NF-κB activation is a turn on for vaccinia virus phosphoprotein A49 to turn off NF-κB activation

Sarah Neidel[1], **Hongwei Ren**[1], **Alice Torres**[1], **Geoffrey L Smith**[1]

[1]University of Cambridge

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Vaccinia virus protein A49 inhibits NF-κB activation by molecular mimicry and has a motif close to the N terminus that is conserved in IκBα, β-catenin, HIV vpu and other proteins. This motif contains 2 conserved serines and, for IκBα and β-catenin, phosphorylation of these serines enables recognition by the E3 ubiquitin ligase β-TrCP. Binding of IκBα and β-catenin to β-TrCP leads to their ubiquitylation at upstream lysines and thereafter proteasome-mediated degradation. In contrast, HIV vpu and VACV A49 lack these lysines and so are not degraded. This report demonstrates that A49 is phosphorylated at serine 7, but not serine 12, and that this is necessary and sufficient for binding β-TrCP and antagonism of NF-κB activation. Phosphorylation of A49 S7 occurs when NF-κB signaling is activated by addition of IL-1β, or overexpression of TRAF6 or IKKβ, the kinase needed for IκBα phosphorylation and activation of NF-κB signaling. Thus A49 shows beautiful biological regulation for it becomes an NF-κB antagonist upon activation of NF-κB signaling. The virulence of viruses expressing mutant A49 proteins, or lacking A49 (vΔA49), were tested. vΔA49 was attenuated compared to wild type, as reported previously, but viruses expressing A49 that cannot bind β-TrCP, or that binds β-TrCP constitutively, each had intermediate virulence. Therefore, A49 promotes virulence by inhibiting NF-κB activation and by another unknown mechanism independent of S7 phosphorylation and NF-κB antagonism. Lastly, a virus lacking A49 was a more potent vaccine than wild type virus.

Vaccinia virus | nuclear factor kappa B | virus immune evasion | innate immunity

Introduction

Vaccinia virus (VACV) is a member of the *Orthopoxvirus* genus of the *Poxviridae* (1). Like other poxviruses, VACV has a large, complex virion, a cytoplasmic site of replication (2) and a dsDNA genome encoding about 200 genes (3). Orthopoxvirus genomes have a highly conserved central region and more variable termini (4). The central region encodes proteins needed for replication, whilst the terminal regions encode proteins that affect virus virulence, host range and immunomodulation. Many of the latter proteins are dispensable for replication in cell culture, but suppress innate immunity and are important *in vivo* (5). These immunomodulatory proteins are numerous and many target the same signaling pathway. For instance, VACV encodes at least ten proteins that inhibit activation of nuclear factor kappa B (NF-κB) (5, 6). This article concerns one NF-κB inhibitor, protein A49.

A49 is a small intracellular protein that contributes to virus virulence (7). A49 has a B cell lymphoma (Bcl)-2-like fold (8) and is one of 11 Bcl-2-like proteins encoded by VACV. Some of these mimic cellular Bcl-2 family proteins with anti-apoptotic activity. For instance, proteins N1 (9-11) and F1 (12) inhibit apoptosis (10, 11, 13, 14). However, VACV Bcl-2 proteins B14, A52 (15) and A46 (16, 17) do not inhibit apoptosis, but inhibit other innate immune signaling pathways (18-22). A49 most closely resembles myxoma virus protein M11, an anti-apoptotic protein (23), but does not bind the cellular pro-apoptotic Bcl-2 proteins bound by M11 (8).

A49 inhibits activation of the interferon (IFN)-β promoter (7) by blocking NF-κB signaling via molecular mimicry (7). Near its N terminus, A49 contains 2 serines that are conserved in several proteins, such as IκBα and β-catenin (24), and as viral proteins HIV vpu (25, 26) and rotavirus non-structural protein 1 (NSP1) (27). For IκBα, these serines are phosphorylated by IKKβ that is activated during NF-κB signaling. Once phosphorylated, IκBα is recognised by the E3 ubiquitin ligase, beta-transducin repeat-containing protein (β-TrCP) (24), which ubiquitylates upstream lysine residues leading to proteasomal degradation of IκBα (28). This releases the NF-κB subunits p65 and p50 into the nucleus.

A49 binds to β-TrCP and prevents ubiquitylation of phosphorylated (p)-IκBα and thereby stabilises it (7). A49 also stabilises another β-TrCP substrate, β-catenin, leading to activation of the wnt signaling pathway (29). The interaction of A49 with β-TrCP requires either or both of serines 7 and 12 for mutation of both residues to alanine prevented binding to β-TrCP and NF-κB antagonism (7). In contrast, mutation to glutamic acid enhanced binding to β-TrCP and increased NF-κB antagonism, suggesting A49 needs phosphorylation to be an NF-κB inhibitor.

Here A49 is shown to be phosphorylated on S7, but not S12, and this is necessary and sufficient for binding to β-TrCP and antagonism of NF-κB activation. Further, A49 is phosphorylated when NF-κB signaling is activated. Therefore, A49 functions to inhibit NF-κB signaling conditionally, when this signaling pathway is activated. VACVs expressing mutant A49 unable to bind β-TrCP and antagonise NF-κB signaling, or expressing A49 binding β-TrCP constitutively, each had intermediate virulence between WT virus and a virus lacking the *A49R* gene (vΔA49). This indicates that A49 promotes virulence by inhibiting NF-κB activation and another function. Lastly, a VACV lacking A49 was more immunogenic than WT virus and provided better protection against VACV challenge.

Significance

Vaccinia virus (VACV) encodes many proteins that inhibit the innate response to infection. One example is protein A49 that inhibits NF-κB activation by binding to the E3 ligase β-TrCP. Here we show that A49 is phosphorylated on serine 7 and that this is necessary for binding β-TrCP and inhibition of NF-κB activation. Further, this phosphorylation occurs when the NF-κB pathway is stimulated and the kinase IKKβ is activated. Thus, A49 shows beautiful biological regulation, for activation of the pathway activates the virus inhibitor of the pathway. The significance of this is seen in vivo. For VACVs expressing A49 S7A or S7E are less virulent than wild type virus but more virulent than a virus lacking A49.

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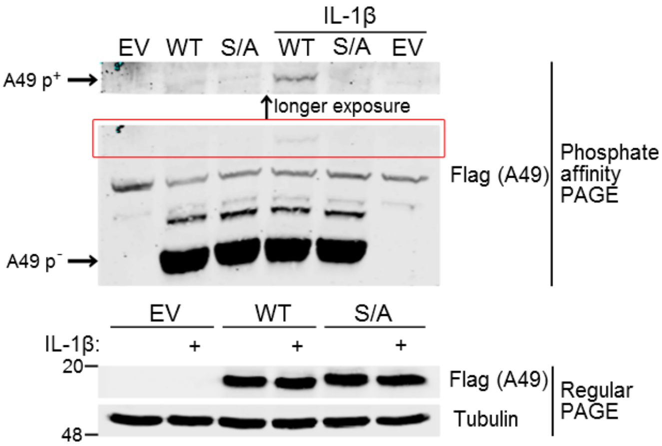

Fig. 1. A49 is phosphorylated upon stimulation of NF-κB signaling. HeLa cells were transfected with codon-optimised, FLAG-tagged wildtype A49 (WT) or A49 mutant S7/12A (S/A) or an empty vector (EV) control. After 24 h, cells were stimulated for 30 min with 50 ng/ml IL-1β, or left unstimulated, before lysis in 50 mM Tris pH 7.4, 250 mM NaCl, 0.5% NP-40, supplemented with protease inhibitors and NaF. Cleared lysates were mixed with SDS loading buffer, boiled and analysed immediately by Phosphate affinity PAGE, to separate phosphorylated (p^+) from unphosphorylated (p^-) A49, and regular PAGE, followed by immunoblotting.

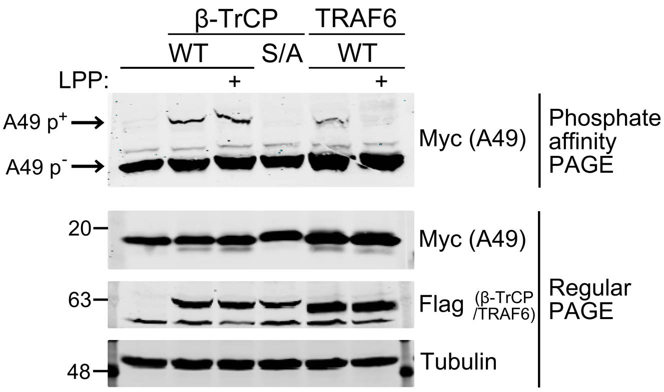

Fig. 2. Phosphorylated A49 is protected from de-phosphorylation by β-TrCP. HEK-293T cells were transfected with codon-optimised, myc-tagged wildtype A49 (WT) or A49 mutant S7/12A (S/A) and FLAG-tagged β-TrCP or TRAF6. Cells were lysed in Phosgel lysis buffer supplemented with protease inhibitors and NaF and treated with Lambda protein phosphatase (LPP) or not as indicated. Samples were then mixed with SDS loading buffer, boiled and analysed immediately by Phosphate affinity PAGE, to separate phosphorylated (p^+) from unphosphorylated (p^-) A49, and regular PAGE, followed by immunoblotting.

Results

A49 is phosphorylated

The cellular proteins β-catenin and IκBα are phosphorylated to enable efficient binding to β-TrCP, and the structure of β-TrCP bound to p-β-catenin shows extensive interactions between the phosphate groups of β-catenin and the β-TrCP binding pocket (30). To examine if A49 is also phosphorylated, a phos-tag was introduced into polyacrylamide gels as described (31). Phosphorylated proteins bind the phos-tag and so migrate more slowly during gel electrophoresis. Plasmids expressing codon-optimised, FLAG-tagged, WT A49, or in which serines 7 and 12 are changed to alanines (S7/12A) (7), were transfected into HeLa cells in parallel with empty vector (EV). These cells were left untreated or treated with interleukin (IL)-1β before harvesting and analysis by phosphate affinity PAGE and regular SDS-PAGE and immunoblotting (Fig. 1). The level of WT and mutant A49 detected by anti-FLAG antibody were similar with or without IL-1β stimulation (bottom). However, the phosphate affinity gel showed a slower migrating band that was present with WT A49 protein, but not S7/12A, and only after IL-1β stimulation (Fig. 1 top). Compared to the non-phosphorylated A49 protein, the phosphorylated (p)-A49 band is weak, suggesting only a small fraction of A49 is phosphorylated, or that, in the absence of binding partner, it is de-phosphorylated.

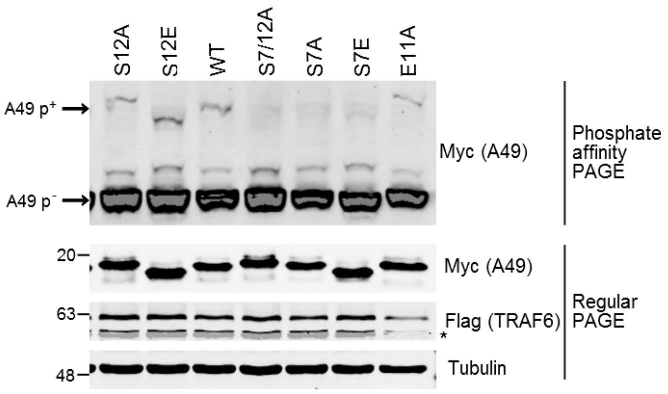

Fig. 3. . A49 is phosphorylated on serine 7, but not serine 12. HEK-293T cells were transfected with codon-optimised, myc-tagged wildtype A49 (WT) or A49 mutants and FLAG-tagged TRAF6. Cells wer lysed in IP buffer (Methods) and cleared cell lysates were mixed with SDS loading buffer, boiled and analysed immediately by Phosphate affinity PAGE, to separate phosphorylated (p^+) from unphosphorylated (p^-) A49, and regular PAGE. The star marks a non-specific signal detected by the anti-FLAG antibody.

To confirm this extra band is p-A49, samples were treated with phosphatase (LPP). In this case, myc-tagged WT and S7/12A mutant A49 were expressed in HEK-293T cells, together with FLAG-tagged β-TrCP. In parallel, WT A49 was transfected either alone or with FLAG-tagged TRAF6 to activate NF-κB signaling. SDS-PAGE showed expression of FLAG-tagged β-TrCP or TRAF6, and myc-tagged A49 proteins (Fig. 2 bottom). In the phosphate affinity gel, there was a slower migrating A49 band, seen only when the pathway was activated by TRAF6 (compare lanes 1 and 5), and treatment with LPP caused loss of this band (lane 6). When β-TrCP was overexpressed, p-A49 was seen without pathway stimulation and was resistant to de-phosphorylation, suggesting A49 - β-TrCP interaction is stable and protects p-A49 from de-phosphorylation (lanes 2 and 3). The stabilisation induced by β-TrCP overexpression may explain why p-A49 was detectable without addition of IL-1β or expression of TRAF6 to activate the pathway.

A49 is phosphorylated on serine 7 only

To investigate which serines are phosphorylated, additional mutant A49 proteins were constructed. These comprised S7A, S12A, and also S7E, S12E and E11A. S7E and S12E were included because, previously, a mutant A49 with both these serines mutated to glutamic acid (S7/12E) bound to β-TrCP strongly and was a stronger inhibitor of NF-κB activation (7). E11A was included because, compared to IκBα, A49 contains an extra aa prior to S12. Since this residue is glutamic acid, a phosphomimetic, this might substitute for a phospho-serine at S12. WT and mutant myc-tagged A49 alleles were transfected into cells together with FLAG-tagged TRAF6 and cell lysates were analysed as above (Fig. 3). SDS-PAGE showed the expression and slightly differing electrophoretic mobility of these mutants (lower panel). The phospho-affinity gel showed a p-A49 band with WT, S12A, S12E and E11A, but not with S7/12A, S7A, or S7E (Fig. 3 top). Therefore, S7 but not S12 is phosphorylated.

Phosphorylation of A49 is needed for binding β-TrCP and inhibition of NF-κB activation

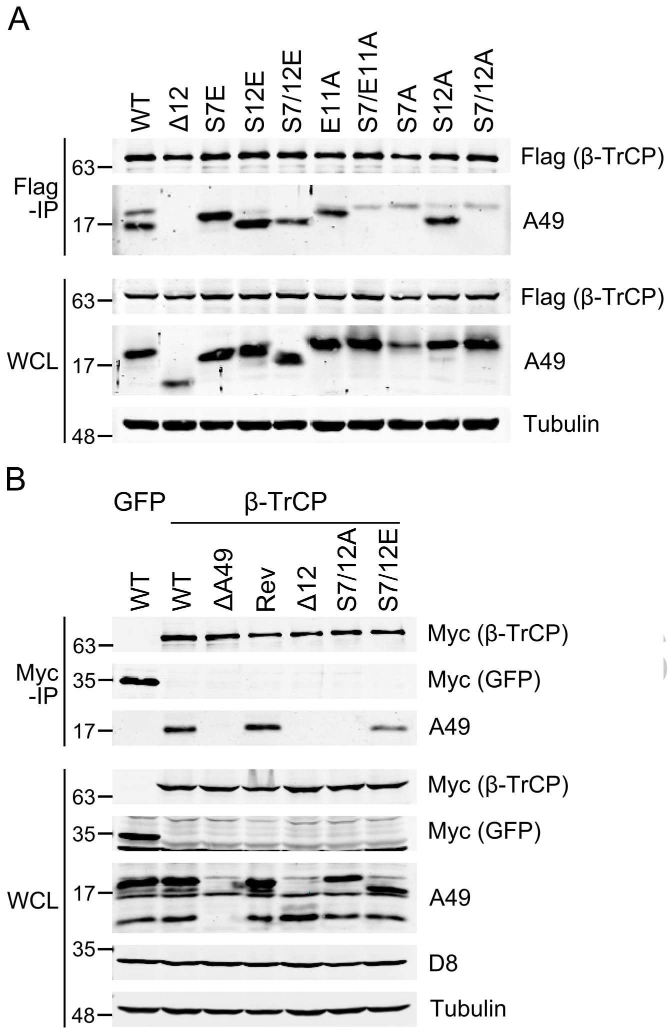

Fig. 4. . p-A49 co-immunoprecipitates with β-TrCP. A) HEK-293T cells were transfected with FLAG-tagged β-TrCP and untagged, codon-optimised wildtype A49 (WT) or its mutants. Cells were lysed after 24 h in IP buffer supplemented with protease and phosphatase inhibitors and cleared lysates were incubated for 16 h with a FLAG-affinity gel. Bound proteins were washed with lysis buffer and eluted with SDS loading buffer before they were analysed by immunoblot, together with the whole cell lysate (WCL). B) HEK-293T cells were transfected with plasmids expressing either myc-tagged GFP, or myc-tagged β-TrCP. After 24 h, the cells were infected at 5 p.f.u./cell with the indicated VACVs for 16 h. Cells were then treated as in A), with the exception that a myc-affinity gel was used.

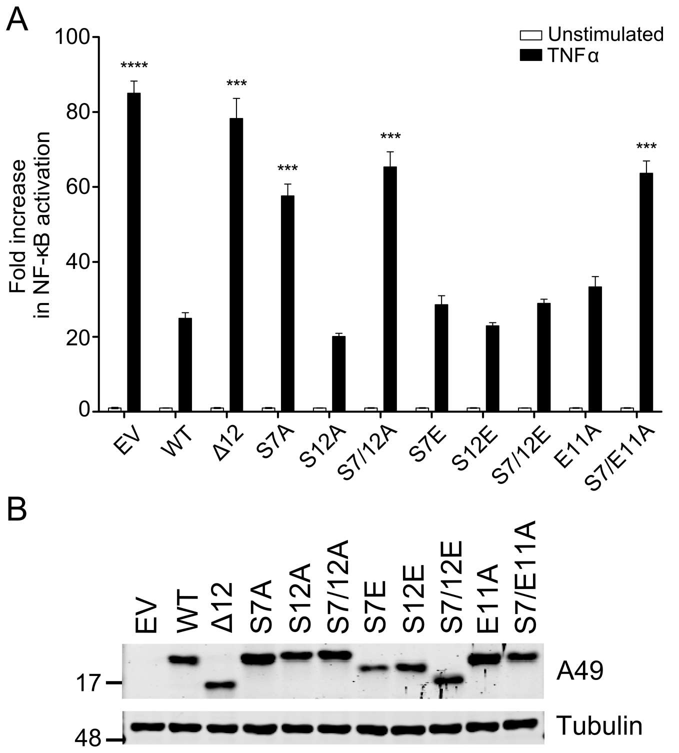

Fig. 5. Mutation of serine 7 abolishes inhibition of NF-κB activation by A49. A) HEK-293T cells were transfected with NF-κB-luc, TK-Renilla and plasmids encoding WT or mutant A49 proteins, or EV. After 24 h cells were stimulated by addition of 15 ng/ml TNFα for 8 h. Luciferase activity was measured in cell lysates. Triplicate samples were analysed for each condition. Data are expressed as the mean fold induction of the firefly luciferase activity normalised to renilla values for the stimulated versus unstimulated samples. Data are then expressed as the EV value compared to the test sample +/- Standard error of the mean. ** $p<0.01$, * $p< 0.05$, ns not, significantly different. B) Immunoblot showing the expression levels of A49 and α-tubulin analysed in A).

The ability of these A49 mutants to bind β-TrCP and inhibit NF-κB signaling was tested. HEK-293T cells were transfected with FLAG-tagged β-TrCP and untagged, codon-optimised WT A49 or mutants. The FLAG-tagged β-TrCP was immunoprecipitated from cell lysates and analysed by immunoblotting with anti-FLAG and anti-A49 polyclonal antibody (7) (Fig. 4A). Note the slightly different electrophoretic mobility of these untagged A49 proteins compared to the myc-tagged A49 proteins shown in Figure 3. All mutants with S7, or S7E, were immunoprecipitated by β-TrCP, and all mutants lacking S7 (or S7E) were not, or only very poorly. Thus S7 phosphorylation, or E at this position, is needed for binding β-TrCP. Similar analyses in which myc-tagged β-TrCP or myc-tagged GFP were transfected into cells that subsequently were infected with WT or A49 mutant viruses, also showed that WT A49 or A49 S7/12E were co-precipitated with β-TrCP, whereas A49 Δ12 or A49 S7/12A were not (Fig. 4B). Equal infection and equal loading were shown using antibody to VACV protein D8 or α-tubulin, respectively.

The ability of A49 mutants to block NF-κB activation was measured using a NF-κB responsive promoter linked to luciferase, as described (7). WT A49 and mutants S12A, S7E, S12E, S7/12E and E11A all inhibited NF-κB activation, but S7A, S7/12A, S7/E11A and a mutant lacking the first 12 aa (Δ12) did not (Fig. 5A). Immunoblotting of cell lysates with anti-A49 antibody and anti-α-tubulin confirmed equivalent expression of these proteins and equal loading (Fig. 5B). In summary, the phosphorylation of A49 occurs at S7 and this is critical for A49 to bind β-TrCP and antagonise NF-κB signaling.

IKKβ can phosphorylate A49

Stimulation of NF-κB signaling via addition of IL-1β, or overexpression of TRAF6 caused phosphorylation of A49 (Figs. 1 & 2). Both stimuli cause phosphorylation and activation of IKKβ leading to phosphorylation of IκBα. Under these conditions, A49 was phosphorylated, suggesting that IKKβ might phosphorylate A49. To test this, cells were transfected with myc-tagged A49 together with FLAG-tagged TRAF6, IKKα, IKKβ or NEMO and cell lysates were analysed by SDS-PAGE or phosphate affinity PAGE (Fig. 6). SDS-PAGE confirmed expression of all these proteins, except for IKKα, that was barely detectable. Phospho-affinity PAGE showed that expression of IKKβ induced strong phosphorylation of A49 and that TRAF6 was less effective. Trace amounts of p-A49 were also seen with low expression of IKKα, but no phosphorylation of A49 was observed when NEMO was expressed. In parallel, substitution of WT A49 for the S7/12A

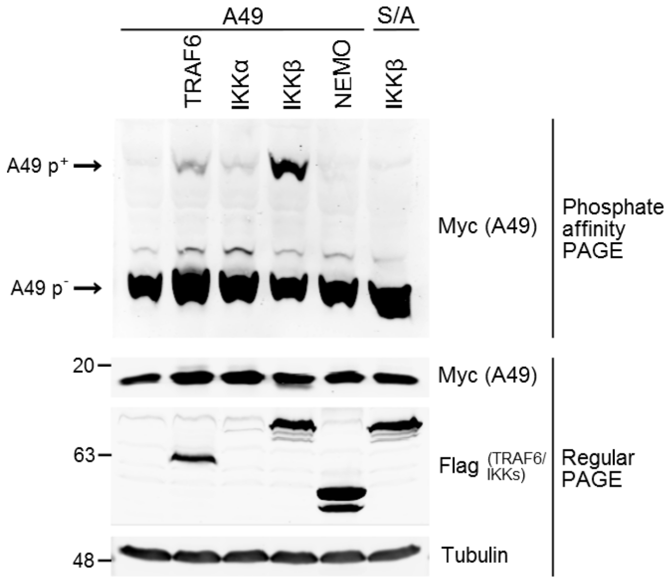

Fig. 6. A49 is phosphorylated after activation of NF-κB signaling at or downstream of the IKK complex. HEK-293T cells were transfected with codon-optimised, myc-tagged A49 alone or together with FLAG-tagged TRAF6, IKKα, IKKβ or NEMO. As a control cells were co-transfected with the S7/12A mutant of A49 and IKKβ. Cells were lysed in Phos-gel buffer supplemented with NaF and cleared lysates were mixed with SDS loading buffer, boiled and stored at -80 °C before analysis by Phosphate affinity PAGE, to separate phosphorylated (p$^+$) from unphosphorylated (p$^-$) A49, and regular PAGE.

mutant confirmed lack of A49 phosphorylation even when IKKβ was expressed. In summary, IKKβ can induce phosphorylation of A49.

VACV expressing non-phosphorylated A49 is less virulent than wild type virus

A virus mutant lacking gene *A49R*, vΔA49, was attenuated in a murine intranasal model (7). To determine if the ability of A49 to bind β-TrCP and so inhibit NF-κB signaling is the only reason for attenuation, recombinant VACVs bearing A49 S7/12A, S7/12E or lacking the first 12 aa (Δ12) were constructed and the virulence of these viruses was tested by measuring weight change after infection (Fig. 7A). Notably, all these mutant viruses had intermediate virulence that was statistically different from both WT and deletion mutant. For the A49 S7/12E mutant virus, the mutant *A49R* gene was replaced with the WT gene (A49 S7/12E-rev). The virulence of this virus was the same as WT (Fig. 7B), confirming that the intermediate virulence of the S7/12E virus was due to A49 mutation. For the other mutant viruses (S71/12A and Δ12), additional revertant viruses were not made, but instead complete genome sequencing confirmed that there were no other mutations compared to WT. Collectively, these analyses showed that A49 binding to β-TrCP and thereby inhibiting NF-κB is important for virulence. However, because viruses with mutant A49 proteins unable to inhibit NF-κB were more virulent that vΔA49, A49 has an additional function that contributes to virulence. Surprisingly, the virulence of the A49 S7/12E mutant virus was also intermediate. This A49 protein binds β-TrCP and inhibits NF-κB signaling at least as efficiently as WT A49 (7) and yet the virus has reduced virulence. A possible explanation is binding β-TrCP constitutively, whether or not the NF-κB pathway is activated. This might prevent non-phosphorylated A49 unbound by β-TrCP from having another function. This would be consistent with the observation that a virus expressing A49 that cannot bind β-TrCP has intermediate virulence.

Deletion of A49 increases VACV immunogenicity

In several cases loss of one immunomodulator from VACV increases virus immunogenicity despite virus attenuation. To investigate if this was true for A49, mice immunised with vA49WR or vΔA49 were challenged intranasally 42 d later with WT VACV strain WR. Animals immunised with vΔA49 had significantly lower weight loss compared to WT virus (Fig. 7C). The viruses with mutated A49 proteins were intermediate between WT and vΔA49, but these small differences did not reach statistical significance (Supplementary Figure 1).

Discussion

This article reports that VACV protein A49 is phosphorylated on serine 7 when the NF-κB signaling pathway is turned on. Addition of IL-1β, or overexpression of TRAF6 or IKKβ, caused A49 phosphorylation and this enabled p-A49 binding to β-TrCP. Sequestration of β-TrCP by p-A49 prevents β-TrCP recognizing other substrates such as p-IκBα (7) or p-β-catenin (29) so they remain stable. β-TrCP has several other substrates, for instance CDC25 (32), that might also be affected. For β-catenin, its stabilisation leads to activation of the wnt signaling pathway (29). For p-IκBα, its stabilisation causes retention of cytoplasmic NF-κB p65 and p50 and no transcription from NF-κB dependent promoters. Since the ability of A49 to bind to β-TrCP is dependent on phosphorylation, and phosphorylation can be achieved by IKKβ activation, the activation of NF-κB signaling is a turn on for A49 to turn off NF-κB activation. This represents beautiful biological regulation for without the need to inhibit NF-κB, A49 remains unbound to β-TrCP and free to undertake other function(s).

In vivo evidence indicates that A49 has a second function. The virulence of VACV expressing mutant A49 that is not phosphorylated, no longer binds β-TrCP and no longer inhibits NF-κB activation, is intermediate between WT and vΔA49. If inhibiting NF-κB activation was the sole function of A49, a virus expressing A49 unable to be phosphorylated would likely have virulence equivalent to vΔA49. Analysis of VACV expressing A49 S7/12E provides further evidence for another A49 function. A49 S7/12 binds β-TrCP constitutively, but despite this, VACV expressing S7/12E A49 is attenuated compared to WT. This attenuation may arise from A49 being constitutively bound to β-TrCP and so unable to bind to other substrates and mediate other functions. An alternative hypothesis, that transient early activation of NF-κB is beneficial for the virus, seems less likely because the virus expresses many other NF-κB inhibitors early after infection.

Possible other functions for A49 include bringing other substrates to β-TrCP for ubiquitylation and proteasomal degradation, rather as HIV Vpu induces degradation of CD4 (26). Such function would be lost by mutation of A49 to prevent β-TrCP binding. Alternatively, A49 might bind to other substrates without inducing their degradation to inhibit or modify their function. Some rotavirus NSP1 proteins bind to β-TrCP and modify its function like A49, whereas other NSP1 proteins induce degradation of β-TrCP (as well as IRF3 and IRF7) (33). Further proteomic screens for additional A49 binding partners either in the presence of proteasomal inhibitors, or using A49 mutants unable to bind β-TrCP are needed to identify other targets.

A49 is one of several virus proteins that contain the motif SXXXS and mimic cellular proteins with this motif. Such virus proteins include HIV vpu (26), Epstein-Barr virus latent membrane protein 1 (34) and the NSP1 protein from some rotavirus strains (33). These viral proteins, like the cellular counterparts, are phosphorylated and thereafter are recognized by β-TrCP. The cellular proteins such as IκBα and β-catenin contain lysine residues just upstream of the SXXXS motif and these are ubiquitylated by β-TrCP leading to proteasomal degradation. This phosphorylation, ubiquitylation and degradation are cellular mechanisms to control activation of the NF-κB and wnt signaling pathways. In contrast, the virus proteins lack upstream lysines and so are not ubiquitylated following phosphorylation and remain bound to β-TrCP, blocking its engagement with other substrates.

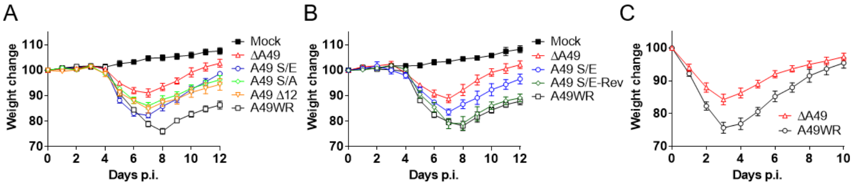

Fig. 7. Virulence and immunogenicity of VACV A49 mutant viruses. A) Female Balb/c mice (6-8 weeks old, n=5) were infected intranasally with 5 × 10^3 p.f.u. of the indicated viruses (10 µl / nostril) and their weight was monitored daily. Weight data are expressed as the percentage ± SEM of the mean weight of the same group of animals on day 0. Statistical analyses compared each mutant virus with each other and with vA49 and vΔA49 viruses using 2-way ANOVA. B) As in A) except using VACV WT (A49WR), A49 S7/12E (A49 S/E), ΔA49, and A49 S7/12E-rev (A49 S/E-rev). A49WR and A49 S/E-rev were not statistically different. C) Mice immunised with VACV WT or ΔA49 were challenged i.n. with 1 × 10^7 p.f.u. of wild-type VACV WR at 42 d p.i. Mice were monitored as in A). Statistical analyses using 2-way ANOVA showed the difference between these two groups was significant; p=0.012. Data shown in A) and C) are from one representative experiment out of two and in B) are from a single experiment.

A49 differs from some other β-TrCP substrates in being phosphorylated on only one of the two serines (S7) and this is sufficient for binding β-TrCP. In contrast, phosphorylation of p105 on both serines 927 and 932 is needed for recognition by β-TrCP and subsequent degradation (35). Other β-TrCP substrates can be recognized without phosphorylation (36). In cases where phosphorylation is needed for binding to β-TrCP, the kinases mediating phosphorylation may differ. For instance, HIV Vpu is phosphorylated by casein kinase-2 (CK-2) at S52 and S56 (37), whereas IKKβ can induce phosphorylation of VACV A49, although other kinases may also do so. Exactly how p-A49 fits into the IκBα-binding pocket of β-TrCP is unknown, but phosphorylation of S7 is essential and a phospho-mimetic residue at S12 is tolerated. A49 differs from most cellular and viral proteins in containing an extra residue between the 2 serines, and shares this property with p105. Removal or substitution of this residue does not prevent binding to β-TrCP.

Overexpression of β-TrCP, increased p-A49 levels without activation of NF-κB signaling by upstream stimuli. This suggests that some A49 phosphorylation may occur without pathway activation, due to constitutive low IKKβ activity or activity of other kinases, and that p-A49 is stabilized by binding β-TrCP. Consistent with this, A49 is resistant to de-phosphorylation when β-TrCP is overexpressed. In contrast, at endogenous β-TrCP levels, A49 may be dephosphorylated by phosphatases, so only a small fraction of A49 is phosphorylated in steady state.

Lastly, A49 is one of more than 10 VACV proteins that all inhibit NF-κB activation, and which when deleted individually from VACV cause an *in vivo* phenotype (5). Intuitively, attenuation from loss of an inhibitor seems improbable if 10 other inhibitors remain. A possible explanation for this paradox is that these NF-κB inhibitors are multi-functional and the other function(s) are not compensated by the other NF-κB inhibitors. For instance, N1 inhibits NF-κB activation and apoptosis (10, 11), and evidence for more than one function for A49 is presented here.

In conclusion, VACV protein A49 is phosphorylated on serine 7 and this is required for binding to β-TrCP and inhibition of NF-κB signaling. Viruses expressing A49 unable to bind to β-TrCP, or that bind β-TrCP constitutively, have virulence intermediate between WT and A49 deletion mutant. Thus A49 is a conditional inhibitor of NF-κB and is only activated when needed, namely when NF-κB signalling is activated.

Materials and Methods

Cells

HEK-293T cells BSC-1 cells were grown in DMEM (Gibco) supplemented with 10% heat-treated (56 °C, 30 mins) fetal bovine serum (FBS, Harlan-Sera Lab), 100 U/ml penicillin and 100 µg/ml streptomycin (P&S). HeLa cells and RK_{13} cells were grown in mimimum essential medium (MEM, Gibco) supplemented with 10% FBS and P&S. HeLa cells were also supplemented with non-essential aa (Gibco).

Viruses

Vaccinia virus (VACV) strain Western Reserve (WR) and derivatives lacking the *A49R* gene, vΔA49, or in which the *A49R* gene was re-inserted into vΔA49, vA49-Rev, were described (7). Additional VACVs expressing mutant A49 proteins were constructed by transient dominant selection (38) as described for vA49-Rev (7) using plasmids bearing mutant A49R alleles. Viruses were grown on RK_{13} cells and titrated by plaque assay on BSC-1 cells. For *in vivo* work, viruses were purified from cytoplasmic extracts of infected cells by sedimentation through a cushion of 36% (w/v) sucrose (39).

Codon optimisation and site-directed mutagenesis

A version of gene *A49R* codon-optimised for expression in human cells was purchased from Thermo Fisher Scientific. Mutations in either the WT *A49R* gene for expression from VACV, or the codon-optimised *A49R* gene for expression in mammalian cells, were made using the QuickChange ® Site-Directed Mutagensis Kit (Agilent). Changes were confirmed by DNA sequencing.

Reporter gene assays

Reporter gene assays in HEK-293T cells using NF-κB-firefly luciferase and TK-renilla were done as described (7). These plasmids were transfected into cells together with plasmids expressing WT or mutant A49 proteins and the following day cells were stimulated with TNF or IL-1β (as indicated) and the levels of luciferase activity determined. The fold induction of luciferase activity following pathway stimulation (normalised to renilla control) were calculated compared to unstimulated control. Statistical analyses compared the fold induction with empty vector (EV) to test sample.

Genome sequencing

DNA was extracted from viruses that had been purified from infected cells by sedimentation through 2 sucrose cushions (36% w/v) and the complete genome sequence was determined by Illumina technology. The VACV-A49-S/A DNA gave 232,436 reads with a mean coverage density of 273 per nucleotide, the VACV-A49Δ12 had 294,154 reads with a mean coverage of 423, and the WT-VACV was also sequenced with 401,667 reads and a mean coverage of 556.

Mobility shift detection of phosphorylated proteins

HEK-293T cells were transfected and or stimulated as described in figure legends. Prior to harvest, cells were washed with PBS on ice, scraped into 1 ml PBS, collected by centrifugation at 500 *g* for 5 min at 4 °C and then lysed in 180 µl Phosgel lysis buffer (20 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100) with 10 mM NaF (New England Biolabs) if phosphatase treatment was performed. After 15 min on ice, the lysate was cleared by centrifugation at 18,845 *g* for 20 min at 4 °C and the supernatant was mixed with 6 x loading buffer (final concentration 50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.1% bromophenol blue) and either stored at -80 °C or analysed immediately by SDS- PAGE.

Phosphate affinity SDS-PAGE

Polyacrylamide gels were prepared with Bis-Tris/HCl buffer at pH 6.8 (31) and addition of Phos-tag™ acrylamide (40). Separating gels (10% acrylamide) were made with 357 mM Bis-Tris-HCl, 50 mM Phos-tag acrylamide and 100 mM $Zn(NO_3)_2$ and stacking gels (4% acrylamide) were made with 357 mM Bis-Tris-HCl, without Phos-tag or $Zn(NO_3)_2$. For the running buffer, fresh 1 M sodium bisulphite (Sigma) solution was prepared, filtered and mixed with 20 x MOPS buffer prior to electrophoresis to give final concentrations of 50 mM MOPS, 50 mM Tris, 0.1% (w/v) SDS and 5 mM sodium bisulphite.

Immunoblotting of Phos-tag™ gels

After electrophoresis the gel was twice soaked in NuPAGE® transfer buffer (Thermo Scientific) supplemented with 10% (v/v) methanol and 5 mM sodium bisulphite for 15 min. Transfer and subsequent procedures were the same as described (7). The antibodies used for immunoblotting and the positions of molecular mass markers (kDa) are indicated on the figures.

Phosphatase treatment of cell lysates

Cell lysates (39 µl), prepared as above, were mixed with 5 µl $MnCl_2$ (New England Biolabs) and 5 µl 10 x PMP buffer (New England Biolabs). Samples that were lysed in the absence of NaF also received 1 µl lambda protein phosphatase (LPP, New England Biolabs). After incubation at 30 °C for 30 min, 6 x SDS loading buffer was added and samples were analysed by Phos-tag PAGE.

Immunoprecipitation

Cells were lysed in IP buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM CaCl2, 0.1% NP-40, 10% glycerol) supplemented with protease inhibitors (cOmplete EDTA-free, Roche) and phosphatase inhibitors (PhosSTOP, Roche) where indicated. Lysates were cleared by centrifugation and then incubated with anti-FLAG M2 affinity resin (Sigma) at 4 °C for 4 h or with anti-c-Myc agarose resin (Santa Cruz) at 4 °C overnight, washed 4 times with lysis buffer, mixed with protein loading buffer and analysed by SDS-PAGE.

Antibodies and cytokines

The following antibodies were used: rabbit polyclonal anti-A49 (diluted 1:1,000) (7), mouse monoclonal AB1.1 against VACV protein D8 (diluted 1:1,000) (41), rabbit polyclonal anti-FLAG (diluted 1:1,000) (Sigma-Aldrich F7425), mouse monoclonal anti-myc (diluted 1:1,000) (Cell-Signaling (B11), mouse monoclonal anti-α-tubulin (diluted 1:1,000) (Millipore DM1A). TNFα and IL-1β were purchased from Peprotech.

In vivo experiments in murine models

VACV was purified from cytoplasmic extracts of infected cells by two rounds of sedimentation through 36% (w/v) sucrose at 32,900 *g* for 80 min. Virus was re-suspended in 10 mM Tris-HCl pH 9 and stored at -80 °C. Virus used for infections was diluted in PBS containing 1% bovine serum albumin and the titer of the diluted virus that was used to infect mice was determined by plaque assay on the day of infection. Female BALB/c mice ($n = 5$, 6-8 weeks old) were anaesthetised and inoculated intranasally (i.n.) in both nostrils with 5×10^3 p.f.u. and the body weight was measured daily thereafter (42). For the challenge experiments, mice that had been immunised i.n. were challenged i.n. 6 weeks later with 1×10^7 p.f.u. of wild-type VACV WR. Mice were monitored daily to record body weight.

Statistical analysis

Data were analysed using an un-paired Student's *t*-test, with Welch's correction where appropriate, or a two-way ANOVA test where appropriate using the GraphPad Prism statistical software (GraphPad Software, San Diego, CA). Statistical significance is expressed as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are representative of at least 2 independent experiments.

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1. Fenner F, Wittek R, & Dumbell KR (1989) *The Orthopoxviruses* (Academic Press Ltd, London) p chapter 4.
2. Moss B (2013) Poxviridae. *Fields Virology*, ed Knipe DMH, P. M. (Lippincott Williams & Wilkins, Philadelphia), 6th Ed Vol 2, pp 2129-2159.
3. Goebel SJ, *et al.* (1990) The complete DNA sequence of vaccinia virus. *Virology* 179:247-266, 517-263.
4. Gubser C, Hue S, Kellam P, & Smith GL (2004) Poxvirus genomes: a phylogenetic analysis. *J Gen Virol* 85:105-117.
5. Smith GL, *et al.* (2013) Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. *J Gen Virol* 94:2367-2392.
6. Sumner RP, Maluquer de Motes C, Veyer DL, & Smith GL (2014) Vaccinia virus inhibits NF-kappaB-dependent gene expression downstream of p65 translocation. *J Virol* 88:3092-3102.
7. Mansur DS, *et al.* (2013) Poxvirus targeting of E3 ligase beta-TrCP by molecular mimicry: a mechanism to inhibit NF-kappaB activation and promote immune evasion and virulence. *PLoS Pathog* 9:e1003183.
8. Neidel S, *et al.* (2015) Vaccinia virus protein A49 is an unexpected member of the B-cell Lymphoma (Bcl)-2 protein family. *J Biol Chem* 290:5991-6002.
9. Aoyagi M, *et al.* (2007) Vaccinia virus N1L protein resembles a B cell lymphoma-2 (Bcl-2) family protein. *Protein Sci* 16:118-124.
10. Cooray S, *et al.* (2007) Functional and structural studies of the vaccinia virus virulence factor N1 reveal a Bcl-2-like anti-apoptotic protein. *J Gen Virol* 88:1656-1666.
11. Maluquer de Motes C, *et al.* (2011) Inhibition of apoptosis and NF-kappaB activation by vaccinia protein N1 occur via distinct binding surfaces and make different contributions to virulence. *PLoS Pathog* 7:e1002430.
12. Kvansakul M, *et al.* (2008) Vaccinia virus anti-apoptotic F1L is a novel Bcl-2-like domain-swapped dimer that binds a highly selective subset of BH3-containing death ligands. *Cell Death Differ* 15:1564-1571.
13. Postigo A, Cross JR, Downward J, & Way M (2006) Interaction of F1L with the BH3 domain of Bak is responsible for inhibiting vaccinia-induced apoptosis. *Cell Death Differ* 13:1651-1662.
14. Wasilenko ST, Banadyga L, Bond D, & Barry M (2005) The vaccinia virus F1L protein interacts with the proapoptotic protein Bak and inhibits Bak activation. *J Virol* 79:14031-14043.
15. Graham SC, *et al.* (2008) Vaccinia virus proteins A52 and B14 Share a Bcl-2-like fold but have evolved to inhibit NF-kappaB rather than apoptosis. *PLoS Pathog* 4:e1000128.
16. Fedosyuk S, *et al.* (2016) Vaccinia virus immunomodulator A46: A lipid and protein-binding scaffold for sequestering host TIR-domain proteins. *PLoS Pathog* 12:e1006079.
17. Fedosyuk S, Grishkovskaya I, de Almeida Ribeiro E, Jr., & Skern T (2014) Characterization and structure of the vaccinia virus NF-kappaB antagonist A46. *J Biol Chem* 289:3749-3762.
18. Bowie A, *et al.* (2000) A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. *Proc Natl Acad Sci U S A* 97:10162-10167.
19. Chen RA, Ryzhakov G, Cooray S, Randow F, & Smith GL (2008) Inhibition of IkappaB kinase by vaccinia virus virulence factor B14. *PLoS Pathog* 4:e22.
20. Harte MT, *et al.* (2003) The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. *J Exp Med* 197:343-351.
21. Stack J & Bowie AG (2012) Poxviral protein A46 antagonizes Toll-like receptor 4 signaling by targeting BB loop motifs in Toll-IL-1 receptor adaptor proteins to disrupt receptor:adaptor interactions. *J Biol Chem* 287:22672-22682.
22. Stack J, *et al.* (2005) Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1-receptor adaptors and contributes to virulence. *J Exp Med* 201:1007-1018.
23. Kvansakul M, *et al.* (2007) A structural viral mimic of prosurvival Bcl-2: a pivotal role for sequestering proapoptotic Bax and Bak. *Mol Cell* 25:933-942.
24. Winston JT, *et al.* (1999) The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. *Genes Dev* 13:270-283.
25. Vincent MJ & Abdul Jabbar M (1995) The human immunodeficiency virus type 1 Vpu protein: a potential regulator of proteolysis and protein transport in the mammalian secretory pathway. *Virology* 213:639-649.
26. Margottin F, *et al.* (1998) A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. *Mol Cell* 1:565-574.
27. Davis KA, Morelli M, & Patton JT (2017) Rotavirus NSP1 Requires Casein Kinase II-Mediated Phosphorylation for Hijacking of Cullin-RING Ligases. *MBio* 8.
28. Kanarek N & Ben-Neriah Y (2012) Regulation of NF-kappaB by ubiquitination and degradation of the IkappaBs. *Immunol Rev* 246:77-94.
29. Maluquer de Motes C & Smith GL (2017) Vaccinia virus protein A49 activates Wnt signalling by targetting the E3 ligase beta-TrCP. *J Gen Virol*.
30. Wu G, *et al.* (2003) Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. *Mol Cell* 11:1445-1456.
31. Kinoshita E & Kinoshita-Kikuta E (2011) Improved Phos-tag SDS-PAGE under neutral pH conditions for advanced protein phosphorylation profiling. *Proteomics* 11:319-323.
32. Kanemori Y, Uto K, & Sagata N (2005) Beta-TrCP recognizes a previously undescribed nonphosphorylated destruction motif in Cdc25A and Cdc25B phosphatases. *Proc Natl Acad Sci U S A* 102:6279-6284.
33. Graff JW, Ettayebi K, & Hardy ME (2009) Rotavirus NSP1 inhibits NFkappaB activation by inducing proteasome-dependent degradation of beta-TrCP: a novel mechanism of IFN antagonism. *PLoS Pathog* 5:e1000280.
34. Tang W, Pavlish OA, Spiegelman VS, Parkhitko AA, & Fuchs SY (2003) Interaction of Epstein-Barr virus latent membrane protein 1 with SCFHOS/beta-TrCP E3 ubiquitin ligase regulates extent of NF-kappaB activation. *J Biol Chem* 278:48942-48949.
35. Lang V, *et al.* (2003) betaTrCP-mediated proteolysis of NF-kappaB1 p105 requires phosphorylation of p105 serines 927 and 932. *Mol Cell Biol* 23:402-413.
36. Chowdhry S, *et al.* (2013) Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* 32:3765-3781.
37. Schubert U, *et al.* (1994) The human immunodeficiency virus type 1 encoded Vpu protein is phosphorylated by casein kinase-2 (CK-2) at positions Ser52 and Ser56 within a predicted alpha-helix-turn-alpha-helix-motif. *J Mol Biol* 236:16-25.
38. Falkner FG & Moss B (1990) Transient dominant selection of recombinant vaccinia viruses. *J Virol* 64:3108-3111.
39. Unterholzner L, *et al.* (2011) Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7. *PLoS Pathog* 7:e1002247.
40. Kinoshita E, Kinoshita-Kikuta E, Takiyama K, & Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics* 5:749-757.
41. Parkinson JE & Smith GL (1994) Vaccinia virus gene A36R encodes a M(r) 43-50 K protein on the surface of extracellular enveloped virus. *Virology* 204:376-390.
42. Alcami A & Smith GL (1992) A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell* 71:153-167.