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Short Communication

Lysine residues of interferon regulatory factor 7 affect the replication and transcription activator-mediated lytic replication of Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8

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Kaposi's sarcoma-associated herpesvirus (KSHV) infection goes through latent and lytic phases, which are controlled by the viral replication and transcription activator (RTA). Upon KSHV infection, the host responds by suppressing RTA-activated lytic gene expression through interferon regulatory factor 7 (IRF-7), a key regulator of host innate immune response. Lysine residues are potential sites for post-translational modification of IRF-7, and were suggested to be critical for its activity. In this study, we analysed the 15 lysine residues for their effects on IRF-7 function by site-directed mutagenesis. We found that some mutations affect the ability of IRF-7 to activate interferon (IFN)-α1 and IFN-β promoters, to suppress RTA-mediated lytic gene expression and to repress KSHV reactivation and lytic replication. However, other mutations affect only a subset of these four functions. These findings demonstrate that the lysine residues of IRF-7 play important roles in mediating IFN synthesis and modulating viral lytic replication.

Kaposi's sarcoma-associated herpesvirus (KSHV) is the aetiological agent of Kaposi's sarcoma, the most common neoplasm in AIDS patients (Chang *et al.*, 1994). KSHV infection goes through lytic and latent phases. Latency enables the virus to establish persistent infection (Moore & Chang, 2003), whereas lytic reactivation enables the virus to spread from the lymphoid compartment to endothelial cells (Grundhoff & Ganem, 2004; Katano *et al.*, 2001). The switch from latency to lytic replication is controlled by the viral replication and transcription activator (RTA) (Gradoville *et al.*, 2000; Lukac *et al.*, 1998; Sun *et al.*, 1998). RTA activates a number of viral genes, and RTA binding sites have been identified throughout the genome (Chen *et al.*, 2009; Ziegelbauer *et al.*, 2006). Eight KSHV genes have been shown to be direct transcriptional targets of RTA in the absence of *de novo* protein synthesis (Bu *et al.*, 2008). RTA-mediated lytic gene expression can be modulated by interferon regulatory factor 7 (IRF-7), a key regulator of type-I interferon (IFN)-dependent innate immune response (Honda *et al.*, 2005; Naranatt *et al.*, 2004; Zhang & Pagano, 1997, 2002). IRF-7 activates cellular defence genes, including the IFN genes, IFN-stimulated genes and chemokine genes, via the IFN-stimulation response element (Tamura *et al.*, 2008). Upon KSHV infection, IRF-7 competes with RTA for viral gene promoters to modulate viral transactivation (Wang *et al.*, 2005). The function of IRF-7 can in turn be regulated by post-translational modifications including phosphorylation (Caillaud *et al.*, 2005; Marie *et al.*, 2000), acetylation (Caillaud *et al.*, 2002), ubiquitination (Kawai *et al.*, 2004; Ning *et al.*, 2008; Yu *et al.*, 2005) and SUMOylation (Chang *et al.*, 2009; Kubota *et al.*, 2008). Lysine residues are potential sites for acetylation, ubiquitination and SUMOylation, and may serve as targets to modulate the functions of IRF-7.

In this study, we performed site-directed mutagenesis of IRF-7 lysine residues and determined the functional effect of these mutations. Each of the 15 lysine residues, K45, K50, K61, K92, K120, K179, K209, K296, K303, K341,

†These authors contributed equally to this work.

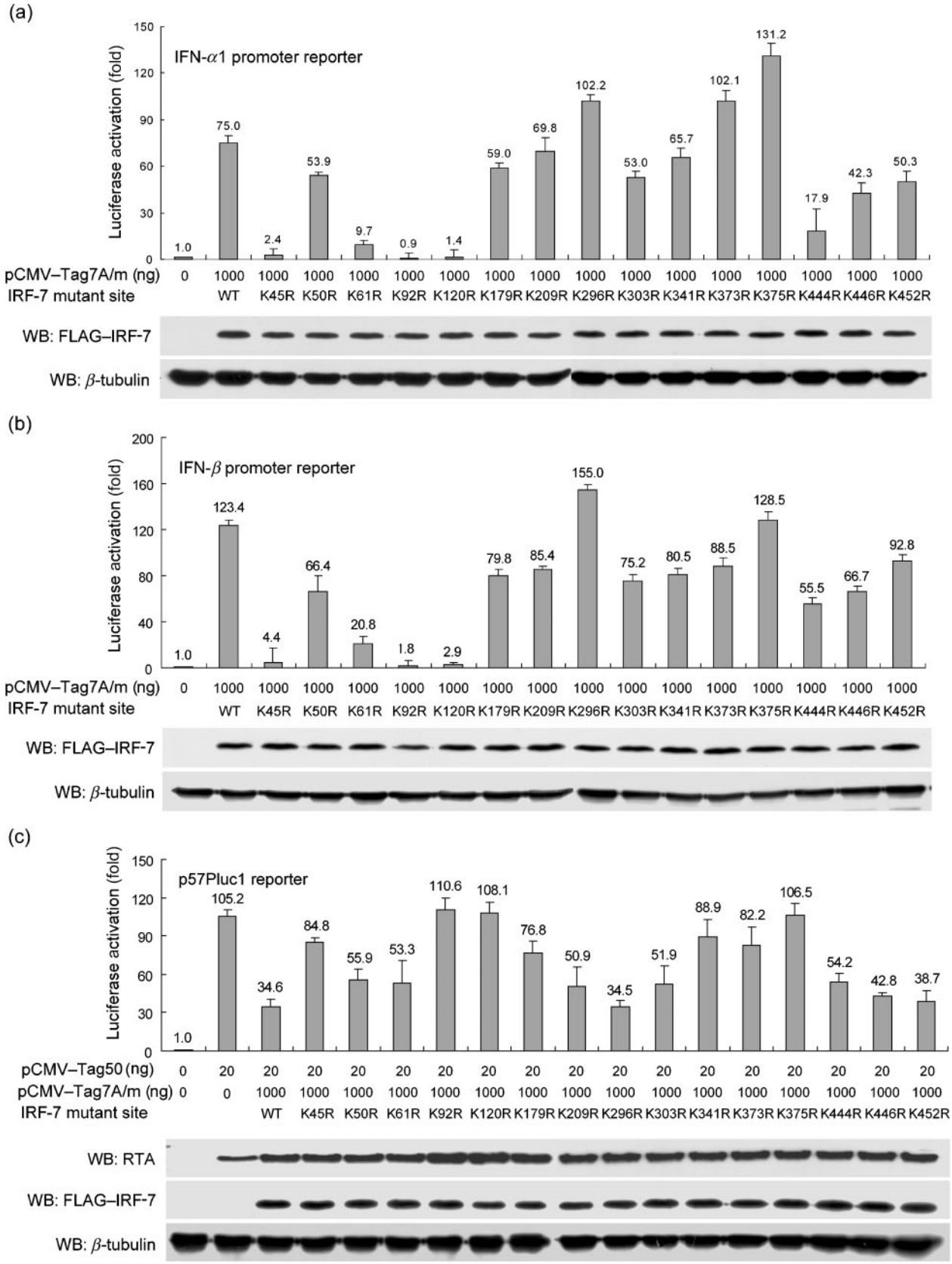
A supplementary table is available with the online version of this paper.

K373, K375, K444, K446 and K452, were mutated individually to arginine, using primers listed in Supplementary Table S1 (available in JGV Online). To evaluate whether these mutations affect the stimulation of IFN promoters, 293T cells (human embryonic kidney cells) were co-transfected with IFN-α1 promoter- (nt −110 to +10) or IFN-β promoter (nt −280 to +20)-driven luciferase reporter constructs, and an expression plasmid encoding wild-type IRF-7 or IRF-7 with a mutated lysine residue. Luciferase activities were measured (Fig. 1a). As expected, wild-type IRF-7 showed a 75-fold activation of the IFN-α1 promoter. However, when various IRF-7 mutants were tested the effects varied (Table 1). The K45R, K92R and K120R mutants could barely stimulate the IFN-α1 promoter (2.4-, 0.9- and 1.4-fold, respectively), and the K61R mutant stimulated very weakly (9.7-fold). The activation due to mutants K50R, K179R, K303R, K444R, K446R and K452R was much weaker than for wild-type IRF-7 (53.9-, 59.0-, 53.0-, 17.9-, 42.3- and 50.3-fold, respectively). However, the activation of mutants K209R and K341R was similar (69.8- and 65.7-fold, respectively), while mutants K296R, K373R and K375R were more effective (102.2-, 102.1- and 131.2-fold, respectively) than the wild-type. Wild-type IRF-7 activated the IFN-β promoter 123.4-fold, whereas K45R, K92R and K120R mutants were barely able to activate this promoter (4.4-, 1.8- and 2.9-fold, respectively). Mutant K61R activated the IFN-β promoter very weakly (20.8-fold). The activation shown by mutants K50R, K179R, K209R, K303R, K341R, K373R, K444R, K446R and K452R was also weaker than that for wild-type IRF-7 (66.4-, 79.8-, 85.4-, 75.2-, 80.5-, 88.5-, 55.5-, 66.7- and 92.8-fold, respectively). However, mutants K296R and K375R showed 155.0- and 128.5-fold activation, respectively, and were stronger than the wild-type (Fig. 1b, Table 1). Western-blot analyses revealed that the variations in activation of the IFN promoters were not due to differential expression of wild-type or mutated IRF-7 (lower panels of Fig. 1a, b).

To examine whether IRF-7 lysine residue mutations affect the repression of RTA-mediated transactivation of viral promoters, the ORF57 promoter-derived luciferase-reporter p57Pluc1 (Duan *et al.*, 2001), an RTA expression plasmid (Wang *et al.*, 2001) and wild-type or mutant IRF-7 plasmids were co-transfected into 293T cells (Fig. 1c). RTA activated the ORF57 promoter by 105.2-fold, and the activation was reduced to 34.6-fold when IRF-7 was expressed. Variation in the mutants' abilities to repress RTA activity was observed (Table 1). This repression was abolished completely for mutants K92R, K120R and K375R (110.6-, 108.1- and 106.5-fold, respectively) and weakened markedly for mutants K45R, K179R, K341R and K373R (84.8-, 76.8-, 88.9- and 82.2-fold, respectively). Mutants K50R, K61R, K209R, K303R and K444R also had much smaller repressive activities (55.9-, 53.3-, 50.9-, 51.9- and 54.2-fold, respectively). The repressive activities of mutants K296R, K446R and K452R were similar to that of wild-type IRF-7 (34.5-, 42.8- and 38.7-fold, respectively). Variation in repression capacity was not due to differential expression of wild-type and mutant IRF-7 protein (lower panel of Fig. 1c). Interestingly, we also observed an increase in RTA protein level in the presence of IRF-7 and the various mutants thereof, but no obvious induction of expression from the CMV promoter by IRF-7 was detected (data not shown). It is possible that IRF-7 and its various mutants may interact with RTA at the protein level to affect the stability of RTA (Yu *et al.*, 2005).

Since mutation of lysine residues in IRF-7 affected its repression of RTA function, lysine residues in IRF-7 may play a role in the regulation of KSHV lytic replication. To test this hypothesis, Vero cells containing rKSHV.219 were utilized to study KSHV reactivation from latency. rKSHV.219 is a recombinant KSHV that expresses GFP from the EF-1α promoter and red fluorescent protein (RFP) from the KSHV lytic polyadenylated nuclear (PAN) RNA promoter. The expression of GFP indicates the presence of latent infection, whereas RFP expression indicates lytic gene expression and viral reactivation (Vieira & O'Hearn, 2004). RTA expression plasmid, wild-type or mutant IRF-7, or the IRF-7 RNA interference plasmid were transfected into the rKSHV.219-containing Vero cells. Control cells were transfected with an empty vector lacking RTA or IRF-7 coding regions. At 48 h after transfection, cells were fixed with 2.5 % glutaraldehyde and observed with a laser-scanning confocal microscope (FluoView1000S; Olympus). The presence of green fluorescence showed the presence of latent KSHV and a small amount of red signal represented a low level of spontaneous lytic reactivation, demonstrating that the RFP

Fig. 1. Lysine residues of IRF-7 affected the activation of IFN promoters and the suppression of KSHV ORF57 transactivation. (a, b) Lysine residues of IRF-7 modulate the activation of IFN-α1 and IFN-β promoters. Expression plasmids of wild-type (WT) or each of the lysine-residue mutants of IRF-7 (1 μg), and IFN-α1 promoter (nt −110 to +10) or IFN-β promoter (nt −280 to +20)-driven luciferase reporter (50 ng) were co-transfected into 293T cells. Luciferase activities were measured with a Luciferase Assay system (Promega) and transfection efficiency was normalized by using the pCMV-β expression plasmid (100 ng) as an internal control. Numbers above each bar represent fold activation and are normalized to unity, relative to the case where IRF-7 is absent. Results are the means from three independent experiments and SD are shown. Western blots (WB) were performed with anti-FLAG M2 (Stratagene) and anti-β-tubulin (Sigma–Aldrich) antibodies. (c) Lysine residue mutants affect the capacity of IRF-7 to repress KSHV RTA-mediated transactivation of the ORF57 promoter. 293T cells were co-transfected with ORF57 promoter reporter p57Pluc1 (50 ng), pCMV-Tag50 (20 ng) and an expression plasmid of WT or mutant IRF-7 (1 μg). The activation multiples shown are normalized to unity, relative to the case where RTA is absent. WB were performed with anti-RTA (Qin *et al.*, 2010), anti-FLAG M2 and anti-β-tubulin antibodies.


(a)
IFN-α1 promoter reporter
Luciferase activation (fold)
150
120
90
60
30
0
1.0
75.0
2.4
53.9
9.7
0.9
1.4
59.0
69.8
102.2
53.0
65.7
102.1
131.2
17.9
42.3
50.3
pCMV–Tag7A/m (ng) 0 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000
IRF-7 mutant site WT K45R K50R K61R K92R K120R K179R K209R K296R K303R K341R K373R K375R K444R K446R K452R
WB: FLAG–IRF-7
WB: β-tubulin
(b)
IFN-β promoter reporter
Luciferase activation (fold)
200
160
120
80
40
0
1.0
123.4
4.4
66.4
20.8
1.8
2.9
79.8
85.4
155.0
75.2
80.5
88.5
128.5
55.5
66.7
92.8
pCMV–Tag7A/m (ng) 0 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000
IRF-7 mutant site WT K45R K50R K61R K92R K120R K179R K209R K296R K303R K341R K373R K375R K444R K446R K452R
WB: FLAG–IRF-7
WB: β-tubulin
(c)
p57Pluc1 reporter
Luciferase activation (fold)
150
120
90
60
30
0
1.0
105.2
34.6
84.8
55.9
53.3
110.6
108.1
76.8
50.9
34.5
51.9
88.9
82.2
106.5
54.2
42.8
38.7
pCMV–Tag50 (ng) 0 20 20 20 20 20 20 20 20 20 20 20 20 20 20 20 20 20
pCMV–Tag7A/m (ng) 0 0 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000 1000
IRF-7 mutant site WT K45R K50R K61R K92R K120R K179R K209R K296R K303R K341R K373R K375R K444R K446R K452R
WB: RTA
WB: FLAG–IRF-7
WB: β-tubulin

expression was not activated in the absence of RTA and IRF-7 (Fig. 2a, control). Transfection with RTA strongly activated latent KSHV to produce lytic replication, as demonstrated by the high level of RFP expression (Fig. 2a, RTA). The reactivation was suppressed by the transient expression of wild-type IRF-7 (Fig. 2a, IRF-7). When the expression of IRF-7 was silenced by RNA interference plasmid pGE1–IRF-7i (Wang *et al.*, 2005) the suppression of viral reactivation was reversed, suggesting that the suppression by IRF-7 was specific (Fig. 2a, 7i). Variation in the levels of suppression of KSHV reactivation was observed when various IRF-7 mutants were examined (Table 1). Mutants K92R (Fig. 2a, K92R), K446R and K452R abolished the suppression of KSHV reactivation. The suppression activities of mutants K45R (Fig. 2a, K45R), K50R, K61R, K209R, K296R, K303R, K341R, K373R, K375R and K444R were all very weak. However, mutant K120R retained strong suppression function (Fig. 2a, K120R) and mutant K179R behaved in almost the same way as wild-type IRF-7. These results demonstrated that some lysine residues affected IRF-7-mediated repression of KSHV reactivation while others did not.

To investigate the significance of lysine-residue mutations of IRF-7 in the regulation of KSHV lytic replication, we used tagged rKSHV.219 virus to study the effects of wild type and mutant IRF-7, on lytic viral replication in 293T cells. Some 293T cells were transfected with RTA plasmid alone to stimulate lytic replication. Alternatively, to test the effects of IRF-7 on infection, 293T cells were co-transfected with RTA and wild-type or mutant IRF-7, or the IRF-7 RNA interference plasmid, before infection by rKSHV.219 virus (Fig. 2b). As expected, no green fluorescence was observed without rKSHV.219 infection (not shown); GFP expression was seen only upon rKSHV.219 infection, but no lytic replication (RFP expression) was seen without the transfection of RTA (Fig. 2b, control). Lytic virus replication was observed in the presence of RTA, demonstrating that RTA activated viral lytic replication (Fig. 2b, RTA only). However, RFP expression was repressed by the expression of wild-type IRF-7 (Fig. 2b, RTA + IRF-7). When expression of IRF-7 was silenced by pGE1–IRF-7i, the repression of RFP expression by IRF-7 was reversed (Fig. 2b, 7i). The effect of different IRF-7 mutants on RFP expression was then examined (Table 1). Mutants K92R (Fig. 2b, RTA + K92R), K303R, K444R and K446R could not suppress RTA-activated RFP expression. Suppression of RFP expression by mutants K45R (Fig. 2b, RTA + K45R), K61R, K179R, K209R, K296R, K341R, K375R and K452R was very weak, whereas suppression by mutants K50R, K120R (Fig. 2b, K120R) and K373R was almost as good as for wild-type IRF-7. These results suggested that several lysine residues of IRF-7 played critical roles in the regulation of KSHV lytic replication.

The use of rKSHV.219 may have some limitations. RFP expression is under the control of the KSHV PAN promoter, which may be activated by transfected RTA (Song *et al.*, 2001). Also, the endogenous *rta* gene can be activated, since RTA can auto-activate its promoter (Deng *et al.*, 2000). It is possible that transfected IRF-7 may affect RTA-mediated activation of the PAN promoter. In addition, IRF-7 may affect endogenous IRF-7 target genes,

Table 1. Lysine-residue mutations affect IRF-7 function

++, IRF-7 function abolished; +, IRF-7 function weakened; –, wild-type like or IRF-7 function enhanced.

Mutant	Activation of:		Suppression of:		
	IFN-α1 promoter	IFN-β promoter	RTA-mediated ORF57 transactivation	KSHV reactivation in rKSHV.219-containing Vero cells	KSHV lytic replication in 293T cells
K45R	++	++	+	+	+
K50R	+	+	+	+	–
K61R	++	++	+	+	+
K92R	++	++	++	++	++
K120R	++	++	++	–	–
K179R	+	+	+	–	+
K209R	–	+	+	+	+
K296R	–	–	–	+	+
K303R	+	+	+	+	++
K341R	–	+	+	+	+
K373R	–	+	+	+	–
K375R	–	–	++	+	+
K444R	+	+	+	+	++
K446R	+	+	–	++	++
K452R	+	+	–	++	+

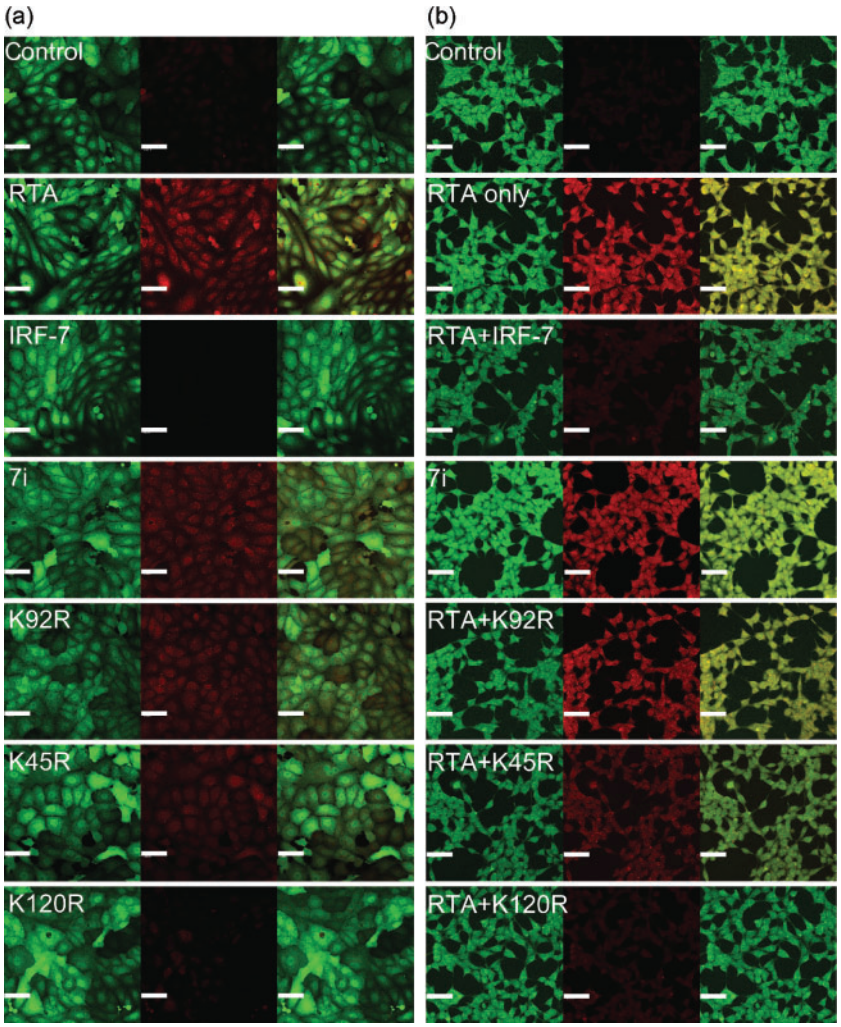


Fig. 2. Lysine residues affected IRF-7-mediated repression of KSHV reactivation and lytic replication. (a) Confocal images of the effect of IRF-7 on the reactivation of rKSHV.219 in Vero cells. rKSHV.219-containing Vero cells were transfected with pCMV–Tag2 vector (control), pCMV–Tag50 (RTA), or wild-type/mutant IRF-7 (panels IRF-7, K92R, K45R and K120R) and pGE1–IRF-7i (7i). Twenty-four hours post-transfection, expression of GFP (green) and RFP (red) was recorded. All of the confocal images of GFP or RFP, respectively, were captured using identical parameters. The overlay images of GFP and RFP are also shown (yellow). (b) Confocal microscope images of 293T cells infected by rKSHV.219. The 293T cells were transfected with pCMV–Tag2 vector (control) or pCMV–Tag50 (RTA only), or co-transfected with pCMV–Tag50 and WT or mutant IRF-7 (panels RTA+IRF-7, RTA+K92R, RTA+K45R and RTA+K120R) and pGE1–IRF-7i (7i). One day after transfection, 293T cells were infected with rKSHV.219. Expression of GFP and RFP was recorded 2 days after infection. All of the confocal experiments using rKSHV.219-containing Vero cells and rKSHV.219-infected 293T cells were repeated, blindly and independently, and the same patterns were observed. Bars, 50 μm.

including the IFN genes, which may affect RTA-mediated activation of the PAN promoter. Additional experiments with untagged KSHV will be needed to substantiate our findings. The effect of IRF-7 lysine residues on the modulation of other viral promoters and on combinations of multiple viral and cellular gene functions should also be investigated.

We have undertaken a comprehensive site-directed mutagenesis and functional-analysis study of the effect of mutating each lysine residue of IRF-7 on the modulation of IRF-7 function. Lysine residues in the DNA-binding domain (DBD) are critical for the functioning of IRF-7. Mutants K92R, K45R and K61R abolished, or weakened significantly, all four functions of IRF-7: activation of IFN promoters, suppression of KSHV lytic-gene expression and repression of viral reactivation and lytic replication. Mutants K50R and K120R affected a combination of these four functions. The crystal structure of the IRF-7 DBD (Panne *et al.*, 2007) indicates that K92 and K45 are located in the α2 and L1 domains in the vicinity of the DNA double helix and may be important for direct DNA binding (Zhang *et al.*, 2005). K120 and K61 are located in the β4 and α2 domains, which are folded towards the inside of the molecule, and may not bind DNA directly. Several lysine residues (such as K444, K446 and K452) in the activation domain are also essential for IRF-7 function. Ning *et al.* (2008) reported that the triple mutant of IRF-7 (K444, K446, K452) abolished IRF-7 transactivation of the IFN-α4 promoter, and the K444, K446 or K452 single mutants dramatically reduce the activity of IRF-7. Our results further demonstrate that a single lysine mutation can weaken IRF-7 function markedly.

Currently, we cannot determine whether the effects of lysine-residue mutations on IRF-7 function are due to structural alterations or post-translational modifications. We have observed that all 15 mutants of FLAG-tagged IRF-7 localized in both the nuclei and the cytoplasm of 293T cells, whereas wild-type IRF-7 predominantly localized in the cytoplasm (data not shown). Further studies will be needed to determine the effect of lysine residues on the molecular properties of IRF-7 and whether any of the affected lysine residues have post-translational modifications.

In conclusion, lysine residues affect the function of IRF-7: mutants K45R, K61R, K92R and K120R failed to stimulate IFN promoters; mutants K92R, K120R and K375R abolished IRF-7 repression of the RTA-activated ORF57 promoter; mutants K92R, K446R and K452R abolished repression of viral reactivation in rKSHV-containing Vero cells; and mutants K92R, K303R, K444R and K446R could not suppress RTA-activated lytic replication in a single-cycle infection experiment. To our knowledge, this is the first report to describe the systematic mutation of each and every lysine residue of IRF-7 and to have tested their effect on IRF-7 function. Our findings demonstrate the importance of lysine residues in IRF-7-regulated IFN synthesis and in innate immunity against viral lytic replication. An understanding of the structural function of IRF-7 will decipher how hosts counteract KSHV infection via the IFN pathway.

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Supplementary Table S1. Primers used for the mutagenesis of IRF-7

The underlined sequences indicate the codons for arginine residues.

IRF-7 mutant	Primer sequence
K45R	5′-GTTTCCGCGTGCCCTGGAGGCACTTCGCGCGCAAGGAC-3′
	5′-GTCCTTGCGCGCGAAGTGCCTCCAGGGCACGCGGAAAC-3′
K50R	5′-CACTTCGCGCGCAGGGACCTGAGCGAGGCCGACGCGCGCATC-3′
	5′-GATGCGCGCGTCGGCCTCGCTCAGGTCCCTGCGCGCGAAGTG-3′
K61R	5′-CTGAGCGAGGCCGACGCGCGCATCTTCAGGGCCTGGGCTGTG-3′
	5′-CACAGCCCAGGCCCTGAAGATGCGCGCGTCGGCCTCGCTCAG-3′
K92R	5′-ACTGCGGAGCGCGCCGGCTGGAGAACCAACTTCCGCTGCGCACT-3′
	5′-AGTGCGCAGCGGAAGTTGGTTCTCCAGCCGGCGCGCTCCGCAGT-3′
K120R	5′-ACTCGGGGGACCCGGCCGACCCGCACAGGGTGTACGCGCTCAG-3′
	5′-CTGAGCGCGTACACCCTGTGCGGGTCGGCCGGGTCCCCCGAGT-3′
K179R	5′-CAGCTGGTGACAGGGGGGACCTCCTG-3′
	5′-CAGGAGGTCCCCCCTGTCACCAGCTG-3′
K209R	5′-GATCCAGTCCCAACCAGGGCTCCTGGAGAGGGACAAG-3′
	5′-CTTGTCCCTCTCCAGGAGCCCTGGTTGGGACTGGATC-3′
K296R	5′-GTGACCATCATGTACAGGGGCCGCACGGTGCT-3′
	5′-CAGCACCGTGCGGCCCCTGTACATGATGGTCAC-3′
K303R	5′-ACAAGGGCCGCACGGTGCTGCAGAGGGTGGTGGGACAC-3′
	5′-GTGTCCCACCACCCTCTGCAGCACCGTGCGGCCCTTGT-3′
K341R	5′-GAGCTCCCGGACCAGAGGCAGCTGCGCTACAC-3′
	5′-GTGTAGCGCAGCTGCCTCTGGTCCGGGAGCTC-3′
K373R	5′-CTGTGGGCCCGGCGCATGGGCAGGTGCAAGGTGTAC-3′
	5′-GTACACCTTGCACCTGCCCATGCGCCGGGCCCACAG-3′
K375R	5′-GCATGGGCAAGTGCAGGGTGTACTGGGAGGTG-3′
	5′-CACCTCCCAGTACACCCTGCACTTGCCCATGC-3′
K444R	5′-GCTGGGAGGCCCAGGGAGAAGAGCCTGGTC-3′
	5′-GACCAGGCTCTTCTCCCTGGGCCTCCCAG-3′
K446R	5′-GAGGCCCAAGGAGAGGAGCCTGGTCCTGGTGAAG-3′
	5′-ACCAGGACCAGGCTCCTCTCCTTGGGCCTCCCAG-3′
K452R	5′-CTGGTCCTGGTGAGGCTGGAACCCTGGCTGTG-3′
	5′-CAGAGCCAGGGTTCCAGCCTCACCAGGACCAG-3′

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