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Rift Valley fever virus NSs inhibits host transcription independently of the degradation of dsRNA-dependent protein kinase PKR

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ABSTRACT

Rift Valley fever virus (RVFV) encodes one major virulence factor, the NSs protein. NSs suppresses host general transcription, including interferon (IFN)-β mRNA synthesis, and promotes degradation of the dsRNA-dependent protein kinase (PKR). We generated a novel RVFV mutant (rMP12-NSsR173A) specifically lacking the function to promote PKR degradation. rMP12-NSsR173A infection induces early phosphorylation of eIF2α through PKR activation, while retaining the function to inhibit host general transcription including IFN-β gene inhibition. MP-12 NSs but not R173A NSs binds to wt PKR. R173A NSs formed filamentous structure in nucleus in a mosaic pattern, which was distinct from MP-12 NSs filament pattern. Due to early phosphorylation of eIF2α, rMP12-NSsR173A could not efficiently accumulate viral proteins. Our results suggest that NSs-mediated host general transcription suppression occurs independently of PKR degradation, while the PKR degradation is important to inhibit the phosphorylation of eIF2α in infected cells undergoing host general transcription suppression.

Introduction

Rift Valley fever virus (RVFV) belonging to the family *Bunyaviridae*, genus *Phlebovirus* is a mosquito-borne zoonotic pathogen endemic to sub-Saharan Africa, and has spread into Egypt, Madagascar, Saudi Arabia and Yemen (Pepin et al., 2010; Swanepoel and Coetzer, 2004). Humans infected with RVFV suffer from febrile illness with occasional complications such as hemorrhagic fever, encephalitis or blindness (Ikegami and Makino, 2011). RVFV infection of pregnant ruminants causes high rates of abortion and fetal malformation (Swanepoel and Coetzer, 2004). Because of its potential impact on public health and agriculture, RVFV is classified as Category A Priority pathogen by NIH/NIAID, and overlap select agent by CDC/USDA in the U.S. (Bird et al., 2009; Mandell and Flick, 2010). Currently, there are no commercially available vaccines outside endemic countries, and there are no effective therapeutics to treat RVF patients. MP-12 is the only strain excluded from select agent rule, and can be handled in BSL-2 laboratories.

RVFV genome is comprised of a tripartite negative-stranded RNA genome named S-, M-, and L-segments (Schmaljohn and Nichol, 2007). In addition to viral structural proteins; i.e., N, L, Gn and Gc proteins, RVFV encodes two nonstructural proteins (NSs and NSm) and the less characterized 78 kD protein. NSs protein, encoded in the S-segment, is a major virulence factor of RVFV, and has three functions; (1) suppression of the general host transcription by sequestering TFIIH p44 subunit proteins (Le May et al., 2004) and by promoting the degradation of TFIIH p62 subunit proteins (Kalveram, Lihoradova and Ikegami, 2011), (2) degradation of dsRNA-dependent protein kinase (PKR) (Habjan et al., 2009; Ikegami et al., 2009), and (3) inhibition of the IFN-β promoter activation through sin3A-associated protein (SAP30) (Le May et al., 2008). MP-12 strain encodes functional NSs gene, which inhibits host general transcription and promotes degradation of PKR (Billecocq et al., 2008; Ikegami et al., 2009; Ikegami et al., 2006; Kalveram, Lihoradova and Ikegami, 2011). In addition, NSs is responsible for cell cycle arrest at either G0/G1 or S phase, as well as DNA damage response via ataxia-telangiectasia mutated (ATM) (Austin et al., 2012; Baer et al., 2012). NSs interacts with pericentromeric γ-satellite sequence and induces defect of chromosome cohesion and segregation (Mansuroglu et al., 2009).

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Little is known about the role of PKR degradation in RVFV life cycle. Habjan et al. showed that the RVFV clone 13 strain, lacking a functional NSs, replicates efficiently in PKR-knockout mice (Habjan et al., 2009). Our study demonstrated that cells infected with rMP12-rLuc (lacking NSs) resulted in increased levels of eIF2α phosphorylation, as compared to those infected with parental MP-12 (encoding NSs), in the presence of actinomycin D (ActD; transcription inhibitor) (Ikegami et al., 2009). On the other hand, phosphorylation of eIF2α was significantly suppressed in the presence of MP-12 NSs or PKRΔE7 (a dominant-negative form of human PKR). These results suggest that the PKR degradation by MP-12 NSs alleviates the negative effect of host transcription suppression to maintain viral translation. Although RVFV NSs encodes both host transcription suppression function and PKR degradation function, it is not clear whether host transcription suppression occurs independently of PKR degradation, or vice versa, and NSs-mediated transcription suppression creates cellular environment which requires PKR degradation for an efficient viral translation.

We hypothesize that host transcription suppression and IFN-β gene suppression occur independently of PKR degradation, while PKR degradation is important to inhibit eIF2α phosphorylation under NSs-mediated host transcription suppression. In this study, we generated and characterized a novel NSs mutant that does not promote PKR degradation while inhibiting host general transcription. Our results suggest that NSs-mediated host transcription suppression occurs independently of PKR degradation, and cells undergoing host general transcription by NSs induce early eIF2α phosphorylation by viral replication when PKR degradation does not occur. Thus, PKR degradation plays an important role for an efficient viral protein synthesis in RVFV-infected cells.

Results

Generation of MP-12 NSs mutants by alanine substitution.

To study the significance of PKR degradation in cells infected with RVFV, we tried to generate an MP-12 encoding a mutant NSs that does not promote PKR degradation yet inhibits host general transcription. Since no structural analyses are available for the NSs protein, we performed random screening of NSs mutants. Initially, 11 serial in-frame truncations of 25 amino acids were introduced into MP-12 NSs, and the resulting NSs mutants were functionally analyzed. Those NSs truncated mutants lacked PKR degradation function, as well as IFN-β gene suppression (Head, Kalveram and Ikegami, 2012). In an attempt to generate mutants lacking only one of known functions of NSs, we next randomly substituted one or two charged residues of NSs with alanine to create single or double point mutants. Among 10 recombinant MP-12 viruses characterized (R15A/R16A, R47A, R103A, D124A, E156A/D157A, R164A, E169A, R173A, D184A, E188A), R15A/R16A, R47A, R103A, D124A, R164A and R173A mutants inhibited IFN-β gene in MRC-5 cells. Among the mutants inhibiting IFN-β gene, only R173A did not promote degradation of PKR in VeroE6 cells, while R15A/R16A, R47A, R103A, D124A and R164A still promoted PKR degradation in VeroE6 cells (data not shown). We designated the R173A mutant as rMP12-NSsR173A, and further characterized the phenotypes to know the NSs functions in detail. We first characterized the plaque phenotype because MP-12 forms varied sizes of plaques with clear cytopathic effect (CPE), while MP-12 lacking NSs forms turbid plaques with minimum CPE (Ikegami et al., 2006). R15A/R16A, R47A, D124A, E156A/D157A, R164A, E188A formed MP-12-like plaques, while E169A and D184A formed uniformly turbid plaques, and R103A formed a mixture of MP-12-like plaques and turbid plaques (data not shown). The rMP12-NSsR173A formed uniformly smaller plaques than those of MP-12 with clear cytopathic effect (CPE) (Fig. 1).

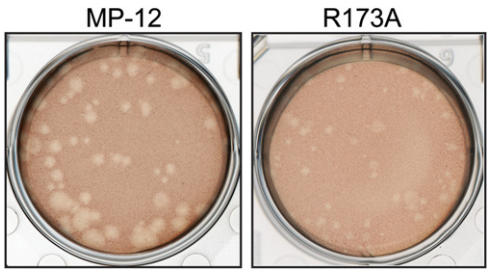

Fig. 1. rMP12-NSsR173A virus does not promote PKR degradation. Plaque phenotype of MP-12 (left) and rMP12-NSsR173A virus (right) on VeroE6 cells at 3 dpi.

The rMP12-NSsR173A induces PKR-mediated eIF2α phosphorylation

We examined the viral protein synthesis and eIF2α phosphorylation level in cells infected with rMP12-NSsR173A . Type-I IFN-competent mouse embryonic fibroblast cells (MEF/wt cells) were mock-infected or infected with MP-12, rMP12-C13type, rMP12-NSsR173A, or rMP12-mPKRN167 virus at an moi of 3. Cell lysates were collected at 6 and 12 hpi, and analyzed by Western blot (Fig. 2A and B). We included rMP12-C13type or rMP12-mPKRN167 (Lihoradova et al., 2012) which lack all NSs functions or specifically inhibit mouse PKR while still inducing IFN-β, respectively, as controls. We used mouse MEF cells to be able to further characterize the effect in cells lacking PKR (MEF/PKR$^{0/0}$ cells) in later experiments. As expected, cells infected with MP-12 caused a degradation of PKR at 6 hpi, and phosphorylation status of eIF2α was not significantly changed at 6 or 12 hpi. Cells infected with rMP12-C13type increased the abundance of phosphorylated eIF2α (1.63 times) at 12 hpi, and the accumulation of Gn and Gc was 25% and 31% less than those of MP-12-infected cells, respectively, while accumulation of N protein was still similar to that in cells that were infected with MP-12 . On the other hand, cells that were infected with rMP12-NSsR173A showed a visible band of PKR, while phosphorylated eIF2α was significantly increased by 1.87 times ($p=0.0185$, vs. MP-12) at 6 hpi, and the accumulation of Gn, Gc, and N proteins was 53%, 41%, and 38% less than those of MP-12-infected cells at 6 hpi, respectively. At 12 hpi, cells infected with rMP12-NSsR173A still had abundant phosphorylated eIF2α and less abundant Gn, Gc, and N. Levels of phosphorylated eIF2α in cells infected with rMP12-mPKRN167 virus were not significantly increased, and the abundance of Gn, Gc, and N proteins were similar to those of MP-12-infected cells. These results suggested that rMP12-NSsR173A does not induce PKR degradation, and increases the abundance of phosphorylated eIF2α faster than rMP12-C13type .

Since the abundance of R173A NSs proteins was lower than that of MP-12 NSs at 6 and 12 hpi, it was possible that low level of R173A NSs accumulation could result in a lack of PKR degradation. To address this, we analyzed early time points (i.e., 3, 4 and 5 hpi in MEF/wt cells mock-infected or infected with MP-12 or rMP12-NSsR173A). At 3 hpi, PKR abundance was lower in cells infected with MP-12 than those infected with rMP12-NSsR173A at 3 hpi (unpaired *t*-test, $p=0.029$) due to an increase of PKR abundance in cells infected with rMP12-NSsR173A (Fig. 2C and D). However, the abundance of PKR was clearly decreased at 4 hpi in cells infected with MP-12 compared to mock-infected cells (unpaired *t*-test, $p=0.033$ vs. mock-infected cells at 4 hpi). Thus, MP-12 NSs could start degrading PKR as early as 4 hpi in MEF/wt cells. We compared the PKR level of MP-12-infected cells at 4 hpi and that of rMP12-NSsR173A-infected cells at 5 hpi. The abundance of R173A N and NSs at 5 hpi was approximately 82.6% and 55.9% of that of MP-12 N and NSs at 4 hpi, respectively. The abundance of PKR in cells infected with rMP12-NSsR173A at 5 hpi was significantly higher than that in cells infected with MP-12 at 4 hpi (unpaired *t*-test, $p=0.033$). Compared to mock-infected cells at