Conserved Herpesviral Kinase Promotes Viral Persistence by Inhibiting the IRF-3-Mediated Type I Interferon Response

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SUMMARY

A conserved herpesviral kinase, designated ORF36 in murine γ-herpesvirus 68 (MHV-68), plays multiple vital roles in the viral life cycle. Here, we show by screening mutant viruses that ORF36 counteracts the antiviral type I interferon (IFN) response. ORF36 specifically binds to the activated form of interferon regulatory factor 3 (IRF-3) in the nucleus, inhibiting IRF-3 interaction with the cotranscriptional activator CBP and thereby suppressing the recruitment of RNA polymerase II to the interferon β promoter. The anti-IFN function of ORF36 is conserved among herpesvirus subfamilies, although the conserved kinase activity is not absolutely required for this function. MHV-68 lacking ORF36 induces a greater interferon response and is attenuated in vitro and in vivo, where acute viral infection in the lung and latency in the spleen are compromised. Our data suggest that herpesviruses have evolved within their conserved kinase an anti-IFN activity critical for evasion of host immunity and for persistence.

INTRODUCTION

Viral infection induces a variety of immune responses in the host that control viral replication. In the absence of pre-existing adaptive immunity, the non-specific innate immune responses are critical for restricting viral invasion and replication. Type I interferons (IFNs), a family of cytokines that includes IFN-α and multiple IFN-β species, are likely to be the most critical components of the innate immune defense against viruses. The secretion of IFNs by virus-infected cells is the foremost step of activating an antiviral state through autocrine and paracrine signaling (Taniguchi and Takaoka, 2002). Direct antiviral effects of IFN include inhibition of viral RNA expression (Li et al., 1998), degradation of viral mRNA (Player and Torrence, 1998), inhibition of viral protein synthesis, and induction of apoptosis (Garcia et al., 2006). Indirectly, IFN activates lymphocytes, NK cells, and macrophages, and it enhances antigen presentation on the cell surface (Stark et al., 1998). Therefore, IFNs are the main component of host antiviral defense, which links the innate and adaptive wings of the host immune system (Mossman and Ashkar, 2005).

IFNs have been shown to be induced by different pathways, including Toll-like receptor (TLR)-dependent (TLR-3,-4, -7, and -9) and independent (RIG-I and Mda5) pathways (Hiscott, 2007). Those inducing signals are thought to activate latent transcription factors in the cytoplasm, such as interferon regulatory factors (IRFs) and nuclear factor kappa B (NF-κB). The activated transcription factors translocate into the nucleus and activate IFN promoters in both a temporally and spatially controlled manner. Among those transcription factors, IRF-3 is the central player in the IFN system. IRF-3 is expressed constitutively but activated immediately upon viral infection (Hiscott, 2007). Virus infection activates cellular kinases, such as TBK-1, and leads to the phosphorylation of IRF-3. The phosphorylation induces a conformation change in IRF-3, which relieves autoinhibitory intramolecular binding within IRF-3. The unphosphorylated form of IRF-3 forms a dimer, which translocates into the nucleus, and then recruits the CBP/p300 transcriptional coactivator and activates promoters, including IFN-β promoter. Eventually, virus-induced phosphorylation and activation of IRF-3 lead to the proteasome-mediated degradation of IRF-3 (Hiscott, 2007).

The produced IFN-β is secreted, and it binds to IFN receptors on cell surfaces. This binding causes cascades of phosphorylation events of Janus kinase (JAK) as well as signal transducer and activator of transcription (STAT) proteins, leading to the nuclear translocation of phosphorylated STAT complex (Katze et al., 2002). Binding of the complex to the IFN-stimulated response element (ISRE) within several promoters initiates transcription of interferon-stimulated genes (ISGs) (Mossman and Ashkar, 2005; Taniguchi and Takaoka, 2002). The protein products of these ISGs subsequently function to control virus replication and propagation. One of the induced ISG products, IRF-7, is further activated by virus infection and induces the full array of IFNs cooperatively with IRF-3, resulting in the induction of a larger subset of ISGs (Hiscott, 2007).
Viruses have evolved under the hostile pressure of the host's antiviral immune responses. In proportion to the importance of the IFN system in controlling viral infection and replication, viruses have developed a variety of strategies to antagonize the system. Herpesviruses are large DNA viruses that can establish a lifelong persistence in a host by evading the host immune surveillance and can cause various diseases during their persistent infection. Thus, understanding the mechanism of herpesviral immune evasion is essential in controlling the herpesviral diseases. To investigate the immune evasion mechanisms of tumorigenic herpesviruses, we used an animal model, murine herpesvirus 68 (MHV-68), which is biologically and genetically related to the human herpesviruses. Through screening of the mutant library of MHV-68 in IFN receptor knockout mice, we found that the conserved herpesviral kinase ORF36 suppresses the IFN-mediated response by inhibiting IRF-3. This inhibitory function, although it does not absolutely require the conserved kinase activity, is shared by the ORF36 homologs among herpesvirus families. Therefore, we have identified a function of the conserved herpesviral kinase that is essential for herpesvirus to evade host immune surveillance and persist in a host.

RESULTS

Identification of ORF36 as a Necessary Gene of MHV-68 to Counteract IFN Response

To systematically analyze the role of viral genes in virus-host interaction, we generated a random insertion mutant library of MHV-68 by in vitro Mu transposase-mediated signature-tagged mutagenesis (Song et al., 2005). The signature tag allows simultaneous screening of multiple mutants in vivo by tagging each mutant with a unique short DNA sequence for PCR-mediated identification and quantification. We conducted in vivo screening to identify viral genes that counteract antiviral IFN response, which is the first line of host immune defense. A pool of MHV-68 mutants was infected into BALB/c mice or type I interferon receptor knockout mice (IFNAR−/−) mice. Each mouse was infected with a total of 500 plaque-forming units (pfu) of mixed mutant viruses with distinct tags (50 pfu/mutant). The in vivo growth of mutants was analyzed for acute replication in the lung at 7 days postinfection (dpi) and for establishment of latency in the spleen at 14 dpi by real-time quantitative PCR (q-PCR) as previously described (Song et al., 2005). After comparing the growth of each mutant in two groups of mice, one mutant virus was identified to show significant attenuation (<1/50 of average growth of mutants) in normal BALB/c mice and enhanced growth (>10-fold higher than average growth of all mutants) in IFNAR−/− mice in both acute replication in the lung and latency in the spleen (Figures 1A–1B). The mutant virus had a transposon insertion in the orf36 region (nucleotide 52925 in U97553, corresponding to the 27th amino acid [aa] in the total 437 aa protein).

To confirm the screening result using the pooled mutant infection, BALB/c and IFNAR−/− mice were infected with 50 pfu of either wild-type (WT) or ORF36 null transposon mutant (36T) MHV-68. With 50 pfu/mouse infection, the peak time of acute replication in the lung was determined as 9 dpi in a separate experiment. Thus, at the peak time of acute replication in the lung and latency in the spleen (14 dpi), the organs were harvested and analyzed for infectious/reactivatable virus and viral genome copy number. The 36T was attenuated in normal BALB/c mice (<1/400 of WT in both acute and latent replication) (Figures 1C–1D). However, its attenuation was significantly rescued in IFNAR−/− mice (<1/10 of WT in acute and <1/50 of...
WT in latent replication). Therefore, the attenuation of 36T in normal mice and the partial recovery of its attenuated growth in IFNAR−/− mice are due to the intrinsic growth defect of 36T in vivo, rather than by an unexpected effect of infection with pooled mutants. Furthermore, these data validate the effectiveness of our screening method of using the pool of mutants to identify critical viral genes in virus-host interaction.

### Lack of ORF36 Delays the Establishment of Splenic Latency

To further analyze the role of ORF36 in the establishment of MHV-68 latency, we generated a mutant virus with a nonsense mutation on the 30th aa of ORF36 (36S) and analyzed the establishment of splenic latency at 14 and 21 dpi after intranasal infection with WT or 36S. In the spleen of 36S-infected mice at the peak of latency (14 dpi), latently infected cells were almost undetectable (Figure 2A). This represents at least a 104-fold decrease in latency compared with that of the WT virus. There was no evidence of the latency-associated splenomegaly normally driven by MHV-68, which resembles lymphoproliferative responses that occur in humans during infectious mononucleosis (Sunil-Chandra et al., 1994) (Figure 2B). However, the analysis of the number of latently infected cells at 21 dpi showed that 36S established latency similar to WT. These data indicate that the lack of ORF36 expression delays, but does not completely impair, the establishment of γ-herpesvirus latency.

Next, we analyzed the 36S for its capacity to elicit an adaptive T cell immune response, which has been known to control the in vivo replication of MHV-68 (Braaten et al., 2009). To monitor the virus-specific CD8 T cell response, we used tetrameric reagents against two well-defined MHV-68 epitopes, ORF6487–495/Dβ and ORF61524–531/Kβ. The absolute numbers of ORF6487–495/Dβ- and ORF61524–531/Kβ-specific CD8 T cells at the mucosal site of infection (lung) (Figure 2C) were reduced 10- to 100-fold in 36S-infected mice at 14 dpi, but no differences in the size of the response were found at 21 dpi. The analysis of spleen cells (Figure 2D) showed that the numbers of MHV-68-specific CD8 T cells were slightly reduced in 36S-infected mice at 14 dpi, but they were comparable in 36S- or WT-infected mice at 21 dpi, a time point when 36S- and WT-infected mice show similar levels of latency (Figure 2A). These data suggest that the magnitude of the early MHV-68-specific CD8 T cell response is reduced in 36S-infected mice as a consequence of its reduced replication in lung and delayed latency in spleen.

### ORF36 Inhibits IRF-3-Mediated Activation of Interferon β Promoter

Virus-infected cells produce a mixture of IFNs depending on the cell type. In general, fibroblast and epithelial cells predominantly produce IFN-α, whereas dendritic cells, leukocytes, and macrophages express multiple IFN-β (Katze et al., 2002). For efficient IFN response, IFN-β-producing cells mainly rely on autocrine feedback, but IFN-α-producing cells constitutively express IRF-7 to rapidly produce high levels of IFN-β (Mosman and Ashkar, 2005). Since intranasal infection of MHV-68 can reach both types of cells, we first investigated the effect of ORF36 expression on the activity of IFN-β promoter, which is a common denominator in both systems (Hiscott, 2007).

While the IFN-β promoter was induced by the known transactivators, such as IRF-3, IRF-7, or NF-kB, ORF36 specifically inhibited the IRF-3-mediated activation of IFN-β promoter in a dose-dependent manner (Figure 3A). These data suggest that ORF36 may inhibit the production of IFN-β through IRF-3. We further pursued this possibility by testing upstream activators of IRF-3 signal transduction pathway, such as TANK-binding kinase 1 (TBK-1), Toll-like receptor 4 (TLR-4), or Sendai virus infection. TBK-1 phosphorylates and activates IRF-3 upon viral infection (Sharma et al., 2003). TLR-4 detects microbial invasion and activates IRF-3 pathway (in this study, we used a constitutively activated form of TLR-4 by the conjugation with extracellular domain of CD4 [Doyle et al., 2002]). Sendai virus infection is a potent activator of IFN pathway (Lin et al., 1998). As shown in Figure 3B, all three inducers successfully activated IFN-β promoter, but ORF36 inhibited these activations in a dose-dependent manner. These data suggest that ORF36 can inhibit the antiviral signal transduction pathway from viral invasion detection to IFN-β promoter activation by inhibiting the function of IRF-3.

Consistently, ORF36 inhibited the downstream reporter (ISRE-luc; IFN stimulatory response element) of IFN receptor signaling when it was activated by the TBK-1 overexpression, suggesting that the inhibitory effect of ORF36 can significantly reduce the
production of the downstream antiviral effectors (Figure 3C). Furthermore, the inhibitory effect of ORF36 on IFN-β promoter can be reversed by the overexpression of either TBK-1 or IRF-3 (Figure 3D). In contrast, the inhibition by the dominant-negative form of IRF-3 (IRF-3 dDBD, which lacks the DNA-binding domain but maintains interaction and activation domain) can be reversed by IRF-3, but not by TBK-1. This prompted us to test whether ORF36 inhibits the function of IRF-3 directly.

**ORF36 Inhibits the Interaction between IRF-3 and CBP**

To further elucidate the inhibitory mechanism of ORF36, we investigated the effect of ORF36 on each step of IRF-3-mediated activation of IFN-β promoter. We first found that increasing the expression of ORF36 has no effect on the expression level of IRF-3 and the phosphorylation of IRF-3 mediated by TBK-1 (Figure S1A available online). In fact, activated IRF-3 by TBK-1 overexpression translocates into the nucleus, but ORF36, which localizes in the nucleus, did not affect this translocation (Figure 4A). Consistently, when we activated IFN-β promoter with IRF-3(5D), which mimics the phosphorylated and activated form of IRF-3 by mutation of the five serine/threonine at the C terminus to aspartic acid (Lin et al., 1999), ORF36 can inhibit IRF-3(5D)-mediated activation of IFN-β promoter (Figure S1B). Altogether, these data clearly demonstrate that ORF36 inhibits a step downstream of IRF-3 nuclear translocation.

To test whether ORF36 can inhibit the transactivation function of IRF-3 bound to DNA by a heterologous DNA-binding domain, we analyzed the activation of a reporter controlled by the GAL4-binding site and the GAL4-DBD fused form of IRF-3. ORF36 still inhibited the activation of the reporter (Figure S1C), implying that ORF36 interferes with the transactivation function of IRF-3 independently of the DNA-binding activity of IRF-3. Next, we examined whether ORF36 can physically interact with IRF-3. Although we could not detect any interaction between ORF36 and the inactive native form of IRF-3, a physical interaction was observed between ORF36 and IRF-3(5D), representing an activated form of IRF-3 (Figure 4B). These data suggest that ORF36 may bind to the activated IRF-3 during the viral infection.

Therefore, to further confirm the interaction between active IRF-3 and ORF36 during the natural infection of MHV-68, we constructed a recombinant MHV-68, in which triple FLAG epitope coding sequence is inserted into the 5' of the ORF36 gene in MHV-68 genome, thus expressing FLAG-tagged ORF36 during viral replication. Infection of the recombinant...
**Figure 4. Molecular Interaction between IRF-3 and ORF36**

(A) Subcellular localization of IRF-3 upon TBK-1/ORF36 expression in 293T cells. Anti-FLAG and anti-mouse Alexa405 (blue) for FLAG-tagged ORF36; anti-HA-FITC (green) for HA-tagged TBK-1; anti-IRF-3 and anti-rabbit Cy3 (red) for endogenous IRF-3.

(B) Interaction between ORF36 and the native or activated form of IRF-3 by coimmunoprecipitation assay. The 293T cells were transfected with the plasmids expressing the indicated proteins (bottom) and lysed and analyzed at 48 hr posttransfection as described in method. IP, immunoprecipitation using antibody against indicated epitope. IB, immunoblot using antibody against indicated epitope.
MHV-68 at high MOI caused the phosphorylation/activation of endogenous IRF-3, and, consistent with Figure 4B, the FLAG-tagged ORF36 expressed from the virus bound to this slower-migrating phosphorylated IRF-3 (Figures 4C and S1E). These data suggest that, during the natural infection of MHV-68, the host may detect/inhibit the infection of MHV-68 through the activation of IRF-3, but the ORF36 of MHV-68 can counteract this antiviral defense of host by directly binding to the phosphorylated/activated IRF-3. Furthermore, purified ORF36 from bacteria can bind to in vitro translated IRF-3(35D), supporting the direct interaction between ORF36 and the activated IRF-3 (Figure S2A).

Through the binding assay of IRF-3 deletion mutants to ORF36, we found that the IRF association domain (IAD, aa 193–350) of IRF-3 is necessary and sufficient for binding to ORF36 (Figure S2B). The IAD domain has been shown to be required for binding to CBP as well as dimerization (Qin et al., 2005), suggesting that the binding of ORF36 to IRF-3 may inhibit the interaction between the activated IRF-3 and CBP inside the nucleus. Therefore, we investigated whether the physical binding between ORF36 and IRF-3 affects the interaction between IRF-3 and CBP. When the activated form of IRF-3 was coexpressed with CBP, both IRF-3 and CBP were coinmunoprecipitated reciprocally. However, in the presence of ORF36, the amounts of coinmunoprecipitated IRF-3 and CBP were significantly reduced (Figure S1D). Indeed, when we overexpressed TBK-1, endogenous IRF-3 bound to CBP. ORF36 inhibited this interaction, suggesting that ORF36 may inhibit the recruitment of CBP by IRF-3 to IFN-β promoter (Figure 4D).

We further analyzed the recruitment of CBP and RNA polymerase II (Pol II) to IFN-β promoter by performing chromatin immunoprecipitation (ChIP) analysis of IFN-β promoter using anti-CBP and anti-Pol II antibody. As shown in Figure 4E, overexpression of TBK-1 induced occupancy of IFN-β promoter with both CBP and Pol II. However, when ORF36 was coexpressed, the recruitment of both CBP and Pol II was significantly reduced, indicating that ORF36 inhibited the recruitment of general transcription complex to IFN-β promoter by activated IRF-3. Moreover, there was more recruitment of CBP and Pol II to IFN-β promoter in the mutant virus-infected cells than in the wild-type infected cells. Taken together, all of the data suggest that ORF36 binds to active IRF-3 in the nucleus and inhibits the IRF-3-mediated recruitment of general transcription complex to IFN-β promoter, thus inhibiting IFN-β production.

Anti-IFN Function of ORF36 Is Conserved in Herpesviruses

ORF36 is a kinase conserved among all of the subfamilies of herpesvirus, e.g., α (herpes simplex virus [HSV] UL13, β (human cytomegalovirus [HCMV] UL97), and γ (Epstein-Barr virus [EBV] BGLF4 and Kaposi’s sarcoma-associated herpesvirus [KSHV] ORF36) (Gershburg and Pagano, 2008). We tested whether this anti-IFN function of ORF36 is also conserved. When we expressed the homologs of ORF36, all of the homologs inhibited the TBK-1-mediated activation of IFN-β promoter in a dose-dependent manner (Figure 5A). Further, when the production of IFN-β was induced by treating cells with double-strand RNA (ds polyIC), mimicking the infection of RNA viruses, all of the homologs suppressed the activation of IFN-β promoter (Figure 5D). The ORF36 homologs also suppressed the production of endogenous IFN-β and downstream effectors (e.g., Mx1) after selecting the transfected cells by drug resistance, due to the low efficiency of transfection (Figure 5B). Collectively, these data suggest that the anti-IFN function of ORF36 may be also conserved among the conserved herpesviral kinases.

Subsequently, the next question was whether this inhibitory activity requires the conserved kinase function of ORF36. We constructed a kinase null mutant of ORF36 by mutating the invariantly conserved lysine residue in catalytic core to glutamine (K107Q), and the kinase activity is indeed knocked out in the mutant (Tarakanova et al., 2007). This kinase null mutant of ORF36 still inhibited the IFN-β reporter, although less potently than it did with the wild-type (Figure 5B–5D). Consistently, the part of ORF36 containing conserved kinase domain was sufficient, but not necessary, for binding to IRF-3 (Figure S2C). The same inhibition was also observed with the kinase null mutant of EBV BGLF4 (K102I) (data not shown). Although there is a possibility that the kinase activity may indirectly contribute to the anti-IFN function of ORF36, these data suggest that the kinase activity of ORF36 is not absolutely required for inhibition of IFN-β promoter by ORF36.

Kinase-Dependent/Independent Function of ORF36 Is Required for Efficient Replication of MHV-68

The ChIP data suggest that MHV-68 without functional ORF36 induces more transcription complex recruitment to IFN-β promoter, which will lead to more IFN-β production. Previously, we have found that 36T was attenuated even in vitro cell culture systems (Song et al., 2005). Thus, we hypothesized that MHV-68 without functional ORF36 may induce more antiviral IFN response than wild-type, and this may lead to the attenuation of ORF36 null viruses. Indeed, ORF36 null mutant viruses induced more IFN-β and ISRE response than did wild-type at the same multiplicity of infection (MOI = 0.05) (Figure S3A). This induction was proportional to the extent of viral replication because both WT and the mutants induced more IFN reporter activity at higher MOI (data not shown). To further confirm the specific phenotype of ORF36 null mutants, we generated additional mutant viruses: another nonsense mutant of ORF36 (N36S, nonsense mutation on the 107th aa), the kinase null mutant of ORF36 (36KN, K107Q), and wild-type revertant of ORF36 null mutant (36R). Like the other ORF36 null mutants,
N36S induced more IFN response than did wild-type (Figure 6A). However, both 36KN and 36R showed similar phenotype to wild-type, supporting the specificity of ORF36 null phenotypes.

Next, we investigated the importance of antiviral IFN response in controlling the in vitro replication of MHV-68 by generating stable cell lines that express either IRF-3 dDBD or IRF-3(5D). All ORF36 null mutants, but not 36R, showed attenuated growth in the parental cell line. Their attenuated growth was significantly rescued in the cells expressing IRF-3 dDBD and further reduced in the cells expressing IRF-3(5D) (Figures 6B and S3B). Furthermore, the growth of 36T and 36S was also attenuated in primary mouse embryonic fibroblast (MEF) cells, but this attenuation was also rescued in MEF cells derived from IFNAR−/− mice (Figure S3B). Interestingly, 36KN also showed similarly attenuated growth like ORF36 null mutant viruses in vitro (Figure 6B), although it did not significantly induce IFN response (Figure 6A). Collectively, these data suggest that ORF36 is required for MHV-68 to counteract IFN response and to grow efficiently in vitro.

IFN production in vivo (Weslow-Schmidt et al., 2007). Knocking out ORF36 may not be enough to increase IFN in the entire lung to measurable levels by our assay systems, but it may cause a significant difference in IFN level only at the microenvironment level. Thus, we attempted to measure the transcript level of ISGs, such as Mx1 and IRF-7, in the same RNA harvested from either WT or 36S-infected lung, we detected a similar level of Mx1 and IRF-7 (Figure 6C), showing that 36S can induce the same level of ISG response to WT even when its replication was severely attenuated in vivo. Taken together, these data suggest that MHV-68 without ORF36 may induce more antiviral IFN response in vivo as well as in vitro than does wild-type at the same level of viral replication.

Next, we investigated whether the same phenomenon happens in vivo. When we measured IFN levels in the lung of WT and 36S-infected mice, however, we could not detect any significant level of IFN-β production by ELISA to measure secreted IFN-β or by q-PCR to measure the transcription of IFN-β (data not shown). This may be due to the extremely low level of IFN-β production after MHV-68 infection because it is known that MHV-68 is a poor inducer of IFN-β in vivo (Weslow-Schmidt et al., 2007). Knocking out ORF36 may not be enough to increase IFN in the entire lung to measurable levels by our assay systems, but it may cause a significant difference in IFN level only at the microenvironment level. Thus, we attempted to measure the transcript level of ISGs, such as Mx1 and IRF-7, in vivo. We harvested RNA from either WT or 36S-infected lung, we detected a similar level of Mx1 and IRF-7 (Figure 6C), showing that 36S can induce the same level of ISG response to WT even when its replication was severely attenuated in vivo. Taken together, these data suggest that MHV-68 without ORF36 may induce more antiviral IFN response in vivo as well as in vitro than does wild-type at the same level of viral replication.

We next examined the phenotype of ORF36 null mutants by infecting wild-type and mutant MHV-68 into normal and IFNAR−/− mice. During the acute replication of MHV-68 in the lung, 36R replicates just like WT, but both N36S and 36KN...
were significantly attenuated (~1/1000 and ~1/100 of WT by infectious viral titer, respectively) (Figure 6D). However, N36S, but not 36KN, could be partially rescued in the IFNAR−/− mice (~1/100 by infectious viral titer). Therefore, during acute infection in the lung, it appears that the anti-IFN function of ORF36 is largely mediated by a kinase-independent mechanism. These data further suggest that the kinase activity of ORF36 may play another role in the replication of MHV-68 in the lung, independently of anti-IFN function.

We also examined the level of viral latency established in the spleen at 14 dpi. The 36R behaved indistinguishably from WT, while both N36S and 36KN were attenuated in normal mice (Figure 6E). However, in contrast to the acute replication in the lung, the attenuation of both mutants could be mostly rescued in the IFNAR−/− mice. Thus, the kinase activity of ORF36 seems to be mainly responsible for the anti-IFN function during the latency in the spleen. Altogether, these data indicate that the anti-IFN function of ORF36 is essential for viral infection in the host and can be mediated through both kinase-dependent and -independent mechanisms. Furthermore, the fact that the attenuation of N36S could not be fully restored in the IFNAR−/− mice suggests additional in vivo roles of ORF36 other than the anti-IFN function (Lee et al., 2007; Tarakanova et al., 2007).

The Critical Role of ORF36 in the Normal Replication Kinetics of MHV-68

For the continuous monitoring of the interaction between MHV-68 and the host, we recently developed a bioluminescent imaging system using a recombinant MHV-68, in which viral M3 promoter drives firefly luciferase expression (M3FL). Our results suggest that the replication kinetics of M3FL is similar to that of parental wild-type MHV-68 and that M3FL is an effective model for studying the in vivo interaction of γ-herpesvirus with its host (Hwang et al., 2008). To further examine the systemic infection of MHV-68 without ORF36, we generated the ORF36 null stop codon mutant in the background of M3FL (M3FL-36S). Normal BALB/c mice were intranasally infected with 5 × 10^5 pfu of either wild-type M3FL or M3FL-36S, and bioluminescent images were obtained every other day postinfection. In contrast to the normal progression of wild-type from lung to spleen, which is from the primary site of infection to the major reservoir of viral latency, the mutant virus showed relatively normal acute replication in the lung but could not progress to the spleen (Figure 7). This imaging result is consistent with the virological assays presented in Figures 1 and 6. Furthermore, increasing the inoculum dose by 10,000-fold (compared to 50 pfu shown in Figure 1) cannot overcome the attenuation caused by the loss of ORF36.

However, when IFNAR−/− mice were infected with 5 × 10^5 pfu of the same viruses, there was no significant difference between the two viruses in replication or distribution. Eventually, IFNAR−/− mice infected with either M3FL or M3FL-36S succumbed to infection during 6–8 dpi (Figure 7). This systemic analysis of infection in vivo clearly demonstrated that the defective replication/progression of MHV-68 without ORF36 can be rescued by inhibiting IFN response. Moreover, these data suggest that ORF36 is the gene important for MHV-68 to counteract antiviral IFN response in order to replicate efficiently inside immune-competent host/cells.

DISCUSSION

The critical role of the IFN system in defending the host from viral invasion can be attested by the diverse antagonistic strategies of various viruses. Herpesviruses have developed multiple ways to counteract this critical antiviral host response (Mossman and Ashkar, 2005; Stevenson and Efstatou, 2005). Here, we identified a function of a conserved herpesviral protein that antagonizes IFN response during viral replication through the systematic screening of MHV-68 mutant library in mice with different genetic backgrounds. As in this study, screening pools of mutant viruses in hosts with different genetic backgrounds will provide an efficient screening environment with competition among viruses, which will expedite the identification process. Furthermore, by combining this genetic screening with bioluminescence whole-body imaging, screening and validation processes can be accelerated.

Many viral and cellular proteins have been shown to be phosphorylated by ORF36 homologs, including ORF36 itself and elongation factor 1α. Multiple functions of ORF36 homologs have been proposed in the various stages of viral life cycle, such as cellular and viral gene regulation, nuclear egress, virus maturation and replication, chromosome condensation, and tissue tropism (Asai et al., 2007; Gershburg and Pagano, 2008; Hamza et al., 2004; Izumiya et al., 2007; Kawaguchi and Kato, 2003; Lee et al., 2007; Michel and Mertens, 2004). The ORF36 also plays a major role in sensitizing γ-herpesvirus to nucleoside analog drugs, such as ganciclovir (Davis et al., 2007). However, the biological significance of many of the proposed targets and functions has not been clearly demonstrated in the life cycle of the virus during natural infection in vivo. In this study, we identified a function of ORF36 via an unbiased screen. The biological significance of this function in the virus life cycle can be demonstrated by the attenuation of the ORF36 null mutant in normal cells and mice and the significant rescue of that attenuation in IFNAR−/− cells and mice.

Intriguingly, ORF36 interacts only with active IRF-3, which localizes in the nucleus. The finding is further supported by the nuclear localization of ORF36 (Figure 4A) and the binding of ORF36 to IAD of IRF-3 (Figure S2B), which is buried by the auto-inhibitory elements in inactive IRF-3 (Qin et al., 2005). It may represent the beauty of viral evolution: the virus targets only the activated form of IRF-3, rather than all IRF-3 molecules. The majority of IRF-3 in the cell is inactive form. By targeting activated IRF-3 specifically, the virus can achieve the inhibitory goal more efficiently with a small amount of ORF36 proteins.

Through the point and deletion mutant study of ORF36 protein in vitro, we found that the kinase domain of MHV-68 ORF36 is sufficient, but not necessary, for this inhibition (Figures 5 and S2C). Consistently, the mutant MHV-68 without functional kinase did not significantly induce the production of IFN response (Figure 6A). However, MHV-68 with the kinase null mutation did not replicate competently both in vitro and in vivo, and this attenuated growth was significantly rescued when the antiviral IFN response was compromised (Figures 6B, 6D, and 6E). These data suggest that ORF36 counteract antiviral IFN responses by both kinase-dependent and -independent manners. Although the kinase activity was not absolutely required for ORF36 to interact with IRF-3 and to suppress the...
induction of IFN-β promoter by the activated IRF-3, the kinase activity may affect other cellular or viral proteins indirectly to reduce the antiviral effect of IFN, augmenting direct inhibitory effect of ORF36 on IRF-3. This may be more crucial in the real life cycle of MHV-68, in which multiple viral proteins act together to subvert the antiviral host defense for efficient replication.

Moreover, the fact that the ORF36 null mutant could not be fully rescued in the IFNAR−/− mice (Figure 6D and 6E) suggests that, in addition to antagonizing the host IFN responses, ORF36 has other functions that are important for viral replication in vivo. This is consistent with the notion that ORF36 may have multiple functions that are important for the efficient replication of MHV-68, such as the initiation of DNA damage response and chromosome condensation (Lee et al., 2007; Tarakanova et al., 2007).

IFN response is an intruder-alerting system used by host cells to defend themselves, so invading viruses need to subvert this alarm system immediately to infect and replicate successfully. It has been shown that herpesvirus virions, especially glycoproteins on the envelope mediating attachment and fusion of virus to the host cells, induce interferon response (Barchet et al., 2002; Compton et al., 2003; Dalod et al., 2002; Lund et al., 2003; Morrison, 2004; Simmen et al., 2001). Although ORF36 is an early protein expressed several hours after virus entry (Ebrahimi et al., 2003; Martinez-Guzman et al., 2003), it was also detected in the infectious virion (Asai et al., 2006; Bechtel et al., 2005; Bortz et al., 2003; Overton et al., 1992; Varnum et al., 2004; Zhu et al., 2005). Therefore, it can be released into the cytoplasm of host cells immediately upon viral entry and may downregulate the host IFN response to modify the host cell physiology in favor of viral gene expression and replication. This function will be further enhanced after ORF36 is expressed in the infected cells. Alternatively, but not exclusively, ORF36 may also be needed for the latent virus to efficiently reactivate. In fact, ORF36 is one of the genes directly activated from latent KSHV in a hypoxia-induced reactivation (Haque et al., 2006). IFN response is an intruder-alerting system used by host cells to defend themselves, so invading viruses need to subvert this alarm system immediately to infect and replicate successfully.

Figure 6. The Role of ORF36 and Its Kinase Activity in the IFN Induction and Replication of MHV-68 In Vitro and In Vivo
(A) The induction of IFN response by WT, N36S, 36KN, and 36R. NIH 3T3 cells were infected with WT, N36S, 36KN, or 36R virus at MOI = 0.05. At 24 hr post-infection, cell supernatant and RNA extracted from the infected cells were analyzed for endogenous IFN-β production by ELISA (left) and transcription level of IFN-β (middle) and Mx1 (right) by q-PCR. For ELISA, samples from three independent experiments were combined and tittered.

(B) The multistep growth curve of WT, N36S, 36KN, and 36R. Samples from two independent experiments were combined, tittered, and shown here.

(C) Transcript level of ISGs in the lung after WT or 36S MHV-68 infection examined by q-PCR. Viral genome and cellular actin transcript level were also measured as control. Data are represented as mean ± SD.

(D) Acute replication in the lung after intranasal infection of the indicated viruses in normal C57BL/6 (left) and IFNAR−/− (right) mice.

(E) Latency establishment in the spleen after intranasal infection of the indicated viruses in normal C57BL/6 (left) and IFNAR−/− (right) mice. I.C., infectious center. sp., splenocytes.

Figure 7. Spatial and Temporal Progression of M3FL and M3FL-36S Replication In Vivo
(A) Bioluminescence imaging of in vivo replication of wild-type M3FL and mutant M3FL-36S in normal mice. Images at different time points are shown to represent the peak of acute infection in the lung (D6 with 5 × 10⁵ pfu/mouse), the transition of replication from the lung to the spleen (D10), and the establishment of viral replication in the spleen (D14).

(B) Bioluminescence imaging of in vivo replication of wild-type M3FL and mutant M3FL-36S in IFNAR−/− mice.

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has been shown to regulate the reactivation of latent MHV-68 (Barton et al., 2005). Without IFN, MHV-68 reactivated more readily, suggesting that IFN reduces the efficiency of latent virus reactivation. To maintain a lifelong persistent infection, MHV-68 would need to reactivate to transmit and replenish the latent pool. Thus, MHV-68 needs to overcome the restraining effect of IFN when it reactivates; ORF36 may also be required in this process.

**Innate Immunity Affecting Adaptive Immune Response**

Herpesvirus replication is significantly affected by IFN response both in vitro and in vivo (Barton et al., 2005; Mossman and Ashkar, 2005). Depletion of IFN in wild-type mice during the establishment of latency does not enhance MHV-68 reactivation compared to the reactivation of MHV-68 in IFNAR−/− mice, suggesting that IFN-mediated innate and adaptive immune responses during the acute infection of MHV-68 may be required to control the reactivation of MHV-68 (Barton et al., 2005). These data imply that the effect of IFN responses is beyond the immediate nonspecific immune defense against viral invasion, further affecting the later stage of viral replication and persistent infection in vivo. IFNs induced during viral infection have been considered to be an important link between the innate and adaptive immune responses to viruses. For example, IFNs stimulate antigen-presenting cells to initiate crosspriming for the activation of CD8+ T cell response (Durand et al., 2004; Le Bon et al., 2003) and promote proliferation and maintenance of CD8+ cytotoxic T lymphocytes (CTL) (Agello et al., 2003; Marrack et al., 1999). Given the critical role of T lymphocytes in controlling MHV-68 replication in vivo (Braaten et al., 2005; Sparks-Thissen et al., 2004), it is possible that the anti-IFN function of ORF36 is required not only to breach the immediate innate immune defense upon viral infection/reactivation, but also to hinder the development of adaptive T cell response.

The early reduction of the size of the virus-specific T cell response, induced by a replication-attenuated 36S, correlates with a critical role of the magnitude of antigenic stimulation for T cell activation and differentiation (Wherry et al., 1999). Intriguingly, the ratio of the virus-specific CD8 T cell response per virus titer is higher for 36S than for WT (Figures 2C and 2D). Further, MHV-68 without functional ORF36 induced more IFN response in vitro (Figure 6A) and a similar level of IFN response in vivo (Figure 6C) in comparison with WT even when its replication was attenuated. Therefore, it is tempting to speculate that the lack of an anti-IFN function of ORF36 in 36S contributes to induce an enhanced innate antiviral state with higher IFN signaling in vivo (Figure 6C) that also will add to the expansion of T cells (Kolumam et al., 2005), even in the presence of very low levels of replicating virus. One prediction based on this idea is that a herpesvirus vaccine without ORF36 will be safer and more effective since it will replicate less and still generate a strong T cell response.

The outcome of a herpesvirus infection depends on the delicate balance between the strength of the host immune system and the ability of the virus to counteract. The identification of viral genes responsible for different immune evasion strategies of herpesviruses will provide not only a new ground for basic mechanistic research but also more opportunities to develop new preventive and therapeutic approaches against persistent herpesviral infection and associated diseases.
Cell Host & Microbe

Herpesviral Kinase Inhibits IFN through IRF-3


