Molecular Genetic Analysis of Human Herpes Virus 8-encoded Viral FLICE Inhibitory Protein-induced NF-κB Activation*

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The human herpes virus 8 (HHV8)-encoded viral FLICE inhibitory protein (vFLIP), also known as K13, is known to activate the NF-κB pathway, a property not shared by other vFLIPS. Previous studies have demonstrated that HHV8 vFLIP K13 interacts with several cellular signaling proteins involved in NF-κB activation, such as receptor-interacting protein, NF-κB-inducing kinase, IκB kinase (IKK) 1, IKK2, and NF-κB essential modulator (NEMO). In this report we have used cell lines deficient in the above proteins to investigate the mechanism of NF-κB activation by HHV8 vFLIP K13. We demonstrate that receptor-interacting protein and NF-κB-inducing kinase are dispensable for vFLIP K13-induced NF-κB DNA binding and transcriptional activation. On the other hand, vFLIP K13-induced NF-κB DNA binding is significantly reduced, although not absent, in cells deficient in IKK1, IKK2, and NEMO. Furthermore, vFLIP K13-induced NF-κB transcriptional activity is only weakly present in IKK1-deficient cells and almost completely absent in those deficient in IKK2 and NEMO. HHV8 vFLIP K13-induced NF-κB activation in IKK1- and IKK2-deficient fibroblasts could be rescued by wild type but not by the kinase-inactive mutants of IKK1 and IKK2, respectively. Consistent with the above results, vFLIP K13-induced NF-κB activation could be effectively blocked by chemical inhibitors of the kinase activity of IKK1 and IKK2. Thus, a cooperative interaction of all three subunits of the IKK complex is required for maximal NF-κB activation by HHV8 vFLIP K13.

These virally encoded death effector domain-containing proteins function as death effectors and play a critical role in the treatment of disorders caused by abnormal NF-κB activation, a property shared by other vFLIPs. Previous studies have demonstrated that HHV8 vFLIP K13 possesses the unique ability to activate the NF-κB pathway that is not shared by the vFLIP E8 from equine herpes virus 2 and the vFLIP MC159L from molluscum contagiosum virus (10). We recently demonstrated that vFLIP K13 can co-immunoprecipitate with several protein kinases known to be involved in NF-κB activation, such as RIP, NIK, IKK1 (or IKKα), IKK2 (or IKKβ), and NEMO (or IKKγ) (10, 11). Furthermore, vFLIP K13 was found to physically associate with a ∼700-kDa IκB kinase (IKK) complex composed of IKK1, IKK2, and NEMO, which is known to play a crucial role in the NF-κB pathway. In the present study, we have focused on the functional contribution of the above interactions of vFLIP K13 to NF-κB activation.

**MATERIALS AND METHODS**

**Plasmids and Cell Lines—**Plasmids containing IKK1, IKK1 kinase mutant, IKK2, IKK2 kinase mutant, and pcDNA3-K13-FLAG have been described previously (10). Wild type, IKK1−/−, IKK2−/−, and NEMO−/− murine embryonic fibroblast (MEF) cells were obtained from Drs. Inder Verma and Richard Gaynor. Wild type and NIK−/− MEFs have been described previously (12) and were obtained from Dr. Robert Schreiber. These cell lines were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (13). Both of these media were supplemented with an antibiotic-actinomycotic solution (Invitrogen) containing 10 IU/ml penicillin, 10 μg/ml streptomycin, 25 μg/ml amphotericin B. Cells from exponentially growing cultures were used for all of the experiments. A retrovirus construct containing C-terminal FLAG epitope-tagged HHV8 vFLIP K13 was generated in MSCV neo-based retroviral vector. Amphotropic and ecotropic viruses were generated and used for infection as described previously (11). The cells were selected in the presence of 1 mg/ml of G418 (Invitrogen).

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1 The abbreviations used are: HHV8, human herpes virus 8; vFLIP, viral FLICE inhibitory protein; NEMO, NF-κB essential modulator, NIK, NF-κB-inducing kinase; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; IKK, IκB kinase; RIP, receptor-interacting protein; MEF, mouse embryonic fibroblast.
Role of RIP in HHV8 vFLIP K13-induced NF-κB Activation—RIP is a serine-threonine protein kinase, which has been shown to play an essential role in NF-κB activation via TNF receptor 1 (13, 15). We have previously demonstrated that RIP and vFLIP K13 physically interact with each other upon transient transfection in 293T cells (10). More recently, we demonstrated that endogenous RIP co-immunoprecipitates with vFLIP K13 from the lysates of H460 cells expressing the latter protein in a stable fashion (11). To study the role of RIP in HHV8 vFLIP K13-induced NF-κB activation, we took advantage of a mutant clone of Jurkat cells deficient in this protein (13). We used retrovirally mediated gene transfer to generate polyclonal populations of wild type and RIP-deficient Jurkat cells expressing an empty vector or FLAG-tagged vFLIP K13. Western blot analysis confirmed equivalent expression of the vFLIP K13 in the wild type and RIP-deficient Jurkat cells (Fig. 1A). We subsequently used an electrophoretic mobility shift assay to examine the ability of vFLIP K13 to activate the NF-κB pathway in the two cell lines. Expression of vFLIP K13 in the wild type Jurkat cells was found to lead to constitutive NF-κB activation as measured by the gel shift assay, which was absent in the empty vector-infected cells (Fig. 1B). However, vFLIP K13 could effectively induce NF-κB DNA binding activity in the RIP-deficient cells as well, thereby suggesting that RIP is not involved in this process (Fig. 1B). To test the possibility that the observed NF-κB complexes in RIP-deficient cells are transcriptionally active, we transiently transfected an empty vector or a vFLIP K13-expressing vector in wild type and RIP-deficient Jurkat cells and measured NF-κB transcriptional activation using an NF-κB luciferase reporter assay. As shown in Fig. 1C, transient transfection of vFLIP K13 in wild type Jurkat cells led to significant NF-κB activation as compared with the empty vector transfected cells. Consistent with the gel shift assay, transient transfection of vFLIP K13 led to an equivalent increase in NF-κB activation as compared with control vector in RIP-deficient cells as well. Collectively, the above results argue against the involvement of RIP in K13-induced NF-κB DNA binding or transcriptional activities.

Role of NIK in HHV8 vFLIP K13-induced NF-κB Activation—We have previously demonstrated that HHV8 vFLIP K13 can co-immunoprecipitate with NIK when both of these proteins are overexpressed in 293T cells (10). Involvement of NIK in vFLIP K13-induced NF-κB activation was also supported by the ability of dominant-negative mutants of NIK to block vFLIP K13-induced NF-κB activation in a luciferase reporter assay (10). However, the above conclusions were based on assays involving transient transfection of different plasmids and, as such, could have been influenced by the artifacts introduced by overexpressed proteins. Furthermore, the role of NIK in NF-κB activation is enigmatic. For example, although a dominant-negative mutant of NIK can block NF-κB activation via TNF receptor 1, subsequent studies using NIK−/−MEFs have argued against the involvement of this kinase in TNF receptor 1-induced NF-κB. To re-examine the involvement of NIK in vFLIP K13-induced NF-κB activation in a more physiological setting, we took advantage of mouse embryonic fibroblast cells from NIK−/−animals. As we did earlier, we used retrovirally mediated gene transfer to generate polyclonal populations of wild type and NIK-deficient cells expressing an empty vector or FLAG-tagged vFLIP K13 and confirmed equivalent expression of vFLIP K13 protein using Western blot analysis (Fig. 2A). Subsequently, we used gel shift and luciferase reporter assays to examine the status of the NF-κB pathway in the various cellular populations. As shown in Fig. 2 (B and C), we found near equivalent activation of the NF-κB pathway in vFLIP K13-expressing wild type and NIK−/−MEFs using the gel shift and luciferase reporter assays, thereby arguing against the involvement of NIK in vFLIP K13-induced NF-κB DNA binding or transcriptional activation.

Role of IKKs in HHV8 vFLIP K13-induced NF-κB Activation—We have previously reported that vFLIP K13 physically associates with a ~700-kDa IKK signalosome complex consisting of IKK1, IKK2, and NEMO. To investigate whether or not all of the subunits of the IKK complex are required for vFLIP K13-induced NF-κB, we took advantage of mouse embryo fibro-
blast cells deficient in IKK1, IKK2, and NEMO. As we did earlier, we generated stable clones of the above cells expressing an empty vector or vFLIP K13 and confirmed the expression of the K13 protein by Western blot analysis (Fig. 3A). To determine the effect of K13 expression on the activation of the NF-κB pathway, we initially performed a gel mobility shift assay. As shown in Fig. 3B, this assay demonstrated increased NF-κB DNA binding activity in the vFLIP K13-expressing wild type MEFs, which was absent in the vector-expressing cells. However, unlike the situation with the RIP- and NIK-deficient cells, significantly reduced NF-κB DNA binding activity was observed in vFLIP K13-expressing IKK1−/−, IKK2−/−, and NEMO−/− MEFs (Fig. 3B). Interestingly, some residual NF-κB DNA binding activity was observed in K13-expressing IKK1−/−, IKK2−/−, and NEMO−/− deficient cells, suggesting that none of these subunits is absolutely essential for K13-induced NF-κB DNA binding activity (Fig. 3, B and C). A gel supershift assay revealed that similar to the wild type MEFs, the residual NF-κB complexes present in the vFLIP K13-expressing IKK1−/−, IKK2−/−, or NEMO−/− MEFs were primarily composed of p65 and p50 subunits (Fig. 3C).

The above results were further supported by an NF-κB-based luciferase reporter assay. As shown in Fig. 3D, transient transfection of vFLIP K13 in the wild type MEF cells led to significant activation of the NF-κB reporter activity, which was markedly reduced in the IKK1-deficient cells and nearly completely absent in IKK2- and NEMO-deficient cells. Collectively, the above results suggest that optimal NF-κB activation by vFLIP K13 requires the activities of all the three subunits of the IKK complex.

Role of the Kinase Activities of IKK1 and IKK2 in vFLIP K13-induced NF-κB Activation—After confirming the involvement of the catalytic subunits of the IKK complex in vFLIP K13-induced NF-κB activation, we sought to determine the requirement of their kinase activities in the above process. For this purpose, IKK1− or IKK2-deficient MEFs were transiently transfected with an empty vector or vFLIP K13 along with either wild type or kinase-inactive mutants of IKK1 or IKK2, and NF-κB activation was measured using the luciferase reporter assay. As shown in Fig. 4A, vFLIP K13 could activate NF-κB in the IKK1-deficient MEFs when co-expressed with the wild type IKK1 but not with the kinase-inactive IKK1. Similarly, vFLIP K13-induced NF-κB activation in the IKK2-deficient MEFs could be partially restored upon co-transfection of the wild type IKK2 but not its kinase-inactive mutant (Fig. 4B). The lack of complete complementation of K13-induced NF-κB activity by transfection of wild type IKK2 in IKK2-deficient cells is probably due to failure to faithfully reproduce the stoichiometry of various IKK subunits present in the wild type cells.

Inhibitors of the Kinase Activity of the IKK Complex Block vFLIP K13-induced NF-κB Activation—We next sought to determine whether the inhibitors of the kinase activity of the IKK complex might be used to inhibit vFLIP K13-induced NF-κB activation. For this purpose, we generated a subclone of the HEK293 cell line, designated 293NF-B-Luc, with stable expression of an NF-κB-driven luciferase reporter construct. Subsequently, we used retrovirally mediated gene transfer to generate polyclonal derivatives of this subclone with stable expression of either an empty vector or vFLIP K13 (Fig. 5A). Consistent with its ability to activate the NF-κB pathway, vFLIP K13-expressing 293NF-B-Luc cells demonstrated 50-fold higher levels of luciferase activity as compared with empty vector-expressing cells (Fig. 5B).

We next used the vector- and vFLIP K13-expressing 293NF-B-Luc cells to study the effect of inhibitors of the IKK complex on K13-induced NF-κB activation. Recent studies suggest that aspirin, As2O3, arsenite, and phenylarsine oxide are potent inhibitors of the kinase activities of the IKK complex (16–19). As shown in Fig. 5C, treatment of 293NF-B-Luc vFLIP K13 cells with the above chemicals led to significant inhibition of the luciferase reporter activity, which was comparable in magnitude with that achieved with lactacystin and MG132, two proteasome inhibitors that are known to block NF-κB activation via diverse pathways by blocking IκB degradation. Taken together, the above results support the hypothesis that vFLIP K13-induced NF-κB activation requires the kinase activity of the IKK complex. Furthermore, these results suggest that selective inhibitors of the IKK kinase activity might have a role in the treatment of disorders caused by abnormal activation of the NF-κB pathway by vFLIP K13.

**DISCUSSION**

Based on its sequence homology to the prodomain of caspase 8, vFLIP K13 was believed to function mainly as an inhibitor of caspase 8 recruitment and activation during death receptor signaling (7). However, we subsequently demonstrated that vFLIP K13 could activate the NF-κB pathway (10, 11), thereby extending the range of its biological activities. NF-κB activation by vFLIP K13 has been recently confirmed by two inde-
dependent groups (20, 21). In addition to vFLIP K13, HHV8 is known to encode for two other proteins with potential for NF-κB activation: vGPCR (viral G protein-coupled receptor) and K1 (22, 23). However, among these proteins, only vFLIP K13 is expressed in latently infected primary effusion lymphoma cell lines, making it a prime candidate for the persistent NF-κB activation present in the HHV8-infected primary effusion lymphoma cell lines (11, 24).

Because of the presence of two death effector domains in vFLIP K13, a likely model for its NF-κB inducing ability could have involved its interaction with the death effector domain-containing adaptor protein FADD and subsequent reverse signaling via the death receptors. However, we have previously demonstrated the ability of vFLIP K13 to activate the NF-κB pathway in FADD-deficient MEF cells, thereby arguing against this possibility (10). Another potential mediator of vFLIP K13-induced NF-κB activation is RIP, which is known to play a key role in NF-κB activation via TNF receptor 1 (13, 15) and is known to physically interact with vFLIP K13 (10). However, our results with RIP-deficient Jurkat cells argue against this possibility and suggest that vFLIP K13 induces NF-κB activation by interacting with the downstream kinases of this pathway.

NIK is a serine threonine kinase of the mitogen-activated protein kinase pathway that was suspected to play a role in vFLIP K13-induced NF-κB activation based on the ability of

Fig. 3. NF-κB DNA binding activity is reduced in vFLIP K13-expressing IKK1-, IKK2-, and NEMO-deficient MEFs. A, wild type (WT), IKK1−/−, IKK2−/−, and NEMO−/− cells were transduced with an empty retroviral vector or a vector expressing FLAG epitope-tagged vFLIP K13. Expression of the transduced proteins was confirmed by Western blot analysis with a rabbit polyclonal antibody against the FLAG epitope tag. The blot was reprobed with an antibody against actin to ensure equal loading of all lanes. B, electrophoretic mobility shift assay demonstrating increased NF-κB DNA binding activity in K13-expressing wild type MEFs and reduced activity in IKK1-, IKK2-, and NEMO-deficient cells. The position of the induced NF-κB complexes is marked by an arrow, while an asterisk marks the position of the constitutive complexes. The numbers below the blots represent the relative intensity of the induced NF-κB complexes after normalization with the respective constitutive complexes. C, electrophoretic mobility shift assay demonstrating subunit composition of NF-κB complexes seen in vFLIP K13-expressing MEF cells derived from wild type, IKK1−/−, IKK2−/−, and NEMO−/− animals. Supershift assay was performed using control antisera (lanes 3) or antisera against p52 (lanes 4), p50 (lanes 5), p65 (lanes 6), Rel B (lanes 7), and c-Rel (lanes 8) subunits of NF-κB. The position of the induced NF-κB complexes is marked by an arrow, while an asterisk marks the position of the constitutive complexes. The supershifted bands are marked by arrowheads. The numbers below the blots represent the relative intensity of the induced NF-κB complexes after normalization with the respective constitutive complexes. D, vFLIP K13-induced NF-κB transcriptional activity in the wild type, IKK1−/−, IKK2−/−, and NEMO−/− cells as measured by a luciferase-based reporter assay. MEF cells were transiently transfected with an empty vector or vFLIP K13 along with an NF-κB/luciferase reporter construct (75 ng/well) and a Renilla reporter construct (75 ng/well), and the experiment was performed as described for Fig. 2C. The values shown are averages of one representative experiment of two in which each transfection was performed in duplicate.
IKK1 in cytokine-induced activation of the canonical NF-κB pathway (35, 40, 41). The role of the IKK complex in vFLIP K13-induced NF-κB activation has been suspected based on our prior study, which demonstrated that vFLIP K13 could physically associate with a 700-kDa IKK signalosome complex containing IKK1, IKK2, and NEMO/IκKB (11). This study also demonstrated that vFLIP K13 was incapable of NF-κB activation in a NEMO-deficient mouse pre-B cell lymphoma cell line, thereby demonstrating an essential role for NEMO in this process (11). In the present study we demonstrate that both the catalytic subunits of the IKK complex, i.e., IKK1 and IKK2, also contribute to vFLIP K13-induced NF-κB DNA binding and transcriptional activities. Thus, all three subunits of the IKK complex are required for maximal NF-κB activation via vFLIP K13.

In the current study we have noted some residual NF-κB DNA binding activity in vFLIP K13-expressing IKK1-, IKK2-, and NEMO-deficient MEF cells. The results with IKK1-deficient cells were expected because a majority of published studies have found significant cytokine-induced NF-κB DNA binding activity in IKK1-deficient cells (35, 40, 41). On the other hand, there is conflicting data about the presence of residual cytokine-induced NF-κB DNA binding activity in IKK2-deficient cells, which may be due to the use of different cell types in different studies. Thus, although one study reported a nearly complete absence of interleukin-1 and TNF-α-induced NF-κB DNA binding activity in IKK2-deficient embryonic stem cells (36), two studies reported significant residual NF-κB DNA binding activity in IKK2-deficient MEFs (36, 38). Finally, the presence of residual NF-κB DNA binding activity in K13-expressing NEMO-deficient MEFs is surprising because it has been previously reported that cytokine-induced NF-κB DNA
binding activity is completely lacking in these cells (37, 38, 42). It is conceivable that the difference in the two results reflects the fact that vFLIP K13 induces NF-κB DNA binding activity via a mechanism distinct from cytokines. Alternatively, it is also possible that vFLIP K13 induces NF-κB DNA binding activity via two pathways, one involving NEMO (and IKK2) and the other involving IKK1, and full NF-κB DNA binding activity requires synergetic interaction between these two pathways. In this regard, it is pertinent to note that unlike our results with MEFs in the current study, we have previously observed a complete lack of NF-κB DNA binding activity in 1.3E2 cells, a NEMO-deficient subclone of 70Z/3 mouse pre-B cell lymphoma cell line (11). A possible explanation for the difference in the results between the two studies may lie in the absence of IKK1-induced NF-κB binding activity in the 1.3E2 cells. It is also conceivable that the residual NF-κB DNA binding activity seen in the vFLIP K13-expressing IKK1−/−, IKK2−/−, and NEMO−/− cells reflects the involvement of an as yet unidentified signaling pathway. Finally, as discussed above, K13 is known to make a multisubunit signalsome complex with IKK1, IKK2, and NEMO/IKKγ (11). It is possible that K13 is able to induce the formation of a partial IKK complex under the conditions when one or the other IKK subunit is missing. The residual NF-κB DNA binding activity observed in the IKK1−/−, IKK2−/−, and NEMO-deficient cells in the current study could simply reflect the activity of such a partial IKK complex. Unlike NF-κB DNA binding activity, NF-κB transcriptional activity was only weakly present in IKK1-deficient cells and was absent in IKK2 and NEMO-deficient cells. The lack of K13-induced NF-κB activation in IKK2−/− and NEMO-deficient cells is consistent with previous studies demonstrating the requirement for these proteins in TNF-induced NF-κB transcriptional activity (34–39). On the other hand, the results with IKK1-deficient cells were somewhat unexpected because earlier studies have suggested a lack of role for this kinase in transcriptional activation of the canonical NF-κB pathway (35, 40, 41). However, two recent studies, which were published while this manuscript was in preparation, demonstrated that IKK1 plays an important role in cytokine-induced NF-κB transcriptional activation via histone H3 phosphorylation (43, 44). It is conceivable that IKK1-mediated histone H3 phosphorylation also contributes to K13-induced NF-κB transcriptional activity.

Abnormal activation of the NF-κB pathway has been shown to play a role in the pathogenesis of several human malignancies, and several inhibitors of this pathway are in clinical use or in various stages of development (45, 46). A common mechanism of action of the NF-κB inhibitors currently in clinical development is to block the degradation of IκBα by inhibiting the activity of proteasome (47, 48). However, proteasome is required for breakdown of several regulatory proteins, which may contribute to the side effects of such inhibitors. In this regard, our result suggesting a major role for IKK1 in vFLIP K13-induced constitutive NF-κB activation presents potential opportunities for the development of molecularly targeted drugs for the treatment of HHV8-associated malignancies. However, future development of this approach may depend on further analysis of the physiological role played by IKK1 and the consequences of its inhibition on normal cellular physiology.

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