INTRODUCTION

Vaccinia virus (VACV) was used as a vaccine against variola virus (smallpox) infection (Moss, 2013). Despite the elimination of smallpox in 1980, VACV remains important to the medical community. It is now being used as a vector for vaccines against other infectious diseases and as an oncolytic virus (Gilbert, 2013; Sánchez-Sampedro et al., 2015). In addition, VACV remains an excellent model for understanding immune responses to complex pathogens (Sette et al., 2009). As such, the continued study of VACV identifies key virus–host interactions that can be exploited for the benefit of human health.

The NF-κB transcription factor is critical for the host antiviral defence because it activates genes involved in immunity and inflammation (Bhatt et al., 2014). The RelA (p65) and NF-κB1 (p50) heterodimer is the best-studied member of this family (Bhatt & Ghosh, 2014). NF-κB is sequestered in the cytoplasm by IκBα (Hinz et al., 2012). Upstream proteins (e.g. TNF receptor-1) activate the IκB kinase (IKK) complex (Hayden et al., 2014) and IKK phosphorylates IκBα (Hinz et al., 2014). As a consequence, IκBα is polyubiquitinated by the SCFβ-TrCP E3–ubiquitin ligase complex and degraded by the 26S proteasome (Hinz et al., 2012). The freed NF-κB then migrates to the nucleus (Hayden et al., 2008), where it undergoes several post-translational modifications that are required for its transcriptional activity, such as acetylation of RelA (Huang et al., 2010). Originally, it was thought that RelA must be acetylated by CBP/p300 prior to NF-κB binding to its promoter. However, recent publications demonstrate that, for some genes, RelA migrates to a promoter already bound by CBP/p300 (Mukherjee et al., 2013). In these cases, RelA is acetylated after it binds to the DNA-CBP/p300 complex (Mukherjee et al., 2013). Regardless, once the promoter is occupied by NF-κB, transcription is initiated when the adjacent transcriptional start site is occupied by RNA polymerase II and a set of general transcription factors (Orphanides et al., 1996).

The VACV K1 protein is a 31 kDa protein that possesses nine ankyrin repeat domains (ARDs) (Li et al., 2010). During virus infection, the presence of K1 correlates with the presence of IκBα and absence of NF-κB activation (Shisler et al., 2004). This implies that K1 acts at or upstream of IκBα (Shisler & Jin, 2004). K1 also inhibits PKR (Willis et al., 2009), a multi-functional cellular protein that activates NF-κB (Zamanian-Daryoush et al., 2000), in addition to...
PKR’s main role of inhibiting host cell translation (Yim et al., 2014). Willis et al. (2011) went on to show that, during virus infection, PKR activation is the cause of NF-κB activation. Thus, in the infected cell environment, K1 antagonizes PKR activation, and this upstream event is responsible for K1 inhibition of NF-κB (Willis et al., 2011).

Aravalli et al. (2008) showed that ectopic K1 inhibits Toll-like receptor 2 (TLR2)-induced NF-κB activation in primary murine macrophages. Although this may also involve PKR activation (Cabanáski et al., 2008), it also raised the possibility that K1 may inhibit NF-κB in a PKR-independent function. To further query whether K1 indeed possesses an alternative strategy to antagonize NF-κB, we used two strategies to uncouple PKR and NF-κB activation: we expressed K1 independently of infection and used reagents that minimized PKR activation. Using these systems, data revealed that K1 localized to the nucleus and prevented CBP/p300-induced RelA acetylation and RelA–CBP interactions.

RESULTS

K1 protein inhibits NF-κB activation independently of virus infection

To examine whether K1 inhibited NF-κB via a PKR-independent mechanism, K1 was expressed ectopically in cells as a strategy to uncouple PKR activation and NF-κB activation. NF-κB transcriptional activation was evaluated by using luciferase reporter assays. We used pNF-κB luciferase reporter gene (Shisler & Jin, 2004; Fig. 1(a, b)), or pIL-8 luciferase reporter gene (Da Silva Correia et al., 2002), as shown in Fig. 1(c). Controls included cells co-transfected with empty vector (pCI) or with a plasmid expressing a dominant-negative IkBα molecule, a protein that cannot be phosphorylated by IKK and thus remains associated with NF-κB to prevent NF-κB activation (Van Antwerp et al., 1996).

Fig. 1(a, b) shows that K1 inhibited NF-κB activation induced by either TNF or MyD88 overexpression, respectively, albeit to a lesser extent than the dominant-negative (DN) IkBα molecule. Because TNF- and MyD88-induced NF-κB signal transduction pathways overlap from the point of IKK activation and downwards, this implied that K1 may be inhibiting an event occurring post-IKK activation. K1 was stably expressed in cells for the duration of the experiments, and K1 expression itself did not activate NF-κB. K1 also inhibited reporter activity of a luciferase gene controlled by the cellular CXCL8 promoter, a promoter that contains an NF-κB-binding site as shown in Fig. 1(c). Additional luciferase assay was performed in which activation of the IRF3 transcription factor was examined by overexpressing TBK1 (Gates et al., 2016). As shown in Fig. 1(d), the presence of K1 did not inhibit IRF3 activation, showing that K1 specifically targets NF-κB activation. The cFLIP L protein, which is known to inhibit IRF3 activation (Gates & Shisler, 2016), was expressed in parallel to show that conditions were conducive for activating and detecting IRF3 transcriptional activation.

RT-qPCR was used to evaluate the transcription of the NF-κB-regulated CXCL8 and IL-6 genes, as shown in Fig. 1(e–g), as an alternative measure of NF-κB activation. We were interested in examining IL-6 gene expression because induction of IL-6 gene expression is reported to be PKR-independent during virus infection, thus providing another opportunity to examine PKR-independent NF-κB activation. TNF stimulated the transcription of the CXCL8 and IL-6 genes, as expected. In contrast, CXCL8 and IL-6 mRNA levels were significantly lower in K1-expressing 293T and HeLa cells, as shown in Fig. 1(e, f), respectively. CXCL8 and IL-6 gene expression was also reduced in cells expressing the DN form of IkBα, as expected. CXCL8 mRNA levels were also lower in HeLa cells, although this decrease was not statistically significant (Fig. 1g). These data demonstrated that ectopically expressed K1 inhibited NF-κB activation under different conditions and in more than one cell type.

K1 protein inhibits NF-κB activation in a PKR-independent manner

To further test the possibility that K1 possesses a PKR-independent NF-κB inhibitory function, we compared NF-κB activation in normal HeLa cells and a HeLa cell line derivative (PKRkd) that reduces PKR protein levels by 95% (Zhang et al., 2008). Similar to data in Fig. 1(d), K1 inhibited TNF-induced NF-κB activation in normal HeLa cells. This same inhibition was observed in PKRkd cells, as shown in Fig. 2(b). We noticed that TNF stimulation of luciferase activity was lower in PKRkd versus parental HeLa cells, and this is consistent with one report showing that PKR plays a role in the TNFR1–NF-κB signalling pathway (Takada et al., 2007). Phorbol 12-myristate 13-acetate (PMA) was used as an alternative stimulator of NF-κB in Fig. 2(c, d) because it activates NF-κB independently of PKR (Zhou et al., 2005). We confirmed that PKR protein levels are reduced in PKRkd cells as compared to HeLa cells in Fig. 2(e), similar to ours and others’ observations (Willis et al., 2009; Zhang et al., 2008). Indeed, PMA-induced luciferase activity levels were similar in HeLa and PKRkd cells, demonstrating that the absence of PKR did not alter cellular responsiveness to PMA. Importantly, K1 continued to antagonize PMA-induced NF-κB activation to a similar extent in both cell lines. While, in some cases, K1 protein levels were slightly lower in stimulated versus unstimulated cells, K1 still inhibited luciferase activity. These data suggest that K1 inhibits NF-κB activation in a PKR-independent mechanism.

IkBα degradation occurs in the presence of K1

To characterize the portion of the NF-κB pathway blocked by K1, we initially examined the effect of K1 on IkBα degradation (Fig. 3). Observations of Figs 1 and 2 suggested that using
TNF to stimulate NF-κB would minimize PKR activation, and this system continued to be used. It is well known that TNF–TNFR1 interactions trigger rapid IκBα degradation in several cell lines (Hayden & Ghosh, 2014). This same temporal degradation was observed in pCI-transfected cells (Fig. 3). Interestingly, K1 did not block IκBα degradation in all three cell lines tested. This phenotype was surprising because a 2004 publication from our lab shows that K1 prevents IκBα degradation when a DN IκBα was used to query whether K1 affects NF-κB nuclear translocation when a DN IκBα to inhibit RelA nuclear translocation (Chen et al., 1995). However, K1 did not inhibit NF-κB migration to the nucleus. RelA-containing band densities were similar in nuclear extracts (NEs) from pCI- versus pHA-K1-transfected cells. This implies that K1 does not inhibit NF-κB nuclear translocation.

The localization of K1 was also examined. K1 was predicted to be a cytoplasmic protein because it has no obvious NLS signals. However, K1 proteins were observed in similar amounts in the cytoplasmic extract (CE) and NEs (Fig. 4). This translocation event was inhibited by a cellular signal, as Fig. 4 shows that a DN IκBα was overexpressed, a result expected because DN IκBα remains bound to NF-κB to inhibit RelA nuclear translocation (Chen et al., 1995). However, K1 did not inhibit NF-κB migration to the nucleus. RelA-containing band densities were similar in nuclear extracts (NEs) from pCI- versus pHA-K1-transfected cells. This implies that K1 does not inhibit NF-κB nuclear translocation.

NF-κB translocates to the nucleus in the presence of K1

IκBα degradation unmasks the nuclear localization sequence (NLS) of RelA, thus allowing NF-κB to translocate to the nucleus (Hayden & Ghosh, 2014). Since K1 did not prevent IκBα degradation, a logical question was whether K1 prevented NF-κB nuclear translocation. In this case, a cellular fractionation assay separating cytoplasmic- and nuclear-localized proteins was used to query whether K1 affected NF-κB nuclear translocation. There was a visible increase in nuclear-localized RelA in TNF-treated versus unstimulated pCI-transfected cells (Fig. 4). This translocation event was inhibited when a DN IκBα was overexpressed, a result expected because DN IκBα remains bound to NF-κB to inhibit RelA nuclear translocation (Chen et al., 1995). However, K1 did not inhibit NF-κB migration to the nucleus. RelA-containing band densities were similar in nuclear extracts (NEs) from pCI- versus pHA-K1-transfected cells. This implies that K1 does not inhibit NF-κB nuclear translocation.

The localization of K1 was also examined. K1 was predicted to be a cytoplasmic protein because it has no obvious NLS signals. However, K1 proteins were observed in similar amounts in the cytoplasmic extract (CE) and NEs (Fig. 4). Amounts of K1 did not visibly change when cells were stimulated (Fig. 4).
This suggested that K1 was not migrating into the nucleus as part of an NF-κB complex. These same extracts were also probed with antiserum recognizing β-tubulin and Poly ADP Ribose Polymerase (PARP) proteins to demonstrate successful separation of nuclear and cytoplasmic proteins. Note that the expression of a DN IκBα molecule greatly reduced RelA/p65 inability to inhibit RelA nuclear translocation because the signal decreased if an excess of oligonucleotides containing wild-type NF-κB consensus sites was present. However, K1 did not inhibit NF-κB–DNA interactions. This signal was specific for NF-κB–DNA interactions, as demonstrated by the increase in values for the assay. This event was blocked in cells over-expressing a DN form of IκBα. However, K1 did not inhibit NF-κB–DNA interactions. This signal was specific for NF-κB because the signal decreased if an excess of oligonucleotides containing wild-type NF-κB consensus sites was present (WT competitor), but not when an excess of oligonucleotides with mutant NF-κB–binding sites (mutant competitor) was present. Note that these controls, where excess WT or mutant competitor nucleotides was present, were added to lysates only from pCI-transfected cells that received 30 min TNF for 2 h or (c, d) 50 ng ml⁻¹ PMA for 8 h. Results are presented as fold induction of luciferase activity relative to that of untreated, pCI-transfected cells. Lysates were also examined for HA-K1 protein expression. (e) PKR protein level in HeLa and PKRkd cell lysates. These experiments are a representation of experiments performed at least three times. *P<0.05, compared to unstimulated, pCI-transfected cells.

**K1 allows NF-κB to interact with an oligonucleotide containing NF-κB-binding sites**

Data from Fig. 4 suggested that the antagonistic mechanism of K1 could be associated with K1 nuclear localization. In the nucleus, NF-κB binds to a conserved DNA sequence present in the promoter of its target genes. The TransAM assay is one method used to detect and quantitatively measure transcriptionally active NF-κB–DNA interactions (Fig. 5). As expected, TNF treatment significantly increased NF-κB–DNA interactions, as demonstrated by the increase in values for the assay. This event was blocked in cells over-expressing a DN form of IκBα. However, K1 did not inhibit NF-κB–DNA interactions. This signal was specific for NF-κB because the signal decreased if an excess of oligonucleotides containing wild-type NF-κB consensus sites was present (WT competitor), but not when an excess of oligonucleotides with mutant NF-κB–binding sites (mutant competitor) was present. Note that these controls, where excess WT or mutant competitor nucleotides was present, were added to lysates only from pCI-transfected cells that received 30 min TNF treatment.
Vaccinia K1 inhibits RelA acetylation

K1 inhibits CBP/p300-mediated NF-κB activation and prevents RelA acetylation

The above data suggested that K1 allowed NF-κB to enter the nucleus and interact with its promoter but inhibited NF-κB transcriptional activation. One explanation for this pattern would be if K1 inhibited RelA acetylation, a post-translational modification required for NF-κB activation. This modification is performed by the CBP/p300 family of proteins (Gerritsen et al., 1997). To test this hypothesis, we first queried whether K1 could inhibit NF-κB activation triggered by overexpression of RelA and p300, conditions known to induce NF-κB activation (Gerritsen et al., 1997). K1 inhibited NF-κB activation, as measured by expression of NF-κB regulated CXCL8 (IL-8) and NFKBIA (IkBα) genes, as shown in Fig. 6(a, b), respectively. These data support the hypothesis that K1 acts at or downstream of RelA acetylation.

CBP, like its p300 orthologue, binds to and acetylates RelA at lysine 310 (Mukherjee et al., 2013). This can occur either before or after NF-κB interacts with κB-binding sites in a promoter (Mukherjee et al., 2013). To query whether K1 prevented this post-translational modification, RelA and CBP were overexpressed to trigger and detect RelA acetylation (Chen et al., 2015). This approach was chosen because it has proven difficult by us and others to detect acetylation of endogenous RelA (Chen & Chen, 2015). As expected, acetylation at lysine 310 was observed in pCI-transfected cells, as shown in Fig. 6(c). However, this acetylation was greatly reduced in K1-expressing cells, as observed in both HeLa (Fig. 6c) and PKR^ΔΔ cells (Fig. 6d). It was also

![Fig. 3. IkBα degradation occurs in the presence of K1. (a) 293T, (b) HeLa or (c) PKR^ΔΔ cells were transfected with 2000 ng of pCI or pHA-K1 for 24 h prior to incubation with regular medium (UN) or medium containing 10 ng ml⁻¹ TNF for 15 or 30 min. Cellular lysates were separated by SDS-PAGE and then transferred to PVDF membranes. Membranes were probed with anti-IκBα antibodies, and then the same blots were re-probed with anti-β-actin antiserum. A separated set of immunoblotted proteins was used to detect HA-K1 protein levels. These experiments are a representation of experiments performed at least three times.](image)

![Fig. 4. NF-κB translocation to the nucleus occurs in the presence of K1. 293T cells were transfected for 24 h with 1500 ng pCI, pIkBα-DN or pHA-K1. Cells were incubated in medium either lacking (UN) or containing TNF (20 ng ml⁻¹) for 30 or 60 min, and then lysed in a manner that separated cytoplasmic from nuclear proteins. A portion of extracts containing cytoplasmic or nuclear proteins was probed for the presence of the RelA subunit of the NF-κB heterodimer using immunoblotting. The same samples were also probed for the presence of K1 proteins by immunoblotting. Each sample was also probed with anti-β-tubulin and anti-PARP antibodies to serve as markers of cytoplasmic content and nuclear content, respectively. These experiments are a representation of experiments performed at least three times.](image)
observed that K1 protein levels slightly decreased when increased amounts of plasmids RelA and CBP were present. Nevertheless, K1 still inhibited RelA acetylation. Thus, the data in Fig. 6 support the hypothesis that K1 targets RelA acetylation to prevent NF-κB activation in a PKR-independent mechanism.

One possibility for this lack of RelA acetylation would be a lack of CBP–RelA interactions in K1-expressing cells. To test this possibility, PKRkd cells were examined for RelA–CBP interactions using co-immunoprecipitations, using the same conditions as in Fig. 6(c). Results are shown in Fig. 6(d). In vector-transfected cells, RelA–CBP interactions were detected when RelA and CBP were overexpressed. This interaction was greatly reduced when the K1 protein was present. This was a specific property of K1 because the expression of another poxviral NF-κB inhibitory protein (M2) did not inhibit this interaction and did not inhibit RelA acetylation (Fig. 6e)(Gedey et al., 2006). M2 is not expected to inhibit RelA–CBP interactions because M2 interferes with MEK-directed phosphorylation of ERK2, a kinase that targets the IKK complex for NF-κB activation (Gedey et al., 2006), thus acting on a signal transduction step prior to RelA–CBP interactions. Multiple attempts were made to co-immunoprecipitate K1 with RelA or with CBP under several different conditions. However, under no conditions tested could we reproducibly detect such interactions.

**DISCUSSION**

Several data indicate that K1 inhibits NF-κB activation in a PKR-independent manner. K1 retained its NF-κB inhibitory function when using stimuli that do not trigger PKR activation (e.g. PMA or co-expression of RelA and CBP/p300) or using a cell line with dramatically decreased PKR levels. Since K1 allowed IκB degradation and NF-κB nuclear translocation, it was likely that K1 prevented a signal transduction event that occurs after these events. There are two lines of evidence that K1 targets RelA acetylation. First, K1 inhibited RelA/p300-induced luciferase reporter gene expression. Second, K1 inhibited p300-induced acetylation of RelA. It was also observed that RelA-CBP co-immunoprecipitations no longer occurred in the presence of K1, suggesting that K1 prevents interactions of proteins necessary for NF-κB activation. Unfortunately, we could not reliably detect K1–RelA or K1–CBP interactions in eukaryotic cells by co-immunoprecipitations. Thus, we cannot rule out the possibility that K1 may act indirectly to prevent RelA–CBP interactions.

Interestingly, K1 did not prevent NF-κB from binding to oligonucleotides containing κB-binding sites. Originally, it was thought that CBP/p300 interacts with NF-κB prior to NF-κB binding to its consensus DNA sequence. However, a recent publication from Mukherjee et al. (2013) shows that there is a higher preponderance of promoters constitutively loaded with CBP/p300. One such gene found to be controlled by this latter mechanism is IκBα, a gene we examined here. Thus, one model based on these data is that K1 allows NF-κB to bind to its promoter sequence, but then prevents p300/CBP from interacting with NF-κB. Mukherjee et al. (2013) did not report whether the IL-8 gene, an additional gene we examined, was controlled in a similar manner. Thus, whether K1 acts in a similar manner or via a different mechanism remains the focus of future studies.

K1 possesses nine ARDs (Li et al., 2010). These 33-residue repeats are important for mediating protein–protein interactions in other ARD-containing proteins (Li et al., 2006; Mosavi et al., 2004). Two viral ARD-containing proteins that inhibit NF-κB are African swine fever virus A238 and cowpox CP77 proteins (Chang et al., 2009; Revilla, 1998), and these bind to RelA. However, two lines of evidence suggest K1 does not interact with RelA. First, we did not observe increased nuclear K1 protein levels in TNF-treated cells, implying that K1 does not enter the nucleus as an NF-κB-binding partner. Second, we did not consistently observe K1–RelA co-immunoprecipitations in pHA–K1-
transfected cells. It should be noted that the Orf poxvirus 002 protein also inhibits RelA acetylation by binding to RelA (Diel et al., 2011). However, Orf 002 lacks ARDs, suggesting that K1 and Orf 002 share functional similarities despite lacking similarity at the amino acid level (Diel et al., 2011).

Along with K1, there are other viral ARD proteins (vankyrins) encoded by poxviruses, herpesviruses and polydnaviruses (Bitra et al., 2012; Dreyfus et al., 2011; Herbert et al., 2015). Most of the poxvirus vankyrins also possess an additional F-box motif (referred to as a PRANC (Poxvirus Repeat of Ankyrin C terminus) domain) (Herbert et al., 2015). This F-box motif allows these poxviral ARDs to bind to Skp1, a member of the E3 SCF–ubiquitin ligase complex. This occurs for multiple proteins encoded by Ectromelia virus (EVM002, EVM005, EVM154 and EVM165) (Burles et al., 2014; Van Buuren et al., 2008), Orf virus (OV008, OV123, OV126, OV128 and OV129) (Sonberg et al., 2008), Myxoma virus (M-T5, M148, M149 and M150) (Werden et al., 2009), VACV 68k (Sperling et al., 2008), fowlpox virus FPV014 (Buttigieg et al., 2013), monkeypox virus 003 (Mohamed et al., 2009), variola virus G1R (Mohamed et al., 2009) and cowpox virus proteins CP77 (Chang et al., 2009) and CPXV006 (Mohamed et al., 2009). For the ectromelia virus proteins with PRANC domains, interaction with Skp1 is sufficient to antagonize the normal ubiquitination function of the SCF E3–ligase complex and inhibit IκBα degradation (Burles et al., 2014). K1 is unique in that it does not possess a partial or full F-box motif. This implies that K1 cannot interfere with IκBα ubiquitination, a hypothesis supported by the observation that K1 allowed IκBα degradation. Interestingly, the variola virus G1R, cowpox virus 006, ectromelia EVM002 and monkeypox virus 003 ANK/F-box proteins also bind to and cleave p105 to prevent formation of the RelA/p50 complex (Mohamed et al., 2009). It is unlikely that K1 targets p105 processing because NF-κB still binds to an oligonucleotide containing κB-binding sites (Fig. 5), suggesting that a functional RelA/p50 complex is in the nucleus.

Here, we show that K1 inhibits RelA acetylation and RelA–CBP interactions, suggesting that K1 performs its inhibitory
function in the nucleus. VACV encodes multiple inhibitors of NF-κB including M2, A46, A52, K7, B14, N1, C4, A49 and E3 (Bowie et al., 2000; Chen et al., 2008; Diperma et al., 2004; Ember et al., 2012; Gedey et al., 2006; Mansur et al., 2013; Myskiw et al., 2009; Schröder et al., 2008; Shisler & Jin, 2004). Most of these proteins act prior to NF-κB nuclear translocation. For example, M2, A46, A52, K7 and E3 proteins inhibit cytoplasmic proteins that stimulate IKK (Bowie et al., 2000; Gedey et al., 2006; Myskiw et al., 2009; Oda et al., 2009; Schröder et al., 2008). B14 binds to cytoplasmic IKK and inhibits its activation (Chen et al., 2008). N1 has also been reported to interact with the IKK complex (Diperma et al., 2004). However, this interaction has been disputed (Chen et al., 2008). Similarly, C4 acts at a step at or downstream of IKK activation but prior to NF-κB nuclear translocation (Ember et al., 2012). A49 prevents IκBα ubiquitination and degradation by binding to cytoplasmic βTrCP (Mansur et al., 2013). Thus, K1 is the first VACV protein to act in the nucleus to prevent NF-κB activation. K1 is unique also because the other known NF-κB inhibitory proteins expressed by VACV do not possess ARDs (Smith et al., 2013). It should be noted that Sumner et al. (2014) reported that an as-yet- unidentified early VACV protein inhibits an NF-κB signal transduction event that occurs post-RelA nuclear translocation. The construct used for their studies (vv811ΔA49) lacks K1L, suggesting that K1 may not be the sole VACV protein that has antagonistic function in the nucleus.

The subcellular location of K1 was studied here for the first time. It was present in both the cytoplasm and nucleus of unstimulated and stimulated cells. Because K1 is a small protein (31 kDa), it may be able to enter the nucleus by diffusion (Wang & Brattain, 2007). No obvious NLS is present in K1. Two other ARD-containing proteins (myxoma virus M150 protein and cellular IκBα) use an ARD repeat to localize to the nucleus (Camus-Bouclainville et al., 2004). Thus, a similar mechanism for K1 may be present, which is a future direction for our research.

K1 was identified as a host range gene over 30 years ago (Drillien et al., 1981; Perkus et al., 1986). Other functions have been ascribed to K1, including binding to the VACV C10 protein (Mccraith et al., 2000), ACAP2 (Meng et al., 2006) and SAMD9 (Sivan et al., 2015). K1 also inhibits PKR activation (Willis et al., 2009, 2011), and inhibits a cellular factor whose expression is controlled by IRF1 (Meng et al., 2012). These functions map to different regions of K1. For example, K1 residues 47, 51, 82, 83 and 85 are important for VACV replication in HeLa cells, a cell line where either K1 or C7 is sufficient for virus replication (Li et al., 2010; Meng & Xiang, 2006). A mutant K1 that no longer allowed virus replication (mutations at residues 82, 83, 85; S2C2) was still interacted with ACAP2, showing that these two molecular functions of K1 were not related to each other (Meng & Xiang, 2006). A future direction of our work is to identify the K1 region responsible for RelA acetylation as a means to better understand how K1 inhibits RelA activation.

The ARD is one of the most abundant motifs in nature (Mosavi et al., 2004), and these motifs are the basis for protein–protein interactions that control a variety of cellular signal transduction pathways and functions. ARDs are 33-residues in length, comprising two short α-helices which are connected by β-turns (Mosavi et al., 2004). Sequential ARDs in proteins stack together to form an L-shaped domain. Unlike enzymes, ARD-containing proteins do not recognize specific sequences in their target proteins. Instead, their ability to bind to a certain protein is due to the orientation of ARDs that, when stacked together, form a surface that exposes a binding site unique for its target protein (Li et al., 2006). The solving of the crystal structure of K1 revealed novel aspects of this viral ARD in contrast to cellular ARD proteins (Li et al., 2010). Namely, the residues critical for K1s host range function are exposed on the convex surface of its ankyrin repeats (Li et al., 2010). This makes K1 unusual; all other ARDs use residues in the concave surface to interact with binding partners. DARPin (new design ankyrin repeat proteins) are being explored as a novel alternative strategy to mAbs to treat diseases such as measles and HIV (Friedrich et al., 2013; Schweizer et al., 2008). DARPinS are an attractive alternative to antibodies as they provide several advantages including absence of aggregation, high stability, smaller size, simpler architecture and low-cost production (Stumpp et al., 2007). Thus, the continued exploration of ARDs like K1 may provide a rational basis for improving DARPinS.

METHODS

Cells and plasmids. Human embryonic kidney 293T (293T) cells and human cervical carcinoma (HeLa) cells were obtained from the American Type Culture Collection (ATCC). HeLa cells in which >95 % of PKR was knocked down (PKRkd) were obtained from Dr Charles Samuel (Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara) and were characterized previously (Zhang et al., 2008). Cells were cultured in Eagle’s minimal essential medium supplemented with 10 % FBS, 2 mM l-glutamine, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin.

Plasmid pHAK1 consists of a haemagglutinin epitope-tagged K1L gene inserted into the pCI expression vector (Willis et al., 2011). A plasmid encoding an IκBα DN (pIκBα-DN) was provided by Dr Dean Ballard (Vanderbilt University). (Scherer et al., 1995). pV5-M2 plasmid expresses a V5 epitope-tagged M2 protein (Gedey et al., 2006). Plasmids pIFN-κBLuc, pIL-1Luc and pIRF-3Luc encode the firefly luciferase gene under the control of a synthetic NF-κB promoter (Promega), the IL-8 promoter (Da Silva Correia & Ulevitch, 2002) or four copies of the PRDIII promoter sequence specific for IRF3 (Randall et al., 2014), respectively. Plasmid pRl–null encodes a promoter-less sea pansy luciferase gene (Promega). Plasmid pMyD88 was obtained from Dr Richard Tapping (Department of Microbiology, University of Illinois). pcFLIP was provided by J. Cohen (National Institutes of Health). Plasmid Tbk1 was a kind gift from S. Balachandran (Fox Chase Center). Plasmid pGFP–RelA was provided by Dr Lin Feng-Chen (Department of Biochemistry, University of Illinois) (Chen et al., 2001). A plasmid encoding an FLAG-tagged version of CBP protein (pFLAG-CBP) was obtained from Dr David P. Lebrun (Queen’s University) (Hyndman et al., 2012). Plasmid HA-p300 was obtained from Dr Den Rock (Department of Pathobiology, University of Illinois) (Diel et al., 2011). All plasmids were
Cells were transfected with the amounts of plasmids indicated in the figure (50 ng ml⁻¹ medium or medium containing TNF-α; Sigma) at the indicated times. In some cases, NF-κB or IRF3 activation was instead triggered by co-transfection of cells with plasmids containing MyD88 or TBK1 genes, respectively. All transfections were performed in triplicate. Cells were lysed in 1× passive lysis buffer (PLB; Promega) and lysates were analysed for firefly and sea pansy luciferase activity. For each lysis, the ratio of firefly luciferase activity to sea pansy luciferase activity was calculated to correct for differences in transfection efficiency. The triplicates were averaged. The resultant values were used to compare the expression of the firefly luciferase gene in stimulated cells to that present in unstimulated, pCI-transfected cells, whose value was set to one. Values are shown as mean±SD. Student’s t-test was used to determine the statistical significance of inhibition of luciferase activity by K1 or DN IeBeK proteins versus unstimulated, pCI-transfected cells. Statistically significant inhibition of luciferase activity is indicated by asterisks (*P<0.05).

Protein expression levels in lysates from luciferase assays were analysed by immunoblotting. Briefly, 30 µg of protein from each lysis was separated by using SDS-PAGE and transferred to PVDF membranes. Membranes were probed with mouse monoclonal anti-HA antibody (1 : 1000; Sigma-Aldrich) or anti-pKR (1 : 1000; Santa Cruz) and then HRP-conjugated goat anti-mouse IgG (1 : 5000; Thermo Scientific) to detect K1 protein expression levels. Antibody–antigen reactions were detected by using chemiluminescence reagents (Thermo Scientific) and autoradiography.

Quantitative reverse transcriptase PCR. Transcription of the CXCL8 (IL-8), NKFBIA (IκBα), IL-6 and ACTB (β-actin) genes in the presence or absence of K1 protein expression was evaluated by RT-qPCR (quantitative reverse transcriptase PCR). 293T or HeLa cells were seeded in six-well plates at 5×10⁴ or 1×10⁵ cells well⁻¹, respectively. Cells were transfected with the amounts of plasmids indicated in the figure legends, in triplicate. After 24 h, 293T or 2 h (HeLa) with medium lacking or containing 20 ng ml⁻¹ TNF. Alternatively, cells were co-transfected with pGFP-RelA (500 ng) and pHA-p300 (500 ng) and 1000 ng pCI or pHA-K1 for 24 h prior to collection instead of using TNF as a stimulator of NF-κB activation. Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen) following the manufacturer’s instructions. From each sample, 500 ng of RNA was incubated with Oligo-dT and the M-MuLV Reverse Transcriptase (New England BioLabs). The reaction was incubated at 42 °C for 60 min, and then terminated by heating at 85 °C for 5 min. The resultant cDNA was diluted 1 : 5 in DNase- and RNase-free water. Then, 2 µl of diluted cDNA were used for qPCR, using a Mastercycler ep realplex (Eppendorf) and SsoFast EvaGreen Super Mix (BioRad) as per the manufacturer’s instructions. qPCR was performed using the following program: 95 °C for 2 min, 40 cycles of 95 °C for 5 s and 55 °C for 10 s. Changes in expression level of each gene were calculated by the 2⁻∆ΔCt method (Livak et al., 2001). For normalization, β-actin mRNA quantities for each cDNA sample were measured, and then each value was normalized to that of unstimulated, pCI-transfected cells. Data are presented as means±SD from a minimum of three independent experiments. Student’s t-test was used to determine statistically significant differences in mRNA expression levels as compared to unstimulated, pCI-transfected cells. The following designed primers were used to detect β-Actin cDNA: 5'-AGTGGGTACACCCCTTCT-3' and 5'-ACC TTCACGTTTCCGATT-3'. Primers designed to detect CXCL8 cDNA were 5'-CCAAGGGCAAGAATATC-3' and 5'-GAGTAAAGTG TGGCTAATAC-3'. Primers designed to detect IeBeK cDNA were 5'-ATCGCCCTACATTGGTGTC-3' and 5'-ACCATGGGGTGCAGTCAC TC-3'. Primers 5'-TGAAGATGAGGAAAGCAACG-3' and 5'-CA TTTGGTGTGGTCAGG-3' were used to detect IL-6 cDNA.

Immunoblotting. Protein concentration of lysates (including those used for luciferase activity assays) was determined by bicinchoninic acid (BCA; Pierce), with the exception of experiments in which cytoplasmic and nuclear fractions were assayed. In this case, the 660 nm protein assay (Pierce) was used instead of the BCA assay. Regardless, for each experimental sample, an equal amount of protein from each lysate was electrophoretically separated using SDS-PAGE. Proteins were transferred to PVDF membranes, and membranes were blocked in 5 % (w/v) milk in Tris-buffered saline and Tween 20 (TBST; 150 mM NaCl, 50 mM Tris base and 0.05 % Tween 20) for at least 30 min at room temperature. Membranes were incubated in the primary and secondary antibodies listed below. Antibody reactions on membranes were detected by using chemiluminescence reagents (Amersham and Thermo Scientific) and autoradiography.

For the detection of IeBeK protein levels, subconfluent cellular monolayers of 293T HeLa or PKRkd cells in six-well plates were transfected with 2000 ng of either pCI or pHA-K1. After 24 h, supernatants were aspirated and cells were incubated in fresh medium either lacking or containing TNF (10 ng ml⁻¹; Roche) for 15 or 30 min. Next, cellular monolayers were dislodged by trypsinization, and cells were collected by centrifugation. Cellular pellets were suspended in CE buffer containing Halt protease inhibitors for 15 min (Oie et al., 2001; Shisler & Jin, 2004). Lysates were centrifuged and clarified supernatants were removed to fresh tubes. Ten micrograms of cytoplasmically extracted protein from each sample were evaluated for IeBeK protein levels by using immunoblotting, in which immunoblots were incubated with rabbit monoclonal anti-IeBeK antibody (1 : 2500; Cell Signaling) and then HRP-conjugated goat anti-rabbit IgG (1 : 10 000; Calbiotech). Blots were re-probed with rabbit monoclonal anti-β-actin antibody (1 : 5000; Sigma) and then HRP-conjugated goat anti-rabbit IgG (1 : 10 000; Calbiotech). An additional, identical set of immunoblotted lysates was used to detect K1 protein expression, in which PVDF membranes containing 30 µg of protein from each sample were incubated with mouse monoclonal anti-HA antibody (1 : 1000; Sigma-Aldrich) and then HRP-conjugated goat anti-mouse IgG (1 : 5000; Thermo Scientific). Acetylation of the RelA protein at residue 310 was detected as follows. 1×10⁶ HeLa cells were transfected with 250 or 500 ng of pGFP-RelA and pFLAG-CBP or pCI. In addition, cells were co-transfected with 1000 ng of pCI or pHA-K1. After 24 h, cellular monolayers were dislodged by trypsinization and collected by centrifugation (18 000 rcf for 1 min). Cellular pellets were resuspended and lysed in acetyl lysis buffer (ALB; 25 mM Tris/HCl pH 7.5, 250 mM NaCl, 1 % NP-40 and 1 mM EDTA) for 20 min on ice. Lysates were centrifuged (18 000 rcf for 10 min) and clarified supernatants were transferred to new tubes. Samples were analysed by immunoblotting, in which membranes were incubated with rabbit monoclonal anti-acetyl NF-κB RelA (Ly310) antibody (1 : 1000; Cell Signaling). The blot was re-probed with rabbit polyclonal anti-RelA antiserum (1 : 1000; Santa Cruz) to detect unmodified RelA. A duplicate blot was probed with anti-HA antiserum to detect K1 protein expression and actin. CBP was detected in a separate blot using anti-CBP (11 000; Cell Signaling). To detect RelA–CBP interactions, PKRkd cells were transfected with 250 or 500 ng of pGFP-RelA and pFLAG-CBP or pCI. In addition, cells were co-transfected with 1000 ng of pCI, pHA-K1 or pV5-M2 plasmids using TransIT transfection reagent. At 24 h post-transfection, cells from three wells were lysed in 300 µL ALB. A portion of each clarified lysate was removed to a new tube and used to probe protein expression in cellular lysates. The remaining clarified lysates were incubated with anti-CBP antibodies (Cell Signaling) for 6 h. Then
Protein G Sepharose beads (Life Technologies) were added to the mixture and incubated overnight at 4 °C. Next, beads were washed in large volumes of ALB. Beads were mixed with 2× sample buffer containing 2-mercaptoethanol and boiled for 5 min. A portion of each immunoprecipitation reaction was separated by SDS-PAGE. Proteins were transferred electrophoretically to PVDF membranes and membranes were probed with the indicated antiserum described above. The M2 protein was detected using anti-V5 antibody (1 : 1000; Millipore).

Cytoplasmic and nuclear fractionations. 1 × 10⁶ 293T cells were transfected with 1500 ng of either pCI, pIgBe-DN or pHA-K1. At 24 h after transfection, cells were incubated with medium lacking or containing TNF (20 ng ml⁻¹) for 15 or 30 min. Cellular monolayers were detached by trypsinization, processed by centrifugation and processed as described in a previous publication (Shisler & Jin, 2004). Cells were suspended in 50 µl CE buffer and incubated on ice for 15 min. Lysates were centrifuged at 1000 rcf for 5 min, and clarified supernatants were collected in new tubes. The remaining pellet was washed in 200 µl CE and centrifuged (1000 rcf for 5 min) twice. The nuclei-containing pellet was resuspended in 30 µl of NE buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 25 % glycerol) and incubated for 20 min on ice, vortexing samples every 10 min. Samples were centrifuged for 10 min at 18 000 rcf. Supernatants (NEs) were collected and stored at −80 °C. Both CE and NE were evaluated by immunoblotting for the following proteins: tubulin (mouse monoclonal anti-tubulin antibody, 1 : 5000; Abcam), PARP (rabbit polyclonal anti-PARP-1 antibody, 1 : 1000; Santa Cruz), Rea (rabbit polyclonal anti-Rea antibody, 1 : 5000; Santa Cruz) or K1 (mouse monoclonal anti-HA antibody, 1 : 1000; Sigma-Aldrich).

NF-κB-oligonucleotide (TransAM) binding assay. To study NF-κB–DNA interactions, 293T cells were transfected with 1500 ng of pCI, pIgBe-DN or pHA-K1. After 24 h, cells were incubated with medium lacking or containing 20 ng ml⁻¹ TNF for 30 or 60 min. Next, nuclei were isolated and nuclear extracted proteins were generated as described above. Five micrograms of NE were evaluated for the DNA-binding activity of NF-κB using the TransAM NF-κB ReA DNA-binding ELISA kit (Active Motif) following the manufacturer’s instructions. NEs were added to a 96-well plate coated with immobilized oligonucleotides containing an NF-κB consensus site (5’-GGGACCTTTCC-3’). Wells were washed and then incubated with a solution containing a primary antibody recognizing the ReA subunit of the NF-κB heterodimer. This antibody only recognizes the transcriptionally active form of Rea when complexed with DNA. Wells were washed and incubated with solution containing an HRP-conjugated secondary antibody. Wells were washed again and incubated with a developer solution provided with the kit. Reactions were analysed by spectrophotometry at a wavelength of 450 nm. In addition, some wells had an excess amount of immobilized oligonucleotides containing either the regular (wild type) or mutated NF-κB consensus sequence to evaluate non-specific binding of NF-κB in this system. Data shown are a representative experiment of at least three independent experiments, and each experiment was performed with three technical triplicates.

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