Coscoy et al., 2001) interfere with the processing of MHC class I peptides. The ubiquitin–proteasome system has been shown to facilitate entry or transport of incoming influenza virus (Khor et al., 2003), minute virus (Ros et al., 2002; Ros and Kempf, 2004) and murine coronavirus (Yu and Lai, 2005). The release of HIV viral progeny requires proteasome activity, presumably by allowing efficient processing of Gag protein by the viral protease, thereby facilitating virion budding from the plasma membrane (Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000). Additionally, proteasome inhibition markedly reduces coxsackievirus group B3 viral RNA and protein synthesis levels, resulting in a decrease in the release of progeny virus (Luo et al., 2003).

Here we demonstrate that WNV genome amplification (either genome replication or genome translation) is sensitive to disruption of proteasome function. In a large-scale siRNA screen of human genes,

we discovered that silencing two different alpha subunits of the 26S proteasome with siRNAs, PSMA1 and PSMA2, resulted in a dramatic reduction in WNV genome-driven firefly luciferase (FLuc) activity. Additionally, using specific proteasome inhibitors, MG132 and PS1, we demonstrate that inhibition of the proteasome results in a reduction of WNV antigen levels, WNV protein accumulation and progeny virus yield. This inhibition is observed following treatment with these inhibitors both pre- and post-infection, indicating that WNV utilizes the proteasome at a post entry step of its replication cycle.

Results

Silencing proteasome subunits results in a reduction in WNV FLuc, but not VEEV FLuc, activity

To aid in identifying host proteins that facilitate WNV infection and/or genome amplification, a library of siRNAs was screened for its ability to suppress firefly luciferase (FLuc) signal encoded by WNV viral replicon particles (VRPs) (Fayzulin et al., 2006). VRPs are replicons (viral genomes lacking the sequences for the structural genes) packaged into particles with structural proteins that are provided *in trans* by packaging cells. WNV VRPs are particularly useful as they mimic WNV infection and genome amplification in nearly all aspects; however, because the packaged replicons do not encode the structural genes, VRP infections do not produce progeny virions. Thus, VRPs can be used to easily study infection and/or genome amplification independent of assembly and release. Additionally, because they are unable to spread beyond the initially infected cell, VRPs are safe to use under biosafety level 2 (BSL2) conditions.

The siRNA library utilized for these studies was a human druggable genome library purchased from Ambion that consisted of 3 individual siRNAs designed to silence approximately 5500 genes. Huh7 cells stably expressing *Renilla* luciferase (RLuc) were transfected with each siRNA (30 μ M), infected with the WNV VRP expressing FLuc (WNV FLuc VRP) 72 h later, and then assayed for both FLuc (WNV VRP infection/genome amplification) and RLuc (cell number) activity 24 h post infection. These studies identified 50 candidate genes for which transfection of 2 of the 3 individual siRNAs into Huh7 cells resulted in a greater than 60% reduction in RLuc-normalized WNV-dependent FLuc activity and did not result in a reduction in RLuc activity of greater than 40% (data to be published elsewhere).

To validate the 50 candidate genes obtained from screening the 5500 gene library, several confirmatory tests were performed utilizing the siRNAs corresponding to these 50 genes. The three individual siRNAs for each of the 50 genes were assayed for inhibition of WNV FLuc VRP activity in 3 additional human cell lines (HeLa, Hec1B and SK-N-SH cells), and also tested for their ability to reduce β -galactosidase (β -gal) activity encoded by a WNV β -gal VRP (Fayzulin et al., 2006) and to reduce antigen synthesis by 'live' WNV. To ensure that the candidate genes were important specifically for WNV infection, siRNA-transfected cells were infected with a FLuc-encoding Venezuelan equine encephalitis virus (VEEV) VRP and tested for the ability of the siRNAs to inhibit the infection and/or genome amplification of this alphavirus. As a final confirmation, pools of 4 individual siRNAs targeted against each candidate gene were purchased from a second source (Dharmacon) and assayed in the Huh7 cells. These secondary screens resulted in approximately 10 confirmed candidate genes (data to be published elsewhere).

Interestingly, 2 of these 10 confirmed candidate genes were subunits of the proteasome. In fact, 2 of 3 individual siRNAs (Ambion) and the pools of 4 siRNAs (Dharmacon) targeted against two alpha subunits, PSMA1 and PSMA2, were shown to consistently reduce WNV infection or genome amplification (>60% decrease in RLuc-normalized FLuc activity) when transfected into cells with little or no effect on cell number. Fig. 1A shows an example of a typical silencing experiment showing that transfection of a pool of 4 siRNAs (Dharmacon) targeted

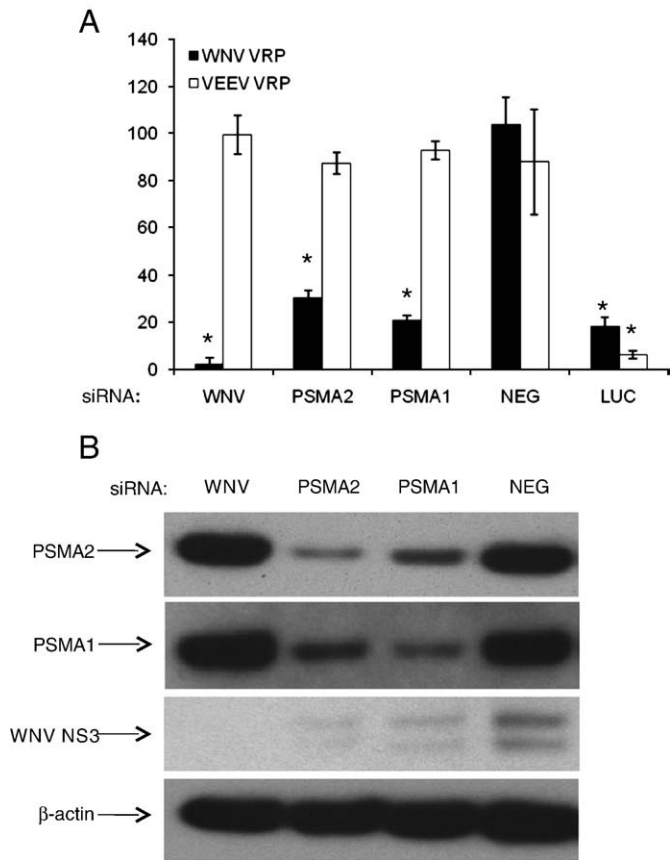


Fig. 1. Silencing PSMA1 or PSMA2 result in a decrease in WNV-induced FLuc activity. (Panel A) Non-targeting, negative control (NEG) siRNA, WNV-specific, FLuc-specific, PSMA1-specific or PSMA2-specific siRNAs were transfected into Huh7 cells, incubated for 72 h and then infected with VEEV FLuc VRP or WNV hFLuc VRP for an additional 16 or 24 h, respectively. Cell monolayers were then assayed for FLuc activity (WNV or VEEV replication levels) and RLuc activity (cell number). Values are shown as % FLuc normalized to % RLuc compared to mock-transfected cells. Error bars represent standard deviations from the mean between three independent experiments and an asterisk represents statistical significance compared to NEG siRNA transfection ($p < 0.05$). (Panel B) Monolayers of siRNA-transfected Huh7 cells were harvested and assayed, by Western blot, for levels of PSMA1, PSMA2, WNV NS3 and β -actin (loading control).

against either PSMA1 or PSMA2 dramatically lowered WNV replicon-driven FLuc activity compared to cells transfected with a non-targeting control siRNA (NEG; Fig. 1A). However, VEEV replicon-driven FLuc activity was not impaired following transfection with either PSMA1 or PSMA2-specific siRNA (Fig. 1A), indicating the specificity of the siRNA interference. Additionally, although FLuc-specific siRNAs reduced FLuc expression derived from both WNV and VEEV VRPs, siRNAs targeted against the WNV genome were, as expected, specific for WNV replicon-driven FLuc activity (Fig. 1A). Transfection with PSMA1 or PSMA2 siRNAs also resulted in a greater than 60% reduction in WNV antigen expression following 'live' WNV infection (data not shown).

To confirm the effectiveness of the siRNAs, Western blot analysis was performed on the siRNA-transfected cell lysates. Analysis indicated that transfection of PSMA1 or PSMA2 siRNA resulted in reduced PSMA1 and PSMA2 expression, respectively, compared to NEG control or WNV-specific siRNA transfection (Fig. 1B). Interestingly, transfection of either PSMA1-specific or PSMA2-specific siRNAs resulted in decreased expression of both subunits, although the expression of the specific target gene always demonstrated greater knockdown. Even so, the silencing of one subunit had an effect on the steady state levels of the other subunit of the proteasome, possibly through the destruction of subunits that are not stabilized by their assembly into the multi-molecule complex or by the ability of one or

more of the siRNAs targeted against PSMA1 will also partially recognize PSMA2, and *vice versa*.

To demonstrate a corresponding decrease in WNV antigen expression following the silencing of PSMA1 or PSMA2, membranes were also probed with a WNV-specific antibody. Results showed a dramatic reduction in the expression of WNV NS3 following transfection with PSMA1, PSMA2 or WNV siRNAs compared to transfection with the NEG siRNA (Fig. 1B).

Pre-treatment of cells with proteasome inhibitors results in reduced WNV genome amplification and protein expression

siRNA technology is a powerful tool for studying gene function; however, due to the potential for off-target silencing effects (Birmingham et al., 2006; Jackson et al., 2003; Lin et al., 2005; Qiu et al., 2005; Scacheri et al., 2004), we validated our results by treating Huh7 or HeLa cells with two different proteasome inhibitors, MG132 and PS1, both of which are potent, cell-permeable, reversible inhibitors of the chymotrypsin-like activity of the 20S proteasome (Borissenko and Groll, 2007).

The cells were treated with MG132 or PS1, infected with WNV hFLuc VRP and then assayed for FLuc activity, WNV antigen accumulation (ELISA) and cell viability. Cell viability/cell number was measured by two methods: the MTT assay and an assay for cell-expressed RLuc activity. To simplify presentation of the results only the MTT values are shown, although RLuc activity correlated well with the MTT values in all cases.

HeLa cells pre-treated with as little as 0.3 μ M of MG132 showed a marked reduction in both WNV-driven FLuc activity and WNV ELISA antigen expression compared to mock- or DMSO-treated cells (Fig. 2A). Additionally, treatment with as little as 0.08 μ M of PS1 (Fig. 2B) resulted in a greater than 70% inhibition in WNV activity compared to the DMSO vehicle treatment. Although the inhibitors appeared to be less effective at inhibiting WNV VRP infection/genome amplification in Huh7 cells compared to HeLa cells, treatment with both MG132 and PS1 resulted in substantially lower FLuc activity and WNV ELISA antigen expression compared to mock or DMSO treatment. Treatment with MG132, which was more effective than PS1 in these cells, led to a greater than 80% reduction in WNV activity with as little as 1.25 μ M (Fig. 2C) while pre-treatment with PS1 at this same concentration resulted in an approximate 60% reduction in WNV activity (Fig. 2D). Consistent with the inability of PSMA-specific siRNAs to impair VEEV VRP-driven FLuc activity, MG132- or PS1-treated Huh7 cells demonstrated little or no reduction in VEEV VRP-driven FLuc activity compared to mock-treated cells (data not shown). Throughout these experiments, only the highest concentration (20 μ M) of MG132 and PS1 in either Huh7 or HeLa cells resulted in any major changes in cellular characteristics or a reduction in cell viability, measured by either MTT assay (Figs. 2A–D) or RLuc activity (results not shown).

To monitor WNV antigen expression by another method and to help ensure the specificity and sensitivity of the ELISA assay, PS1- or MG132-treated HeLa cells were infected with WNV hFLuc VRPs and then the cell monolayers were harvested and assayed for NS3 expression by Western blot analysis. Consistent with the Fig. 2 studies, treatment of HeLa cells with PS1 resulted in a reduction in WNV protein expression at the lowest drug doses tested (Fig. 3A). Additionally, treatment with MG132 resulted in a reduction in WNV protein expression compared to mock- or DMSO-treated cells, although, consistent with our FLuc and ELISA detection methods (see Fig. 2) the MG132-mediated effect on NS3 expression was less than that observed with PS1 treatment (Fig. 3B). The NS3 antibody consistently recognized two bands. The lower band corresponds to the predicted molecular weight of NS3, approximately 70kd, while the top band may represent uncleaved NS2A–NS3 or NS3–NS4A. Levels of β -actin, which served as a loading control, were similar in all treatment groups with the exception of 20 μ M MG132, where a

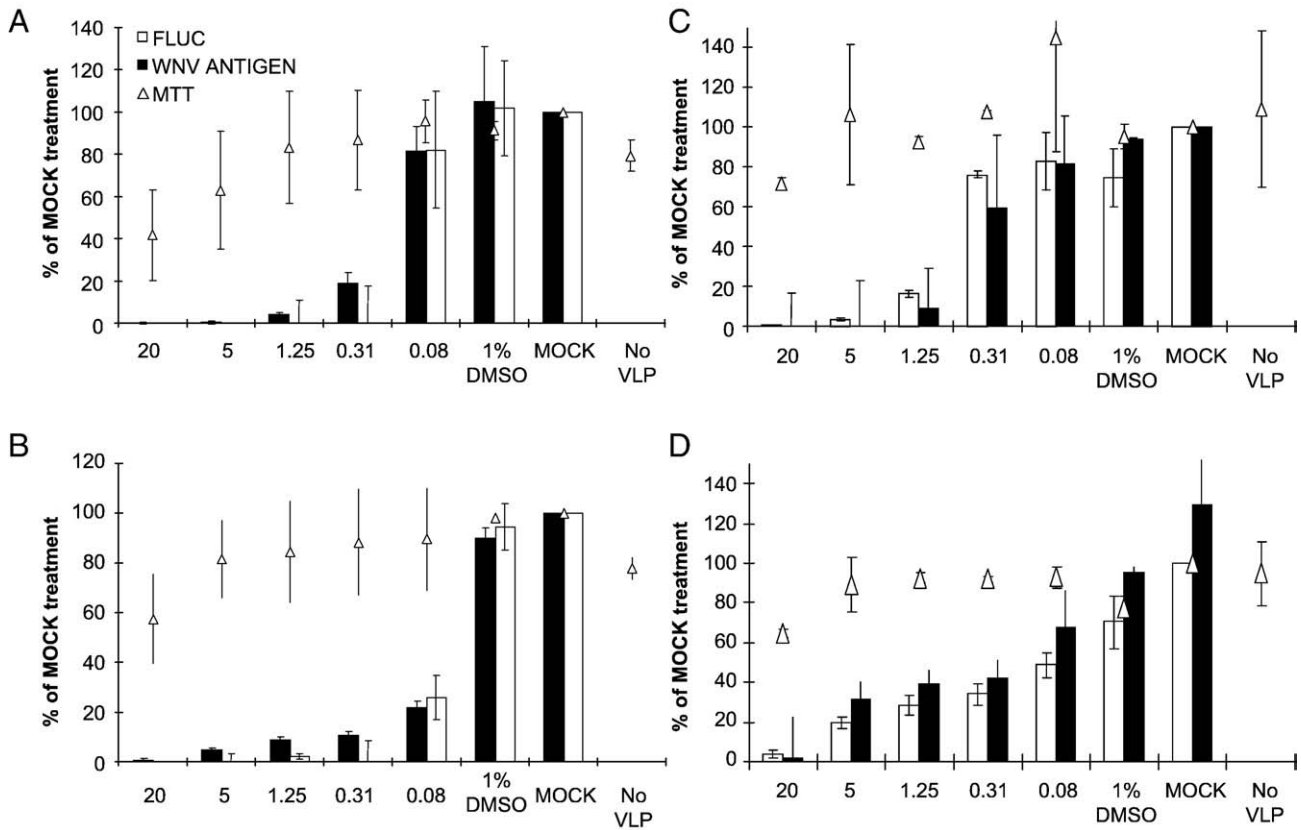


Fig. 2. Pre-treatment of cells with proteasome inhibitors impairs WNV infection/replication. HeLa cells (panels A–B) and Huh7 cells (panels C–D) were pre-treated with the indicated concentrations (0, 0.08, 0.31, 1.25 or 20 μ M) of either MG-132 (panels A, C) or PS1 (panels B, D) prior to WNV hFLuc VRP infection and then assayed for FLuc activity, WNV ELISA antigen expression or cell viability (measured by MTT assay, see Materials and methods). Values are shown as percent of WNV VRP-infected, mock-treated cells and error bars represent standard deviation from the mean between three independent experiments.

reduction in β -actin was observed, consistent with the cytotoxicity observed with this dose. IFA performed on proteasome inhibitor-treated HeLa cells was consistent with the Western blot analysis (Fig. 4). PS1 and MG132 treatment in HeLa cells reduced the detectable levels of both NS1 (Fig. 4A) and NS3 (Fig. 4B), although PS1 appeared to be more effective than MG132, consistent with our previous studies. Interestingly, both PS1 and MG132 appeared to be more effective at blocking NS1 protein accumulation than NS3, although the significance of this is not known (Fig. 4). IFA performed on proteasome inhibitor-treated Huh7 cells showed a similar reduction in both NS1 and NS3 levels (results not shown). DAPI and phalloidin counterstains indicated that inhibitor-treated cells did not have any nuclear abnormalities or any major defects in their overall cellular architecture, arguing against a non-specific effect of the inhibitor on WNV genome amplification.

Since both MG132 and PS1 are reversible inhibitors of the proteasome, it was possible to determine whether removing inhibitor treatment from cells would return WNV infection/genome amplification to levels observed in mock-treated cells. This type of experiment helps to ensure that the inhibitor treatment did not result in any permanent damage to other cellular processes which might be responsible for the inhibitor-induced inhibition of WNV VRP infection or genome amplification. To this end, Huh7 and HeLa cells were pre-treated with MG132 or PS1 for approximately 2 h, after which the drugs were removed. The cells were then infected with WNV hFLuc VRPs following a 2 h 'recovery' period.

Consistent with Fig. 2, Huh7 (Fig. 5A) and HeLa (Fig. 5B) cells that were incubated with MG132 or PS1 demonstrated a dose-dependent reduction in WNV-dependent FLuc activity and WNV ELISA antigen accumulation. However, WNV hFLuc VRP infection/replication in both Huh7 (Fig. 5A) and HeLa (Fig. 5B) cells in which the MG132 and PS1 treatments were washed off was nearly identical to mock- or DMSO-

treated cells for all doses of the inhibitors. These data suggest that treatment with the inhibitors did not have an indirect effect on the cell's ability to support WNV VRP genome amplification.

Inhibition of the proteasome post-infection reduces WNV genome amplification

To ascertain whether the proteasome inhibitors were serving to block WNV VRP at entry or at a later stage of infection, HeLa cells were treated with MG-132 or PI-1 either concurrently with WNV hFLuc VRP infection or at various times post-infection and then assayed for WNV-dependent FLuc activity and WNV antigen accumulation. Surprisingly, treatment with the inhibitors (either MG-132 or PI-1) as late as 12 h post infection resulted in a reduction in WNV-dependent FLuc and antigen accumulation (Figs. 6A, B). In fact, at 0 and 2 h post-infection, the inhibition of WNV activity was nearly to the same extent observed when the drugs were added prior to infection. However, the inhibitory effect of these inhibitors on WNV genome amplification appears to wane at the later times post-infection with only a 50–60% reduction in WNV activity at 12 h post infection for all but the highest doses of each of the drugs (Fig. 6). Consistent with pre-treatment studies, PS1 treatment (Fig. 6B) was more effective than MG132 at all timepoints tested (Fig. 6A).

Proteasome inhibition results in a reduction of WNV reproduction

Although WNV VRPs mimic WNV infection, it was important to examine the effect of proteasome inhibitor treatment on 'live' WNV infection, as well. Since proteasome inhibitor treatment had no effect on the ability of WNV hFLuc VRPs to infect cells, monolayers of HeLa cells were infected with WNV for 1 h prior to MG132 or PS1 treatment

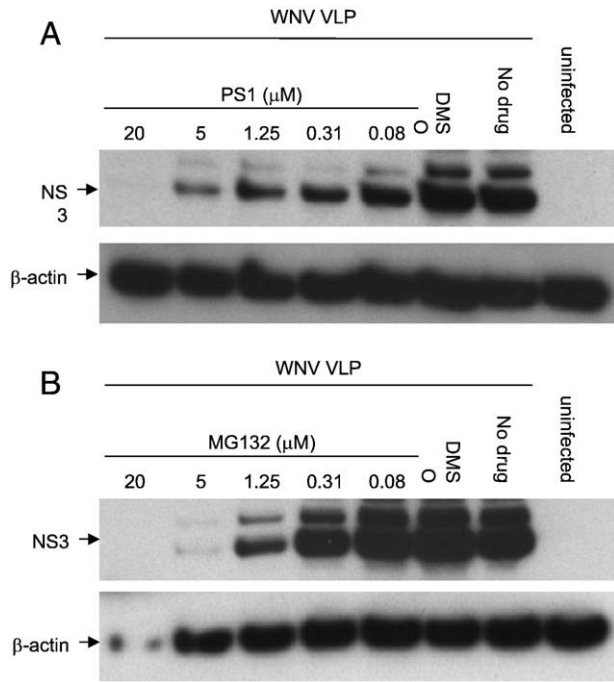


Fig. 3. Inhibition of proteasome results in reduction in the accumulation of WNV proteins. Monolayers of HeLa cells were treated with PS1 (panel A) or MG132 (panel B) at concentrations of 20, 5, 1.25, 0.31, 0.08, 0 μ M or DMSO alone for 1 h prior to infection. Following a 24 h WNV hFluc VRP infection, Western blot analysis was performed on the cell lysates. Membranes were probed for WNV NS3 and β -actin, which served as a loading control.

and assayed for WNV accumulation in the supernatant fluid 24 h later. Titrations performed on supernatants harvested from the treated cells indicated that proteasome inhibitor treatment dramatically reduced the production of infectious WNV particles (Fig. 7). MG132 treatment of HeLa cells, as observed in WNV VRP experiments, was slightly less effective at impairing WNV production compared to PS1 treatment; however, the treated cells still demonstrated a near 80% reduction in secreted infectious particles compared to mock-treated cells with as little as 0.16 μ M (Fig. 7). PS1, on the other hand, demonstrated inhibition of WNV particle release similar to MG132 at a concentration of only 0.032 μ M (Fig. 7). The inhibition of WNV release was consistent with the inhibition of WNV VRP-induced FLuc activity and WNV antigen accumulation. These data indicate that inhibiting proteasome activity in these cell lines severely impairs the WNV genome amplification, suggesting that the proteasome is an important cellular co-factor during WNV replication.

Discussion

Many RNA viruses require specific host factors for efficient replication. WNV is no exception. Beyond the host machinery required for entry and translation, such as receptors present on the cell surface (Chu and Ng, 2004b; Davis et al., 2006; Medigeshi et al., 2008), acidification of endosomes (Chu and Ng, 2004a) and translation factors (Davis et al., 2007), WNV likely requires additional host proteins to establish an environment permissive for efficient replication. For example, the interaction between WNV 3' stem loop structure and two host proteins, TIA-1 and TIAR (Li et al., 2002), has been shown to interfere with the cell's ability to form stress granules, effectively preventing the host cell from shutting down viral and cellular translation (Emara and Brinton, 2007). Here we report that the

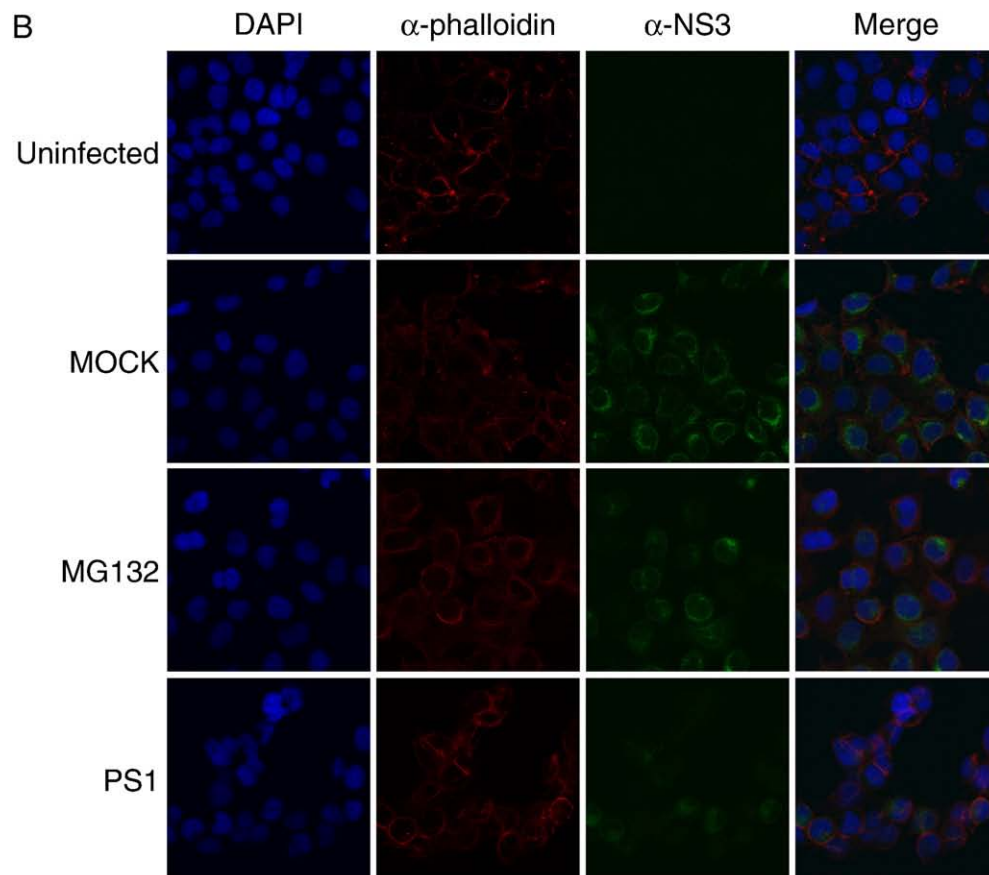
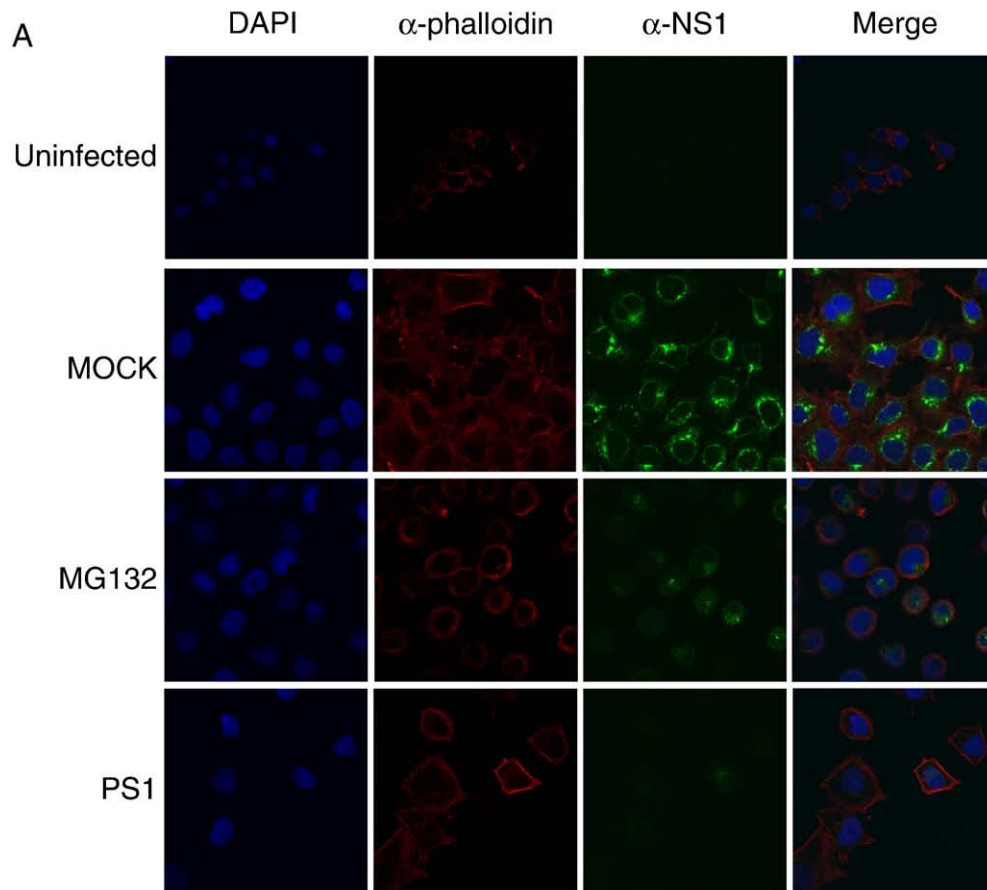
proteasome may serve as an essential co-factor during the WNV replication cycle.

The ubiquitin–proteasome system is the primary mechanism for intracellular, extralysosomal protein degradation. It plays a key role in a variety of cell functions, including cell cycle regulation, antigen processing, transcriptional regulation, apoptosis, signal transduction and transcriptional regulation (Ciechanover, 1994; Glickman and Ciechanover, 2002; Goldberg et al., 2002; Nandi et al., 2006). Because of its involvement in a vast array of functions, it is not surprising that multiple viruses have evolved mechanisms to manipulate this system to its own advantage. Influenza virus has been shown to utilize the ubiquitin–proteasome system for efficient trafficking through the late endosome/lysosome stages of virus entry (Khor et al., 2003). Mouse hepatitis virus (a coronavirus) and minute virus (a parvovirus) have also been shown to utilize the proteasome system for trafficking to the cytoplasm (Yu and Lai, 2005) or the nucleus (Ros et al., 2002; Ros and Kempf, 2004), respectively. Multiple members of the *Herpesviridae* family have evolved different mechanisms to manipulate the ubiquitin–proteasome pathway, whether it is by encoding ubiquitin ligase-like proteins (Coscoy et al., 2001) or by targeting specific proteins for destruction (Coscoy et al., 2001; Kikkert et al., 2001; Parkinson et al., 1999; Prosch et al., 2003). Inhibition of proteasome activity was shown to impair coxsackievirus B3 replication (Luo et al., 2003). A recent report indicated that coxsackievirus-induced activation of the extracellular signal-regulated kinase (ERK) pathway, which is required for its replication (Luo et al., 2002), is reduced following proteasome inhibition (Wong et al., 2007), suggesting a possible mechanism of proteasome action. The ubiquitin–proteasome pathway also appears to be a critical factor in the lifecycle of HIV, as well. Efficient processing of Gag polyprotein – and subsequent release and maturation of HIV particles – is dependent upon ubiquitin–proteasome activities (Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000).

In this study, we describe some of the results from our attempts to identify novel host factors involved in WNV infection and/or genome amplification. Our studies were based on an unbiased screen of host factors, systematically knocking down nearly 5500 human genes using a library of siRNAs. Throughout the initial screening process, and multiple confirmatory screens, silencing multiple different subunits of the proteasome dramatically reduced WNV infection and/or genome amplification. In fact, siRNAs targeted against two alpha subunits, PSMA1 and PSMA2, consistently knocked down WNV VRP infection and/or genome amplification levels by greater than 70% compared to non-targeting negative control siRNAs. A similar screen performed with live WNV also showed that silencing proteasome subunits has a negative effect on WNV infection/genome amplification (Krishnan et al., 2008). Consistent with these results showing that post-translational silencing of two proteasome genes could impair WNV replication, we showed that treatment of cells with micromolar concentrations of two well-characterized proteasome inhibitors (MG132 and PS1) resulted in impaired the activity of WNV VRPs and live WNV, indicating that a functional proteasome is required for WNV genome amplification.

The small number of candidate genes identified (less than 0.2% following all confirmatory tests) was somewhat startling. However, the library consisted of a relatively small subset of genes (5500 genes from the 30,000 genes encoded by the human genome) which are currently known to be involved in many different disease processes. Even within this subset of genes, it is very likely that many were overlooked, due to the presence of compensatory pathways, the expression of homologues or the failure of specific siRNAs to efficiently silence their target. Although silencing many different proteasome subunits had an effect on WNV activity in the initial siRNA

Fig. 4. Proteasome inhibition reduces levels of NS1 and NS3 in HeLa cells. IFA of proteasome inhibitor-treated HeLa cells. Monolayers of HeLa cells were treated with either PS1 (4 μ M) or MG132 (4 μ M) and then infected with WNV hFluc VRPs. Twenty-four hours post infection, the monolayers were fixed and assayed for levels of NS1 (panel A) or NS3 (panel B). The stained monolayers were counterstained for nuclei (blue) and actin (red). NS1 and NS3 are shown in green.



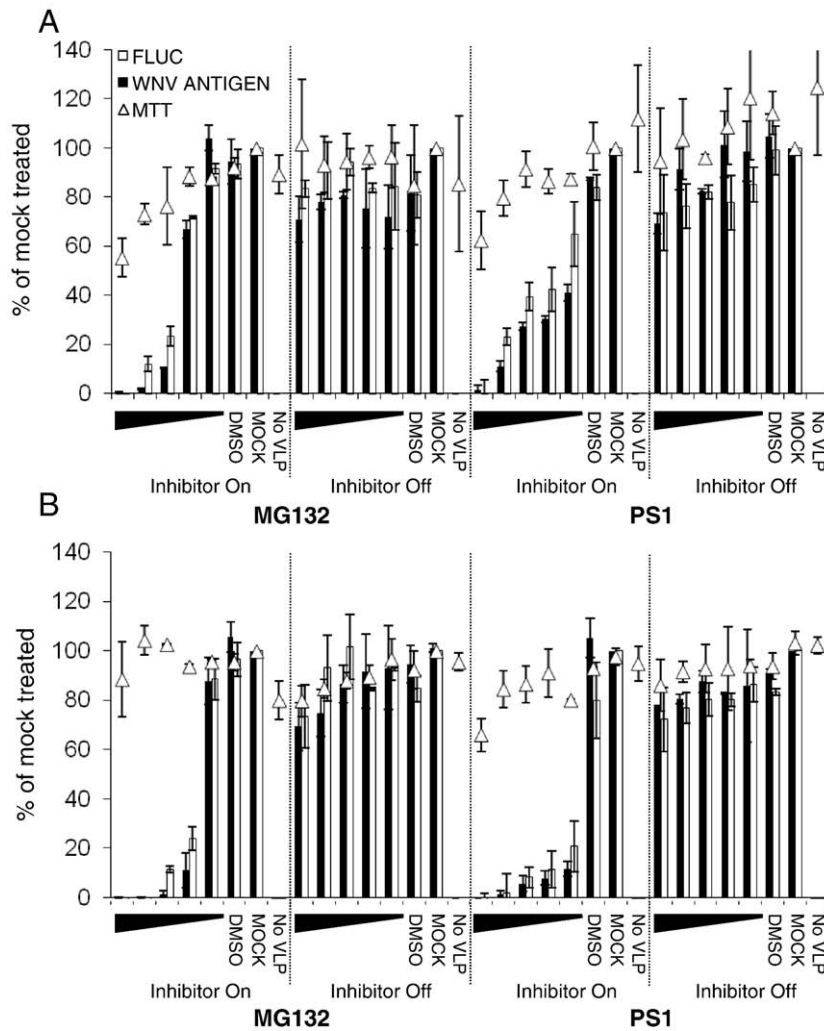


Fig. 5. Removing proteasome inhibitors restores WNV infection/replication. Huh7 cells (panel A) or HeLa cells (panel B) were treated with 20, 5, 1.25, 0.31, 0.08, 0 μ M MG132 or PS1 for 2 h, and then the drugs were removed and the cells were infected with WNV VRPs. Cells were assayed for FLUC activity, WNV antigen expression and cell viability. Values shown as percent of mock and error bars represent standard deviation from the mean between three independent experiments.

library screen we performed, PSMA1 and PSMA2 were the only two genes to consistently knockdown WNV activity in all secondary screens. This was puzzling; however, we believe that this is likely due to the quality of the siRNAs in the initial library. As mentioned above, not all siRNAs are equally effective, especially the ‘first-generation’ siRNAs (which were not optimized or modified to enhance their activity) used in the initial siRNA library. Thus, it is likely that the siRNAs for other proteasome subunits were either not as specific or as effective at silencing their target gene compared to PSMA1 and PSMA2 siRNAs. Because the proteasome is composed of many different subunits we had multiple opportunities to silence its expression. Thus, it appears that this type of multisubunit complex is particularly well suited for discovery with function-based siRNA screening strategies.

As discussed in the Introduction, viruses have been shown to manipulate the ubiquitin–proteasome pathway to enhance viral activities at various stages of the virus lifecycle, including entry and post-entry trafficking, translation, transcription, and maturation/release. Post-treatment of WNV VLP-infected cells with MG132 and PS1 severely impaired WNV VLP genome amplification, indicating that the proteasome was not required for viral entry. In fact, both MG132 and PS1 were effective inhibitors of WNV VLP activity up to 12 h post infection, suggesting that it is unlikely that the proteasome is required for the endosomal trafficking of WNV particles or the uncoating of the viral RNA, as it has been shown for influenza (Khor et al., 2003) and mouse hepatitis virus (Yu and Lai, 2005). The timing of the

WNV life cycle is not well established and is expected to vary between cell types. The process of viral entry and genome unpacking likely occurs within the first 2 h. Because WNV is a positive-stranded RNA virus, the newly unpackaged genome can be transported to cellular membranes and immediately be translated by cellular machinery. Once the viral proteins are produced to sufficient levels, the replication complex can be formed and begin replicating the viral genome, which serves as a source for more templates for translation. In some cell types, we have detected low levels of WNV antigen as little as 12 h post infection, suggesting the presence of active translation and, likely, replication at this time point. Although we do not know precisely how the proteasome is involved in the lifecycle, its effectiveness up to 12 h post infection suggests it is acting at the level of either translation or genome replication.

We have shown that proteasome inhibition results in a reduction in WNV yield, however, we have not tested whether, as seen with HIV (Martin-Serrano, 2007; Schubert et al., 2000; Strack et al., 2000), the proteasome is directly involved in the packaging and/or release of WNV progeny virions. Our studies do not exclude the possibility that the proteasome is involved at these stages of infection; however, our initial studies demonstrating impairment in WNV genome amplification were performed with WNV VRPs, which are not packaged. Thus, the proteasome cannot be involved *solely* in packaging and release of viral progeny. Instead, it is likely that the impairment of WNV particle release that we observed is due to a decrease in either (or both)

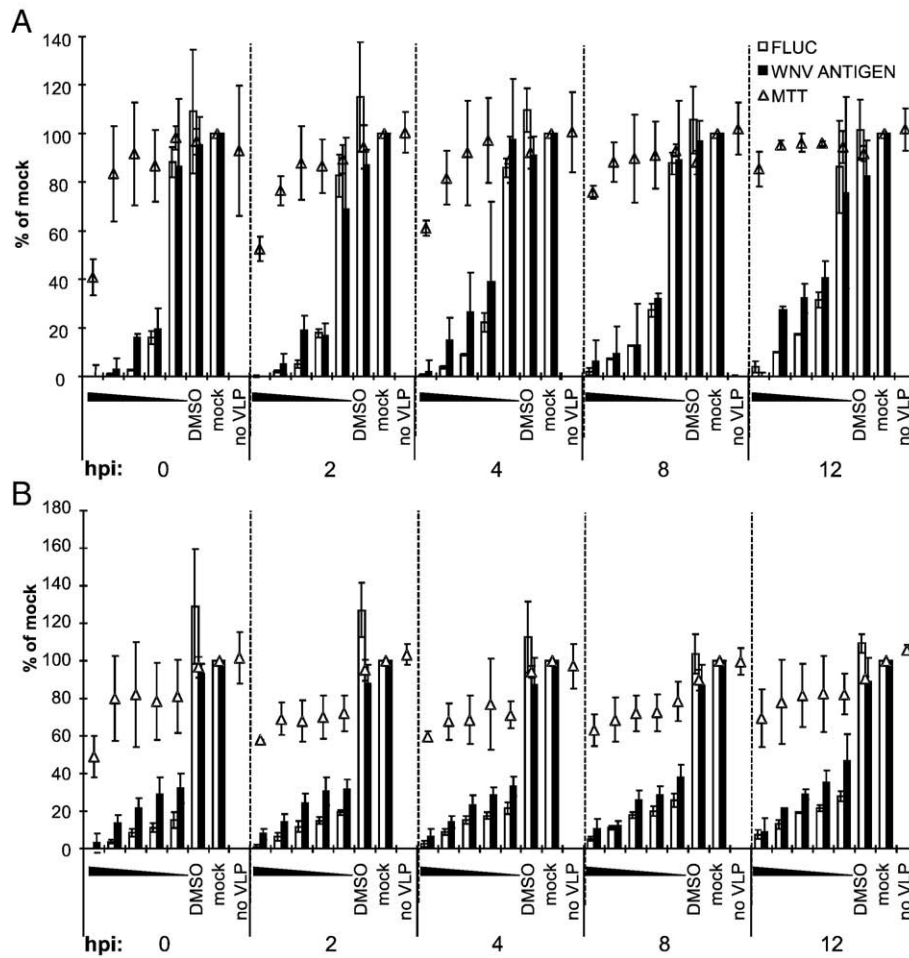


Fig. 6. Inhibition of proteasome results in impairment in WNV translation or genome replication, not WNV entry or unpackaging. HeLa cells were treated with either MG132 (panel A) or PS1 (panel B) concurrent with or at various times post-WNV VLP infection and assayed for FLUC activity, WNV antigen expression and cell viability. Values are shown as percent of mock and error bars represent standard deviation from the mean between two independent experiments.

proteins derived from translation or genomes produced during genome replication.

Consistent with our indirect assays (Fluc and ELISA), total levels of WNV NS3, as measured by Western blot analysis, was dramatically reduced following proteasome inhibitor treatment. The WNV NS2A–NS3 complex serves as the viral protease and is required for the proper processing of the viral polyprotein and subsequent genome replication. It is possible that the proteasome inhibitors, or the proteasome itself, could have an effect on the protease activity of NS2A–NS3. However, although there was a decrease in the expression of all detectable viral proteins, there did not appear to be any abnormal banding patterns associated with impaired protein processing from proteasome inhibitor-treated cells (results not shown). This suggested that it is unlikely that inhibiting the proteasome has a direct negative effect on viral polyprotein processing.

Our IFA studies revealed a curious result. As expected, a decrease in the expression of both NS1 and NS3 in the presence of the inhibitors was readily detected; however, it appeared as though the effect on NS1 expression was more pronounced than the effect on NS3. There could be several reasons for this curious effect. It is possible that the antibody against NS3 is more sensitive than the antibody against NS1, particularly with MG132 treatment. It is also possible that viral proteins have different turn-over rates. The location of NS1 within secretory pathways of the infected cell may also concentrate the antigen, making it more visible than NS3. Nevertheless, our data clearly demonstrate a role for the proteasome at post-entry stages of WNV infection, although we have not yet determined whether the

reduction in antigen accumulation/expression is due to a direct inhibition of viral translation or an inhibition of viral genome replication (which would also reduce translational products).

The reason behind why the proteasome is important is not clear. The ubiquitin–proteasome system is involved in a variety of normal cellular processes, including signal transduction, transcriptional

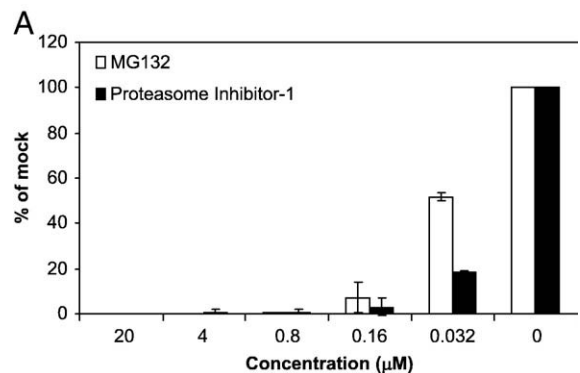


Fig. 7. Proteasome inhibition impairs WNV particle release. Monolayers of HeLa cells were infected with WNV for 1 h, the virus was removed and the indicated concentrations of MG132 or PS1 were added to the infected cells. Twenty-four hours post infection/treatment the supernatants were removed from the treated cells and the titration of each sample was determined by IHC (see Materials and methods). Results are shown as percent virus titer from mock-treated cells. Error bars represent the standard deviation from the mean between three independent experiments.

regulation, the immune response and apoptosis, any of which could be tied to WNV replication. Although WNV has been shown by several groups to interfere with IFN- α/β signaling by preventing STAT1 phosphorylation (Guo et al., 2005; Scholle and Mason, 2005), this impairment has not been shown to be achieved by the degradation of a specific protein. More recently, Medigeshi et al. has demonstrated that WNV induces the degradation of ATF6, a transcription factor associated with the ER, in a proteasome-dependent fashion (Medigeshi et al., 2007). However, their studies were performed with live WNV at 36 h post infection while ours were performed with WNV VRPs at 24 h post infection. In our hands, WNV VRP infection does not cause observable apoptosis at 24 h post infection. Associations between proteasome inhibition of WNV VRP replication genome amplification and the degradation of ATF6 may be of interest to investigate. Nevertheless, it is possible that WNV targets specific protein(s) to the proteasome and that disrupting their degradation has a negative impact on WNV genome amplification. The identity of such proteins will have to be investigated in more detail.

The role of the proteasome could also be linked to the formation of defective ribosomal products (DRiPs). The process of translating the sequences encoded by the viral (or cellular) genome to produce functional proteins has many chances for the introduction of errors. Mistakes include the addition of incorrect amino acids, early (or even late) termination of the translated product or the improper folding or splicing of proteins. These errors result in the formation of DRiPs (Yewdell, 2002; Yewdell et al., 1996; Yewdell and Nicchitta, 2006). It is expected that these incorrect products would be rapidly degraded by the proteasome, as they could potentially interfere with the function of the normal protein or other cellular processes. Thus, an accumulation of WNV-generated DRiPs under conditions of rapid viral polyprotein accumulation within replication complexes could interfere with replication of the viral genome. The removal of these damaged/defective products could be a reason why efficient proteasome function is required for WNV replication.

Materials and methods

Cell lines and their maintenance

Huh7 cells and HeLa cells were propagated in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution. Cells stably expressing *Renilla* luciferase (RLuc) were used for all of these studies (see below), and are referred to simply as HeLa or Huh7 cells throughout these studies. BHK(VEErep/C*-E/pac) cells (Fayzulin et al., 2006), which were utilized for WNV VRP preparations, were maintained in DMEM supplemented with 10% FBS, 1% antibiotic solution and 10 $\mu\text{g}/\text{ml}$ puromycin.

Generation of *Renilla* luciferase-expressing cell lines

RLuc-expressing cells were generated by infecting cell monolayers with a vesicular stomatitis virus (VSV)-lentivirus pseudotype virus expressing RLuc and blasticidin resistance (kindly provided by R Davey, UTMB). Briefly, monolayers of cell (plated in 6-well tissue culture plates) were infected with the pseudotype virus. Twenty-four hours post-infection, 2 $\mu\text{g}/\text{ml}$ of blasticidin was added to the media and the cells were incubated at 37 °C. After approximately 2 weeks under antibiotic selection, pools of the antibiotic-resistant cells were harvested and propagated in normal growth media. To confirm integration of a functional RLuc gene, cells were tested for RLuc activity as described below.

Virus and VRPs

The live WNV utilized in these studies was a low-passage virus recovered from BHK cells transfected with a synthetic RNA derived

from an infectious cDNA clone of a human 2002 isolate from Texas (Rossi et al., 2005). The virus recovered from BHK cells was passed one time in Vero cells to obtain the stock that was used for all experiments.

Two different WNV VRPs were utilized for the siRNA screen: VRPs containing a WNV replicon expressing either a FLuc gene (C-FLuc2A NS1-5) or a β -galactosidase gene (C-bgal2A NS1-5) followed by the FMDV-2A coding region inserted between the C and NS1 encoding regions in the WNV genome (Fayzulin et al., 2006). For the proteasome inhibition studies, WNV hFLuc VRP was utilized instead of WNV FLuc VRP. This VLP contained a replicon identical to C-FLuc2A NS1-5, except that it contained a FLuc gene (derived from Promega) that had been optimized for more efficient expression in human cells. WNV VRPs were generated as described previously (Fayzulin et al., 2006).

The titers of the stock virus and the WNV VRPs were determined by focus formation assay in Vero cells as previously described (Gilfoy and Mason, 2007). VEEV replicons encoding GFP and FLuc under control of two separate subgenomic promoters (VEEre/Luc/GFP) packaged into VRPs as described previously (Volkova et al., 2006) were kindly provided by I. Frolov (UTMB).

Proteasome inhibition and VRP infection

Both MG132 (Calbiochem) and proteasome inhibitor 1 (PS1) (Calbiochem) were initially diluted in DMEM++ to 20 μM and then serially diluted 4- or 5-fold in DMEM++ supplemented with 1% DMSO (diluent control). Fifty microliters of the inhibitor dilutions, 1% DMSO or DMEM++ alone were added to monolayers of Huh7 or HeLa cells (plated in 96 well plates) and incubated at 37 °C for approximately 2 h and then infected with WNV VRPs. For VRP infections, 50 μl of WNV hFLuc VRP was added to each well (on top of the proteasome inhibitor) and incubated for 24 h at 37 °C. Since VRPs cannot spread past the initial infected cell, it was not necessary to remove the VRPs after infection. Unless otherwise noted, the inhibitors remained on the cell monolayers for the entire length of the experiment.

siRNA transfection

Cells were transfected with Dharmacon plus Smartpool non-targeting siRNA negative control (NEG siRNA; Dharmacon), Dharmacon plus Smartpool PSMA1 siRNA (Dharmacon; pool of 4 sequences: GCUAGACUGUUUUGUAAUU, GGGCAGGAUUCUCAAUUU, CCAUUG-GAAUUGUUGGUAU, GAUACCAACACAACGAUUAU) or Dharmacon plus Smartpool PSMA2 siRNA (Dharmacon; pool of 4 sequences: GCAUUAJAGGUUUGGUGUAC, ACACAAGUAGAACCAAUU, GAAUGAGG-GACGACCAUUAU, CAAAUGGUGUGGUUUUAGC). Briefly, siRNAs (to achieve a 30 nM final concentration) were incubated with 0.25 μl DharmaFECT-1 (DF1; Dharmacon) transfection reagent diluted into 25 μl Dharmacon Cell Culture Reagent (DCCR; Dharmacon) for 10–30 min at room temperature. The siRNA-DF1 complexes were added to the wells (25 $\mu\text{l}/\text{well}$ in 96-well plates) and 75 μl of cells at a concentration of 1×10^5 cell/ml was added to the complexes and incubated for approximately 72 h at 37 °C. Following the incubation period, the transfected cells were infected with WNV VRPs as described above. siRNA transfections for all of the secondary confirmatory screens were performed as above. In the case of the Ambion siRNA library, the transfection was similar to that described above with the exception that the siRNAs, which were lyophilized onto 96-well plates, were rehydrated in the plate with the DF1 diluted in DCCR.

Luciferase assays

Media from cell monolayers infected with WNV VRPs expressing FLuc was removed at approximately 24 h post-infection. The cells were mixed with luciferase substrate, a 1:5 mixture of Steady-glo

luciferase substrate (Promega) and lysis buffer (40 mM Tricine, 8 mM magnesium acetate, 33 mM DTT, 0.13 mM EDTA and 0.1% Triton X-100) and agitated for 30 s. Light output from the lysed cells was measured in either a TR717 (Applied Biosystems) or a Centro XS³ LB 960 microplate luminometer (Berthold Technologies). To reduce costs, the Ambion siRNA library and subsequent secondary screens were assayed for FLuc activity using a self-prepared FLuc substrate as described previously (Fayzulin et al., 2006). In some cases, immediately following measurement of FLuc activity, 50 μ l of RLuc substrate (100 mM EDTA, 10 mM Tris, 5 μ g/ml coelenterazine (CTZ; NanoLight Technologies)) was added directly to the wells. The plate was agitated for 30 s and light output from the lysed cells was measured in one of the luminometers described above.

Western blot analysis

Monolayers of siRNA-transfected cells (plated in 96-well plates) were rinsed in phosphate-buffered saline solution (PBS) and harvested in 100 μ l of lysis buffer (0.1% Triton-X-100, 300 mM NaCl and 50 mM Tris, pH 7.69). Protein concentrations were determined by the Bio-Rad D_c protein assay (Bio-Rad). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to a Immobilon polyvinylidene difluoride transfer membrane (Millipore) as described previously (Gilfoy and Mason, 2007). Membranes were probed with mouse anti-actin (Sigma), rabbit anti-PSMA2 (Santa Cruz) or a goat anti-WNV NS3 (R and D Systems) followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary immunoglobulin (IgG) against the origin species for each of the antibodies (KPL). HRP decorated bands were visualized as described previously (Gilfoy and Mason, 2007).

ELISA

WNV antigen expression was measured by ELISA as previously described (Rossi et al., 2005). Briefly, fixed cells were rehydrated in blocking buffer (PBS containing 1% normal horse serum (NHS) and 0.01% Tween-20) and then incubated with an anti-WNV MHIAF polyclonal antibody followed by incubation with an HRP-labeled goat anti-mouse IgG (KPL). 3,3',5,5'-Tetramethylbenzidine (TMB; Sigma) was added to the plate and, following color development, the reaction was stopped by the addition of 1 M HCl. Absorbance was measured at 450 nm.

Cell viability assay

Cell viability was determined by a standard MTT assay. In short, monolayers of treated cells were incubated with 10 mg/ml methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma) solution for 3 h at 37 °C. Following incubation, the solution was removed and 100% isopropanol was added to solubilize the metabolized MTT product. The plates were then agitated for 15 min at room temperature, and absorbance of the MTT product was measured at 560 nm.

WNV yield reduction assay

Yield reduction assays were performed as previously described (Gu et al., 2006). Briefly, monolayers of HeLa cells were infected with WNV (MOI=0.05) for approximately 1 h at 37 °C followed by treatment with dilutions of either MG132 or PS1 diluted in MEM++ containing 1% DMSO. Cell supernatants harvested from the treated cells were assayed for virus yield on Vero cell monolayers as described (Gu et al., 2006).

Immunofluorescence assay (IFA)

Monolayers of HeLa cells were treated with MG132 or PS1 and infected with WNV hFLuc VLP as described above. Treated cell

monolayers were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton-X-100. The fixed monolayers were incubated in blocking buffer (2% bovine serum albumin, 5% normal horse serum, 10 mM glycine in PBS) for 30 min and then probed using the following monoclonal and polyclonal antibodies: mouse anti-NS1 (Eiji; Kobe University, Kobe, Japan), goat anti-NS3 (R and D Systems), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) and Alexa Fluor 488 donkey anti-goat IgG (Invitrogen). Following secondary antibody incubation, the cells were counterstained with DAPI (500 ng/ml) and Alexa Fluor 594-conjugated phalloidin (R. Davey, UTMB) for nuclei and actin filaments, respectively. The monolayers were then mounted using VectaShield (Vector Laboratories) and stained cells were analyzed with a 1.0 Zeiss LSM 510 UV META Laser Scanning Confocal Microscope at the UTMB Infectious Disease and Toxicology Optical Imaging Core Facility.

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