Kaposi’s Sarcoma-associated Herpesvirus (KSHV) Oncoprotein K13 Bypasses TRAFs and Directly Interacts with the IκB Kinase Complex to Selectively Activate NF-κB without JNK Activation*

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Kaposi’s sarcoma-associated herpesvirus (KSHV; 2 also known as human herpesvirus 8), is the etiological agent of Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman’s disease. KSHV-encoded K13 protein is one of the few viral proteins to be expressed in latently infected PEL and KS spindle cells (1, 2), and is believed to play a major role in viral oncogenesis. The K13 protein contains two homologous copies of a Death Effector Domain (DED) that is also present in the prodomain of caspase 8 (also known as FLICE). Proteins with two DEDs have been discovered in other viruses as well and include MC159L and MC160L from the Molluscum contagiosum virus and E8 from the equine herpesvirus 2. These viral DED-containing proteins were originally believed to protect virally infected cells from death receptor-induced apoptosis by blocking the recruitment and/or activation of caspase 8/FLICE and, as such, were collectively referred to as viral FLICE inhibitory proteins or vFLIPs (3). However, subsequent studies demonstrated that K13 is unique among the vFLIPs in possessing the ability to activate the NF-κB pathway (4–7), and it utilizes this pathway to promote cellular survival, proliferation, transformation, and cytokine secretion (8–13).

Considering the key role played by K13-induced NF-κB activation in the pathogenesis of KSHV-associated malignancies, the molecular interactions involved in the activation of this pathway have been the subject of several recent studies, including from our laboratory. Interaction between vFLIPs K13, MC159L, and E8 and the proteins involved in NF-κB signaling pathway was initially characterized by us using transient transfection-based overexpression of various epitope-tagged proteins (4). Using this assay, we reported that while all three vFLIPs could interact with TRAF1, TRAF2, RIP (receptor-interacting protein 1), and NIK (NF-κB-inducing kinase), only K13 interacted with the IKKs (4). Based on these results, it was proposed that the interaction of K13 with the IKK complex, which consists of IKK1, IKK2, and Nemo, may play a critical role in its ability to activate the NF-κB pathway (4). This hypothesis was confirmed in a follow-up study, which utilized cells with near physiological levels of K13 and endogenously expressed IKK proteins, and demonstrated that K13 gets recruited to a 700-kDa IKK signalosome complex consisting of IKK1, IKK2, and Nemo, which possesses the ability to phosphorylate IkBα and activate the NF-κB pathway (5). The functional involvement of IKK1, IKK2 and Nemo in K13-induced NF-κB activation was subsequently confirmed by genetic studies with mouse embryonic fibroblast (MEF) cells deficient in these kinases.

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2 The abbreviations used are: KSHV, Kaposi’s sarcoma associated herpesvirus; DED, death effector domain; EMSA, electrophoretic mobility shift assay; GST, glutathione-S-transferase; KS, Kaposi’s sarcoma; NF-κB, nuclear factor κB; PEL, primary effusion lymphoma; IKK, IκB kinase; TRAF, tumor necrosis factor receptor-associated factor; JNK, c-Jun N-terminal kinase; MEF, mouse embryonic fibroblast; 4-OHT, 4-hydroxytamoxifen; RIP-1, receptor-interacting protein 1; NIK, NF-κB-inducing kinase; ERαTAM, ligand-binding domain of mutated estrogen receptor; vFLIP, viral FLICE-inhibitory protein; IκB, inhibitor of κB; HA, hemagglutinin.
These studies also revealed that the upstream kinases of the NF-κB signaling pathway, such as RIP-1 and NIK, are dispensable for this process (14). The above results were independently confirmed by Field et al. (7) who used a yeast two-hybrid system and further demonstrated an interaction between K13 and Nemo in vitro using the GST pull-down assay and in vivo using mass spectroscopy. Taken together, these studies supported a model according to which K13 activates the NF-κB pathway by directly interacting with and activating the IKK complex, thereby bypassing the upstream components of the NF-κB signaling pathway, such as TRAFs, RIP, and NIK. This model was further supported by results showing that, unlike TRAFs, K13 does not activate the JNK pathway in 293 cells (10, 15).

The TNF receptor-associated factor (TRAF) family of adapter proteins have been shown to play an important role in the activation of the NF-κB and JNK pathways by different members of the TNF receptor family and Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) (16). The involvement of TRAFs in K13 signaling was examined in detail in a recent study, which reported the presence of a TRAF-interacting motif, PYQLT, in the second DED of K13 (17). K13 mutants with point substitutions of two conserved residues in this motif not only failed to interact with TRAF2 and Nemo but also showed a near complete loss of NF-κB reporter activity (17). Furthermore, siRNA-mediated silencing of TRAF2, but not other TRAF family members, disrupted the interaction between K13 and Nemo and significantly inhibited the endogenous NF-κB activity in a PEL cell line (17). Finally TRAF3 was also found to play a key role in K13-induced NF-κB and JNK activation, although it was not needed for mediating the interaction between K13 and Nemo (17). Collectively, the above studies led to a new model of K13 signaling according to which K13 requires TRAF2 for its interaction with the IKK complex, and both TRAF2 and TRAF3 are required for K13-induced NF-κB and JNK activation (17, 18).

Because the NF-κB and JNK pathways play opposing roles in the natural history of KSHV infection (19, 20), the effects of K13 on these pathways and the underlying molecular interactions have important implications for our understanding of viral life cycle and for the development of targeted therapies against KSHV-associated malignancies. As such, in this study, we have carried out a detailed analysis of K13-induced NF-κB and JNK activation and re-examined the role of TRAFs in these processes.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cell Lines, and Antibodies**—Plasmid encoding FLAG-tagged K13 and HA-tagged TRAF2 have been described previously (4). K13-P93L, -Q95R, and -T97L mutant constructs were generated using the QuickSite mutagenesis kit (Stratagene) and the sequence confirmed by automated sequencing. Retroviral constructs containing C-terminal FLAG epitope-tagged wild-type K13 and mutants were generated in MSCV neo-based retroviral vector and used for infection as previously described (5). Primary effusion lymphoma (PEL) cell lines (BC-1, BC-3 and BCBL-1), and Namalwa (B-cell lymphoma) cells were obtained from the American Type Culture Collection. Wild type and TRAF2−/− murine embryonic fibroblast (MEF) were provided by Dr. Wen-Chen Yeh (21). 293-K13-ER(TAM)-NF-κB-Luc cells were generated by infecting 293NF-κB-Luc cells with a retroviral vector expressing the K13-ER(TAM) fusion protein (12) followed by selection with G418. Rabbit polyclonal antibodies against IKKα, IKKβ, Nemo/IKKγ, TRAF2, TRAF3, TRAF5, and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against FLAG and tubulin, control mouse IgG beads and FLAG beads were obtained from Sigma.

**Luciferase Reporter Assay**—293T cells were transfected in a 24-well plate with various test plasmids along with an NF-κB luciferase reporter construct (75 ng/well) and a pRSV/LacZ (β-galactosidase) reporter construct (75 ng/well) as described previously (4). Cells were lysed 24–36 h later, and extracts were used for the measurement of firefly luciferase and β-galactosidase activities, respectively. Luciferase activity was normalized relative to the β-galactosidase activity to control for the difference in the transfection efficiency. Transfection of MEF cells and measurement of luciferase activity was performed essentially as described previously (14).

**RNA Interference (RNAi)**—siRNA oligos against TRAF2 and TRAF3 were purchased from Santa Cruz Biotechnology. p65 and a control siRNAs have been described previously (22). siRNA oligonucleotides (80 nM) were transfected using calcium phosphate, as described previously (23).

**Co-immunoprecipitation Assay**—Cells were lysed in buffer A (20 mM sodium phosphate (pH 7.4), 150 mM NaCl) containing 1% Triton-X 100, and 1 EDTA-free mini-protease inhibitor tablet per 10 ml (Roche Applied Science). Cell lysates were incubated for 1 h at 4 °C with 10 μl of FLAG (Sigma) or control mouse IgG beads precoated with a supersaturated casein solution. Beads were washed twice with buffer A, once with a high salt wash buffer (buffer A with 500 mM NaCl) and again with buffer A. Bound proteins were eluted by boiling the beads for 5 min in SDS sample buffer, separated by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by Western blot.

For transient transfection based co-immunoprecipitation studies, 2 × 10⁶ 293T cells were plated in a 100-mm plate and co-transfected 18–24 h later with 1 μg/plate of each epitope-tagged construct along with 1 μg of a GFP-encoding plasmid (pEGFP) by calcium phosphate coprecipitation. Twenty-four hours post-transfection, cells were lysed, and the co-immunoprecipitation assay was carried out as described above.

**Electrophoretic Mobility Shift Assay**—To determine NF-κB activation, we prepared nuclear extracts and performed electrophoretic mobility shift assay as described previously (24). The bands obtained were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD).

**RESULTS**

**Structural and Functional Analyses of the TRAF2-interacting Motif**—Guasparr et al. (17) recently used a computer model of the second DED of K13 and reported that the Pro⁹⁵ and Gln⁹⁵ residues in the putative TRAF2-binding motif (PYQLT) are exposed on the surface of the molecule and available for interaction with TRAFs. To confirm these results, we used comparative modeling to generate a three-dimensional model of the
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Fig. 1A, the two tandem DEDs in the complete K13 form a dumbbell-shaped structure with each DED forming the weight at either end. We next mapped the location of the putative TRAF2-interacting motif onto our K13 model. Contrary to the expected surface location for a motif involved in intermolecular interactions, we discovered that the putative TRAF2-interacting motif of K13 is buried in the interface between its DED1 and DED2, and is not readily accessible for interaction with TRAF2 or other signaling proteins (Fig. 1, B–D).

Although the structural analysis argued against a major role for the PYQLT motif in K13 interactions, a functional role of this motif in K13 signaling has been supported by previous mutagenesis studies (17). Thus, two K13 mutants, P93L and Q95R, which contain mutations of two conserved residues in the PYQLT motif, were reported to be severely impaired in their ability to activate an NF-κB reporter construct when expressed in the Namalwa cells, while the mutant T97L was found to retain this activity (17). To resolve the discrepancy between our structural analysis and the previously reported functional studies, we used site-directed mutagenesis to generate the P93L, Q95R, and T97L mutants and tested their ability to activate the NF-κB pathway using a luciferase-based reporter assay. Remarkably, we discovered that all three K13 mutants were comparable to the wild-type protein in their ability to activate an NF-κB luciferase reporter construct when transfected into 293T cells (Fig. 2A). Equivalent expression of the transfected constructs was confirmed by immunoblotting with an antibody against the FLAG epitope tag, which was present in the K13 constructs (Fig. 2B).

It is conceivable that K13 utilizes different signaling intermediates to activate NF-κB in different cell types and, therefore, the role of TRAF2-interacting motif in K13 signaling is cell type-dependent. To rule out this possibility, we used retroviral-mediated gene transfer to stably express K13 in three B cell lymphoma-derived cell lines: Namalwa, BC-3, and BCBL-1. The latter two cell lines are derived from KSHV-infected PEL and represent physiologically relevant models to study K13 signaling. Retroviral-mediated expression of K13 led to constitutive NF-κB activation in all three cell lines as measured by an electrophoretic mobility shift assay (EMSA) (Fig. 2C). More importantly, the three K13 mutants, P93L, Q95R, and T97L, activated the NF-κB pathway to a similar extent as the wild-type protein in the three tested cell lines (Fig. 2C). Taken together with the studies using 293T cells, these results demonstrate that the mutations in the putative TRAF-interacting motif have no major impact on the ability of K13 to activate the NF-κB pathway.

complete K13 molecule (i.e. containing both DED1 and DED2) based on the recently resolved structure of vFLIP MC159L from the M. contagiousum virus as a template (25, 26). As shown in

FIGURE 1. Mapping of the putative TRAF2-interaction motif onto the molecular surface of K13. Schematic representation of the structure of K13 protein (residues 12–172) as shown by space-fill (A and C) and ball and stick (B and D) representations, respectively. Basic residues are shaded blue, and acidic residues are shaded red. The putative TRAF2-interacting motif, shown in yellow, is buried in the interface between DED1 and DED2 and is not available for surface interactions. Comparative modeling of K13 structure was done using SWISS-MODEL program (34), and structure visualized using Cn3D version 4.1 (35).

FIGURE 2. The putative TRAF2-interaction motif (PYQLT) is not involved in K13-induced NF-κB activation. A, 293T cells were transfected with the indicated constructs and NF-κB activity measured by luciferase reporter assay. The values shown are averages (mean ± S.E.) of one representative experiment out of three in which each transfection was performed in duplicate. B, Western blots shows equivalent expression of the transfected constructs. Tubulin blot shows equal protein loading. C, electrophoretic mobility shift assay demonstrating equivalent increase in NF-κB DNA binding activity in the Namalwa, BCBL-1, and BC-3 cell lines stably expressing K13-WT, K13-P93L, K13-Q95R, and K13-T97L, respectively. The positions of the induced NF-κB complexes are marked by asterisks. The numbers below the panels represent the relative intensity of the induced NF-κB complexes, as measured by the ImageJ software. Lower panel, expression of K13 does not influence SP1 binding activity.
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FIGURE 3. TRAF2 does not interact with K13 and is not required for the recruitment of K13 to the IKK complex. A and B, cell lysates (C.L.) prepared from Namalwa and BCBL-1 cells expressing an empty vector or the indicated FLAG-tagged K13 constructs were immunoprecipitated (I.P.) using control (C) or FLAG (F) antibody beads and co-immunoprecipitated IKK complex subunits were detected by immunoblotting with antibodies against IKKα/β and Nemo/IKKγ, respectively, as described previously (5). The blots were re-probed with TRAF2, TRAF3 and TRAF5 antibodies to check the interaction of K13 with TRAFs. C, cell lysates (C.L.) prepared from BC-3 and BCBL-1 cells expressing an empty vector or FLAG-tagged K13 were immunoprecipitated using control (C) or FLAG (F) beads and the presence of interacting proteins detected by Western blotting with the indicated antibodies. D, 293T cells were transfected with K13-FLAG and cFLIP-FLAG along with either an empty vector or TRAF2-HA. Approximately 36 h post-transfection, cells were lysed, and cell lysates (C.L.) were immunoprecipitated using control (C) or FLAG (F) antibody beads and the interaction of cFLIP and K13 with TRAF2 was detected by Western blotting.

The impaired NF-κB activation by the P93L and Q95R mutants has been attributed to their reduced ability to interact with the IKK complex (17). Therefore, we re-examined the interaction of the wild-type and the mutant K13 proteins with the components of IKK complex using a co-immunoprecipitation assay. Consistent with the published results (5, 7), we readily detected an interaction between stably expressed wild-type K13 and endogenously expressed IKK complex subunits by co-immunoprecipitation assays (28), we carried out a parallel experiment examining the interaction between FLAG-tagged cFLIP, and a combination of cFLIP and K13. As such, in preliminary experiments we optimized the conditions of transfection and used the least amounts (1 μg/2 × 10^6 cells) of the two plasmids for transfection that yielded easily detectable amounts of the two epitope-tagged proteins for interaction studies. Additionally, because TRAF2 is known to interact with cFLIP in both co-immunoprecipitation and yeast-two hybrid assays (28), we carried out a parallel experiment examining the interaction between FLAG-tagged cFLIP, and TRAF2-FLAG as a positive control. As shown in Fig. 3D, while we readily detected an interaction between overexpressed cFLIP and TRAF2, under similar experimental conditions no such interaction was observed between overexpressed K13 and TRAF2. Taken together with the studies involving endogenously expressed TRAF2, these results demonstrate that K13 does not interact with TRAF2.

TRAF2 Does Not Mediate the Recruitment of K13 to the IKK Complex—There is a controversy in the published literature regarding the requirement for TRAF2 in mediating the interaction between K13 and the IKK complex. Thus, Field et al. (7) observed a direct interaction between K13 and Nemo/IKKγ using GST pull-down and yeast two-hybrid assays. In contrast, Guasparr et al. (17) reported that TRAF2 is required for mediating the interaction between K13 and Nemo/IKKγ. To resolve this apparent discrepancy between the two studies, we generated stable clones of wild-type and TRAF2−/− MEFs expressing an empty vector and FLAG-tagged K13 (Fig. 4A). Subsequently, we used a co-immunoprecipitation assay to study the interaction between K13 and Nemo/IKKγ in the absence and presence of TRAF2. Consistent with our previous studies with PEL cells, we readily detected an interaction between K13 and Nemo/
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While the preceding studies ruled out the involvement of TRAF2 in mediating the interaction between K13 and IKK complex, we still considered that TRAF2 plays a functional role in K13-induced NF-κB activation without physically interacting with it. Consistent with the above model, K13-induced NF-κB activation in 293T and PEL cell lines could be blocked by overexpression of a dominant-negative mutant of TRAF2 (4, 17). Although the above studies can be criticized for the experimental artifacts introduced due to nonspecific binding and titering away of critical components of the NF-κB signaling pathway by the overexpressed TRAF2 mutant, a role for TRAFs in K13 signaling was also suggested by inhibition of constitutive NF-κB and JNK activities in KSHV-infected BC-3 cell line upon siRNA-mediated knock-down of TRAF2 and TRAF3 (17). To definitively address the involvement of TRAF2 in K13-induced NF-κB activation, we compared the ability of K13 to induce NF-κB activation in the wild-type and TRAF2−/− MEFs. As shown in Fig. 5A, transient transfection of K13 led to equivalent increase in NF-κB activity in the wild-type and TRAF2−/− MEFs as measured by a luciferase-based reporter assay. Similarly, stable expression of K13 in the wild-type and TRAF2−/− MEFs led to a similar increase in the NF-κB binding activity over vector-expressing cells, as measured by EMSA (Fig. 5B). It was conceivable that rather than influencing the absolute amounts of NF-κB complexes, TRAF2 affects the subunit composition of NF-κB complexes induced by K13. To rule out this possibility, we examined the subunit composition of NF-κB complexes induced by K13 in the wild-type and TRAF2−/− cells using a gel super-shift assay. As shown in Fig. 5C, this assay revealed that the NF-κB complex induced by K13 consisted of p50, p65, and RelB subunits in both the wild-type and TRAF2−/− MEFs.

To confirm the above results and to provide an independent proof of the lack of involvement of TRAF2 and TRAF3 in K13-induced NF-κB activation, we used 293NF-κB-Luc-K13-ER TAm cells, which express stably integrated copies of an NF-κB luciferase reporter construct and a K13-ER TAm fusion construct (12). The mutated estrogen receptor (ERTAm) in the K13-ER TAm fusion construct does not bind to the physiological ligand estrogen but binds with very high affinity to the synthetic ligand 4-OHT (4-hydroxytamoxifen) and regulates the activity of K13 in a 4-OHT-dependent fashion (12). As shown in Fig. 5D, treatment of 293NF-κB-Luc-K13-ER TAm cells with 4-OHT led to a ~5-fold induction in the NF-κB luciferase activity, which was effectively blocked by siRNA against the p65 subunit of NF-κB. However, under similar experimental conditions,
siRNAs targeting TRAF2 or TRAF3 had no significant effect on K13-induced NF-κB luciferase activity (Fig. 5D). Equivalent and specific knock-down of p65, TRAF2 and TRAF3 by their respective siRNAs was confirmed by immunoblotting with the respective antibodies (Fig. 5E). Taken together with the studies using TRAF2−/− MEFs, the above results confirm the lack of involvement of TRAF2 and TRAF3 in K13-induced NF-κB activation.

**K13 Fails to Activate the JNK Pathway in PEL Cells**—Although NF-κB activation is known to promote KSHV latency, JNK/AP1 activation has been reported to stimulate lytic replication (19, 20). The role of K13 in JNK/AP1 activation has been controversial. K13 has been shown to lack the ability to activate the JNK pathway in two studies (10, 15), which is consistent with its role as a latent protein that promotes viral latency through NF-κB activation. However, K13 has also been reported to activate the JNK pathway and this activity has been attributed to its interaction with the TRAFs (11, 17). Our results showing a lack of involvement of TRAF2 and 3 in K13-induced NF-κB pathway prompted us to re-examine the role of K13 in JNK activation. For this purpose, we generated stable clones of BCBL-1 cells expressing the K13-ERTAM fusion protein (Fig. 6A) and compared the effect of 4-OHT treatment on K13-induced NF-κB and JNK pathways, respectively. As shown in Fig. 6B, treatment of BCBL-1 K13-ERTAM cells with 4-OHT led to robust activation of the NF-κB pathway as measured by p65/RelA DNA binding activity. In contrast, under similar experimental conditions, induction of K13 activity by 4-OHT failed to increase the DNA binding of transcription factors c-Fos, c-Jun, Jun-D, and ATF2 (Fig. 6, C–F), which are known downstream targets of the JNK pathway. Similarly, 4-OHT treatment failed to induce c-Jun phosphorylation as measured by a c-Jun “pull-down” kinase assay (not shown). Thus, consistent with its lack of interaction with the TRAFs, K13 lacks the ability to activate the JNK pathway in PEL cells.

**DISCUSSION**

Constitutive NF-κB activation is seen in several viral-induced lymphoproliferative disorders and has been attributed to the activity of viral-encoded proteins that target different steps of the NF-κB signaling pathway (29). The EBV-encoded LMP-1 behaves as a constitutively activated tumor necrosis factor receptor that activates the NF-κB and JNK pathways via the recruitment of different TRAF family members (30). In con-
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...Tax protein activates the NF-κB pathway at a distal step by directly interacting with the IKK complex via its Nemo subunit (31). In this report we demonstrate that the putative TRAF interaction motif has no functional role in K13-induced NF-κB and TRAF2 neither interacts with K13 nor is required for localizing it to the IKK complex. Similarly, TRAF3 is not needed for K13-induced NF-κB activation. Thus, our results suggest that, similar to HTLV-1 Tax, K13 bypasses the upstream components of the NF-κB signaling pathway to directly interact with the IKK complex, resulting in selective NF-κB activation. This model is consistent with previous studies demonstrating a direct interaction between K13 and Nemo/IKKγ in GST-pull down and yeast two-hybrid assays (7), and by the lack of involvement of the upstream kinases of the NF-κB signaling pathway, such as RIP-1 and NIK, in K13-induced NF-κB activation (5, 6, 14).

Our results are at variance with the recent study by Guasparri et al. (17) which identified a TRAF-interacting motif in K13 and reported that both TRAF2 and TRAF3 are required for K13-induced NF-κB and JNK activation. Although we have no simple explanation for the discrepancy between the two studies, the following points are worth consideration. First, while our computer model indicated that the putative TRAF-interacting motif in K13 is buried in the interface between DED1 and DED2 and is not available for surface-interactions, Guasparri et al. (17) reported that this motif is exposed on the surface and is available for interaction with the TRAFs. It is important to point out that the computer model used by Guasparri et al. (17) was based only on the second DED of K13. Because the putative TRAF-interacting motif of K13 is buried in the interface between the two DEDs, it is conceivable that exclusion of DED1 from their model contributed to the erroneous impression that the putative TRAF-interacting motif is surface-exposed. Second, we were unable to confirm one of the major conclusions of Guasparri et al. that TRAF2 and TRAF3 are required for K13-induced NF-κB and JNK activation. Guasparri et al. based this conclusion on an experiment in which siRNA-mediated silencing of TRAF2 and TRAF3 resulted in the inhibition of constitutive NF-κB activity and JNK phosphorylation in the BC-3 cells. However, it is possible that the observed inhibitory effect of TRAF2 and TRAF3 siRNAs on NF-κB and JNK activities in the BC-3 cells reflected inhibition of signaling pathways other than those activated by K13, a notion supported by our results showing that K13 does not activate the JNK pathway. Finally, another key finding of the study by Guasparri et al. was that the P93L and Q95R mutants of K13 have lost the ability to activate the NF-κB pathway, which provided one of the strongest arguments supporting a functional role of the putative TRAF-interacting motif in K13-induced NF-κB. This conclusion was based on a transient transfection-based luciferase reporter assay in the Namalwa cells (17). However, we were unable to confirm the lack of NF-κB activation by the P93L and Q95R mutants using a similar luciferase-based reporter assay. Although, we have no clear explanation for the loss of NF-κB activity by the P93L and Q95R mutants in the study by Guasparri et al., it is conceivable that the known variability in the level of expression of the transfected constructs following transient-transfection might have played a role. To avoid such an experimental artifact in our study, we confirmed the results of our transient transfection-based luciferase reporter assay with EMSA on nuclear extracts derived from three different lymphoma cell lines stably expressing the wild-type and mutant K13 constructs. Remarkably, we observed that in all three cell lines the mutant proteins were as effective as the wild-type K13 in activating the NF-κB pathway. Moreover, in contrast to the report by Guasparri et al., we found no decrease in the ability of the mutant proteins to interact with Nemo. Taken collectively, our results clearly demonstrate that mutations in the putative TRAF-interacting motif have no detrimental effect on the ability of K13 to activate NF-κB or interact with Nemo, and underscore the importance of using independent assays and multiple cell lines for confirming key research findings.

Our results have important implications for a better understanding of the regulation of KSHV life-cycle. The NF-κB and JNK/AP1 pathways are known to result in opposing outcomes in cellular physiology as well as in the natural history of KSHV infection (32). Thus, while NF-κB activation has been reported to support viral latency (19), JNK/AP1 activation is known to promote lytic replication (20). As such, bypassing TRAFs to selectively activate the NF-κB pathway through direct interaction with the IKK complex might be a clever strategy employed by KSHV to promote latency. Finally, K13-induced NF-κB activation is believed to play a key role in the survival, proliferation, and transformation of KSHV-infected cells (8–13, 33), and has become an important therapeutic target for the development of drugs for the treatment of KSHV-associated malignancies. A better understanding of the molecular interactions involved in K13-induced NF-κB activation will greatly facilitate the design of high-throughput screening strategies for the isolation of compounds capable of inhibiting this pathway.

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