University of Alberta

A Large Deletion Virus Reveals the Presence of Previously Uncharacterized Vaccinia Virus Inhibitors of NF-κB Signaling

by

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ABSTRACT

The classical Nuclear Factor kappa B (NF-κB) signaling pathway is an important regulator of inflammation and innate immune responses. Poxviruses, including vaccinia virus, encode multiple immune evasion proteins, including a growing number of NF-κB inhibitors. To determine if additional vaccinia virus gene products disrupted NF-κB signaling, we utilized VV811, a mutant virus missing 55 open reading frames and devoid of the known inhibitors of TNFα-induced NF-κB activation. NF-κB nuclear translocation was inhibited in VV811 infected cells stimulated with TNFα. Furthermore, VV811 infection suppressed IκBα degradation and resulted in accumulation of phosphorylated IκBα in cells stimulated with TNFα. Co-immunoprecipitation assays demonstrated that the inhibitory IκBα-p65-p50 complex was intact in VV811 infected cells, and, significantly, treatment with AraC revealed the involvement of late protein synthesis in stabilization of IκBα. This work indicates that unidentified inhibitors of NF-κB exist in vaccinia virus and illustrates the importance of NF-κB activation in the antiviral response.

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LIST OF ABBREVIATIONS

μg	Microgram
μL	Microlitre
μM	Micromolar
ALCAM	Activated leukocyte cell adhesion molecule
ANK	Ankyrin repeats
AraC	Cytosine arabinofuranoside
ASFV	African swine fever virus
ATCC	American Type Culture Collection
Bcl-2, 3	B-cell lymphoma 2, 3
BGMK	Baby green monkey kidney
BHK	Baby hamster kidney
BTB	Bric-a-Brac, Tramtrack, Broad complex
CD4	Cluster of differentiation 4
CEV	Cell-associated enveloped virions
cm	centimetre
CPXV	Cowpox virus
Crm	Cytokine response modifier
DAPI	4',6-diamidino-2-phenylindole
DD	Death domain
DDX3	Dead box helicase protein
DED	Death effector domain
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreiotol
EBV	Epstein Barr virus
ECTV	Ectromelia virus
EEV	Extracellular enveloped virions
eIF2α	Eukaryotic initiation factor 2 alpha
ER	Endoplasmic reticulum
ERK2	Extracellular signal-regulated kinase 2
FADD	Fas-associated via death domain
FBS	Fetal bovine serum
GRR	Glycine-rich region
h	Hour
HC	Heavy chain
HIV-1	human immunodeficiency virus 1
HLH	Helix loop helix motif
Hsp	Heath shock protein
HSV-1	Herpes simplex virus 1
HTLV-1	Human T-cell leukemia virus 1
IκBα,β, ε, γ, δ, ζ, NS	Inhibitor of NF-κB alpha, beta, gamma, delta, zeta, negative selection

ICP	Infected cell protein
IEV	Intracellular enveloped virions
IFNα/β, γ	Interferon alpha/beta, gamma
IKKα, β, γ, ε	IκB kinase alpha, beta, gamma, epsilon
IL-1β	Interleukin 1 beta
IL-18	Interleukin 18
IMP	Inflammation modulatory protein
IMV	Intracellular mature virions
IRAK	IL-1R-associated kinase
IRF3, 7	Interferon regulatory factor 3, 7
KD	Kinase domain
LC	Light chain
LMP1	Latent membrane protein 1
LPS	Lipopolysaccharide
LZ	Leucine zipper
mA	Milliamperes
MFI	Mean fluorescence intensity
mg	Milligram
MG132	Z-Leu-Leu-Leu-al
MHC-I	Major histocompatibility complex type 1
min	minute
mJ	Millijoule
mL	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
MVA	Modified vaccinia virus strain Ankara
MyD88	Myeloid differentiation primary reponse gene 88
NBD	NEMO-binding domain
NCS	Newborn calf serum
Nedd8	Neural precursor cell expressed developmentally down-regulated protein 8
Nef	Negative factor
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
ng	nanogram
NIK	NF-κB inducing kinase
NLS	Nuclear localization sequence
nm	nanometre
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly adenosine diphosphate polymerase
PBS	Phosphate buffered saline
PEST	Region rich in proline, glutamate, serine and threonine
PKR	dsRNA-dependent protein kinase
PMA	Phorbol myristate acetate
RHD	Rel-homology domain

RhoA	Ras homolog gene family member A
RIP1	Receptor interacting protein 1
$SCF^{\beta TrCP}$	Skp1-cullin1-F-box, beta transducin repeat containing protein
SDS	Sodium dodecyl sulfate
SPICE	Smallpox inhibitor of complement enzymes
SSC	Saline sodium citrate
TAB	TAK1 binding protein
TAD	Transcriptional activation domains
TAK1	Transforming growth factor beta-activated kinase 1
TANK	TRAF family member-associated NF-κB activator
Tat	Trans-activator of transcription
TBK1	TANK binding kinase 1
TBST	Tris-buffered saline containing Tween-20
TIR	TLR/IL-1R domain
TIRAP	TIR-containing adapter protein
TLR	Toll-like receptor
TLR/IL-1R	Toll-like receptor/interleukin-1 receptor
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain protein
TRAF	TNFR-associated factor 2
TRAM	TRIF-related adapter molecule
TRIF	TIR-containing adaptor inducing interferon beta
U	Units
UBD	Ubiquitin-binding domain
USP7	Ubiquitin specific peptidase 7
UV	Ultra violet
V	Volts
VCP	Vaccinia virus complement control protein
vFLIP	Viral FADD-like IL-1β converting enzyme inhibitory protein
Vpr	Viral protein R
Vpu	Viral protein U
VV	Vaccinia virus
VVCop	Vaccinia virus strain Copenhagen
WD40	Tryptophan and aspartic acid repeats
ZF	Zinc finger

CHAPTER 1: Introduction

1.1 The NF-κB pathway

Nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) transcription factors are key regulators of inflammation, innate immunity and apoptosis (115, 228, 266, 311). The classical NF-κB signaling pathway is activated by a wide variety of stimuli including pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β), as well as viral and bacterial pathogen-associated molecular patterns such as double stranded RNA and lipopolysaccharide (LPS) (227, 228, 265, 314). In general, the classical NF-κB pathway is triggered via cell surface receptors that, upon binding to a ligand, lead to the activation of multiple kinases and ubiquitin ligases and result in the activation of the inhibitor of NF-κB (IκB) kinase (IKK) complex composed of a regulatory component and active kinases (Figure 1-1). The IKK complex is responsible for phosphorylating the IκB protein that is bound to the NF-κB transcription factor in the cytoplasm, and the IκB protein is subsequently degraded. After release of the NF-κB transcription factor by its inhibitory protein, the pathway culminates in the transcription of genes important for initiating an anti-viral response. As a result of NF-κB activation, various cytokines, chemokines, adhesion molecules, and anti-apoptotic proteins are produced that contribute to the recruitment of inflammatory mediators and the initiation of the immune response (26, 228).

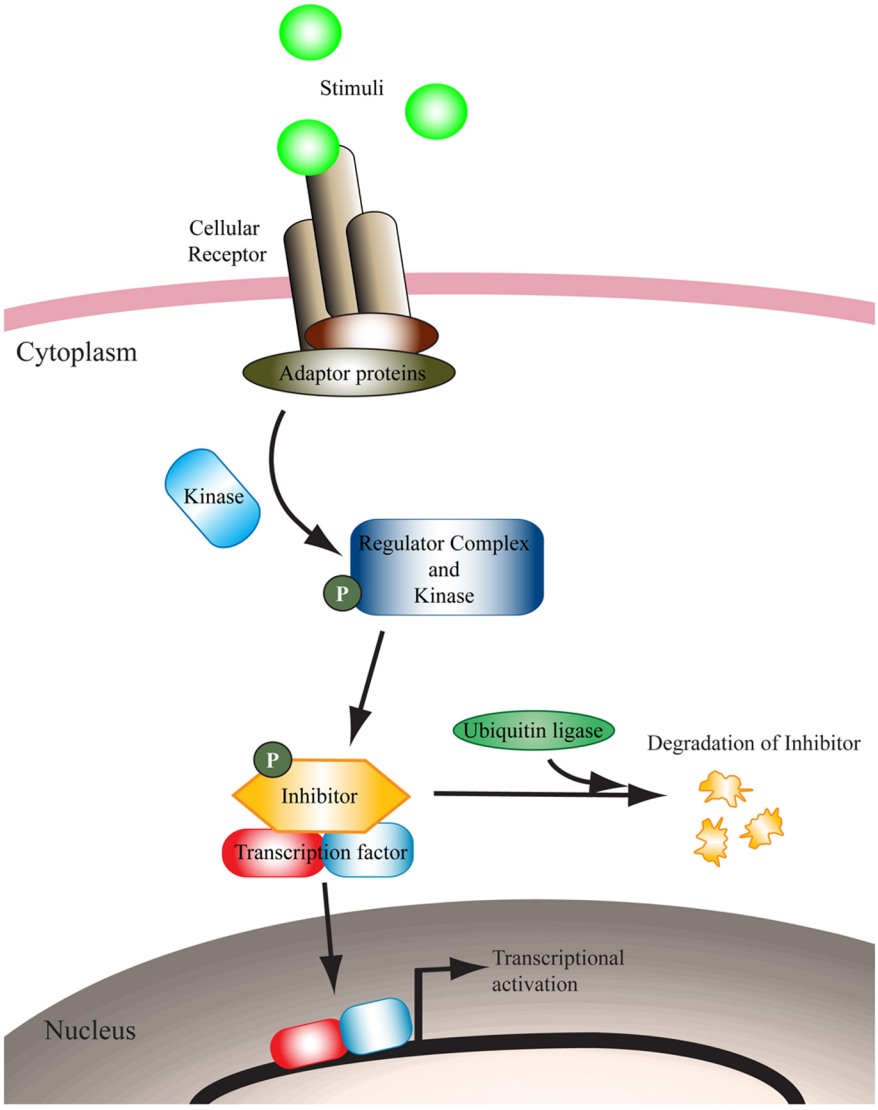


Figure 1-1. Simplified NF-κB signaling pathway.

Upon binding of a ligand, the cellular receptor is stimulated to recruit adaptor proteins to initiate the pathway. A signaling cascade from the receptor complex culminates in the activation of the regulator complex. The inhibitor protein holds the NF-κB transcription factors in an inactive form in the cytoplasm. The regulator complex phosphorylates the inhibitor protein, leading to poly-ubiquitination by a ubiquitin ligase and degradation of the inhibitor. Degradation of the inhibitor results in release of the transcription factor which translocates to the nucleus and binds DNA to direct transcription of target genes.

1.1.1 The NF-κB family of proteins

There are five members of the NF-κB family of transcription factors: p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100 (Figure 1-2A) (115, 311). All of the NF-κB subunits contain a Rel-homology domain (RHD) at the N-terminus, which contains regions responsible for NF-κB subunit dimerization, DNA binding, nuclear localization, and interaction with the IκB inhibitor proteins (20, 89, 119, 182, 221). The NF-κB subunits form homo- or heterodimers with other NF-κB subunits, and three of the NF-κB members, p65, RelB and c-Rel, contain C-terminal transcriptional activation domains (TAD) that direct target gene expression (12, 106, 115, 253, 260, 311). Since the other NF-κB family members, p50 and p52, do not contain TADs they must form dimers with TAD-containing NF-κB subunits to initiate target gene transcription (46, 148, 149, 236, 260, 311). RelB additionally contains a leucine zipper (LZ) motif which is also involved in transcriptional activation (72).

The majority of p50 is produced through co-translational processing of the precursor protein p105 (175, 229). Upon NF-κB activation, p50 is also produced by degradation of the C-terminal portion of p105 through the phosphorylation, multiple mono-ubiquitination and selective proteasomal degradation of p105 (53, 54, 82, 160, 188, 225, 229, 255). Additionally, activation of the NF-κB pathway can lead to complete degradation of p105 by the 26S proteasome (53, 117). The p52 NF-κB family member is co-translationally processed from the precursor protein p100, and p52 is also produced through selective degradation upon

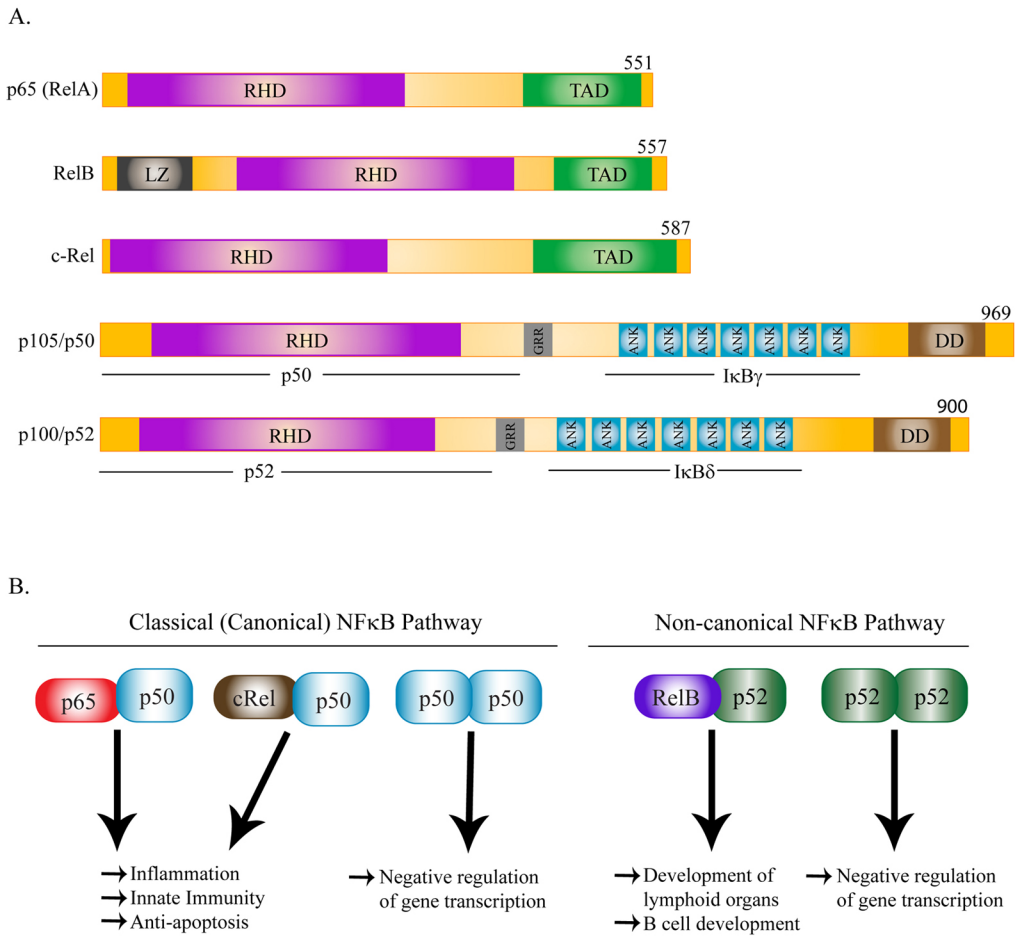


Figure 1-2. Members of the NF-κB transcription factor family.

(A.) The five NF-κB transcription factors are p65 (RelA), RelB, c-Rel, p105/p50 and p100/p52. All members contain a Rel-homology domain (RHD) at their N-termini which contains a nuclear localization signal and is also responsible for DNA binding, NF-κB subunit dimerization, and IκB binding. p65, RelB and c-Rel also contain a C-terminal transcriptional activation domain (TAD) that can direct gene transcription, and RelB contains a leucine zipper motif (LZ) which is involved in transcriptional activity. p105 and p100 are selectively processed to the NF-κB subunits p50 and p52, respectively, and contain glycine-rich regions (GRR) which act as proteolytic stop signals, as well as death domains (DD). The ankyrin repeats (ANK) in p105/p50 and p100/p52 mediate binding to NF-κB transcription factors. (Adapted from Perkins, 2007; Vallabhapurapu and Karin, 2009). **(B.)** NF-κB family members form various dimers to direct specific subsets of genes for transcription upon activation of the NF-κB pathway. This is not an exhaustive representation, as other dimers with unclear roles have been reported.

activation of the noncanonical NF-κB pathway (57, 86, 120, 267). The glycine rich region (GRR) present in both p105 and p100 acts as a processing stop signal to regulate the selective degradation of p105 and p100 to p50 and p52, respectively (22, 120, 176, 226).

The members of the NF-κB family, although very similar, have non-redundant functions. This is exemplified by the fact that different single and double knockouts of NF-κB members in mouse models display differing phenotypes, and different NF-κB subunit dimer combinations are responsible for transcription of different genes (Figure 1-2B) (26, 93, 125, 234). The most thoroughly studied and most ubiquitously expressed NF-κB dimer is the p65-p50 heterodimer, which translocates to the nucleus upon activation of the classical NF-κB pathway (114, 265, 311). Other NF-κB dimers that have been implicated in the classical NF-κB pathway include c-Rel-p50, c-Rel-p65 and p65-p65 (107, 162, 177, 311). RelB-p52 is the predominant dimer of the noncanonical NF-κB pathway (210, 267, 336), and p50-p50 or p52-p52 homodimers negatively regulate target gene transcription and can be found constitutively in the nucleus (46, 139, 148, 238). Translocation to the nucleus of most NF-κB transcription factors is regulated through interactions with the inhibitors of NF-κB, or IκB family members (10).

1.1.2 The IκB family of proteins

In unstimulated cells, NF-κB dimers are held in an inactive form in the cytoplasm by members of the IκB family of proteins (10, 114). There are eight IκB family members, IκBα, IκBβ, IκBε, IκBγ, IκBδ, IκBζ, IκBNS and Bcl-3 (Figure 1-3) (10, 73, 84, 114, 178, 179, 323, 331, 335). The IκB proteins bind to NF-κB dimers through a series of ankyrin repeats present in all IκB family members (111, 112, 114, 179, 197). The classical IκB family members, IκBα, IκBβ and IκBε, bind p65- and c-Rel-containing NF-κB transcription factors involved in the classical NF-κB pathway (234, 311). IκBα is the most studied member of the IκB family and is commonly bound to the p65-p50 heterodimer (114, 311, 337). IκBβ also binds p65-p50, as well as c-Rel-p50, but is believed to be involved in nuclear regulation of the NF-κB dimer since it has been shown to be bound to p65-p50 on κB sites on DNA (62, 286, 298, 337). Additionally, IκBε binds preferentially to p65-p65 homodimers and c-Rel-p65 heterodimers which are involved in the canonical NF-κB pathway (90, 171, 311, 323). The C-terminal regions of the NF-κB precursor proteins p105 and p100 contain ankyrin repeats and act as inhibitory IκB members; therefore, the internal C-termini of p105 and p100 are referred to as IκBγ and IκBδ, respectively (73, 112, 179, 198, 217, 247, 311). IκBγ binds p65 and c-Rel, as well p50, the degradation product of p105 (179, 198). IκBδ binds RelB, and may also be involved in binding other NF-κB subunits (18, 73). The remaining members of the family, IκBζ, IκBNS

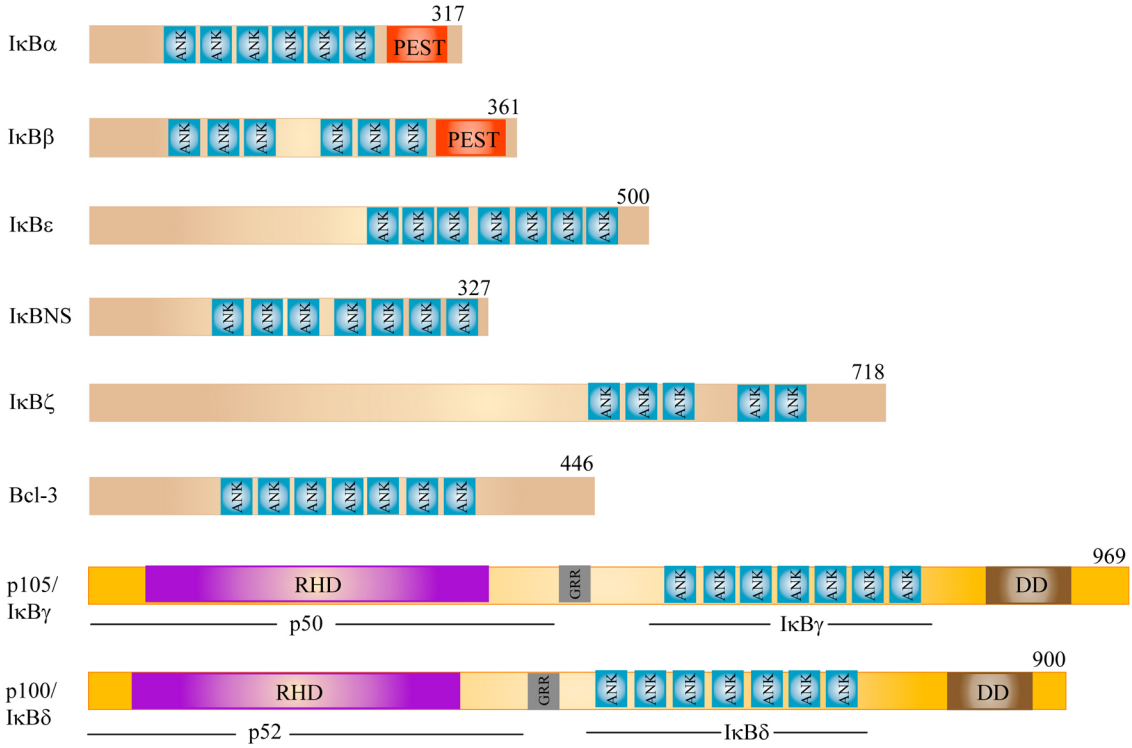


Figure 1-3. Members of the IκB family of proteins.

There are eight IκB family members, IκBα, IκBβ, IκBε, IkBNS, IκBζ, Bcl-3, p105/IκBγ, and p100/IκBδ. Multiple ankyrin repeats (ANK) are responsible for protein-protein interactions, specifically with NF-κB dimers to hold the transcription factors in an inactive state in the cytoplasm of unstimulated cells. IκBα, IκBβ and IκBε are the classical IκB members and the predominant IκB members involved in the classical NF-κB pathway. IκBα and IκBβ also contain N-terminal PEST domains which can be phosphorylated but have unclear effects on IκBα and IκBβ function. The atypical IκB members, Bcl-3, IκBNS and IκBζ, may be involved in nuclear regulation of NF-κB signaling. IκBγ and IκBδ are internal IκB proteins consisting of the C-terminal ankyrin repeats of p105 and p100 respectively. PEST, region rich in proline, glutamate, serine and threonine (Adapted from Perkins, 2007; Vallabhapurapu and Karin, 2009)

and Bcl-3, are atypical in nature and appear to be involved in nuclear regulation of NF-κB signaling (28, 84, 87, 311, 331, 335).

IκB proteins act to retain NF-κB dimers in the cytoplasm by interfering with the nuclear localization signals found in the RHDs of NF-κB transcription factors (20, 89). IκBα-p65-p50 complexes are predominantly found in the cytoplasm of unstimulated cells; however, the complex shuttles between the cytoplasm and the nucleus since IκBα only partially interferes with the nuclear localization signal of p65, and IκBα contains a nuclear export signal (135, 144, 234).

1.1.3 The classical NF-κB pathway

The classical, or canonical, NF-κB pathway can be activated by a variety of stimuli, and leads to the transcription of target genes important for the inflammatory response (228, 311). The main conserved events in the classical pathway are the activation of the IKK complex and the subsequent degradation of IκB resulting in translocation of NF-κB dimers to the nucleus to direct target gene transcription (114, 311). Two different, but parallel, receptor-mediated signaling cascades can lead to activation of the classical NF-κB pathway, namely the stimulation of the tumor necrosis factor receptor 1 (TNFR1) or the toll-like receptor/interleukin-1 receptor (TLR/IL-1R) family of receptors (Figure 1-4) (115, 126, 151, 194).

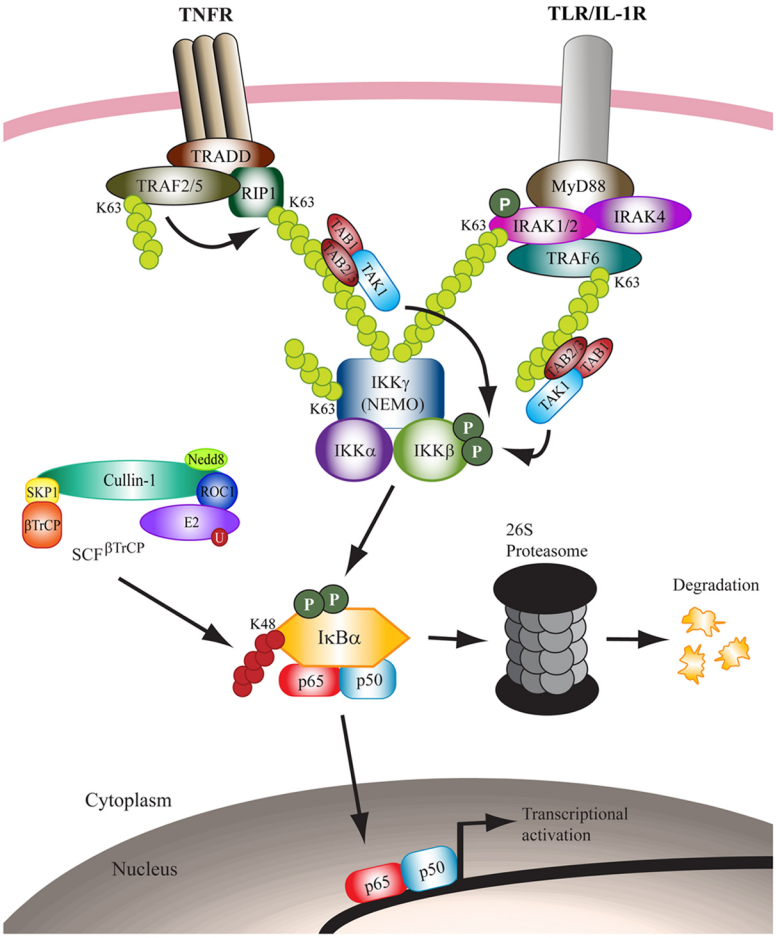


Figure 1-4. The classical NF-κB signaling pathway

Upon binding of a stimulating ligand, TNFR recruits TRADD, RIP1 and TRAF2 or TRAF5. TRAF2 is K63 poly-ubiquitinated and subsequently poly-ubiquitinates RIP1. In the MyD88-dependent TLR/IL-1R pathway, the adapter protein MyD88 is recruited via TIR domains to the TLR/IL-1R. IRAK4 then binds MyD88 and recruits and phosphorylates IRAK1 or IRAK2. IRAK1/2 recruits TRAF6, an ubiquitin ligase which is responsible for K63-linked poly-ubiquitination of IRAK1/2 and itself. IKKγ of the IKK complex (IKKγ-IKKα-IKKβ) binds the K63 poly-ubiquitin chain on RIP1 or IRAK1/2. The ubiquitin-binding proteins TAB2 and TAB3 mediate binding of the TAK1 complex, consisting of TAK1, TAB1 and TAB2 or TAB3, to the K63-linked poly-ubiquitin chain on RIP1 or TRAF6. TAK1 phosphorylates IKKβ once brought into close proximity of the IKK complex. IKKβ phosphorylates IκBα, which targets IκBα for K48 poly-ubiquitination by the Skp1-cullin-1-F-box ubiquitin ligase $SCF^{\beta TrCP}$ and degradation by the 26S proteasome. Once IκBα is degraded the p65-p50 NF-κB dimer is released and translocates to the nucleus due to its exposed NLS. p65-p50 then binds κB sites on DNA and directs target gene transcriptional activation.

To initiate the TNFR1-mediated NF-κB pathway, TNFα binds to TNFR1, leading to the recruitment of the adapter protein TRADD (TNFR1-associated death domain protein), the kinase RIP1 (receptor interacting protein 1) and either TRAF2 (TNFR-associated factor 2) or TRAF5 to the cytoplasmic region of the TNFR1 (Figure 1-4) (130, 131, 250). TRAF2 and TRAF5 are RING-finger containing ubiquitin ligases with redundant functions in NF-κB activation and are believed to be responsible for the poly-ubiquitination of RIP1 (168, 169, 215, 289, 291). RIP1 is required for TNFα-induced NF-κB activation but interestingly, the kinase activity of RIP1 is dispensible for downstream signaling (153, 169, 299).

The classical NF-κB pathway can also be activated through binding of various ligands to the TLR/IL-1R family of receptors. A wide variety of stimuli can activate the TLR/IL-1R pathway including the pro-inflammatory cytokine IL-1β, double stranded RNA, viral single stranded RNA, microbial DNA, and LPS (4, 51, 69, 116, 118, 151, 227). Upon activation, adapter proteins that contain TLR/IL-1R (TIR) domains are recruited to the TIR domains on the cytoplasmic region of the receptor (30, 151, 222). Adapter proteins containing TIR domains include MyD88 (myeloid differentiation primary response gene 88), TRIF (TIR-containing adapter inducing interferon-β), TIRAP (TIR-containing adapter protein) and TRAM (TRIF-related adapter molecule) (151). The most thoroughly characterized TIR-containing protein, MyD88, mediates activation through the IL-1R and multiple TLRs (150, 151). Upon binding of MyD88 to the TIR domain of the activated receptor, the IRAK (IL-1 receptor-associated kinase) family

members IRAK4 and IRAK1 or IRAK2 are also recruited to the receptor (Figure 1-4) (37, 152, 170, 195, 212, 322). IRAK4 binds IRAK1, which is activated by phosphorylation as well as poly-ubiquitination (37, 55, 170, 185, 325). IRAK1 then recruits TRAF6, a RING-finger-containing protein that functions as an ubiquitin ligase (38, 67). In events parallel to TRAF2 and RIP1 in the TNFR1 pathway, TRAF6 is believed to function as the ubiquitin ligase responsible for K63-linked poly-ubiquitination of IRAK1, although other ubiquitin ligases have been proposed (55, 258). In contrast to TRAF2, TRAF6 is also auto-poly-ubiquitinated to mediate IKK activation (147, 167).

The IKK complex is composed of the catalytic subunits IKKα (IKK1) and IKKβ (IKK2), and the regulatory subunit IKKγ (also known as NFκB essential modulator, NEMO) (Figure 1-5) (199, 251). IKKβ is the main kinase involved in regulation of downstream events of the classical NF-κB pathway since it is both necessary and sufficient for activation of the pathway (134, 172). IKKα also functions as a kinase to regulate downstream events in the canonical pathway, and importantly, IKKα is responsible for activation of the noncanonical NF-κB pathway (172, 267).

Activation of the IKK complex in the classical NF-κB pathway involves K63 poly-ubiquitination of the regulatory subunit, IKKγ, and recruitment of the kinases IKKα and IKKβ (Figure 1-4) (199, 235, 251, 293). IKKγ also binds K63 poly-ubiquitin chains through its ubiquitin-binding domain so it is unclear whether the activation of the IKK complex is dependent on poly-ubiquitination of

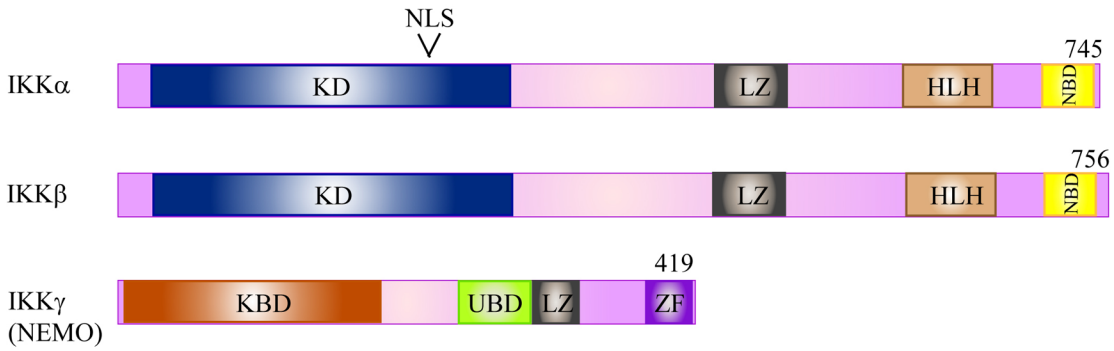


Figure 1-5. Components of the IκB kinase (IKK) complex

Structural domains of the active components, IKKα and IKKβ, as well as the regulatory component IKKγ (NEMO) are displayed. The kinase domain (KD) of IKKα and IKKβ is responsible for kinase activity. Binding of IKKα and IKKβ to IKKγ (NEMO) is mediated by the NEMO-binding domains (NBD) of IKKα and IKKβ and the kinase-binding domain (KBD) of IKKγ (NEMO). The ubiquitin-binding domain (UBD) of IKKγ mediates the association of the IKK complex with K63 poly-ubiquitin chains on RIP1 and TRAF6. The zinc finger (ZF) of IKKγ is postulated to play a role in association with ubiquitinated proteins and activation of IKKβ, and the leucine zipper (LZ) is required for IKK activation. IKKα also contains a nuclear localization sequence (NLS) and is involved in nuclear regulation of NFκB transcription. HLH, helix loop helix motif; LZ, leucine zipper; ZF; zinc finger. (Adapted from Perkins, 2007; Vallabhapurapu and Karin, 2009)

IKKγ, the binding of IKKγ to poly-ubiquitin chains, or both (67, 235). The binding of IKKγ to poly-ubiquitin chains on RIP1 in the TNFR1 pathway or IRAK1 in the TLR/IL-1R pathway is believed to bring the IKK complex into close proximity to the kinase TAK1 (transforming growth factor-β-activated kinase 1) (76, 311, 325, 329). TAK1, along with its regulatory subunits TAB1 (TAK1 binding protein 1) and TAB2 or TAB3, binds to poly-ubiquitinated RIP1 in the TNFR1 pathway and TRAF6 in the TLR/IL-1R pathway (55, 76, 138, 315, 325, 329). The close proximity of TAK1 to the IKK complex facilitates the phosphorylation of IKKβ by TAK1 (325). Upon phosphorylation and subsequent activation of IKKβ, IKKβ phosphorylates the IκB that is bound to the NF-κB dimer, which is most commonly IκBα bound to p65-p50 (199, 328, 339). The phosphorylation of IκBα on serine 32 and serine 36 targets IκBα for polyubiquitination by the Skp1-Cullin1-F-box$^{\beta\text{-Transducin repeat containing protein}}$ ($SCF^{\beta TrCP}$) ubiquitin ligase complex and subsequent degradation by the 26S proteasome (113, 161, 287, 292, 327). Degradation of IκBα releases the p65-p50 NF-κB heterodimer resulting in translocation to the nucleus due to the nuclear localization signal present on p65 (10, 20, 89). The binding of the NF-κB dimer to specific conserved κB target sequences on DNA leads to the expression of genes important for inflammation, innate immunity and inhibition of apoptosis (26, 163, 228, 265, 311). Notably, NF-κB upregulates the production of IκBα,

establishing a regulatory mechanism to prevent prolonged NF-κB activation (63, 285).

In contrast to the classical NF-κB pathway, the noncanonical NF-κB pathway is important for lymphoid organ and cell development and maintenance (26). Upon activation of the noncanonical pathway, NIK (NF-κB inducing kinase) phosphorylates and activates the IκB kinase IKKα, which phosphorylates the NF-κB precursor protein p100, directing p100 to undergo poly-ubiquitination and selective proteasomal degradation leading to the production of the NF-κB subunit p52 (7, 173, 267, 334). The p52-RelB heterodimer translocates to the nucleus and leads to transcriptional activation of genes responsible for chemokine and cytokine production and lymphoid development (26, 321, 336). Whereas the classical NF-κB pathway is dependent on the IKK complex of IKKγ, IKKα and IKKβ, the noncanonical pathway relies solely on IKKα function (26, 234, 267).

1.2 Virus manipulation of the NF-κB pathway

Viruses have developed multiple mechanisms to either activate or inhibit the numerous cellular responses regulated by the NF-κB pathway. NF-κB activation is exploited by viruses to upregulate viral transcription, prevent apoptosis and manipulate the host immune response (Table 1-1) (124, 241). For example, human immunodeficiency virus 1 (HIV-1), Epstein Barr virus (EBV), human papilloma virus type 16 and hepatitis B virus exploit the NF-κB pathway to promote viral transcription by incorporating NF-κB binding sites in viral

Table 1-1: Viral activators of NF-κB signaling

Virus	Protein	Cellular interacting partners	Effect on the NFκB pathway
Adenovirus	E1A 13S	Unknown	Increases IκBα phosphorylation and degradation (261)
		p65	Increases transcription of NF-κB responsive genes (261)
EBV	LMP1	TRAF2, TRAF6, TAK1, RIP1, TRADD	Acts as constitutively active TNFR, leads to persistent NF-κB activation (98, 136, 140, 141, 164, 204, 208, 330)
HIV-1	Tat	Unknown	Induces IκBα degradation, activates NF-κB by unknown mechanism (66)
	Vpr	Unknown	Activates NF-κB by unknown mechanism (252)
	Nef	Unknown	Induces IKKα and IKKβ phosphorylation, increases p65/p50 and p50/p50 dimers (190, 224, 313)
HSV-1	Glycoprotein D	Herpesvirus entry mediator	Activates NF-κB signaling by unknown mechanism (263)
	U_L37	TRAF6	Activates NF-κB by unknown mechanism (180)
	ICP0	NF-κB dimers	NF-κB binds ICP0 promoter, decreases NFκB binding to IκBα promoter and decreases IκBα production, leads to persistent NF-κB activation (6)
	ICP4	Unknown	Required for full NF-κB activation (108)
	ICP27	Unknown	Required for full NF-κB activation (108)
HTLV-1	Tax	IKKγ	Leads to constitutive activation of IKK complex (52, 109, 142, 284)
		NF-κB dimers and p300	Increases transcription of NF-κB responsive genes (23, 24, 181)
MVA	Unknown early protein	Unknown	ERK2 phosphorylated, leads to NF-κB activation (92, 192, 223)

Mechanisms of activation of the NF-κB pathway by viral proteins. (Adapted from Hiscott, 2006). EBV, Epstein-Barr virus; LMP1, latent membrane protein 1; TRAF, tumor necrosis factor receptor associated factor; TAK1, TGF-β-activated kinase 1; RIP1, receptor interacting protein 1; TRADD, TNFR1-associated death domain protein; TNFR, tumor necrosis factor receptor; HIV-1, human immunodeficiency virus 1; Tat, trans-activator of transcription; Vpr, viral protein R; Nef, negative factor; IKK, IκB kinase; HSV-1, herpes simpex virus 1; ICP, infected cell protein; HTLV-1, human T-cell leukemia virus 1; MVA, modified vaccinia Ankara; ERK2, extracellular signal-regulated kinase 2.

promoter regions (123, 124). Viruses use the NF-κB pathway for viral gene expression since NF-κB transcription factors are ubiquitously expressed in cells and are activated upon the initiation of the inflammatory response following virus infection (124, 306). The best characterized example is HIV-1, which contains two NF-κB sites in the long terminal repeat enhancer region that are required for viral transcription (214). Infection with HIV-1 also activates the NF-κB pathway directly through the accessory proteins Tat (trans-activator of transcription), Vpr (viral protein R) and Nef (negative factor), increasing inflammatory cytokine expression and HIV-1 transcription (66, 123, 190, 224, 252, 313). Whereas the mechanism of NF-κB activation by Tat and Vpr is unknown, Nef expression leads to IKKα and IKKβ phosphorylation and an increase in DNA binding of p65-p50 and p50-p50 dimers (66, 190, 224, 252, 313).

Viruses such as human T-cell leukemia virus 1 (HTLV-1) and EBV activate the NF-κB pathway to promote viral transcription as well as inhibit infection-induced apoptosis (123). The HTLV-1 protein Tax interacts with IKKγ, the regulatory protein of the IKK complex, leading to constitutive IKK complex activation and NF-κB induction (52, 109, 142, 284). Furthermore, Tax associates with NF-κB dimers and the transcriptional coactivator p300 in the nucleus, where Tax is acetylated by p300 and promotes transcription of NF-κB responsive genes (23, 24, 181). Upon EBV infection, latent membrane protein 1 (LMP1) acts as a constitutively active TNFR family member mimic and activates both the canonical and noncanonical NF-κB pathways (9, 77, 98, 164, 254). LMP1

interacts with multiple proteins involved in the activation of classical NF-κB, including TRAF2, TRAF6, TAK1, RIP1 and TRADD and activates these signaling molecules, leading to consistent NF-κB activation, although it is debated about which proteins are required (98, 136, 164, 204). Notably, persistent NF-κB activation has been associated with transformation and malignancies during HTLV-1 and EBV infection (34, 68, 141, 284). Therefore various viruses utilize multiple mechanisms to exploit the activation of the NF-κB transcription factor.

While some viruses activate NF-κB for their own advantage, other viruses such as adenoviruses and herpes simplex virus 1 (HSV-1), coordinate both activation and inhibition of the pathway (Tables 1-1 and 1-2). Adenoviruses manipulate the NF-κB pathway through a number of early region proteins including E1A, E1B, E3 14.7K and E3 10.4K/14.5K. NF-κB activation and inhibition by E1A is dependent on the adenovirus serotype as well as the splice variant of E1A. Expression of the adenovirus type 5 E1A decreases TNFα-induced NF-κB transcriptional activity, and reduces IκBα phosphorylation by indirectly inhibiting IKKα and IKKβ (56, 269). Inhibition of NF-κB by E1A is in contrast to the E1A 13S splice variant that activates the NF-κB pathway. Schmitz *et al* (1996) reported that the E1A 13S splice variant from adenovirus type 5 induces NF-κB activation indirectly by leading to IκBα phosphorylation and degradation (261). E1A 13S also associates with, and increases the

Table 1-2: Viral inhibitors of NF-κB signaling

Virus	Protein	Cellular interacting partners	Effect on the NFκB pathway
Adenovirus	E1A 12S	Unknown	Inhibits IκBα phosphorylation and degradation (56, 269)
	E1A (Ad12)	Unknown	Decreases p65 and p50 phosphorylation, inhibits NF-κB binding to DNA (103)
	E1B	Unknown	Inhibits E1A 13S-, PMA-, and TNFα-induced NF-κB activation, decreases NF-κB generation in the nucleus (174, 261)
	E3 14.7K	p50	Decreases p50 binding to DNA (39)
	E3 10.4K/14.5K	Unknown	Decreases TNFR expression, decreases IKK complex activity, inhibits IκBα degradation (83, 88)
ASFV	A238L	NFκB dimer	IκB-like protein, inhibits NF-κB binding to DNA (239, 246, 290)
Coxsackievirus B3	3Cpro	IκBα	Cleaves IκBα, inhibits NF-κB binding to DNA (257, 340)
HSV-1	ICP0	Unknown	Relocates cellular USP7 to deubiquitinate TRAF6 and IKKγ (60)
	ICP27	IκBα	Inhibits IκBα phosphorylation and degradation (155)
Poliovirus	3C protease	p65	Cleaves p65, inhibits transcription of NF-κB responsive genes (218)

Mechanisms of inhibition of the NF-κB pathway by viral proteins. (Adapted from Hiscott, 2006). PMA, phorbol myristate acetate; TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; IKK, IκB kinase; ASFV, African swine fever virus; HSV-1, herpes simplex virus 1; ICP, infected cell protein; USP7, ubiquitin specific peptidase 7; TRAF6, TNFR associated factor.

transactivation ability of, the NF-κB subunit p65 through an unknown mechanism (261). NF-κB induction by E1A 13S, however, is counteracted by expression of E1B (261). E1B also decreases nuclear NF-κB generation and inhibits NF-κB activation induced by TNFα or phorbol myristate acetate, suggesting a highly coordinated mechanism of NF-κB regulation by adenovirus (174, 261). Moreover, the adenovirus protein E3 14.7K diminishes NF-κB transcriptional activity by interacting with p50 resulting in decreased DNA binding of p50 homodimers (39). Finally, the E3 10.4K/14.5K complex inhibits NF-κB activity through yet another mechanism, by reducing cell surface TNFR1 expression as well as decreasing TNFα- and IL-1β-induced IKK complex activity and subsequent IκBα phosphorylation and degradation (83, 88).

HSV-1 also manipulates NF-κB activation and inhibition. HSV-1 infection results in rapid and persistent NF-κB activation that is required for replication of HSV-1 and inhibition of apoptosis (5, 100, 102, 233). NF-κB pathway signaling is activated by the HSV-1 surface protein glycoprotein D which binds to the cellular receptor herpesvirus entry mediator (263). The HSV-1 tegument protein U_L37 also activates NF-κB through an interaction with TRAF6 (180). The mechanisms of NF-κB activation by glycoprotein D and U_L37 are not fully understood (180, 263). Furthermore, p65-containing NF-κB transcription factors are recruited to the promoter of the HSV-1 ICP0 (infected cells protein zero) gene during infection, leading to an increase in ICP0 transcription, and a

decrease in NF-κB binding to the IκBα promoter (6). The subsequent suppression of IκBα production leads to persistent NF-κB activation (6). Interestingly, it was recently shown that ICP0 induces the relocation of the nuclear deubiquitinating enzyme ubiquitin specific peptidase 7 (USP7) to the cytoplasm targeting TRAF6 and IKKγ for deubiquitination (60). Therefore, ICP0 prevents activation of the NF-κB transcription factor since the ubiquitination of TRAF6 and IKKγ are required for NF-κB signaling (60). This dual action of ICP0 appears to exploit the activation of NF-κB for the benefit of the virus, while also inhibiting NF-κB. The HSV-1 proteins ICP27 and ICP4 are also required for full activation of NF-κB during HSV-1 infection (108). ICP27, however, exerts multiple affects on the NF-κB pathway since ICP27 also impedes NF-κB signaling early in infection by interacting with IκBα, inhibiting IκBα phosphorylation and ubiquitination, and thereby preventing NF-κB translocation into the nucleus (155). Overall, HSV-1 balances NF-κB activation to upregulate HSV-1 transcription and also to allow viral replication by preventing apoptosis and innate immune signaling. Overall, adenoviruses and HSV-1 regulate NF-κB signaling through organized activation and inhibition, while other viruses specifically inhibit NF-κB to evade the diverse immune responses regulated by the pathway.

Viruses have evolved multiple mechanisms to inhibit NF-κB induction (Table 1-2). Examples include viruses that have developed mechanisms to

degrade or replace key proteins in the NF-κB pathway. The poliovirus 3C protease cleaves p65, resulting in the production of fragments that act as inhibitors of transcription of NF-κB target genes (218). Alternatively, coxsackievirus B3 protease 3Cpro cleaves IκBα, leading to the nuclear translocation of a fragment of IκBα which directly associates with NF-κB and prevents binding of NF-κB to DNA (257, 340). An interesting example of a virally encoded protein that replaces a cellular protein to inhibit NF-κB signaling is A238L of African swine fever virus. A238L is an IκB-like protein that binds and inhibits the NF-κB heterodimer (239, 246). Initially, infection with African swine fever virus activates the NF-κB pathway, resulting in IκBα degradation (290). A238L functionally replaces cellular IκBα after degradation due to its high homology to porcine IκBα, preventing NF-κB translocation and subsequent gene transcription (246, 290).

Since NF-κB signaling is a key regulator of the inflammatory response to infection, viruses use a wide range of mechanisms to activate or inhibit the NF-κB response. Viruses exploit the activation of the NF-κB pathway by incorporating NF-κB binding sites into enhancer regions to promote viral transcription, or by inducing persistent NF-κB activation to induce viral transcription and inhibit apoptosis. Other viruses coordinate both activation and inhibition of the NF-κB pathway in a highly regulated manner to benefit the virus. A common strategy

utilized by multiple viruses is also inhibition of NF-κB signaling to evade the host immune response regulated by the NF-κB transcription factor.

1.3 Poxviruses

Members of the family *Poxviridae* are large double-stranded linear DNA viruses that infect a wide range of hosts (79, 209). Members of the *Poxviridae* family are divided into the subfamilies *Entomopoxvirinae*, which infect invertebrates, and *Chordopoxvirinae* which infect vertebrates (209). Vertebrate poxviruses are further separated into eight genera: *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, *Suipoxvirus* and *Yatapoxvirus* (209). Various poxviruses infect diverse hosts including primates, rodents, reptiles and multiple bird species, however most poxviruses have very narrow host ranges (79, 209).

Members of the *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus* and *Yatapoxvirus* genera contain viruses that infect humans, including molluscum contagiosum, cowpox virus, variola virus, vaccinia virus, monkeypox virus, orf virus and tanapox virus (79). The genus *Molluscipoxvirus* contains Molluscum contagiosum, a natural pathogen of humans that causes minor localized lesions (79). Infection with molluscum contagiosum can also lead to serious disease in immunocompromised individuals, an increasing problem in individuals with acquired immune deficiency syndrome (79). Members of the genus *Orthopoxvirus* include variola virus, vaccinia virus, cowpox virus, ectromelia

virus and monkeypox virus. All of these viruses, except ectromelia virus, have the ability to infect humans and cause varying degrees of pathology. Monkeypox closely resembles smallpox, but, in contrast to smallpox, primarily leads to disease in children, has a case fatality rate of 10%, and appears to have low person-to-person transmissibility (79, 282). Although monkeypox has a natural reservoir in rodents and is usually seen in West and Central Africa, there was an outbreak of monkeypox in the USA in 2003 caused by human contact with pet prairie dogs infected by a Gambian rat shipped from West Africa (245, 282). It has been proposed that monkeypox outbreaks may increase in frequency and severity in the future (231). Reasons for this include the increasing number of immunocompromised individuals and subsequent contraindications against vaccination, as well as the absence of individuals vaccinated against smallpox, which provides cross-protection against monkeypox infection (231).

Smallpox disease was caused by variola virus, a significant human pathogen that killed more people worldwide than all other infections combined (79, 282). Infection with variola major resulted in the most serious disease, with up to a 30% fatality rate, in contrast to infection with variola minor which had a case fatality rate of less than 1% (79). Variola major infection led to ordinary-type smallpox in immune competent individuals, whereas flat smallpox and hemorrhagic smallpox occurred in those with immune deficiencies and mostly resulted in fatality (79). After a radical vaccination campaign by the World Health Organization, variola virus became the first virus to be eradicated in 1977

(79). Notably, the vaccine used to eradicate variola virus was composed of vaccinia virus, a closely related poxvirus that has been studied extensively as the prototypic member of the *Poxviridae* family (41, 209).

1.3.1 Vaccinia virus and ectromelia virus

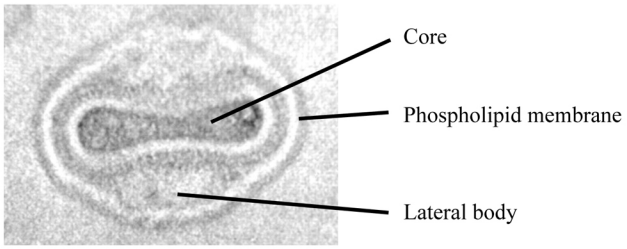
Although the natural host of vaccinia virus (VV) is unknown, the genetic composition of VV and variola virus is highly conserved (32, 209). Since VV can infect humans and has high antigenic similarity to variola virus, infection with VV is able to protect against variola virus challenge (32, 209). Notably, VV does not regularly cause disease beyond the formation of a localized pustule (79). However, serious complications of vaccinia virus infection include progressive vaccinia, which can be fatal in individuals with immunodeficiency; eczema vaccinatum, which affects those with a history of eczema; generalized vaccinia, which can lead to a rash over the whole body,; postvaccinial encephalitis; and ocular vaccinia, which can result in permanent visual impairment (79). Since the eradication of smallpox, the risk of serious morbidity and mortality in the population prevents general vaccination against smallpox (232). The ability of VV to be subjected to various genetic manipulations, along with the similarity between variola virus and VV and the large number of immunomodulatory proteins produced by VV has led to the widespread use of VV as a research tool for studying virus-host interactions (41, 146).

Ectromelia virus (ECTV), another *Orthopoxvirus*, causes mousepox, a lethal infection of susceptible strains of mice (32, 79). Mousepox is a useful animal model for poxvirus infection because ECTV is a natural pathogen of mice with high mortality in susceptible strains, the genetic material of ECTV can be manipulated, and the mouse model is a well-established animal model for research (41, 80, 282). Infection generally originates through the footpad, where viral replication begins locally, eventually leading to swelling and amputation of the foot (79, 80). As early as 8 hours post-infection, the virus travels to the draining lymph nodes. After 3 to 4 days, the virus is released into the bloodstream and spreads to organs such as the spleen and liver. Here the virus undergoes further replication before travelling back into the bloodstream at approximately day 5 post-infection. Susceptible mice, such as A/J, BALB/c and DBA/2 strains, die of as early as 7 days post-infection due to extensive viral replication in major organs leading to organ failure. Resistant mice, such as C57BL/6, C57BL/10 and 129 strains, however, do not develop skin lesions and recover from the viral infection (32, 33, 282).

1.3.2 Structure, life cycle and genome organization of poxviruses

Poxvirus virions have a unique rounded brick-shaped structure that can be seen with a light microscope and, in the case of vaccinia virus, is approximately 350 X 270 nm in size (Figure 1-6A) (75, 209). The virions consist of one or more phospholipid membranes enclosing lateral bodies as well as a core, which

A.



B.

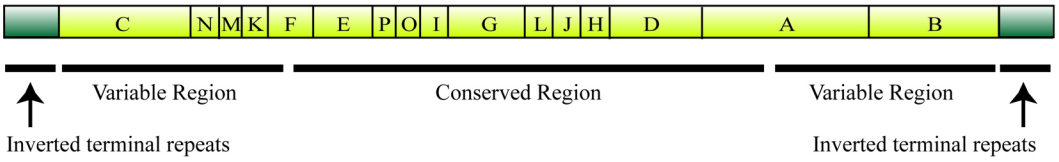


Figure 1-6. Poxvirus structure and genome organization.

(A.) Electron microscopy of a poxvirus virion, displaying the internal features of the virion structure (Hollinshead, 1999). **(B.)** In the 191,738 basepair genome of vaccinia virus, inverted terminal repeats flank variable regions at the ends of the genome and the central conserved region. The letters correspond to the *Hind*III restriction digest map showing the organization of open reading frames (DeFilippes, 1982).

contains the genome and viral proteins required early in infection (128, 209). The linear double-stranded DNA genome of poxviruses ranges in size from 130 to 360 kilobases, with vaccinia virus having a 191,738 basepair genome, and contains terminal hairpin loops due to inverted terminal repeat regions at the ends of the genome (Figure 1-6B) (15, 209, 302). About 200 open reading frames are responsible for the production of viral proteins, and orthopoxvirus open reading frames are named according to the *Hind*III restriction digest mapping of the vaccinia virus genome in 1982 (64, 209). The central conserved region of the genome contains the open reading frames required for viral replication, transcription and structural components, and 90 of these conserved genes are present in all chordopoxviruses that have been sequenced (104, 209, 310). Furthermore, 49 open reading frames in the central region of the genome are conserved in all poxviruses, including entomopoxviruses (104, 209, 310). The terminal regions of poxvirus genomes are variable among poxviruses and encode proteins that are important in virus-host interactions including evasion of the host immune response (209).

The lifecycle of vaccinia virus begins with the binding of infectious virus, extracellular enveloped virions (EEV) or intracellular mature virions (IMV), to a currently unknown receptor on host cells (209, 248) (Figure 1-7). Vaccinia virus enters cells by directly fusing with the cell membrane or, in the case of IMV, enters through endocytosis (43, 248, 301). The virus core is subsequently released into the cytoplasm and transported to the perinuclear region, by travelling

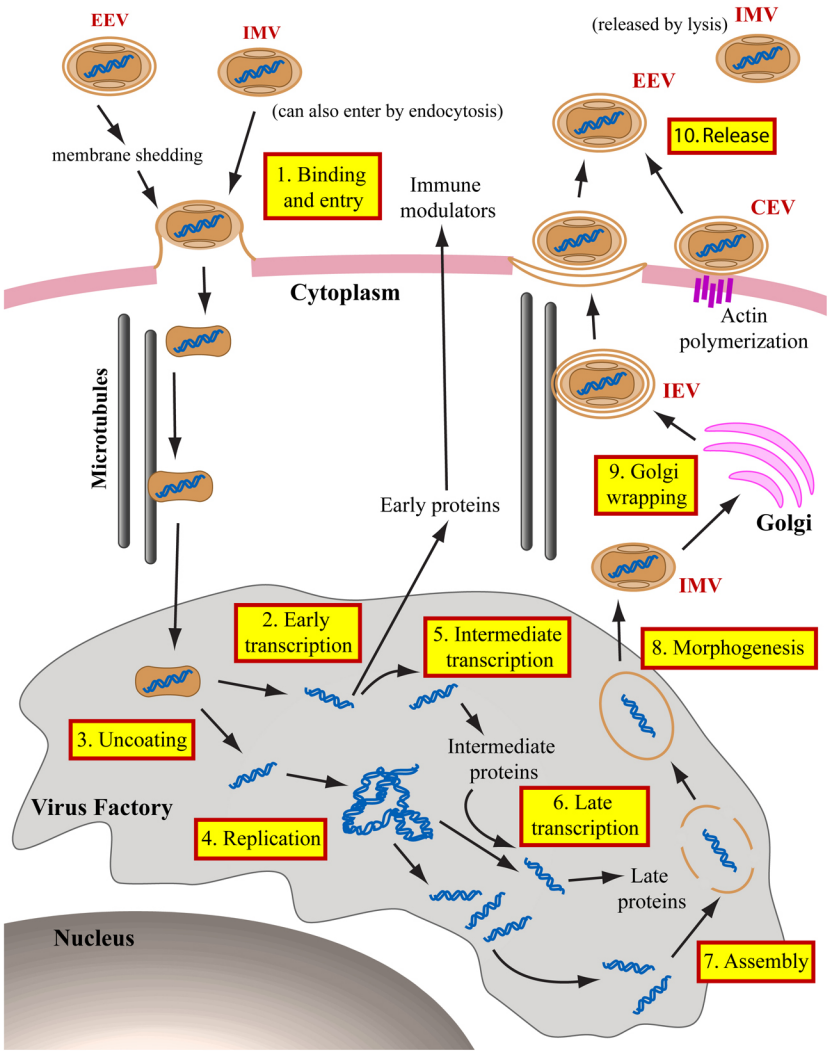


Figure 1-7. Vaccinia virus lifecycle.

Extracellular enveloped virions (EEV) and intracellular mature virions (IMV) attach to the host cell for entry through fusion with the cell membrane or endocytosis **(1.)**. The core is released into the cytoplasm and is transported to the perinuclear region, where uncotaing of the core and early transcription occurs in viral factories **(2. and 3.)**. Early proteins, including many immunomodulatory proteins, are produced. Viral DNA replication begins, as well as intermediate transcription **(4. and 5.)**. Intermediate proteins contribute to late transcription, producing late proteins **(6.)**. Progeny virions composed of late proteins and replicated viral DNA are assembled **(7.)** and undergo morphogenesis **(8.)** into mature IMV. IMV are released by cell lysis or are wrapped in an endosome- or golgi-derived membrane, producing intracellular enveloped virus (IEV) **(9.)**. IEV are transported to the cell membrane on microtubules and fuse with the cell membrane. EEV are released from the cell, and cell-associated enveloped virus (CEV) particles are propelled away from the cell through actin polymerization **(10.)**.

along microtubules (44, 209, 248). This region close to the nucleus of the infected cell is the area where viral replication and assembly occurs and is termed the virus factory (209). The viral DNA-dependent RNA polymerase, along with other viral proteins enclosed in the core, initiates early transcription and the core is uncoated to release viral DNA into the cytoplasm (209, 248). Early transcription leads to the production of early proteins, including those responsible for viral DNA replication, intermediate transcription, and immune modulation (209). Intermediate proteins are involved in late transcription, which also requires DNA replication, and which results in the production of late proteins that comprise the structural components of the progeny virions as well as factors enclosed in the virus particles (209). New virions are assembled from the late proteins and replicated viral DNA, and undergo morphogenesis to produce mature virions (209, 248). IMV are either released upon cell lysis, which is the case for most progeny virions, or are wrapped in a golgi- or endosome-derived membrane and become intracellular enveloped virions (IEV) (121, 209, 248, 259, 300). IEV are further transported to the cell membrane on microtubules and are released from the cell by fusion with the cell membrane, resulting in loss of the IEV outer membrane (127, 209, 317, 318). Infectious EEV are released from the cell, whereas cell-associated enveloped virions (CEV) remain attached to the outside of the cell membrane and are propelled through actin polymerization to infect adjacent cells (25, 59, 127, 209).

1.3.3 Evasion of the immune system by poxviruses

The large genomes of poxviruses encode multiple proteins to manipulate cellular signaling and evade the host immune response. Since the immune system attacks the virus on various fronts, poxviruses counteract these responses in a variety of ways (146, 264). Poxviruses block cytokine, chemokine and complement responses by encoding secreted binding proteins and receptor homologs, such as IL-1β binding proteins, TNF receptor homologs and complement binding proteins (Table 1-3) (2, 132, 157, 158, 230, 249, 280, 307). Poxviruses also inhibit cellular processes in infected cells by encoding proteins that downregulate apoptosis, inflammation and signal transduction pathways, such as mitochondrial apoptosis inhibitors, suppressors of the interferon response, and serine protease inhibitors that suppress pro-inflammatory cytokine activation (Table 1-4) (13, 19, 47, 81, 154, 200, 243, 277, 320). Multiple cellular pathways involved in the immune response are targeted and inhibited by poxviruses to allow for successful infection of the host.

1.4 Poxvirus inhibitors of the NF-κB pathway

Poxviruses express a large number of proteins involved in immune evasion mechanisms. Therefore, not surprisingly, this family of viruses has developed multiple mechanisms to manipulate the classical NF-κB pathway (205). However, infection with an attenuated vaccinia virus, modified vaccinia strain Ankara, MVA, missing the known NF-κB inhibitors is still able to induce

Table 1-3: Secreted poxviral proteins involved in immune evasion

Gene/protein	Virus	Effect
i) Inhibitors of cytokine signaling		
M-T2, vCD30, CrmB,C,D,E	Myxoma, Cowpox	TNFR family member homologs, bind and sequester TNFR ligands to inhibit signaling (132, 184, 230, 256, 276, 307)
B8R	Vaccinia	IFNγ receptor homolog, inhibits IFNγ signaling (3)
B18R	Vaccinia	IFNα/β receptor homolog, inhibits IFNα/β signaling (288)
B15R	Vaccinia	IL-1β receptor homolog, binds IL-1β, inhibits signaling (2, 280)
D7L, MC45L	Ectromelia, Molluscum contagiosum	IL-18 binding protein, binds IL-18, inhibits signaling (27, 278, 333)
ii) Inhibitors of chemokine signaling		
M-T1	Myxoma	Chemokine binding protein, inhibits chemokine activity (101, 166)
M-T7	Myxoma	Chemokine-binding protein, inhibits chemokine activity, IFNγ receptor homolog, binds and inhibits IFNγ (165, 309)
iii) Inhibitors of complement		
VCP, IMP, SPICE	Vaccinia, Cowpox, Variola	Complement binding proteins, inhibit activation of complement (137, 157-159, 203, 249)
iv) Inhibitors of inflammation		
SERP-1	Vaccinia, myxoma	Extracellular serine protease inhibitor, cleaves and inhibits pro-inflammatory mediators (183, 187, 216, 308)

Secreted poxviral proteins involved in immune evasion and their modes of action. Crm, cytokine response modifier; TNFR, tumour necrosis factor receptor; IFNγ, interferon γ; IFNα/β, interferon α/β; IL-1β, interleukin-1β; IL-18, interleukin 18; VCP, vaccinia virus complement control protein; IMP, inflammation modulatory protein; SPICE, smallpox inhibitor of complement enzymes

Table 1-4: Intracellular poxviral proteins involved in immune evasion

Gene/protein	Virus	Effect
i) Cellular receptor downregulators		
M153R	Myxoma	Ubiquitin ligase, downregulates MHC-I, CD4, ALCAM, CD95/Fas, blocks recognition by immune cells (16, 105, 191)
ii) Inhibitors of inflammation		
SPI-3	Vaccinia, Cowpox	Serine protease inhibitor, cleaves and inhibits pro-inflammatory mediators (303, 304)
M013, gp013L	Myxoma, Shope fibroma	Contain pyrin domain, inhibit the inflammasome (74, 145, 242)
iii) Inhibitors of apoptosis		
F1L, M11L, FPV039	Vaccinia, Myxoma, Fowlpox	Mitochondrial inhibitors of Bak and Bax activation and subsequent apoptosis (13, 14, 81, 283, 294, 316, 319, 320)
iv) Inhibitors of interferon induction		
E3L	Vaccinia	Binds dsRNA and PKR, inhibits activation of PKR, NF-κB, IRF3 and IRF7 (47, 213, 271, 277, 332)
K3L	Vaccinia	Homolog of eIF2α, binds PKR and sequesters it to inhibit eIF2α activation, inhibits interferon induction (19, 40, 61)
v) Intracellular viral proteins with multiple functions		
CrmA, SPI-2, SERP-2	Cowpox, Myxoma	Serine protease inhibitors, inhibit caspase-1 processing of IL-1β and IL-18 and inflammation, inhibit granzyme B-mediated apoptosis, CrmA also inhibits caspase-8-mediated apoptosis (71, 78, 154, 200, 240, 243, 295, 296, 305, 342)
vFLIPs (MC159, MC160)	Molluscum contagiosum	Inhibit apoptosis and NF-κB signaling, MC159 binds FADD and procaspase-8 (21, 96, 133, 219, 273, 297)

Intracellular poxviral proteins involved in immune evasion and their modes of action. MHC-I, major histocompatibility complex type I; CD4, cluster of differentiation 4; ALCAM, activated leukocyte cell adhesion molecule; Bcl-2, B-cell lymphoma 2; IL-1β, interleukin-1β; IL-18, interleukin-18; dsRNA, double stranded RNA; PKR, dsRNA-dependent protein kinase; IRF3 and 7, interferon regulatory factors 3 and 7; eIF2α, eukaryotic initiation factor 2α; Crm, cytokine response modifier; vFLIPs, viral FLICE (FADD-like IL-1β converting enzyme) inhibitory proteins; FADD, Fas-associated via death domain

NF-κB activation (223). MVA was passaged over 570 times in chicken embryo fibroblasts and as a result accumulated multiple open reading frame deletions and mutations (8, 201). Upon infection, MVA induces degradation of IκBα as early as 1 hour post infection as well as NF-κB translocation to the nucleus (92, 186). Activation of the NF-κB pathway by MVA occurs through the phosphorylation and activation of dsRNA-dependent protein kinase (PKR) and extracellular signal-regulated kinase 2 (ERK2) (92, 186). Recently, Martin and Shisler (2009) have demonstrated that MVA requires expression of an early gene product for activation of NF-κB in fibroblasts, but this viral protein has yet to be elucidated (192). Notably, in macrophages, NF-κB activation has been shown to occur through the sensing of MVA by the TLR2-TLR6 heterodimer (65). In contrast to MVA, numerous poxviruses of different genera suppress NF-κB signaling (205, 223). Inhibition occurs through the expression of secreted proteins that bind ligands of NF-κB activating receptors, and through intracellular inhibitors that target receptor adapter proteins, the IKK complex, other proteins that activate NF-κB, as well as the NF-κB transcription factor itself (Figure 1-8, Table 1-5) (205).

1.4.1 Secreted poxviral inhibitors of the NF-κB pathway

Secreted proteins produced by various poxviruses have been shown to bind ligands of the TNF receptor and IL-1 receptor, preventing binding of the ligand to the receptor and blocking subsequent signaling pathways (205, 264). Poxviruses encode different families of TNF-binding proteins. The T2-like

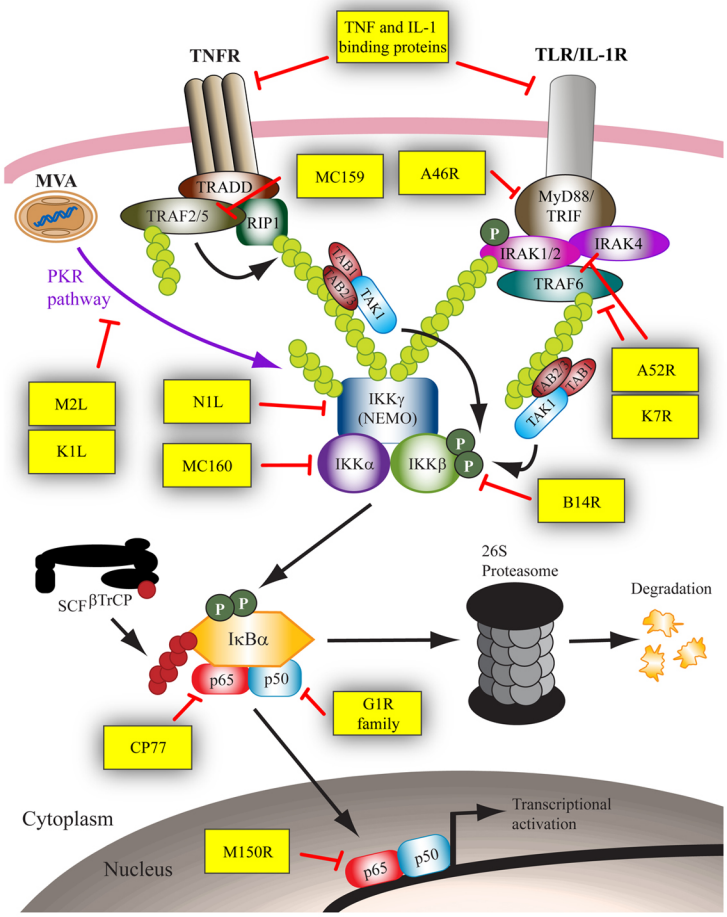


Figure 1-8. Poxviral inhibitors of the NF-κB pathway

Multiple poxviruses express secreted proteins which act as TNFα and IL-1β binding proteins to inhibit the activation of NF-κB through the TNFR and IL-1R. Poxviral proteins that inhibit PKR-mediated NF-κB activation are the vaccinia virus proteins K1L which abrogates IκBα degradation, and M2L which inhibits ERK phosphorylation mediated by MVA-induced PKR activation. Vaccinia virus A46R, A52R, and K7R and Molluscum contagiosum MC159 associate with proteins upstream of the IKK complex. A46R interacts with MyD88, TRIF and other TIR-containing adapter proteins, and A52R and K7R both interact with TRAF6 and IRAK2. Molluscum contagiosum MC159 interacts with TRAF2 and inhibits IκBβ degradation. N1L, B14R and MC160 inhibit the IKK complex. N1L interacts with the IKK complex and inhibits downstream signaling. MC160 inhibits NF-κB by inducing degradation of IKKα. B14R specifically targets IKKβ and inhibits its phosphorylation and subsequent kinase activity. Cowpox virus CP77 contains ankyrin repeats and an F-box and associates with p65 to inhibit translocation to the nucleus. G1R of variola virus and its orthologs also contain ankyrin repeats and an F-box domain and bind p105 to inhibit production of p50. Myxoma virus M150R colocalizes with p65 in the nucleus after TNFα-induced NF-κB activation.

Table 1-5: Poxviral inhibitors of the NF-κB pathway

Protein	Characteristics and binding partners	Effects
i) Secreted inhibitors		
T2, M-T2, CrmB,C, D, E	TNFR1 homologs, bind TNFα	Bind and inhibit TNFα (132, 184, 256, 275, 276, 307)
2L	Binds TNFα	Binds and inhibits TNFα (31)
B15R	IL-1R homolog, binds IL-1β	Binds and inhibit IL-1β (2, 280)
ii) Inhibitors of the PKR pathway to NF-κB activation		
K1L	Contain ankyrin repeats	Reduces MVA-induced PKR phosphorylation and IκBα degradation (186, 272, 324)
M2L	ER-localized	Inhibits MVA-induced ERK2 phosphorylation (92, 122)
iii) Inhibitors of NF-κB that act upstream of the IKK complex		
A46R	Contains TIR domain, interacts with MyD88, TRIF, TIRAP and TRAM	Inhibits TLR/IL-1R pathway (29, 110, 281)
A52R	May contain TIR domain, interacts with TRAF6 and IRAK2	IRAK2 interaction inhibits TLR/IL-1R pathway (29, 42, 110, 152)
K7R	Interacts with TRAF6 and IRAK2	Inhibits TLR/IL-1R pathway (262)
MC159	Contains DED, interacts with TRAF2, also binds RIP1	Inhibits IκBβ degradation, also inhibits PKR (49, 96, 211, 268)
iv) Inhibitors of the IKK complex		
N1L	Interacts with IKKα, β,γ	Inhibits TNFR1 and TLR/IL-1R pathway (70)
B14R	Interacts with IKKβ	Inhibits IκBα phosphorylation induced through TNFR1 and TLR/IL-1R (50)
MC160	Contains DED, interacts with hsp90	Leads to degradation of IKKα (219, 220, 268)
v) Inhibitors of NF-κB family members		
G1R	Contains ankyrin repeats and F-box, interacts with p105	Possibly inhibits p105 processing to p50, inhibits TNFα-induced activation (206)
CP77	Contains ankyrin repeats and F-box, interacts with p65	Inhibits TNFα and IL-1β-induced p65 nuclear translocation, also inhibits MVA-induced PKR activation (48, 186)
M150R	Contains ankyrin repeats and F-box, associates with p65 in nucleus	Inhibits inflammation probably induced by NF-κB activation (35, 36, 196)

Representative proteins encoded by poxviruses that inhibit NF-κB activation and their characteristics. Crm, cytokine response modifier; TNFR1, tumor necrosis factor receptor; TNFα, tumor necrosis factor α; IL-1R, interleukin-1 receptor; IL-1β, interleukin-1β; MVA, modified vaccinia Ankara; PKR, dsRNA-dependent protein kinase; ERK2, extracellular signal-regulated kinase 2; TIR, toll-like receptor/interleukin-1 receptor; MyD88, myeloid differentiation primary response gene 88; TRIF, TIR-containing adapter inducing interferon-β; TIRAP, TIR-containing adapter protein; TRAM, TRIF-related adapter molecule; TRAF6, TNF receptor associated factor 6; IRAK2, IL-1 receptor-associated kinase; DED, death effector domain; RIP1, receptor interacting protein 1; IKK, IκB kinase; hsp90, heat shock protein 90.

family of proteins produced by members of the *Leporipoxvirus* genus, including Shope fibroma virus and myxoma virus, are viral TNFR1 homologs that bind and inhibit the activity of cellular TNF (275, 307). Tanapox virus and other viruses of the genus *Yatapoxvirus* encode the 2L protein, which binds TNF but does not resemble cellular or other viral TNFR1 homologs, so it is termed a TNF-binding protein rather than a TNFR1 homolog (31). A large family of secreted TNF inhibitors are the Crm (cytokine response modifier)-like family of proteins produced by members of the *Orthopoxvirus* genus (205). There are four viral TNFR1 homologs in this family, CrmB, CrmC, CrmD and CrmE, and all are encoded by various strains of cowpox virus (132, 184, 256, 276). CrmB and CrmD have sequence similarity to the T2-like family of proteins, and are able to bind TNFα as well as another member of the TNF-superfamily, lymphotoxin α (184). Variola virus, monkeypox virus, and camelpox virus also encode CrmB-like proteins that bind TNFα and ectromelia virus expresses functional CrmD (97, 184, 278). Notably, only the Lister, USSR and Evans strains of vaccinia virus encode functional CrmC-like TNFR1 homologs that are expressed as soluble or cell-associated proteins, and vaccinia virus strain USSR also expresses a CrmE homolog (1, 244). Other vaccinia virus strains, including vaccinia virus strains Copenhagen and Western Reserve, possess partial open reading frames and encode non functional TNFR1 homologs (1, 99, 129, 307). Virally encoded IL-1 receptor homologs are also secreted by poxviruses (2, 205, 280). Vaccinia virus B15R and cowpox virus B14R encode IL-1R homologs that bind only IL-1β, and

not IL-1α or a natural IL-1R antagonist (2, 280). Ectromelia virus also expresses an IL-1R homolog that binds mouse IL-1β and, to a lesser extent, human IL-1β (278). Secreted proteins produced by poxviruses block association of the activating ligand and its receptor and therefore prevent downstream events including activation of the NF-κB pathway (205). However, most inhibitors of the NF-κB pathway produced by poxviruses function intracellularly to specifically block signaling events downstream of receptor activation.

1.4.2 Poxviral inhibitors of the PKR pathway to NF-κB activation

The vaccinia virus proteins K1L and M2L suppress NF-κB signaling by interfering with the PKR pathway (92, 186, 272). Interestingly, an unidentified early protein expressed by MVA activates the NF-κB pathway upon infection by inducing PKR (186, 192, 223). PKR binds dsRNA in the cytoplasm of an infected cell and, upon activation, PKR phosphorylates and inactivates the translation factor eIF2α, resulting in the inhibition of protein synthesis in response to infection (91). PKR can also stimulate NF-κB activation through a pathway involving binding of PKR to TRAF family members and activation of the IKK complex (94, 95, 338). While examining NF-κB activation by MVA, Shisler and Lin (2004) found that NF-κB was inhibited after inserting a 5.2kb section of wild type Ankara genome into MVA (272). This section of DNA contained seven genes, including K1L and M2L, that were subsequently shown to inhibit the NF-κB pathway (92, 272).

The vaccinia virus K1L protein effectively downregulates, but does not completely abolish, IκBα degradation in MVA-infected cells through an unknown mechanism (272). It was originally hypothesized that K1L interacted with IκBα or the NF-κB heterodimer through its ankyrin repeats but no evidence of these interactions exists, so it was speculated that K1L interacts with or indirectly inhibits the IKK complex (272). Recently, it has been demonstrated that K1L can prevent MVA-induced PKR phosphorylation and activation, and subsequent eIF2α activation, and this relies on the second ankyrin motif of K1L (324). Additionally, MVA expressing K1L was shown to inhibit MVA-induced IκBα degradation, connecting the suppression of NF-κB by K1L with inhibition of PKR. (186).

M2L is unique among the known NF-κB inhibitors of vaccinia virus in that it is located in the endoplasmic reticulum (ER) and requires this ER-localization for inhibition of NF-κB activation induced by infection with MVA (122). The mechanism of inhibition of NF-κB by M2L occurs by preventing phosphorylation of ERK2 and subsequent NF-κB activation (92). Although their mechanisms of NF-κB inhibition are not completely clear, K1L and M2L are examples of poxviral proteins that suppress NF-κB signaling by targeting pathways that lead to NF-κB activation rather than components of the classical NF-κB pathway.

1.4.3 Poxviral inhibitors that act upstream of the IKK complex to inhibit NF-κB

After stimulation of a cellular receptor, the TNFR1 and TLR/IL-1R signaling pathways are mediated by different adapter proteins and kinases that converge at the activation of the IKK complex. The vaccinia virus proteins A46R, A52R and K7R all inhibit the TLR/IL-1R pathway to NF-κB activation. However, A46R, A52R and K7R do not affect the TNFR1 pathway since these viral proteins associate with cellular proteins in the TLR/IL-1R pathway upstream of IKK complex activation. A46R contains a TIR domain and, due to homotypic interactions, associates with other TIR-domain containing proteins including the TLR/IL-1R adapter proteins MyD88, TRIF, TIRAP and TRAM to abrogate NF-κB activation by multiple TLRs and IL-1 (29, 110, 281). Although A52R and A46R have a high degree of nucleotide and amino acid sequence similarity, A46R and A52R are not redundant (29). It is controversial whether A52R contains a TIR domain, but A52R interacts with TRAF6 and IRAK2 at the TLR/IL-1R complex (29, 42, 110). The A52R-TRAF6 and A52R-IRAK2 interactions prevent complex formation at the TLR/IL-1R and inhibit downstream events of NF-κB activation induced by all TLRs (29, 110, 152). The interaction between A52R and IRAK2 is sufficient and required for inhibition of NF-κB by TLRs or IL-1β, but the A52R-TRAF6 interaction is thought to be important for other signaling pathways and not NF-κB (152, 189, 193). Both A46R and A52R are important

for virus virulence, as shown by the decreased virulence in mice, suggesting that inhibition of the TLR/IL-1R pathway is critical for viral pathogenesis (110, 281).

A new member of the vaccinia virus NF-κB inhibitors, K7R, is well conserved within poxviruses and contains 26% amino acid sequence identity and 45% amino acid sequence similarity to A52R (262). K7R inhibits NF-κB reporter activity induced by IL-1β, poly(I:C) and constitutively active TLR4, and it also decreases chemokine expression dependent on NF-κB activation stimulated by LPS and poly(I:C) (262). Similar to A52R, K7R associates with TRAF6 and IRAK2 to inhibit downstream signaling to NF-κB (262). In contrast to A52R, K7R also associates with the dead box helicase protein DDX3 to block interferon signaling through TBK1 (TANK [TRAF family member-associated NF-κB activator]-binding kinase) and IKKε-mediated activation (262). TBK1 and IKKε are IKK-related proteins involved in interferon signaling through regulation of interferon regulatory factors 3 and 7 (85, 270).

MC159, expressed by Molluscum contagiosum virus, contains a death effector domain and has been shown to inhibit NF-κB (211, 268). MC159 inhibits TNFα-induced IκBβ degradation, but not IκBα degradation, and prevents delayed and persistent NF-κB transcriptional activity (211). It is believed that an interaction between MC159 and TRAF2 is responsible for this effect (211). However, MC159 also inhibits PKR activation in the absence of infection and interacts with RIP1, so a mechanism of action remains to be determined (49, 96).

The vaccinia virus proteins A52R, A46R, and K7R suppress NF-κB signaling activated by the TLR/IL-1R pathway, exemplifying the importance of viral inhibition of TLR/IL-1R signaling. Altogether, poxviral proteins that act upstream of the IKK complex inhibit the NF-κB pathway activated specifically via either TNFR1 or TLR/IL-1R to effectively block specific activators of NF-κB signaling.

1.4.4 Poxviral inhibitors of the IKK complex

The IKK complex is the convergence point of the TNFR1 and TLR/IL-1R pathways and therefore an attractive target for viral inhibition of the classical NF-κB pathway. Several poxviral proteins inhibit the IKK complex to suppress downstream events by interacting with components of the IKK complex.

N1L of vaccinia virus was first examined for NF-κB inhibitory ability due to its involvement in vaccinia virus virulence (17, 156). Importantly, N1L exhibits amino acid sequence similarity to A52R, a known inhibitor of TLR and IL-1R mediated NF-κB activation (29, 70, 110). The effect of N1L expression during infection is controversial, as N1L has been argued to either inhibit apoptosis or inhibit NF-κB in different studies (58, 70). N1L exhibits anti-apoptotic activity, structurally resembles a Bcl-2 family member, and co-immunoprecipitates with the pro-apoptotic family members Bid, Bad and Bax, although the interaction with Bax is not reproducible (14, 58). The authors of the aforementioned study also argued that N1L does not inhibit NF-κB signaling (58).

However, N1L has been shown to inhibit the NF-κB pathway stimulated by IL-1β, TNFα and lymphotoxins, as well as NF-κB activation mediated by multiple TLRs and TIR domain-containing adapter proteins, and TRAF2 and TRAF6 (70). N1L associates with the main components of the IKK complex, IKKα, IKKβ, IKKγ (70). However, N1L also associates with other proteins that have been shown to interact with the IKK complex and mediate NF-κB and interferon signaling, including TANK (TRAF family member-associated NF-κB activator), TBK1 and IKKε (70). Interestingly, N1L bound most strongly to TBK1 to inhibit NF-κB signaling, as well as interferon signaling (70).

The intracellular virulence factor B14R from vaccinia virus strain Western Reserve inhibits NF-κB stimulated by IL-1β, TNFα and poly (I:C) (50). Although B14R displays an interaction with the members of the IKK complex, IKKα, IKKβ, and IKKγ, it was found that B14R only co-purified with the IKK complex in size exclusion chromatography experiments when IKKβ was present (50). This observation led the authors to speculate that B14R was specifically interacting with IKKβ to target the IKK complex (50). Furthermore, a vaccinia virus deletion mutant lacking B14R induces increased phospho-IκBα which was reduced by expressing increasing amounts of B14R (50). This relation further implicated the mechanism of action of B14R to be the inhibition of IKKβ activation (50). By interacting with IKKβ and inhibiting the phosphorylation of IKKβ in its activation loop, B14R is able to inhibit NF-κB activation by

preventing phosphorylation of IκBα and thereby inhibiting the subsequent ubiquitination and degradation of IκBα (50).

Molluscum contagiosum virus encodes a death effector domain containing protein, MC160, that inhibits the IKK complex (219, 220, 268). MC160 expression leads to loss of IKKα and disruption of IKK complex formation, resulting in prevention of downstream events in the NF-κB pathway (219). A recent study has demonstrated that interaction between MC160 and heat shock protein 90, which has been implicated in stabilization of IKKα, leads to the degradation of IKKα (220).

1.4.5 Poxviral inhibitors of NF-κB family members

Poxviral proteins that contain ankyrin repeats and F-box domains are becoming an increasingly important group of NF-κB inhibitors. Cellular F-box proteins interact with the SCF (Skp1-cullin-1-F-box) ubiquitin ligase complex, composed of a cullin-1 scaffold, Roc1 ubiquitin ligase, and Skp1 linker protein (Figure 1-9) (11, 202, 274, 341). The interaction between Skp1 of the SCF complex and cellular F-box proteins occurs via the C-terminal F-box, and the F-box proteins bind substrate proteins through a protein-protein interaction domain, commonly leucine rich repeats or WD40 (tryptophan and aspartic acid) repeats (11, 45, 143, 326).

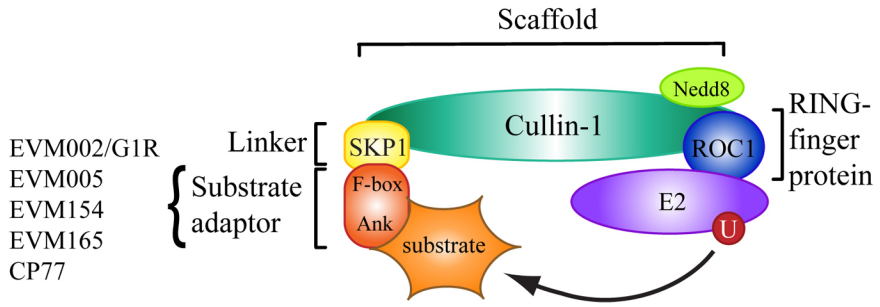


Figure 1-9. Poxviral ankyrin/F-box proteins inhibit the NF-κB pathway

Multi-subunit ubiquitin ligases consist of an active RING-finger containing ligase (ROC1), a scaffold protein (Cullin), and at least one protein which acts as a substrate adapter. A linker protein (Skp1) may also be present connecting the substrate adadpter to the cullin scaffold. The E2 ubiquitin conjugating enzyme binds ROC1, which facilitates the transfer of ubiquitin onto the substrate protein. NEDD8 conjugation activates the ubiquitin ligase. Poxviruses express ankyrin/F-box proteins, including ectromelia virus EVM005, EVM154, and EVM165, the G1R/EVM002 family of proteins, and cowpox virus CP77. These proteins associate with the Skp1 linker protein of the cullin-1 ubiquitin ligase complex and also inhibit the NF-κB pathway.

Recently, it was demonstrated that ectromelia virus encodes four proteins, ECTV002, ECTV005, ECTV154 and ECTV165, that contain N-terminal F-box domains and C-terminal ankyrin repeats, a conformation that is unique to poxviral proteins (143, 196, 279, 312). A highly conserved ortholog of ECTV165, the 68k ankyrin-like protein of MVA, interacts, through its F-box domain, with Skp1 and cullin-1 of the SCF complex, indicating that poxviral ankyrin/F-box proteins are manipulating the ubiquitin-proteasome system in infected cells (279). ECTV002, ECTV005 and ECTV154 associate with Skp1 and conjugated ubiquitin, and ECTV005 colocalizes with cullin-1 and co-immunoprecipitates with cullin-1 and Roc1 (206, 312). Furthermore, the interaction of ECTV005 with Skp1 and cullin-1 is dependent on the presence of the F-box (312). It can therefore be hypothesized that these poxviral ankyrin/F-box proteins interact with the active cellular SCF complex to facilitate ubiquitination of unknown substrate proteins during virus infection. ECTV002, ECTV005, ECTV154 and ECTV165 have been shown to inhibit NF-κB p65 translocation to the nucleus and IκBα degradation upon TNFα or IL-1β treatment (N. van Buuren, *et al*, unpublished data). The precise mechanism of NF-κB inhibition by the ectromelia virus ankyrin/F-box proteins is currently unknown; however, insights have been gained through examination of ECTV002 and its orthologs.

The orthologs of ECTV002 in other pathogenic orthopoxviruses, variola virus, cowpox virus and monkeypox virus, also contain ankyrin repeats and an F-box-like domain (206). The function of ECTV002 and its orthologs was recently

elucidated through study of the ortholog in variola virus, G1R (206). A novel yeast 2-hybrid screen of human cDNA libraries was utilized to search for interactions between human proteins and variola virus proteins not present in vaccinia virus (206). Of the 16 variola constructs screened, G1R was consistently shown to interact with the human protein, p105 (206). The NF-κB family member p50 is produced through co-translational or NF-κB activation-induced degradation of p105 (82, 175, 229). The interaction between G1R and p105 is believed to abrogate activation of the NF-κB pathway by inhibiting the degradation of p105 into p50 (206). Furthermore, the G1R orthologs in other pathogenic poxviruses display binding to p105 (206). G1R and its orthologs interact with Skp1 of the SCF E3 ubiquitin ligase complex, but the significance of this interaction is unknown since no increase in p105 degradation is observed upon G1R expression (206). The ortholog of G1R in cowpox virus, CPXV-006, was further demonstrated to be important for inhibition of pro-inflammatory cytokine production and virus virulence, since infection with a CPXV-006 knockout virus led to increased cytokine production in infected myeloid cells and decreased pathogenesis in a mouse model of infection (207).

Another ankyrin repeat containing protein is cowpox CP77. CP77 interacts with p65 through its ankyrin repeats, although this interaction is not observed during infection (48). Since IκBα also contains ankyrin repeats and binds p65 through these regions, it is believed that CP77 binds p65 after IκBα is degraded to prevent p65 translocation to the nucleus (48). Interestingly, CP77

also binds the SCF ubiquitin ligase complex through an F-box-like domain, and this interaction is required for NF-κB inhibition (48). However, the role of CP77 during virus infection in the absence of other NF-κB inhibitors remains to be fully examined (48). Also, CP77 has been shown to inhibit PKR activation and subsequent IκBα degradation induced by MVA, adding an additional mechanism by which CP77 inhibits NF-κB activation (186).

The M150R protein of myxoma virus also contains ankyrin repeats and an F-box domain and colocalizes with the NF-κB transcription factor p65 in the nucleus after TNFα-induced NF-κB activation (35, 36, 196). M150R was not shown to directly inhibit NF-κB activation through this mechanism, but a virus devoid of M150R displays increased inflammation, which could be explained by loss of NF-κB inhibition (36).

1.5 The large deletion virus VV811

A powerful approach to identify viral proteins that manipulate complex signaling pathways is through the use of large deletion viruses such as MVA and VV811 (8, 237). MVA was utilized to elucidate that the vaccinia virus gene products K1L and M2L inhibit NF-κB activation, by re-introducing regions of vaccinia virus DNA into the MVA genome and analyzing the resulting inhibition of the NF-κB pathway (92, 272). Similarly, VV811 was utilized to reveal the anti-apoptotic ability of the vaccinia virus protein F1L, by examining open reading frames missing from VV811, which was unable to inhibit apoptosis (320).

Whereas MVA was produced by multiple passages through chicken cells and accumulation of mutations, the large deletions in VV811 were engineered (8, 237). To examine the minimal genome required for vaccinia virus replication, selective deletion of the variable terminal regions of the vaccinia virus strain Copenhagen genome was performed (237). Progressive deletions of a total of 55 open reading frames, 38 open reading frames from the left terminus (C23L to F4L) and 17 open reading frames from the right terminus (B13R to B29R), resulted in the generation of VV811 (237). VV811 was able to replicate in cell culture, demonstrating that the deleted open reading frames were not essential for viral replication (237). Since the terminal regions of the vaccinia virus genome encode immunomodulatory proteins, and VV811 is missing a large portion of the genome in these areas, VV811 is useful to potentially identify novel immunomodulatory proteins (209, 237).

1.6 Thesis rationale

Viruses must dampen the immune response to infection in order to successfully infect and replicate in the host. The NF-κB signaling pathway is an important regulator of inflammation, innate immunity and apoptosis, and as a result viruses have evolved multiple mechanisms to manipulate this pathway. Poxviruses are known to encode a plethora of immunomodulatory proteins, and research has shown that vaccinia virus expresses at least seven inhibitors of NF-κB signaling (29, 50, 70, 92, 262, 272). Despite the fact that a number of NF-κB

inhibitors are present in the vaccinia virus genome, we hypothesized that vaccinia virus encodes additional inhibitors of the NF-κB pathway to ensure complete inhibition of NF-κB. In an attempt to determine if other possible NF-κB inhibitors were present in vaccinia virus, we examined the vaccinia virus deletion mutant VV811, which is missing 55 open reading frames (237). The large deletion virus VV811 was utilized because it is devoid of all of the known inhibitors of NF-κB signaling induced by MVA or TNFα (50, 70, 92, 272). We intended to uncover any NF-κB inhibitors masked by the inhibitors of NF-κB expressed by wild-type vaccinia virus. We also aimed to analyze the mechanisms of action of any additional NF-κB inhibitors present in VV811.

Objectives:

1. Determine if there are additional NF-κB inhibitors in vaccinia virus by investigating the deletion mutant VV811 for its ability to inhibit NF-κB signaling.
2. Investigate the mechanism of action of any novel NF-κB inhibitor(s) present in VV811.

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CHAPTER 2: A Vaccinia Virus Deletion Mutant Reveals the Presence of Additional Inhibitors of NF-κB

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A VACCINIA VIRUS DELETION MUTANT REVEALS THE PRESENCE OF ADDITIONAL INHIBITORS OF NF-κB

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Running Title: Vaccinia Inhibits IκBα Degradation

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2.1 Introduction

The nuclear factor kappa B (NF-κB) family of proteins function as transcription factors that regulate a wide range of genes involved in inflammation, innate immunity, and apoptosis (28, 67, 78). The canonical NF-κB pathway is activated by a variety of stimuli, including virus infection, lipopolysaccharide and pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β) (12, 32, 53, 62, 78). In unstimulated cells, the NF-κB dimer, composed of p65 and p50, is found as an inactive form bound to one of the inhibitor of NF-κB (IκB) proteins in the cytoplasm, most commonly IκBα (4, 78). Upon stimulation of the TNF receptor (TNFR) or Toll-like receptor/IL-1 receptor (TLR/IL-1R), signaling cascades are activated which converge at the phosphorylation and activation of components of the IKK (inhibitor of NF-κB kinase) complex, most importantly IKKβ (29, 33, 37, 45, 46, 80). IKKβ phosphorylates IκBα which is subsequently polyubiquitinated by the ubiquitin ligase complex Skp1-cullin-1-F-box protein $SCF^{\beta TrCP}$ and degradated by the 26S proteasome (27, 38, 73, 74, 84). The degradation of IκB releases the NF-κB p65-p50 dimer, which translocates to the nucleus, binds κB sites on DNA and regulates transcriptional activation of target genes (28, 54, 67, 78).

Many viruses manipulate the NF-κB pathway in order to regulate the diverse immune responses regulated by the pathway (31, 32, 62). For example, the enhancer region of human immunodeficiency virus 1 contains two NF-κB

binding sites required for activation of virus transcription (49). Alternatively, viruses such as Epstein Barr virus and human T-cell leukemia virus activate constitutive NF-κB signaling to inhibit apoptosis and support viral transcription (23, 31, 41, 72). Other viruses balance NF-κB activation and inhibition. Upon infection, glycoprotein D and U_L37 of herpes simplex virus 1 (HSV-1) rapidly induce NF-κB activation to promote viral replication and inhibit apoptosis (42, 65). However, the ICP0 protein of HSV-1 redirects the deubiquitinating enzyme, ubiquitin specific peptidase 7, to deubiquitinate TRAF6 and IKKγ to prevent activation of NF-κB (14). Additionally, African swine fever virus encodes an IκB-like protein, A238L, which binds and inhibits the NF-κB heterodimer (58, 60). Viruses have also developed mechanisms to degrade certain proteins in the NF-κB pathway. For example, the poliovirus 3C protease cleaves p65, and coxsackievirus B3 cleaves IκBα resulting in nuclear translocation of a fragment of IκBα and inhibition of NF-κB (50, 87). The regulation of NF-κB by viruses is a common strategy for evading the innate immune response.

Poxviruses are a large family of double-stranded DNA viruses that encode a vast array of proteins that interfere with signaling cascades and anti-viral responses (48, 66). Variola virus, the causative agent of smallpox, is the most well-known member of the family, and mass vaccination campaigns used vaccinia virus, a closely related poxvirus, to globally eradicate smallpox (20). Vaccinia virus, the prototypic member of the poxvirus family, contains approximately 200

open reading frames including the inhibitors of the NF-κB pathway (8, 10, 18, 20, 22, 24, 64, 69).

Recently, a growing list of NF-κB inhibitors has been identified in vaccinia virus (8, 10, 18, 22, 64, 69). The TLR/IL-1R pathway to NF-κB activation is inhibited by A46R, A52R and K7R (8, 64). A46R contains a Toll/IL-1 receptor (TIR) domain, and interacts with other TIR-containing adaptor proteins at the receptor complex (8, 70). A52R and K7R interact with TRAF6 and IRAK2 at the TLR/IL-1R adaptor complex to inhibit downstream NF-κB signaling (8, 26, 64). The vaccinia virus proteins B14R and N1L interact with different components of the IKK complex to inhibit the NF-κB pathway through both the TNFR and IL-1R/TLR pathways. Specifically, B14R prevents the phosphorylation and activation of IKKβ, leading to the inhibition of IκBα phosphorylation, while N1L associates with members of the IKK complex to inhibit NF-κB signaling (10, 18). Finally, K1L suppresses modified vaccinia virus Ankara strain (MVA)-induced PKR phosphorylation and IκBα degradation, whereas the ER-localized protein M2L prevents MVA-induced ERK2 phosphorylation (22, 69, 83). Notably, A52R, K7R, B14R and N1L display structural similarity to the Bcl-2 family of proteins, however, only N1L shows evidence of anti-apoptotic activity (13, 25, 35). Overall, the presence of multiple inhibitors in vaccinia virus that function at different levels of the NF-κB pathway underlines the importance of inhibiting this antiviral response during poxvirus.

A powerful approach to identify viral proteins that manipulate complex signaling pathways is through the use of large deletion viruses such as MVA or VV811 (1, 57, 69). VV811 is missing 55 open reading frames of the parental VVCop genome, lacks the known inhibitors of TNFα-induced NF-κB activation, but contains A46R and A52R, the inhibitors of TLR-mediated and IL-1β-induced NF-κB activation (47, 57). As such, VV811 is an attractive tool to identify previously unknown inhibitors of NF-κB (57). Given the importance of poxviral inhibition of the NF-κB pathway and the growing number of poxvirus encoded NF-κB inhibitors, we sought to determine if other NF-κB inhibitors existed in the vaccinia virus genome by utilizing the large deletion virus VV811, which is devoid of the known inhibitors that regulate TNFα-induced NF-κB activation. We report here that vaccinia virus encodes one or more previously unknown inhibitors of the NF-κB pathway. Infection with VV811 inhibited TNFα-induced p65 translocation to the nucleus and IκBα degradation, even though VV811 lacks the known inhibitors of TNFα-induced NF-κB activation. Additionally, cells infected with VV811 and treated with TNFα demonstrated an accumulation of phosphorylated IκBα. Significantly, VV811 required late gene expression for inhibition of IκBα degradation. Overall, our results indicate that vaccinia virus requires late protein synthesis to regulate IκBα degradation to effectively inhibit NF-κB signaling during infection.

2.2 Materials and Methods

2.2.1 Cell lines

HeLa and CV-1 cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 50U of penicillin per mL (Invitrogen), 50μg of streptomycin per mL (Invitrogen) and 200μM glutamine (Invitrogen) (referred to as Total DMEM). Baby green monkey kidney (BGMK) cells were also obtained from ATCC and were maintained in DMEM supplemented with 10% newborn calf serum (NCS) (Invitrogen), 50U of penicillin per mL (Invitrogen), 50μg of streptomycin per mL (Invitrogen) and 200μM glutamine (Invitrogen).

2.2.2 Antibodies

Antibodies used in the study included rabbit anti-NF-κB p65 (C-20) and rabbit anti-IκBα (C-21) (Santa Cruz Biotechnology Inc.), mouse anti-IκBα (L35A5), rabbit anti-p105/p50 and mouse anti-phospho-IκBα (Ser32/36) (5A5) (Cell Signaling Technology Inc.), mouse anti-β-tubulin (ECM Biosciences), anti-PARP (BD Biosciences), rabbit anti-I5L produced in the Barry Lab as previously described (79), and rabbit anti-E9L which was produced as previously described,

and provided by D. Evans (University of Alberta) (43). Antibodies and dilutions used for experiments are summarized in Table 2-1.

For western blotting, anti-NF-κB p65 (also referred to as anti-p65), anti-IκBα (C-21), anti-phospho-IκBα, and anti-I5L antibodies were used as a concentration of 1:5,000, and anti-β-tubulin, anti-p105/p50, anti-PARP and anti-E9L were used at 1:2,000. For immunofluorescence and confocal microscopy, anti-p65 was used at a concentration of 1:250. For flow cytometry, anti-IκBα (L35A5) was used at a concentration of 1:400, and for immunoprecipitations anti-IκBα (L35A5) was used at 1:200. Secondary antibodies for western blots were peroxidase-conjugated anti-rabbit and peroxidase-conjugated anti-mouse (Jackson ImmunoResearch Laboratories) and were used at a concentration of 1:25,000. The secondary antibody used for immunofluorescence and confocal microscopy was anti-rabbit AlexaFluor 546 (Invitrogen), at a concentration of 1:400. To detect mouse-anti-IκBα (L35A5) by flow cytometry, phycoerythrin-conjugated anti-mouse (Jackson ImmunoResearch Laboratories Inc.) was used at a concentration of 1:1,000.

2.2.3 Viruses

Vaccinia virus strain Copenhagen (VVCop) was provided by G. McFadden (University of Florida, USA). The deletion mutant VV811 was generated as previously described and was provided by E. Paoletti (57). The recombinant virus VV811:Flag-F1L was generated in the Barry lab by John

Table 2-1: Antibodies used in this study

Primary Antibodies

Antibody (anti-)	Species	W.B.	I.F.	Flow	I.P.	Source
β-tubulin	Mouse	1:2,000				ECM Biosciences
E9L	Rabbit	1:2,000				D. Evans
I5L	Rabbit	1:5,000				Barry Lab
IκBα (C-21)	Rabbit	1:5,000				Santa Cruz Biotechnology
IκBα (L35A5)	Mouse			1:100	1.25μg, (2μg for S.S./M.S.)	Cell Signaling Technology
NF-κB p65 (C-20)	Rabbit	1:5,000	1:250			Santa Cruz Biotechnology
NF-κB p105/50	Rabbit	1:2,000				Cell Signaling Technology
PARP	Mouse	1:2,000				BD Biosciences
Phospho-IκBα (Ser34/36) 5A5	Mouse	1:5,000				Cell Signaling Technology

Secondary Antibodies

Antibody (anti-)	Species	W.B.	I.F.	Flow	Source
Rabbit, peroxidase conjugated	Donkey	1:25,000			Jackson Immuno-Research Laboratories
Mouse, peroxidase conjugated	Donkey	1:25,000			Jackson Immuno-Research Laboratories
Rabbit, AlexaFluor 546	Goat		1:400		Invitrogen
Mouse, phycoerythrin-conjugated	Goat			1:1,000	Jackson Immuno-Research Laboratories

W.B., Western blot; I.F., Immunofluorescence; Flow, Flow cytometry;

I.P., Immunoprecipitation; S.S./M.S., Silver Stain/Mass Spectrometry

Taylor (unpublished data). VVCop, VV811 and VV811:Flag-F1L were propagated in BGMK cells and viruses were purified as previously described (71). VVCop was purified by amplifying virus in BGMK cells grown in roller bottles for approximately 24 hours. Cells were collected by treatment with 20mL of SSC (saline sodium citrate), containing 150mM NaCl and 15mM tri-sodium citrate for 20 minutes, and centrifuged at 1,900×g for 5 minutes at 4°C. Cells were resuspended in 20mL of swelling buffer containing 10mM Tris pH 8.0 and 2mM $MgCl_2$. Following three freeze/thaws at -80°C and 37°C, infected cells were dounce homogenized on ice for 100 strokes using a "B" pestle (Bellco Biotechnology), followed by centrifugation at 485×g for 5 minutes at 4°C. Supernatants containing the virus were collected, and the pellets were resuspended in 15mL swelling buffer and dounce homogenized for 60 strokes, centrifuged, and virus-containing supernatants were collected and pooled with previous supernatants. Samples were then centrifuged at 10,000×g for 1 hour at 4°C. Pellets were resuspended in 2mL of DMEM, vortexed, sonicated for 20 seconds with 30 second on/off cycles (Misonix Inc.), and stored at -80°C. VV811 and VV811:Flag-F1L were purified through sucrose cushion purification. VV811 or VV811:Flag-F1L infected cells were harvested as described above, subjected to three freeze/thaws at -80°C and 37°C, and dounce homogenized with a "B" dounce homogenizer for 40 strokes on ice. Homogenized samples were centrifuged at 485×g for 5 minutes at 4°C, and supernatants were collected and sonicated. The volume was brought up to 32mL with 1mM Tris pH 9.0, and

samples were divided into 16mL fractions, which were layered onto 16mL of 36% sucrose in 1mM Tris pH 9.0 in 35mL (25×89mm) polyallomer centrifuge tubes (Beckman) and centrifuged at 35,000×g for 80 minutes at 4°C. Supernatant was aspirated and the virus pellet was resuspended in 500μL of DMEM and stored at -80°C.

Virus titer was determined by infecting BGMK monolayers in 6-well plates with dilutions from 10^{-3}-10^{-8} until plaques were visible. Cells were then washed once with 2mL of phosphate buffered saline (PBS), fixed with 2mL of neutral buffered formalin containing 11% formaldehyde (Sigma-Aldrich), 145mM NaCl, 55mM Na_2HPO_4, and 30mM NaH_2PO_4 for 5 minutes, and stained with 0.1% crystal violet (Sigma-Aldrich) in 20% ethanol for 5 minutes. The number of plaque-forming units per milliliter was then determined.

For experiments with UV inactivated VV811 or UV VVCop, UV-inactivation was performed by treating VV811 or VVCop virus stocks with 200mJ/cm^2 UV light in a Stratalinker UV Crosslinker (Stratagene).

2.2.4 Plaque purification of VV811:Flag-F1L

VV811:Flag-F1L contains Flag-tagged F1L under the control of a synthetic poxviral early/late promoter, as well as a GUS cassette that expresses β-glucoronidase of *Escherichia coli* as a selection marker. Flag-F1L and GUS were inserted into the I4L open reading of VV811 through recombination, using the plasmid pIV113, generously provided by Robert Drillien (Universite Louis

Pasteur, France) (John Taylor and M. Barry, unpublished data) (34). VV811:Flag-F1L recombinants were purified by infecting CV-1 cells with 10^{-2} to 10^{-7} dilutions of virus until plaques were visible, followed by overlay with 1mL of a mixture of 2% low melting point agarose (Sigma-Aldrich), 2×DMEM (2.7% DMEM, 88mM $NaHCO_3$), 166μL of FBS and 800μg of X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucoronic acid) (Rose Scientific Inc.) in dimethylformamide (Sigma-Aldrich). Overlaid virus infected cells were incubated at 37°C until blue coloration was visible. Well isolated blue plaques were picked with a Pasteur pipette, added to 100μL swelling buffer, and subjected to three freeze/thaws at -80°C and 37°C. VV811:Flag-F1L was plaque purified until no white plaques were visible after overlay.

2.2.5 Virus infections

Virus infections (1×10^6 cells per well) were performed in 6-well plates by adding the appropriate amount of virus for the indicated multiplicity of infection (MOI), to 500μL of BGMK media or Total DMEM in each well and rocking every 10 minutes for 1 hour. The media was then aspirated, 2mL of media was added back to each well, and the infection was allowed to progress for the indicated time period. Virus infections of 10cm dishes (7×10^6 cells per dish) were performed by adding virus to 4mL of media, and 10mL of media added back after 1 hour. For microscopy, virus infections were performed on coverslips in

12-well plates (5×10^5 cells per well), with virus added to 200μL of media, and 1mL of media added back after 1 hour.

2.2.6 Treatments

Treatments with 10ng/mL tumour necrosis factor-α (TNFα) (Roche) and 10ng/mL interleukin 1β (IL-1β) (PeproTech Inc.) were used to stimulate NF-κB activation. To inhibit the proteasome, one hour of pre-treatment with 10μM MG132 (Sigma-Aldrich) was utilized as a control for inhibition of IκBα degradation (55, 77). To inhibit DNA replication and subsequent late viral protein synthesis, cytosine arabino-furanoside (AraC) (Sigma-Aldrich) at a concentration of 80μg/mL was added to media after 1 hour of infection to inhibit DNA replication and subsequent late viral protein synthesis (3, 15).

2.2.7 Immunofluorescence microscopy

HeLa cells (5×10^5) were grown on 18mm glass coverslips and infected with the indicated virus at an MOI of 5 for 12 hours at 37°C. Infection was followed by treatment with 10ng/mL of either TNFα or IL-1β for 20 minutes. Cells were washed twice with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 10 minutes at room temperature. After 3 washes with PBS containing 1% FBS, cells were permeabilized in 0.5% NP-40 (Sigma-Aldrich) in PBS for 5 minutes at room temperature. Primary antibody, anti-NF-κB p65 (C-20) at a concentration of 1:250, was applied and incubated overnight at room

temperature. After 3 washes with PBS containing 1% FBS, cells were stained with Alexa Fluor 546 goat anti-rabbit secondary antibody (1:400) (Invitrogen) for 1 hour. Coverslips were mounted on microscope slides with 7μL of 250μg/mL 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) diluted in 4mg/mL n-propyl gallate (Sigma-Aldrich) in 50% glycerol and 50% PBS. Slides were visualized by immunofluorescence microscopy using a Zeiss Axiovert 200M fluorescent microscope with an ApoTome 10 optical sectioning device (Zeiss), and Axiovision software (Zeiss). Alternatively, slides were visualized by confocal microscopy with a Zeiss LSM510 laser scanning confocal microscope and analyzed with LSM510 imaging software (Zeiss).

2.2.8 Cytoplasmic and nuclear extractions

Extracts were prepared through a modified version of a previously described method (40, 52). HeLa cells (2×10^6) were infected at an MOI of 5 for the indicated times, in the presence or absence of 80μg/mL of AraC, followed by treatment with 10ng/mL of TNFα or IL-1β for 20 minutes. Cells were resuspended in 3 volumes of cold cytoplasmic extract buffer containing 10mM HEPES, 10mM KCl, 0.1mM EDTA pH 8.0, 0.1mM EGTA pH 8.0, 1mM dithiothreiotol (DTT), 0.05% NP-40 and protease inhibitor cocktail (Roche), and incubated at 4°C for 30 minutes with constant agitation. Lysed cells were centrifuged at 1,000×g for 5 minutes at 4°C and supernatant (the cytoplasmic extract) was collected and stored at -80°C. The pellet was washed with 5 volumes

of cytoplasmic extract buffer, centrifuged for 5 minutes at 1000×g, and the pellet was resuspended in 3 volumes of nuclear extract buffer containing 20mM HEPES, 25% glycerol, 0.4M NaCl, 1mM EDTA pH 8.0, 1mM EGTA pH 8.0, 1mM DTT and protease inhibitor cocktail, and incubated 20 minutes on ice. Extracts were vortexed for 5 seconds and debris was pelleted at 18 000×g for 10 minutes at 4°C. Supernatants from the nuclear extracts were collected and stored at -80°C.

2.2.9 SDS-PAGE and western blotting

In order to separate isolated proteins, 12% or 15% sodium dodecyl sulfate (SDS) (Fisher Scientific)-polyacrylamide gel electrophoresis (PAGE) was used. SDS sample loading buffer containing 62.5mM Tris pH 8.0, 12.5% glycerol, 2% SDS, 50mM β-mercaptoethanol (BioShop Canada Inc.), and 74.5nM bromophenol blue (BIO-RAD) was added to samples, which were then boiled for 10 minutes, applied to the gels in Mini-PROTEAN3 cells (BIO-RAD) and separated by gel electrophoresis at 150V. Proteins were transferred to nitrocellulose membranes (GE Water and Process Technologies) using a semi-dry transfer apparatus (Tyler Research Corp.) at 420mA for 2 hours in semi-dry transfer buffer, which contains 192mM glycine, 25mM Tris and 20% methanol. Membranes were blocked in 5% skim milk in Tris-bufferd saline containing Tween-20 (TBST), which consists of 20mM Tris (Invitrogen), 150mM NaCl pH 7.5, and 0.1% Tween-20 (Fisher Scientific), for 3 hours at room temperature.

Membranes were probed with the indicated primary antibodies diluted in 5% skim milk in TBST overnight, followed by three 20 minute washes with TBST, and application of secondary antibody diluted in TBST for 1 hour. Enhanced chemiluminescence was used for visualization according to the manufacturer's instructions (GE Healthcare).

2.2.10 Detection of IκBα degradation by flow cytometry

Degradation of IκBα was analyzed by flow cytometry using a modified version of the antibody manufacturer's instructions (Cell Signaling). HeLa cells (1×10^6) were infected at an MOI of 5 for 4 or 12 hours, in the presence or absence of 80μg/mL of AraC, followed by treatment with 10ng/mL of TNFα for 20 minutes. Alternatively, mock infected cells were treated with 10μM MG132 for 1 hour before treatment with TNFα as a control for inhibition of IκBα degradation. Cells were collected by trypsinization, fixed at 37ºC for 10 minutes in 0.5% paraformaldehyde in PBS, and permeabilized on ice for 30 minutes in 90% ice cold methanol. Cells were washed twice with PBS containing 1% FBS, and stained with mouse anti-IκBα (L35A5) (1:400) at room temperature for 1 hour. Cells were washed twice with PBS containing 1% FBS, stained with phycoerythrin-conjugated goat anti-mouse (1:1,000) (Jackson ImmunoResearch Laboratories Inc.) at room temperature for 1 hour, and washed twice with PBS containing 1% FBS. Fluorescence was analyzed by flow cytometry (FACScan; Becton Dickinson) through the FL-2 channel with a 585 nm filter (42 nm band-

pass filter). Data from the collection of 20,000 cells were analyzed using CellQuest software. The mean fluorescence intensity for each sample from three independent experiments was calculated with CellQuest software and averages with standard error were displayed graphically. Whole cell lysates were collected, followed by resuspension of pelleted cells in 25μL of SDS sample loading buffer for western blotting.

2.2.11 Immunoprecipitations

HeLa cells (7×10^6) were collected by trypsinization and lysed for 1 hour at 4°C in 1mL of 1% NP-40 lysis buffer containing 150mM NaCl, 1% NP-40, 50mM Tris pH 8.0, and protease inhibitor cocktail. Samples were centrifuged for 10 minutes at 9,000×g at 4°C and supernatants were collected for immunoprecipitation and lysate samples. Mouse anti-IκBα (L35A5) (1.25μg) was added to the supernatant and incubated at 4°C for 2 hours. Protein G sepharose (GE Healthcare) was added and samples were incubated for 1 hour at 4°C. After 3 washes with 1mL of 1% NP-40 lysis buffer, the immunoprecipitated complexes were resuspended in 60μL SDS sample loading buffer.

2.2.12 Silver Stain and Mass Spectrometry

HeLa cells (1.4×10^7 cells) were mock infected or infected with VVCop or VV811 at an MOI of 5 for 14 hours, and mock treated or treated with 10ng/mL of TNFα for 20 minutes. Immunoprecipitations were performed with anti-IκBα

(L35A5) (2μg), and immunoprecipitated complexes bound to Protein G beads were resuspended in 100μL of SDS loading buffer. Immunoprecipitated samples were separated by SDS-PAGE on a SE600 series 18cm × 16cm electrophoresis apparatus (Hoefer Inc.) at 250-300V until required separation was achieved. To visualize the separated proteins, the polyacrylamide gels were subjected to silver staining. The gels were fixed in 50% methanol and 50% acetic acid for 20 minutes, followed by 15 minutes in 50% methanol and five 5 minute washes with Milli-Q H_2O. After 1 minute incubation with 0.2% sodium thiosulfate (Sigma-Aldrich) and two 1 minute washes with Milli-Q H_2O, the gels were incubated in 0.1% cold silver nitrate (Sigma-Aldrich) for 25 minutes. Gels were then washed twice for 1 minute with Milli-Q H_2O and developed with 0.2% sodium carbonate anhydrous and 0.0148% formaldehyde for a maximum of 10 minutes, until bands were visible. To stop developing, gels were incubated in 5% acetic acid for a minimum of 10 minutes, and then stored in Milli-Q H_2O.

Bands selected for mass spectrometry analysis were excised from the gel with scalpels and stored in 100μL of H_2O at -80°C. Samples were digested with trypsin and analyzed for peptide identification by the Mass Spectrometry Facility at the University of Alberta. Liquid chromatography tandem mass spectrometry was utilized to analyze samples on a Waters Q-Tof Premier (Waters). PEAKS Viewer 5.1 was utilized to examine peptides by the score percentage based on the confidence that the protein identified is correct (PEAKS Score), the amino acids

covered by the peptides corresponding to the protein identified (Coverage), and the number of peptides isolated from the protein identified (Query Matched).

2.3 Results

2.3.1 VV811 inhibits TNFα-induced NF-κB p65 translocation to the nucleus.

The NF-κB pathway is a crucial regulator of inflammation and innate immunity and is tightly regulated by multiple NF-κB inhibitors encoded by vaccinia virus (8, 10, 18, 22, 64, 69, 78). We utilized a large deletion vaccinia virus, VV811, in an attempt to determine if other NF-κB inhibitors were present in the vaccinia virus genome (57). A comparison of the genome of VV811 with the parental wild-type vaccinia virus strain Copenhagen (VVCop) displays the open reading frames missing in VV811 (Figure 2-1) (16, 24, 57). The vaccinia virus mutant VV811 lacks 55 open reading frames from the variable termini of the VVCop genome (57). VV811 possesses the known inhibitors of TLR and IL-1β-induced NF-κB activation, A46R and A52R, but lacks all the currently known inhibitors of TNFα-induced NF-κB activation (57).

To determine if VV811 retained the ability to inhibit TNFα-induced NF-κB activation, microscopy was used to visualize the localization of the p65 subunit of NF-κB upon virus infection (Figure 2-2). HeLa cells were mock infected, infected with VVCop or infected with VV811 for 12 hours. Subsequently, cells were mock treated or treated with TNFα for 20 minutes. Nuclei were visualized by DAPI staining, and infected cells were verified by the

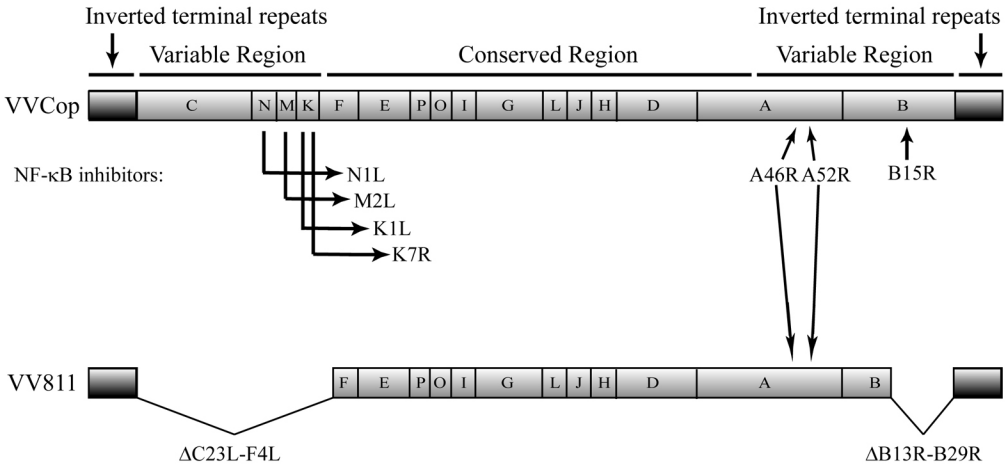

Figure 2-1. VV811 lacks the known inhibitors of TNFα-induced NF-κB activation. Schematics of wild-type vaccinia virus Copenhagen strain (VVCop) and the mutant vaccinia virus VV811 are represented. The letters correspond to the *Hind*III restriction digest map (DeFilippes, 1982). Large deletions in VV811 representing the open reading frames C23L-F4L in the left variable region and B13R-B29R in the right variable region are noted. Locations of the known NF-κB inhibitors are indicated.

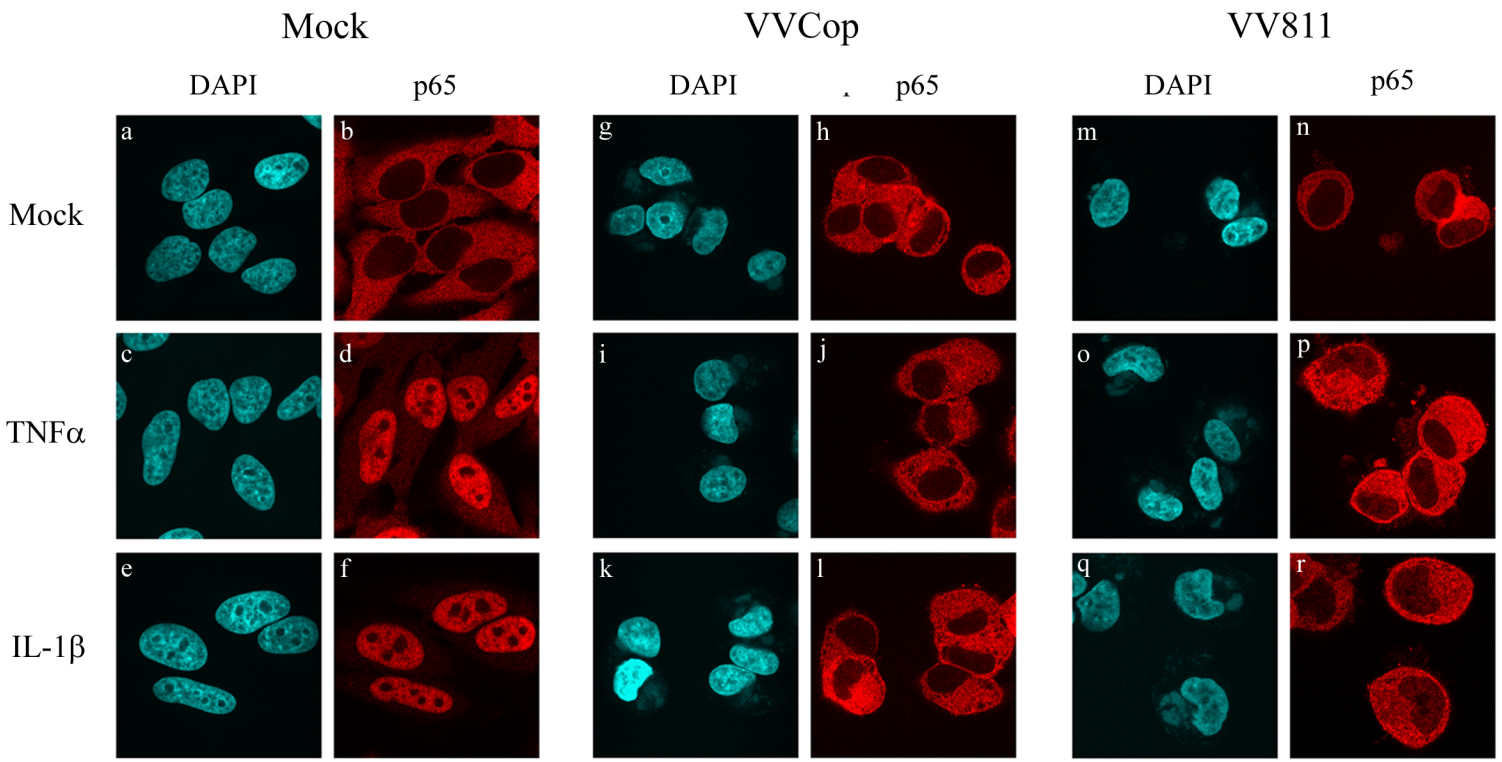

Figure 2-2. VVCop and VV811 infected cells inhibit p65 nuclear translocation. HeLa cells were mock infected (a-f) or infected with VVCop (g-l) or VV811 (m-r) at an MOI of 5 for 12 hours. Following infection, cells were mock treated (a, b, g, h, m, n), or treated with 10ng/ml of TNFα (c, d, i, j, o, p) or IL-1β (e, f, k, l, q, r) for 20 minutes. Localization of endogenous NF-κB p65 was detected using an anti-p65 antibody, and nuclei and virus factories were detected with DAPI and visualized by confocal microscopy.

presence of perinuclear virus factories also visualized with DAPI staining. Staining with an antibody to the p65 subunit of NF-κB revealed that p65 was present in the cytoplasm of mock infected cells (Figure 2-2 panels a, b), while mock infected cells treated with TNFα displayed an accumulation of p65 in the nucleus, as expected (Figure 2-2 panels c, d) (51, 53, 61). Cells infected with VVCop or VV811 in the absence of TNFα stimulation displayed cytoplasmic localization of p65, indicating that infection with both viruses did not stimulate p65 nuclear translocation (Figure 2-2 panels g, h, m, and n). VVCop and VV811 infected cells treated with TNFα demonstrated clear inhibition of p65 translocation into the nucleus, indicating that both viruses inhibited NF-κB activation (Figure 2-2 panels i, j, o, and p). The experiment was repeated by exposing cells to IL-1β. Mock infected cells treated with IL-1β showed a substantial accumulation of p65 in the nucleus, as expected (Figure 2-2 panels e, f) (51, 53, 61). In contrast, VVCop and VV811 clearly inhibited p65 translocation to the nucleus upon IL-1β stimulation (Figure 2-2 panels k, l, q, and r). Overall, infection with VVCop or the deletion mutant VV811 clearly inhibited p65 nuclear translocation induced by TNFα or IL-1β.

As a control, cells were infected with UV-inactivated VVCop (Figure 2-3 panels a-d) or UV-inactivated VV811 (Figure 2-3 panels e-h) and subsequently treated with TNFα. In the absence of TNFα treatment, infection with UV-VVCop (Figure 2-3 panels a, b) or UV-VV811 (Figure 2-3 panels e, f) retained p65 in the cytoplasm. In contrast, upon treatment with TNFα, infection with UV-

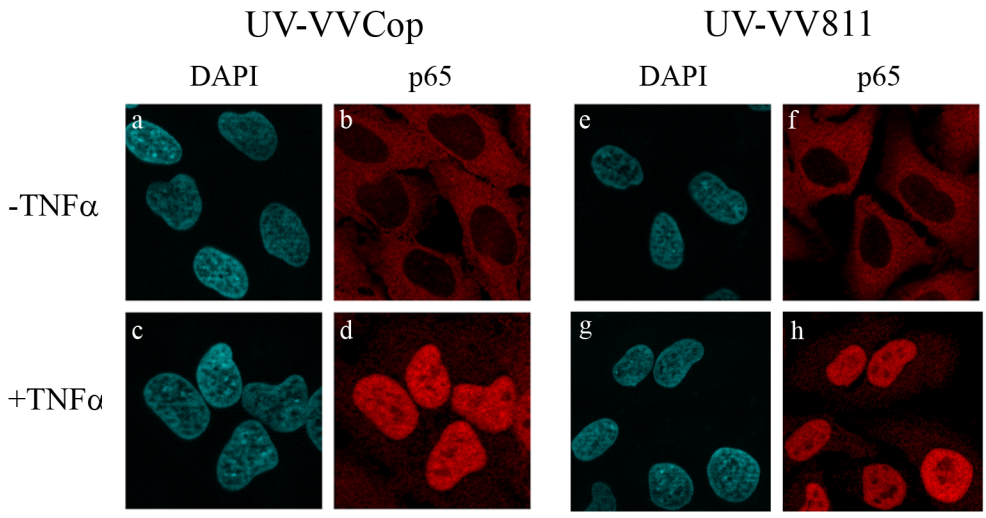

Figure 2-3. UV-inactivated VVCop and VV811 failed to inhibit p65 nuclear translocation. HeLa cells were infected with UV-inactivated VVCop (a-d) or UV-inactivated VV811 (e-h) at an MOI of 5. Twelve hours post infection cells were mock treated (a, b, e, f) or treated with 10ng/ml of TNFα for 20 minutes (c, d, g, h). Endogenous p65 was detected using a p65 specific antibody, and nuclei were detected with DAPI.

VVCop (Figure 2-3 panels c, d) or UV-VV811 (Figure 2-3 panels g, h) demonstrated p65 translocation to the nucleus, indicating the necessity for viral protein production for inhibition of p65 translocation. Nuclear translocation of p65 was quantified by counting cells. Approximately 89 and 86% of mock-infected cells treated with TNFα or IL-1β, respectively, displayed p65 translocation to the nucleus (Figure 2-4). Overall, however, less than 5% of cells infected with VVCop or VV811 treated with TNFα or IL-1β displayed p65 translocation to the nucleus. Strikingly, cells infected with UV-inactivated VVCop or VV811 and treated with TNFα displayed greater than 85% p65 nuclear translocation (Figure 2-4).

To verify that VV811 inhibited TNFα-induced p65 translocation to the nucleus, cytoplasmic and nuclear extractions were generated from HeLa cells that were mock infected or infected with VVCop or VV811. Fourteen hours post infection, cells were mock treated or treated with TNFα for 20 minutes and cytoplasmic and nuclear extracts were western blotted with anti-p65 (Figure 2-5A). In mock infected cells lacking TNFα stimulation, a large amount of p65 was detected in the cytoplasmic fraction with no p65 present in the nuclear fraction, indicative of cells that do not display NF-κB activation. Following TNFα treatment of mock infected cells, p65 accumulated in the nuclear fraction, demonstrating activation of the NF-κB pathway (Figure 2-5A). Significantly, VVCop and VV811 infected cells clearly inhibited p65 translocation into the nuclear fraction following TNFα stimulation (Figure 2-5A). A similar pattern of

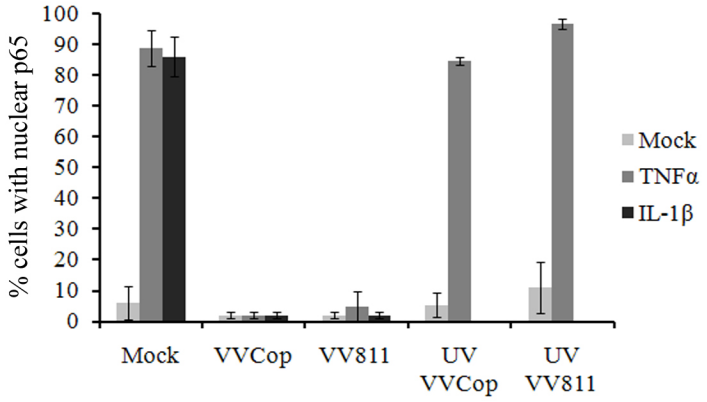

Figure 2-4. Quantification of NF-κB p65 nuclear localization in infected cells. To quantify p65 translocation, cells were counted from three independent experiments and the percentage of cells that exhibited nuclear p65 was calculated. Error bars represent standard deviations.

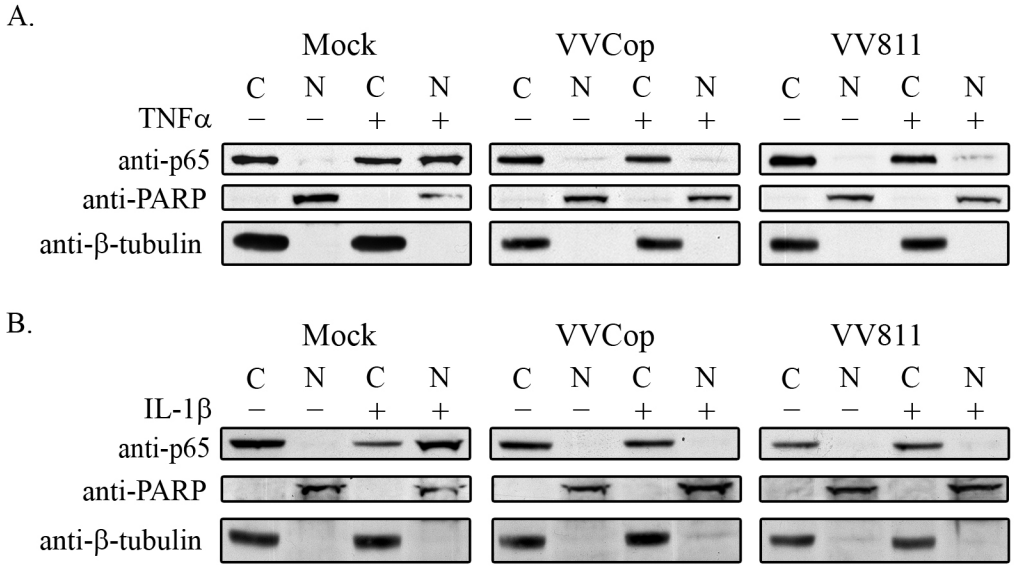

Figure 2-5. p65 is retained in the cytoplasm of VV811 infected cells. HeLa cells were mock infected or infected with VVCop or VV811 at an MOI of 5. Fourteen hours post infection cells were mock treated or treated with TNFα **(A)** or IL-1β **(B)** for 20 minutes and cytoplasmic and nuclear extracts were generated. Cytoplasmic (C) and nuclear (N) extracts were western blotted with anti-p65. Additionally, extracts were western blotted with anti-PARP and β-tubulin which served as controls for nuclear and cytoplasmic extracts.

p65 localization was observed in samples treated with IL-1β (Figure 2-5B). The purity of the cytoplasmic and nuclear extracts was determined by western blotting with anti-PARP (poly adenosine diphosphate polymerase), a nuclear protein, and anti-β-tubulin, a cytoplasmic protein (Figures 2-5A, B) (36, 56). These data further confirmed that, despite lacking the known vaccinia virus encoded inhibitors of NF-κB, VV811 infection still inhibited NF-κB p65 nuclear translocation stimulated by both TNFα and IL-1β, providing further evidence that VV811 contains at least one additional inhibitor of NF-κB.

2.3.2 VV811 inhibits TNFα-induced IκBα degradation.

In unstimulated cells, IκBα retains NF-κB in the cytoplasm (4). Upon NF-κB activation, IκBα is degraded allowing translocation of the NF-κB heterodimer to the nucleus (6, 30). To further establish how VV811 infection manipulates the NF-κB pathway, we examined IκBα degradation in mock infected, VVCop infected or VV811 infected cells (Figure 2-6). In the absence of TNFα stimulation, IκBα was present in cytoplasmic extracts of mock infected HeLa cells, indicative of cells lacking activated NF-κB (Figure 2-6A). Upon treatment with TNFα, mock infected cells demonstrated a loss of IκBα due to degradation (6, 30). In dramatic contrast, cells infected with VVCop or VV811 and stimulated with TNFα retained IκBα, indicating that both VVCop and VV811 infection inhibited the degradation of IκBα (Figure 2-6A). Infection with

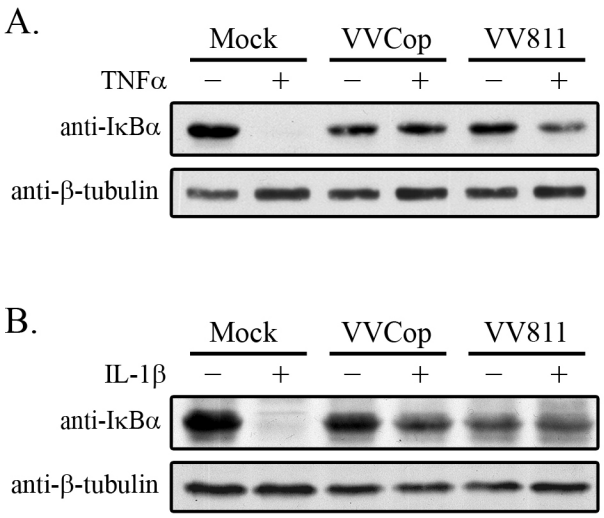

Figure 2-6. IκBα is not degraded in VV811 infected cells. HeLa cells were mock infected or infected with VVCop or VV811 at an MOI of 5. Twelve hours post infection cells were mock treated or stimulated with TNFα **(A)** or IL-1β **(B)** for 20 minutes. Cytoplasmic extracts were western blotted with rabbit anti-IκBα and anti-β-tubulin, as a loading control.

VVCop or VV811 also inhibited IL-1β-induced IκBα degradation (Figure 2-6B), since both viruses contain inhibitors of the IL-1β pathway to NF-κB activation, and these inhibitors act upstream of IκBα degradation (8). The ability of VV811 to inhibit IκBα degradation suggested that the inhibition of NF-κB occurred upstream or at the point of IκBα degradation.

In order to verify the ability of VVCop or VV811 to inhibit IκBα degradation, we used a flow cytometry assay to monitor IκBα levels (Figure 2-7). HeLa cells were mock infected or infected with VVCop or VV811 for twelve hours and subsequently were mock treated or treated with TNFα and expression of IκBα was detected using an antibody specific for IκBα. In the absence of TNFα, HeLa cells (labeled as mock) showed a high level of IκBα (Figure 2-7panel a). In HeLa cells treated with TNFα (labeled as mock + TNFα), the level of IκBα dropped significantly due to the degradation of IκBα (Figure 2-7 panel a) (6, 30). Degradation of IκBα induced by TNFα was rescued by treatment with the proteasome inhibitor MG132 (labeled as MG132 + TNFα), as previously demonstrated (Figure 2-7 panel a) (55, 77). Treatment of HeLa cells with MG132 in the absence of TNFα (labeled as MG132) had no effect on the level of IκBα (Figure 2-7panel a). Notably, however, both VV811 and VVCop infected cells treated with TNFα (labeled as VVCop + TNFα and VV811 + TNFα) displayed no loss of IκBα fluorescence (Figure 2-7 panels b, c), indicating that IκBα was not degraded. Similar results were also found when the experiment was repeated

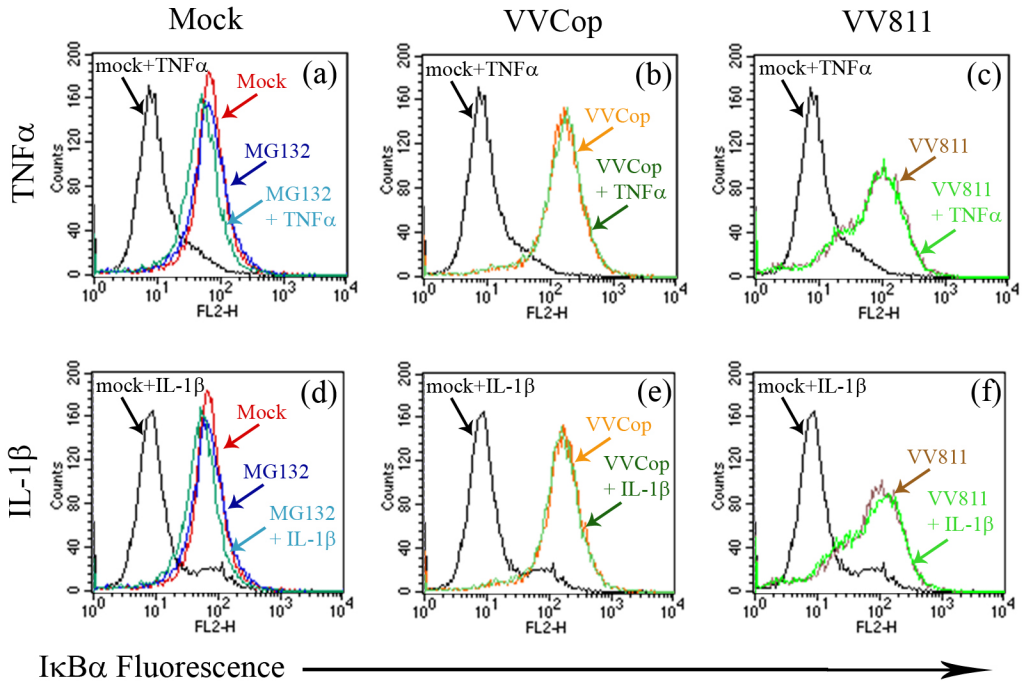

Figure 2-7. VV811 and VVCop inhibit IκBα degradation. HeLa cells were mock infected or infected with VVCop or VV811 at an MOI of 5. Twelve hours post infection cells were mock treated or treated with TNFα **(A)** or IL-1β **(B)** for 20 minutes. IκBα levels were detected using anti-IκBα followed by fluorescently labeled anti-mouse secondary antibody. MG132 was used as a control to inhibit IκBα degradation.

using IL-1β (Figure 2-7 panels d-f). Altogether, the data indicated that cells infected with VV811, which is lacking the known inhibitors of TNFα-induced NF-κB activation, retained the ability to inhibit degradation of IκBα, a crucial event for the activation of the NF-κB pathway.

2.3.3 Inhibition of NF-κB by VV811 is not due to induction of apoptosis

It has previously been demonstrated that VV811 induces programmed cell death, known as apoptosis, upon infection (81). Apoptosis and the NF-κB pathway are tightly linked cellular processes (19, 68). Notably, apoptosis leads to activation of caspase-3, which has been demonstrated to cleave IκBα, resulting in inhibition of NF-κB signaling (5, 39, 59). To examine if the induction of apoptosis by VV811 was responsible for the observed inhibition of the NF-κB pathway, HeLa cells were infected with the recombinant virus VV811:Flag-F1L which expresses F1L, a potent mitochondrial associated inhibitor of apoptosis encoded by vaccinia virus (81). HeLa cells were infected with VV811:Flag-F1L, followed by mock treatment or treatment with TNFα. Cells were subsequently stained with anti-p65 as well as DAPI to detect nuclei and cytoplasmic viral factories. Samples were analyzed by immunofluorescence microscopy (Figure 2-8). VV811:Flag-F1L infected cells not treated with TNFα displayed p65 in the cytoplasm (Figure 2-8 a, b), indicating that infection failed to activate the NF-κB pathway. Furthermore, infected cells treated with TNFα retained p65 in the

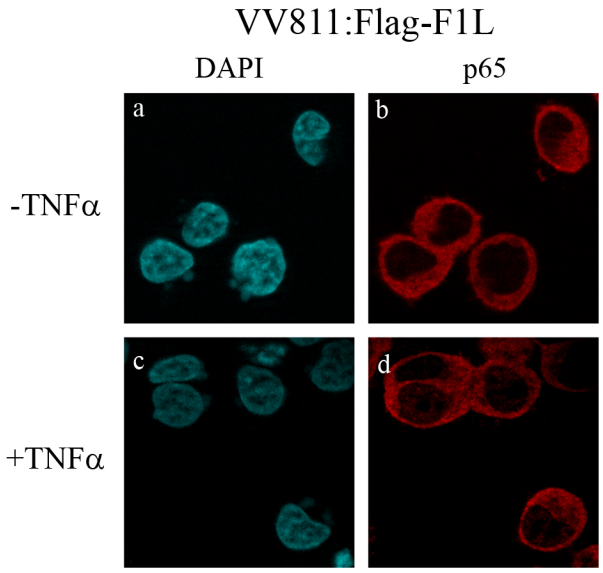

Figure 2-8. VV811 expressing anti-apoptotic protein F1L inhibits p65 translocation to the nucleus. HeLa cells were infected with VV811 expressing F1L (VV811:Flag-F1L) at an MOI of 5 for 12 hours, followed by treatment with TNFα. Cells were stained with anti-p65 and visualized by immunofluorescence microscopy.

cytoplasm indicating that VV811:Flag-F1L inhibited TNFα-induced NF-κB p65 translocation to the nucleus (Figure 2-8 c, d).

Furthermore, to determine if infection with VV811:Flag-F1L also inhibited TNFα-induced IκBα degradation in a similar manner to VV811, flow cytometry was utilized. HeLa cells were mock infected or infected with VV811 or VV811:Flag-F1L for twelve hours, followed by mock treatment or treatment with TNFα, and cells were stained with anti-IκBα (Figure 2-9). Mock infected cells (labeled as Mock) showed a significant level of IκBα which was greatly diminished upon treatment with TNFα (labeled as Mock + TNFα) (Figure 2-9a). Upon stimulation with TNFα and treatment with MG132 (labeled as MG132 + TNFα), IκBα degradation was clearly inhibited, as expected (Figure 2-9a) (55, 77). Cells infected with VV811 and mock treated or treated with TNFα, displayed a high level of IκBα, demonstrating that VV811 inhibited IκBα degradation, as previously demonstrated in Figure 2-7 (Figure 2-9b). Additionally, infection with VV811:Flag-F1L, after mock treatment or treatment with TNFα, also inhibited TNFα-induced IκBα degradation (Figure 2-9c). Together, these results show that VV811:Flag-F1L inhibited NF-κB activation to the same extent as VV811. Therefore, regardless of the effects of apoptosis, VV811 infection inhibited TNFα-induced p65 translocation to the nucleus and degradation of IκBα.

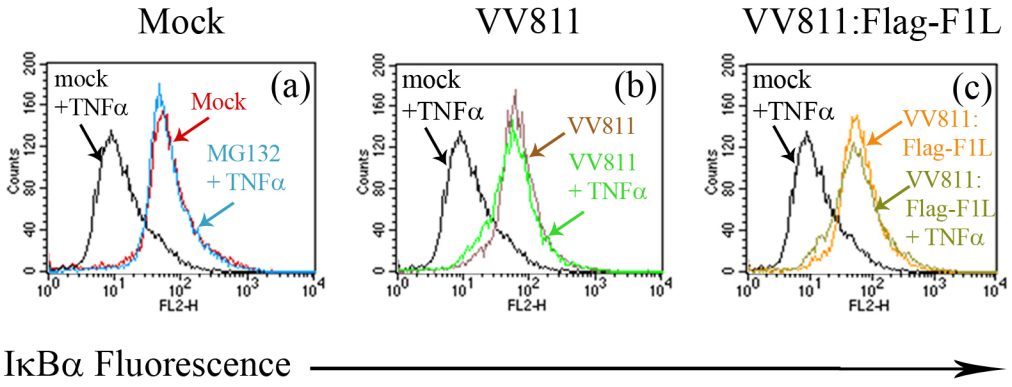

Figure 2-9. Apoptosis does not affect the ability of VV811 to inhibit IκBα degradation. Mock infected cells or cells infected at an MOI of 5 with VV811 or VV811:Flag-F1L for 12 hours were untreated or treated with TNFα for 20 minutes. After staining with anti-IκBα and fluorescently labeled anti-mouse secondary antibody, flow cytometry analysis was performed to examine IκBα fluorescence.

2.3.4 VV811 infection leads to an accumulation of phosphorylated IκBα.

Upon activation of the NF-κB pathway, IκBα is phosphorylated by IKKβ, a member of the IKK complex (46, 85, 86). Phosphorylation of IκBα at serines 32 and 36 signals for poly-ubiquitination, resulting in IκBα degradation by the 26S proteasome (9, 11, 17, 63, 75, 76, 82). To further determine how VV811 infection inhibits the NF-κB pathway, phosphorylation of IκBα was examined. HeLa cells were mock infected or infected with VVCop or VV811 and subsequently mock treated or treated with TNFα. Cytoplasmic extracts were generated and blotted with an antibody specific for phosphorylated IκBα (Figure 2-10A). Mock infected cells treated with TNFα displayed very little accumulation of phospho-IκBα, since IκBα is quickly ubiquitinated and degraded once it is phosphorylated (9, 11). A small amount of phospho-IκBα was detected in VVCop-infected cells treated with TNFα likely due to the presence of multiple virally encoded NF-κB inhibitors, including B15R, which inhibits IKKβ function leading to inhibition of IκBα phosphorylation (Figure 2-10A) (10). Surprisingly, an accumulation of phospho-IκBα was present in the VV811 infected cells treated with TNFα (Figure 2-10A). Since VV811 does not contain B15R, the activity of IKKβ could account for the observed accumulation of phospho-IκBα in VV811 infected cells (46, 57, 86). VV811 infected cells treated with IL-1β also displayed this increase in phospho-IκBα (Figure 2-10B). As a control, cytoplasmic extracts were blotted with an antibody to the vaccinia virus late protein, I5L, to confirm

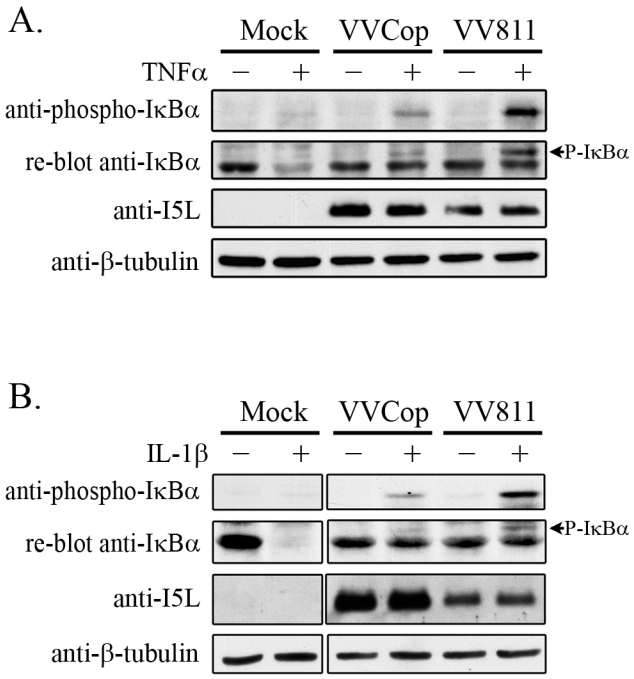

Figure 2-10. Phosphorylated IκBα accumulates in VV811 infected cells treated with TNFα. Cells were mock infected or infected with VVCop or VV811 at an MOI of 5 for 14 hours and treated with TNFα or IL-1β for 20 minutes. Cytoplasmic extracts of samples treated with TNFα **(A)** or IL-1β **(B)** were blotted with anti-phospho-IκBα. The same membranes were re-blotted with anti-IκBα. Samples were western blotted for the vaccinia virus protein I5L as a control for virus infection, and β-tubulin as a loading control.

virus infection and β-tubulin was used as a loading control. Together, this is strong evidence that VV811 expresses an inhibitor of NF-κB that prevents the degradation of phosphorylated IκBα leading to an accumulation of phosphorylated IκBα in infected cells.

To gain insight into the kinetics of phospho-IκBα accumulation during VV811 infection, cells were treated with TNFα for varying amounts of time. Cytoplasmic extracts were generated and blotted for phospho-IκBα and IκBα (Figure 2-11). In mock infected cells, IκBα was present, however, no phospho-IκBα was detected. Treatment with MG132 and TNFα induced accumulation of phosphorylated IκBα, since proteasome inhibition blocks phospho-IκBα degradation (55, 77). In the absence of TNFα, cells infected with VV811 displayed no phospho-IκBα; however, after five minutes of TNFα stimulation a large amount of phospho-IκBα was detected, and the presence of phospho-IκBα was still detected up to 90 minutes of TNFα treatment (Figure 2-11). Additionally, the level of IκBα was maintained in VV811 infected cells. Compared to VV811, VVCop displayed minimal phospho-IκBα present after 20, 45 and 90 minutes of TNFα treatment. Therefore, in contrast to VVCop infected cells, VV811 induced accumulation of phosphorylated IκBα after TNFα treatment, suggesting the presence of an NF-κB inhibitor that acts downstream of IκBα phosphorylation.

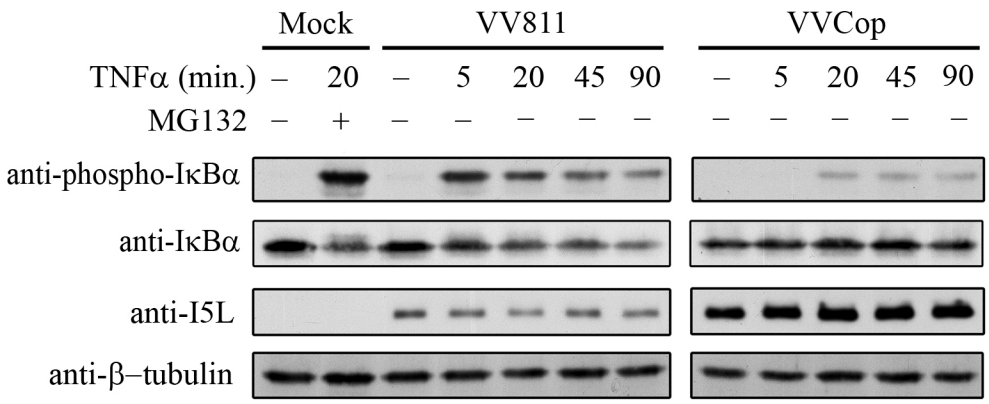

Figure 2-11. VV811 infection induces phospho-IκBα accumulation upon extended TNFα treatment. HeLa cells were mock infected or infected with VV811 or VVCop at an MOI of 5 for 14 hours, followed by mock treatment or treatment with TNFα for 5, 20, 45 or 90 minutes and cytoplasmic extracts were collected. Mock infected cells were also treated with MG132 and TNFα for 20 minutes as a control for accumulation of phospho-IκBα. Cytoplasmic extracts were blotted for phospho-IκBα and IκBα. Anti-I5L was used as a control for virus infection, and anti-β-tubulin was a loading control.

To determine if the accumulation of phospho-IκBα occurred throughout VV811 infection, 4, 8, 12, and 16 hours post infection cells were treated with TNFα for 20 minutes and cytoplasmic extracts were collected (Figure 2-12). Whereas no phospho-IκBα was detected in mock infected cells after either mock treatment or treatment with TNFα, a small amount of phospho-IκBα was detected as early as 4 hours post infection with VV811. Increasing amounts of phospho-IκBα were detected at 8, 12 and 16 hours of VV811 infection (Figure 2-12). VVCop infected cells treated with TNFα displayed only a minimal amount of phospho-IκBα, likely due to the presence of the previously identified virus encoded inhibitors of TNFα-induced NF-κB signaling (10, 18). IκBα levels were maintained in VV811 and VVCop with the exception of VV811 at 4 hours post infection (Figure 2-12). The degradation of IκBα 4 hours post infection with VV811 is due to the absence of the identified vaccinia virus encoded inhibitors of TNFα-induced NF-κB signaling, and also suggests that inhibition of IκBα degradation by VV811 only occurs late in infection. Overall, infection with VV811, followed by TNFα treatment, resulted in an accumulation of phosphorylated IκBα, suggesting that an unknown inhibitor expressed during VV811 infection inhibits the degradation of IκBα.

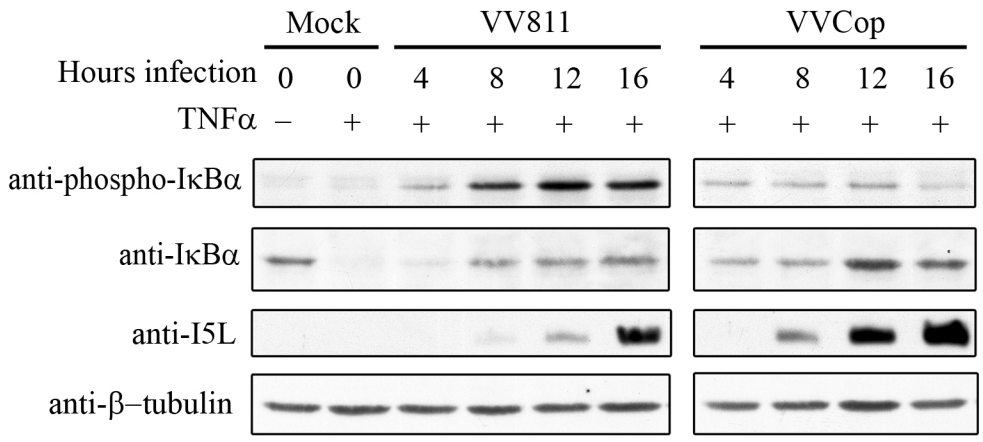

Figure 2-12. Phospho-IκBα persists in VV811 infected cells treated with TNFα. After mock infection or infection with VV811 or VVCop at an MOI of 5, HeLa cells were mock treated or treated with TNFα for 20 minutes. Cytoplasmic extractions were performed and samples were blotted for phospho-IκBα and IκBα. Samples were also blotted with anti-I5L to detect increasing late gene expression during infection, and anti-β-tubulin was used as a loading control.

2.3.5 IκBα remains associated with p65 and p50 in VV811 infected cells.

Upon phosphorylation and degradation of IκBα, the p65-p50 NF-κB heterodimer is released and translocates to the nucleus due to the presence of an exposed nuclear localization signal in p65 (7, 21). Given that IκBα is phosphorylated but not degraded in VV811 infected cells stimulated with TNFα, we sought to determine if IκBα remained bound to the NF-κB heterodimer in the inactive IκBα-p65-p50 complex. HeLa cells were infected with VVCop or VV811 and twelve hours post infection cells were mock treated or treated with TNFα and immunoprecipitated with an antibody specific for IκBα (Figure 2-13A). As expected, immunoprecipitation of IκBα demonstrated binding to p65 and p50 in unstimulated mock infected cells. Immunoprecipitated samples blotted for IκBα confirmed that IκBα was immunoprecipitated in all the samples except for mock infected cells treated with TNFα in which IκBα was degraded, as expected (Figure 2-13A) (6, 30). Importantly, in cells infected with VVCop or VV811 and immunoprecipitated with IκBα, IκBα also immunoprecipitated with p65 and p50 (Figure 2-13A). A significant amount of phospho-IκBα was also immunoprecipitated in mock cells treated with MG132 and TNFα, indicating that inhibition of the proteasome maintained the levels of phospho-IκBα. A small amount of phospho-IκBα was detected in VVCop infected cells treated with TNFα, and conversely, phospho-IκBα was clearly present in VV811 infected cells treated with TNFα, confirming that a portion of the IκBα associated with

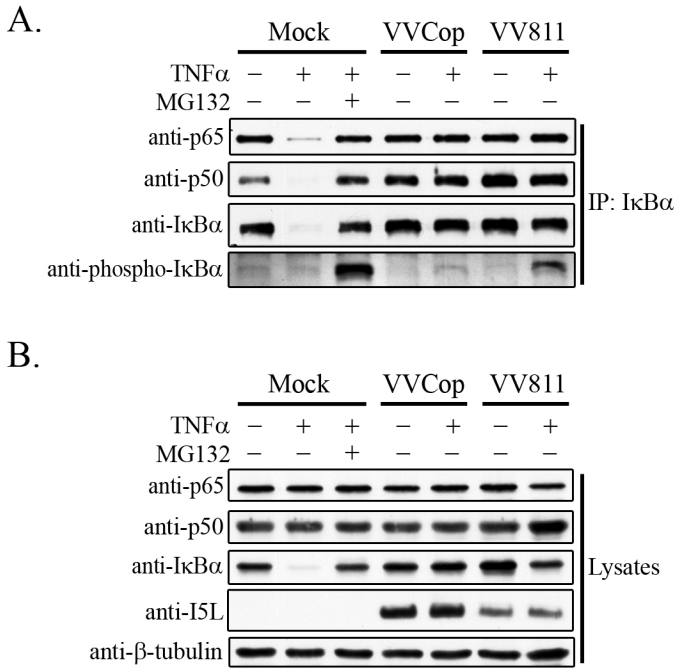

Figure 2-13. NF-κB p65-p50 remains associated with IκBα during VV811 infection. HeLa cells mock infected or infected with VVCop or VV811 at an MOI of 5 for 12 hours, were mock treated or treated with TNFα, lysed in NP-40 lysis buffer and immunoprecipitated with anti-IκBα. Western blotting was performed with antibodies to p65, p50, IκBα and phospho-IκBα. Cell lysates were western blotted for expression of endogenous p65, p50, IκBα the vaccinia virus protein I5L, and β-tubulin as a loading control.

p65 and p50 in VV811 infected cells treated with TNFα was phosphorylated (Figure 2-13A). Cell lysates demonstrated expression of p65, p50 and IκBα in the mock infected and virus infected cells (Figure 2-13B). Blotting with anti-I5L and anti-β-tubulin confirmed virus infection and ensured equal amounts in all samples. The interaction observed between IκBα, p65 and p50 is indicative of the NF-κB inhibitory IκBα-p65-p50 complex. Therefore, in both VVCop and VV811 infected cells stimulated with TNFα, the inactive IκBα-p65-p50 complex remained intact. Furthermore, a portion of the IκBα was phosphorylated in VV811 infected cells stimulated with TNFα. This observation indicates that VV811 expresses a previously unidentified inhibitor of the NF-κB pathway, resulting in inhibition of IκBα degradation after phosphorylation of IκBα.

2.3.6 A late protein expressed by VV811 inhibits IκBα degradation

To effectively inhibit the antiviral immune response many vaccinia virus immune evasion proteins are expressed early during infection (48, 66). To determine if late protein expression contributed to inhibition of TNFα-induced IκBα degradation, HeLa cells were infected with VVCop or VV811 and IκBα fluorescence was examined by flow cytometry in the presence and absence of cytosine arabinofuranoside (AraC), an inhibitor of late viral gene expression (Figure 2-14) (3, 15). As expected, mock-infected cells treated with TNFα (labeled as mock + TNFα) resulted in IκBα degradation (Figure 2-14A panel a).

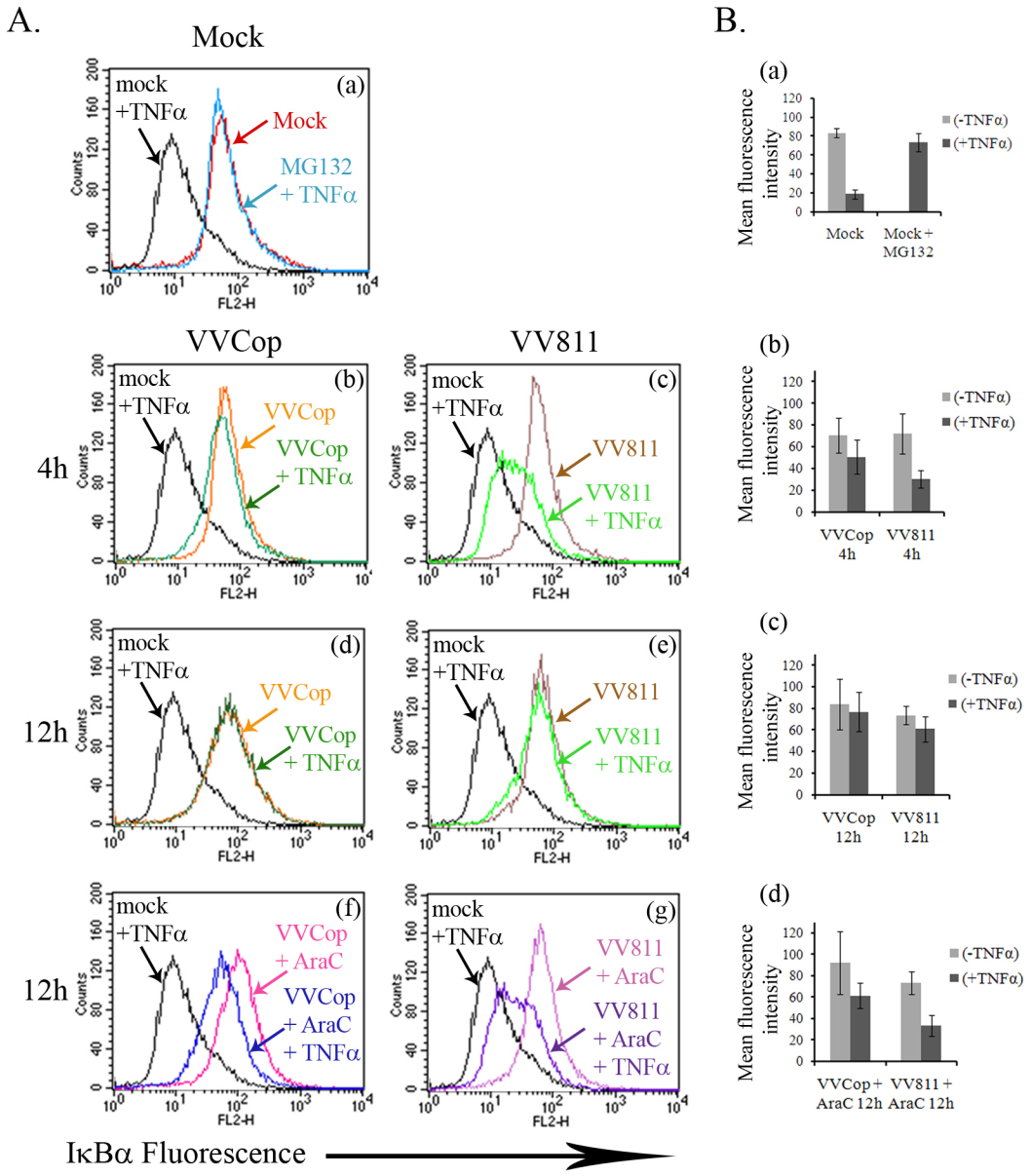

Figure 2-14. Late gene expression is required for inhibition of TNFα-induced IκBα degradation by VV811. **(A)** HeLa cells were mock infected or infected with VVCop or VV811 for 4 or 12 hours or 12 hours in the presence of AraC. Cells were either mock treated, or treated with TNFα for 20 minutes. Cells were treated with 80μg/mL of AraC to inhibit late poxvirus gene expression. Mock infected cells treated with MG132 and TNFα were used as a positive control for inhibition of IκBα degradation. IκBα levels were detected by staining with mouse anti-IκBα, followed by fluorescently tagged anti-mouse secondary antibody, and flow cytometry analysis was performed to examine IκBα fluorescence. **(B)** Quantification of IκBα fluorescence was determined by mean fluorescence intensities represented by the average from three independent experiments. Error bars represent standard deviation.

However, 4 hours post-infection with VVCop or VV811 and after treatment with TNFα, some degradation of IκBα was observed in VVCop infected cells (labeled VVCop + TNFα) and a significant amount of degradation of IκBα was evident in VV811 infected cells (labeled as VV811 + TNFα), suggesting that early gene expression was not sufficient for inhibition of IκBα degradation (Figure 2-14A panels b, c). As previously shown in Figure 2-10, IκBα degradation was inhibited in VVCop and VV811 infected cells 12 hours post infection treated with TNFα (Figure 2-14A panels d, e). In contrast, cells infected with VVCop or VV811 for 12 hours in the presence of TNFα and AraC resulted in loss of IκBα (Figure 2-14A panels f, g). The loss of IκBα in VV811 infected cells was more prominent than in cells infected with VVCop (Figure 2-14A panels f, g). The results of the flow cytometry analysis were also quantified and mean fluorescence intensities of samples were displayed graphically (Figure 2-14B). The average MFI of mock infected cells was 83, cells treated with TNFα was 18 and cells treated with MG132 and TNFα was 73 (Figure 2-14B panel a). After 4 hours of VVCop infection, cells displayed an average MFI of 76 without TNFα treatment, and a decrease to 56 with TNFα treatment (Figure 2-14B panel b). Notably, while cells infected with VV811 for 4 hours displayed an average MFI of 77, after TNFα stimulation, the MFI of VV811 infected cells decreased by more than 50%, to 33 (Figure 2-14B panel b). Twelve hours post infection with VVCop or VV811, the average MFI was 84 or 73, respectively, and after TNFα treatment, the MFI was

76 or 69, respectively (Figure 2-14B panel c). After 12 hours of infection in the presence of AraC, cells infected with VVCop displayed an average MFI of 92, which decreased to 61 after TNFα treatment (Figure 2-14B panel d). Significantly, cells infected with VV811 for 12 hours in the presence of AraC had an average MFI of 73, which decreased to 33 upon treatment with TNFα, closely resembling the MFI displayed by VV811 infected cells at 4 hours post infection (Figure 2-14B panel d).

To confirm that early vaccinia virus proteins were expressed at 4 hours post infection in VVCop and VV811 infected cells, whole cell lysates were collected and blotted for an early protein, E9L (Figure 2-15) (2, 44). As expected, E9L was detected in all VVCop and VV811 infected samples, and E9L expression was not inhibited by AraC treatment (2, 44). Lysates were also blotted for the late protein I5L, to verify that late proteins were expressed 12 hours post infection with VVCop and VV811 and were not expressed in the presence of AraC (Figure 2-15).

To further verify the role of late protein synthesis in the inhibition of the NF-κB pathway, cells were treated with AraC and western blotted for IκBα (Figure 2-16). As expected, unstimulated mock infected cells displayed IκBα, but upon TNFα treatment IκBα was degraded, and treatment with MG132 prior to TNFα treatment retained IκBα (Figure 2-16 lanes 1-3). At 4 hours post infection with VVCop, IκBα was detected (Figure 2-16 lane 4), and upon TNFα treatment, some loss of IκBα was observed (Figure 2-16 lane 5). IκBα was also

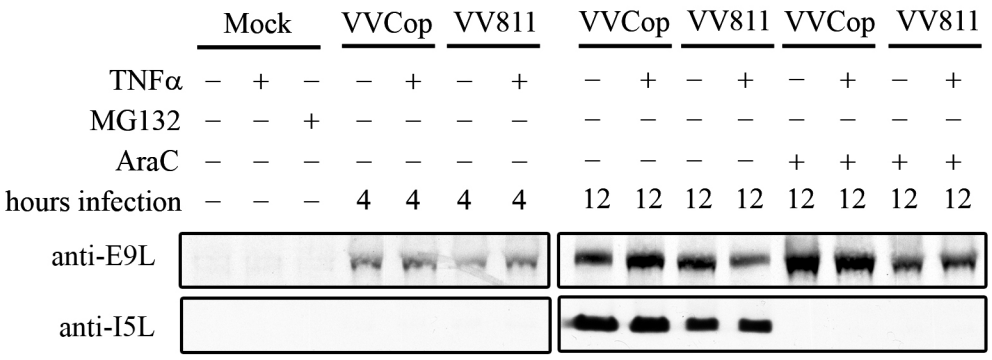

Figure 2-15. Late proteins are not expressed 4 hours post infection or 12 hours post infection in the presence of AraC. Whole cell lysates were collected from samples used for IκBα degradation flow cytometry in Figure 14 and were western blotted for anti-E9L, an early vaccinia virus protein, and I5L, a vaccinia virus late protein. Cells were treated with 80μg/mL AraC to inhibit late gene expression.

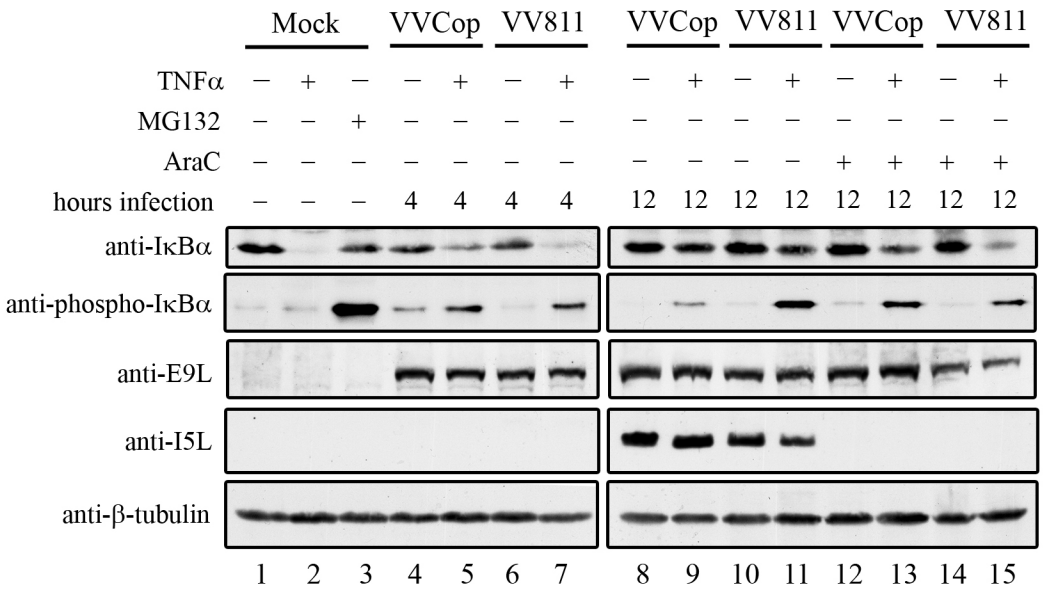

Figure 2-16. A late vaccinia virus protein regulates IκBα degradation. HeLa cells were mock infected or infected with VVCop or VV811 for 4 or 12 hours at an MOI of 5, or 12 hours in the presence of 80μg/mL AraC, and mock treated or treated with TNFα for 20 minutes. MG132 combined with TNFα treatment was used as a positive control for inhibition of IκBα degradation. Cytoplasmic extracts were generated and western blotted with an antibody to IκBα, phospho-IκBα, E9L, I5L, and β-tubulin, a loading control.

present in the cytoplasm of cells infected with VV811 for 4 hours (Figure 2-16 lane 6), however, little IκBα was detected after TNFα treatment (Figure 2-16 lane 7), indicating that both VVCop and VV811 were not fully competent to inhibit TNFα-induced IκBα degradation. After 12 hours of infection with VVCop or VV811, IκBα was maintained in all cytoplasmic extracts in the absence or presence of TNFα stimulation (Figure 2-16 lanes 8 to 11). Upon TNFα stimulation and treatment with AraC, some loss of IκBα was observed in VVCop infected cells (Figure 2-16 lane 13). However, infection with VV811 for 12 hours in the presence of AraC and TNFα resulted in a loss of IκBα which was significantly greater than observed with VVCop infection (Figure 2-16 lane 15). Levels of IκBα were maintained in VVCop or VV811 infected cells treated with only AraC (Figure 2-16 lanes 12 and 14). Along with the flow cytometry data, these results reinforce the notion that late protein synthesis is required to inhibit IκBα degradation.

To examine the affect of AraC treatment on phospho-IκBα accumulation during virus infection, cytoplasmic extracts were also blotted with anti-phospho-IκBα. As expected, phospho-IκBα accumulated in TNFα treated cells infected with VV811, and, notably, phospho-IκBα was maintained even in the presence of AraC (Figure 2-16 lanes 11 and 15). Interestingly, an increase in phospho-IκBα was detected in VVCop infected cells treated with AraC and TNFα (Figure 2-16 lanes 9 and 13), suggesting that additional late gene products regulate

accumulation of phospho-IκBα during infection. To verify that late proteins were not expressed upon treatment with AraC, cytoplasmic extracts were blotted for a late vaccinia virus protein, I5L, and an early vaccinia protein E9L, and the presence of β-tubulin confirmed equal loading. The data indicate that a late vaccinia virus protein(s) is responsible for the observed inhibition of IκBα degradation in VVCop and VV811 infected cells.

2.3.7 IκBα does not interact with viral proteins during VV811 infection

The vaccinia virus protein present in VV811 responsible for inhibition of TNFα-induced NF-κB signaling remained undetermined. However, according to our data it was possible that a viral protein was interacting with IκBα to directly inhibit the degradation of IκBα. To examine this possibility, HeLa cells were infected with VV811 or VVCop for 12 hours, and after mock treatment or TNFα treatment, IκBα was immunoprecipitated and samples were subjected to silver stain (Figure 2-17). Bands corresponding to possible interacting partners with IκBα from the VV811 infected cells were excised and analyzed by mass spectrometry. p65 was detected, corroborating the data demonstrating that IκBα remains associated with p65 during infection, as presented in Figure 2-13 (Table 2-2). Additionally, the NF-κB subunit c-Rel was also found to be associated with IκBα during infection, suggesting that other NF-κB transcription factors besides the typical p65-p50 dimer remain associated with IκBα during infection. Other

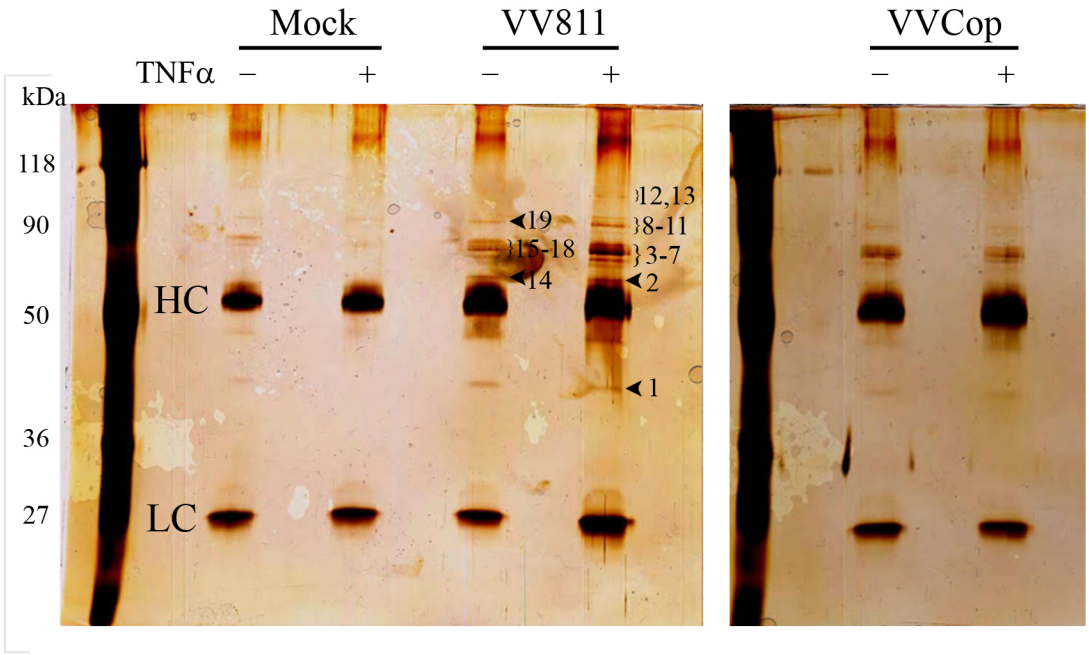

Figure 2-17. IκBα does not interact with any viral proteins during VV811 infection. Mock infected cells or cells infected with VVCop or VV811 at an MOI of 5 for 12 hours were mock treated or treated with TNFα, lysed in NP-40 lysis buffer and immunoprecipitated with anti-IκBα. Silver staining was performed on the samples and bands that were excised and analyzed by mass spectrometry are indicated. kDa, kilodaltons; HC, heavy chain; LC, light chain.

Table 2-2. Proteins that interacted with IκBα during VV811 infection.

Band	Protein	PEAKS Score	Peptide coverage	Query Matches
VV811 + TNFα				
1	**IκBα**	43.3%	4.38%	6
2	**RelA (p65)**	55.4%	15.07%	1
3	**RelA (p65)**	96.4%	12.29%	4
4	**RelA (p65)**	97.0%	12.96%	3
5	**-**	-	-	-
6	**RelA (p65)**	98.8%	19.27%	6
7	**RelA (p65)**	99.1%	35.28%	15
8	**Hsp70**	99.0%	17.19%	10
9	**Hsp70**	99.1%	21.8%	12
	c-Rel	99.0%	18.74%	12
	DDX3X	95.2%	7.67%	3
10	**c-Rel**	99.0%	14.65%	9
	Hsp70	98.2%	7.7%	4
11	**c-Rel**	98.6%	7.67%	4
12	**-**	-	-	-
13	**-**	-	-	-
VV811				
14	**RelA (p65)**	98.2%	17.94%	5
15	**RelA (p65)**	98.9%	12.5%	6
16	**RelA (p65)**	59.2%	18.52%	1
17	**RelA (p65)**	97.5%	10.96%	3
18	**RelA (p65)**	99.1%	27.69%	23
	c-Rel	96.4%	12.36%	5
19	**Hsp70**	99.1%	24.01%	14
	c-Rel	99.1%	18.91%	12

Band numbers corresponding to excised silver stained protein bands that were analyzed by mass spectrometry are displayed with the probable corresponding protein identified. PEAKS score is a percentage calculated by PEAKS Viewer 5.1 based on the probability that the protein identified is correct. Other important parameters include the peptide coverage (the percentage of amino acids in the protein sequence correlated to peptides isolated) and query matches (the number of peptides isolated that are correlated to the protein identified). DDX3X, dead box protein 3X; Hsp70, heat shock protein 70.

cellular proteins that associated with IκBα were dead box protein 3 (DDX3X) and heat shock protein 70 (hsp70) (Table 2-2). No vaccinia virus proteins were found to associate with IκBα during VV811 infection. Therefore, the late protein or proteins that suppress IκBα degradation during VV811 infection remain to be identified.

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CHAPTER 3: Discussion

3.1 Summary

This study was initiated by the intriguing possibility that we could utilize a large deletion vaccinia virus to identify additional inhibitors of the NF-κB pathway. We postulated that more NF-κB inhibitors were present in vaccinia virus due to multiple redundant mechanisms used by poxviruses to manipulate the NF-κB pathway. The observation that infection with VV811 inhibited TNFα-induced p65 translocation to the nucleus, even though VV811 is devoid of the known inhibitors of TNFα-induced NF-κB activation, led us to examine VV811 further in an attempt to discern additional NF-κB inhibitor(s) functions. Through examination of cells infected with VV811 we have determined that at least one previously unidentified inhibitor of TNFα-induced NF-κB activation is present in the genome of VV811, and that late gene expression regulates IκBα degradation.

3.2 Vaccinia virus possesses an uncharacterized inhibitor of TNFα-induced NF-κB signaling

Comparison of VV811 with the parental strain VVCop revealed that VV811 possesses vaccinia virus inhibitors of IL-1β-induced NF-κB activation, A46R and A52R (Figure 2-1). Conversely, VV811 does not contain the NF-κB inhibitors that have been shown to suppress MVA-induced NF-κB activation, K1L and M2L, or the inhibitors shown to block TNFα-induced NF-κB signaling, N1L and B15R (Figure 2-1). Therefore, demonstrating that VV811 infection

inhibited p65 nuclear translocation after TNFα treatment was the first evidence that an additional inhibitor of NF-κB activation in vaccinia virus existed and this inhibitor was present in the large deletion virus VV811 (Figures 2-2, 2-4, 2-5). This inhibitor is in addition to the seven intracellular inhibitors of NF-κB signaling identified in vaccinia virus that all have different mechanisms of action (Figure 1-8, Table 1-1). Furthermore, we demonstrated that UV-inactivated VVCop and UV-inactivated VV811 do not inhibit p65 translocation to the nucleus, providing evidence that viral protein production is necessary for inhibition of p65 nuclear translocation (Figures 2-3, 2- 4). Therefore, a previously uncharacterized vaccinia virus inhibitor of the NF-κB pathway was expressed by the large deletion virus VV811.

An alternative explanation of the inhibition of TNFα-induced NF-κB activation by VV811 is the occurrence of a gain-of-function mutation in VV811. It is possible that, through multiple passages in human and primate cell lines and selective pressure to inhibit NF-κB signaling, VV811 acquired a mutation in an open reading frame that changed the function of the resulting protein to confer NF-κB inhibitory activity. Although this possibility is speculative, it is potentially plausible and should be kept in consideration.

We subsequently determined that VV811 infection inhibits IκBα degradation induced by TNFα stimulation (Figures 2-6, 2-7). The regulation of IκBα has been studied intensely since the discovery of its vital role in NF-κB signaling (7, 26). Activation of the IKK complex results in phosphorylation of the

IKKβ kinase (39, 75). IKKβ phosphorylates IκBα at serines 32 and 36, and this phosphorylation marks IκBα for polyubiquitination by the $SCF^{\beta TrCP}$ ubiquitin ligase (25, 32, 58, 60, 76). Polyubiquitination of IκBα directs IκBα to be degraded by the 26S proteasome, releasing the NF-κB transcription factor and exposing the nuclear localization signal to direct nuclear translocation of NF-κB (9, 21). The degradation of IκBα occurs after the convergence point of the TNFR and the TLR/IL-1R pathways, making it an attractive target for viral inhibition of NF-κB signaling (29). For example, ICP27 expressed by HSV-1 interacts with IκBα to inhibit IκBα phosphorylation and subsequent ubiquitination (31). In addition, the coxsackievirus B3 protease, 3Cpro, cleaves IκBα, producing an inhibitory fragment of IκBα that prevents DNA binding by the NF-κB transcription factor (52, 77). The inhibition of IκBα degradation by VV811 demonstrated that the point of action of the novel inhibitor of NF-κB signaling in vaccinia virus was at or preceding degradation of IκBα.

3.3 The ability of VV811 infection to inhibit NF-κB signaling is independent of the induction of apoptosis

Previous studies in our laboratory have demonstrated that VV811 induces apoptosis (71). At 6 hours post VV811 infection of Jurkat cells, cleaved caspase-3 and cytochrome c release was observed, as well as a small amount of PARP and caspase-9 cleavage, all indicators of apoptosis (71). Wild type vaccinia virus does

not require induction of NF-κB activation to inhibit apoptosis like other viruses such as HTLV-1, EBV and adenoviruses, since vaccinia virus encodes CrmA and F1L, which both inhibit apoptosis (28, 61, 62, 71). The induction of apoptosis, however, has been demonstrated to promote the cleavage of IκBα by caspase-3 (8, 48). The caspase-3 cleaved version of IκBα is not degraded upon activation of the NF-κB signaling pathway, but retains the ability to bind the NF-κB heterodimer and therefore inhibit downstream transcriptional activation (48). A plausible explanation for the observed inhibition of NF-κB activation by VV811 infection is induction of apoptosis (71). The vaccinia virus encoded mitochondrial inhibitor F1L inhibits activation of caspase-3, which has been shown to inhibit NF-κB activation by cleaving IκBα. Therefore, we utilized the recombinant vaccinia virus VV811:Flag-F1L, which expresses a Flag-tagged version of F1L, to demonstrate that apoptosis induction was not responsible for the inhibition of nuclear p65 translocation or IκBα degradation during VV811 infection (Figure 2-8, 2-9). Therefore, apoptosis was not involved in the ability of VV811 to inhibit NF-κB activation.

3.4 VV811 infection inhibits the NF-κB pathway through regulation of IκBα degradation

We further examined the events regulating the degradation of IκBα during infection, including phosphorylation of IκBα. We demonstrated that VV811

infection resulted in an accumulation of phosphorylated IκBα after TNFα stimulation, suggesting that IκBα was phosphorylated but not degraded (Figures 2-10, 2-11, 2-12). Furthermore, the phospho-IκBα accumulation remained evident with prolonged TNFα stimulation and increased with increasing length of infection with VV811 (Figure 2-11, 2-12). Interestingly, in contrast to VV811 infection, only a small amount of phosphorylated IκBα was present in VVCop infected cells treated with TNFα (Figures 2-10, 2-11, 2-12). This is likely due to the presence of the known inhibitors of TNFα-induced NF-κB activation that act upstream of IκBα phosphorylation in VVCop (14, 19). Notably, B15R, also known as B14R in VV strain Western Reserve, prevents the phosphorylation and activation of IKKβ, resulting in inhibition of IκBα phosphorylation (14). Therefore it is not a surprise that little phosphorylated IκBα was observed in VVCop infected cells. However, VV811 lacks the inhibitors of TNFα-induced NF-κB signaling, B15R and N1L, leading us to propose that a novel inhibitor of the NF-κB pathway functions to prevent degradation of phosphorylated IκBα (46).

Surprisingly, accumulation of phosphorylated IκBα was also observed in VV811 infected cells treated with IL-1β (Figure 2-10). VV811 contains A46R and A52R which block IL-1β- and TLR-mediated NF-κB activation. We predicted that A46R and A52R would inhibit activation of the IKK complex and subsequent phosphorylation of IκBα in VV811 infected cells (13, 46). However,

A46R and A52R were characterized in vaccinia virus strain Western Reserve, which contains differences to VVCop, the parental strain of VV811 (13, 22, 56)(www.poxvirus.org/vaccinia_orthologs.asp). The A46R gene in vaccinia virus strain VVCop is truncated by 26 amino acids at the C-terminus with unknown consequences on protein function (13, 22). The A52R gene of VVCop is also slightly different from VVWR A52R, in that it contains a phenylalanine to serine substitution at amino acid 57, also with unknown consequences on protein function (13, 22). Interestingly, all other orthologs of A46R do not possess the same truncation as VVCop A46R, although orthologs to A46R exist in ectromelia virus, cowpox virus, monkeypox virus, variola virus, as well as other poxviruses (www.poxvirus.org). Conversely, orthologs of A52R in other poxviruses including cowpox virus, ectromelia virus, monkeypox virus and variola virus contain the same phenylalanine to serine substitution as VVCop A52R (www.poxvirus.org). If A46R and A52R are not functional in VVCop, IL-1β-induced NF-κB signaling would progress. However, according to our observations of the presence of an unknown inhibitor acting to inhibit degradation of phosphorylated IκBα, IL-1β-induced NF-κB signaling would be blocked prior to IκBα degradation, resulting in an accumulation of phosphorylated IκBα, as observed (Figure 2-10). Overall, the evidence overwhelmingly suggests that a previously uncharacterized protein expressed by vaccinia virus inhibits degradation of IκBα.

Since the phosphorylation of IκBα occurs after the convergence of TNFR and TLR/IL-1R signaling to NF-κB activation, it can be postulated that inhibition of phospho-IκBα degradation during VV811 infection prevents NF-κB activation mediated by the TNFR or TLR/IL-1R pathway. We cannot conclude this, however, without the analysis of VV811 devoid of A46R and A52. Furthermore, it is possible that modulation of a signaling molecule at or upstream of IKK activation may affect degradation of phospho-IκBα after TNFα treatment specifically, through an unknown mechanism. For example, it has been hypothesized that MC159 prevents IκBβ degradation through interaction with a mediator of TNFR but not TLR/IL-1R signaling, TRAF2 (43). The presence of phosphorylated IκBα after TNFα or IL-1β treatment of VV811 infected cells, however, provides evidence that IκBα degradation is inhibited in the same manner after stimulation of the TNFR or TLR/IL-1R pathway.

After IκBα is phosphorylated it is still bound to the NF-κB heterodimer, and the NF-κB transcription factor is only released after polyubiquitination and proteasomal degradation of IκBα (18, 63). Notably, African swine fever virus (ASFV) encodes an inhibitor of NF-κB signaling with a unique mechanism of action (47, 49, 59). Upon ASFV infection, NF-κB activation leads to IκBα degradation; however, A238L, encoded by ASFV, replaces IκBα, sequestering the NF-κB transcription factor in the cytoplasm (47, 49, 59). Our results following infection with VV811 indicated that IκBα was not degraded (Figures 2-

6, 2-7). It was possible, however, that a vaccinia virus protein prevented NF-κB translocation to the nucleus by displacing IκBα from the NF-κB dimer. To examine this possibility, we immunoprecipitated IκBα from VV811 and VVCop infected cells (Figure 2-13). Association of IκBα with both p65 and p50 was detected, and, furthermore, a portion of immunoprecipitated IκBα was phosphorylated (Figure 3-13). Therefore, the inhibitory complex of IκBα-p65-p50 was not disrupted during virus infection, confirming that phosphorylated IκBα bound to the NF-κB dimer. These data provide evidence that inhibition of IκBα degradation by VV811 occurs prior to dissociation of IκBα from the p65-p50 heterodimer.

3.5 An undetermined protein expressed late during vaccinia virus infection inhibits IκBα degradation

Concurrent to the examination of the mechanism of action of NF-κB inhibition by VV811, we attempted to determine the identity of the protein or proteins responsible for the observed inhibition. Vaccinia virus requires inhibition of immune responses to establish infection, therefore most immune evasion proteins are produced early during vaccinia virus infection (42, 54). However, we observed that IκBα was degraded after TNFα- stimulation 4 hours post infection with VV811, and IκBα degradation was inhibited 8 hours post infection (Figure 2-12). This led us to speculate that a protein expressed late

during infection was responsible for inhibition of IκBα degradation. Using flow cytometry, as well as western blotting of cytoplasmic extracts, we determined that late protein expression in VV811 was required for suppression of IκBα degradation during infection (Figures 2-14, 2-15, 2-16). Specifically, a significant amount of IκBα degradation occurred 4 hours post VV811 infection after treatment with TNFα, but this degradation was inhibited 12 hours post infection. Additionally, treatment with AraC, which blocks DNA replication and, subsequently, viral late protein synthesis, resulted in IκBα degradation after 12 hours of VV811 infection followed by TNFα treatment (Figures 2-14, 2-15, 2-16) (6, 17). Notably, late protein expression in VVCop was also required for complete inhibition of IκBα degradation (Figure 2-14, 2-15, 2-16). A small amount of IκBα degradation was observed 4 hours post infection with VVCop and following TNFα treatment, and AraC treatment during 12 hours of VVCop infection also resulted in a small decrease in IκBα after TNFα stimulation; conversely, TNFα-induced IκBα degradation was completely inhibited 12 hours post infection with VVCop in the absence of AraC (Figures 2-14, 2-16). These results indicate that late protein expression is required for complete inhibition of IκBα degradation by vaccinia virus.

The affect of late protein expression on phospho-IκBα accumulation was also examined (Figure 2-16). AraC treatment during VV811 infection resulted in a slight decrease in phospho-IκBα accumulation, but 12 hours post infection in

the presence of AraC, VVCop infection and TNFα treatment resulted in an increase in phospho-IκBα (Figure 2-16). These data suggest that, upon TNFα treatment of VV811 infected cells, IκBα is phosphorylated, but a late protein inhibits IκBα degradation without effecting the phosphorylation of IκBα. Additionally, late protein expression is required for the reduction of phospho-IκBα observed upon TNFα treatment 12 hours post infection with VVCop compared to 4 hours post infection. The activity of a phosphatase expressed by VVCop late during infection, but not expressed by VV811, could account for these results, as de-phosphorylation of phospho-IκBα would explain the reduced phospho-IκBα 12 hours post infection with VVCop. Interestingly, the vaccinia virus encoded phosphatase VH1, encoded by the H1L open reading frame, de-phosphorylates phosphoserine resides and is expressed late during infection, but VH1 is packaged in virions and, as such, exerts its activity immediately upon infection (5, 23, 33). Additionally, VV811 possesses the H1L open reading frame (46). However, VH1 has been shown to de-phosphorylate Stat1 and inhibit interferon γ signaling in vaccinia virus infected cells, demonstrating that de-phosphorylation of signaling proteins is a strategy used by vaccinia virus to evade the immune response (44). The involvement of de-phosphorylation in NF-κB regulation during vaccinia virus infection would be an interesting avenue to pursue in the future.

Vaccinia virus expresses proteins in a temporal manner, beginning with early proteins which include proteins responsible for DNA replication and

subsequent intermediate transcription, intermediate protein expression and late protein synthesis, as well as immune evasion (42). Late proteins are typically structural components of the progeny virions (42). Therefore it was somewhat surprising that a protein with an immune evasion function was expressed late in infection. There are a few examples of late poxviral proteins that are expressed late in infection, including the cowpox secreted TNFα inhibitor CrmC and the myxoma virus anti-inflammatory protein Serp-1 (34, 35, 54, 55, 65). However, all of the currently known inhibitors of NF-κB signaling in vaccinia virus are expressed early during infection to block the rapid immune response elicited after detection of virus infection (5, 54). Our data indicate that an additional inhibitor of NF-κB activation is required late in infection to continue persistent suppression of NF-κB signaling.

In an attempt to identify the late protein(s) responsible for inhibition of IκBα degradation during VV811 infection, we immunoprecipitated IκBα from VV811 infected cells and identified interacting partners by mass spectrometry. Since a viral protein does not appear to displace IκBα from the p65-p50 transcription factor (Figure 2-13), it was possible that a viral protein interacted with IκBα to inhibit its degradation directly while not disrupting the IκBα-p65-p50 complex. IκBα associated with p65, as expected, and, interestingly, IκBα also associated with c-Rel during VV811 infection (Figure 2-17, Table 2-2). Since the NF-κB dimer c-Rel-p50 is also involved in the classical NF-κB pathway and is held in the cytoplasm by IκBα, our data suggest that other IκBα-NF-κB

inhibitory complexes are not disrupted during vaccinia virus infection (67). Therefore, further examination of the effects of vaccinia virus infection on other NF-κB dimers, besides the typical p65-p50 dimer, is warranted. An unexpected interacting partner of IκBα was the dead box protein DDX3 (Table 2-2). This protein stood out in particular due to the recent finding that the vaccinia virus protein K7R inhibits TLR/IL-1R-mediated NF-κB activation but also interacts with DDX3 leading to interruption of interferon regulatory factor signaling and interferon-β production (53). Furthermore, no interaction between IκBα and DDX3 has been reported, and K7R is missing in VV811 (46). The interaction between IκBα and DDX3 may also provide an additional point of cross-talk between NF-κB and interferon signaling, which are tightly linked anti-viral responses (20, 27). Additionally, IκBα interacted with heat shock protein 70 (hsp70) (Table 2-2). Reports have demonstrated that over-expression of hsp70 suppresses NF-κB signaling through interaction of hsp70 with the IKK complex (51). Further examination of the interaction between hsp70 and IκBα during vaccinia virus infection is warranted to elucidate if hsp70 contributes to inhibition of IκBα degradation. Significantly, although multiple cellular proteins interacted with IκBα, no vaccinia virus proteins were associated with IκBα during infection (Table 2-2). This indicated that mass spectrometry was unable to identify vaccinia virus proteins that interacted with IκBα, however, this experiment requires repetition. Alternatively, this suggests that the protein responsible for

inhibition of IκBα degradation in VV811 is acting indirectly, without interacting with IκBα.

3.6 Possible inhibitors of the NF-κB pathway present in VV811

It is possible that the inhibition of IκBα degradation is an indirect effect of viral exploitation of another cellular pathway. The best example of this is the role of vpu (viral protein U) in HIV-1. Vpu is responsible for directing degradation of CD4 during HIV-1 infection (37, 73). Vpu binds the F-box protein βTrCP, and, instead of acting as a substrate like other proteins that interact with βTrCP, vpu is not degraded, but binds CD4 and mediates CD4 polyubiquitination by $SCF^{\beta TrCP}$ to lead to the degradation of CD4 in the ER (37). However, vpu competitively binds βTrCP, significantly reducing the degradation of cellular proteins normally targeted by $SCF^{\beta TrCP}$, demonstrated by the detection of an accumulation of β-catenin and phospho-IκBα (10, 12). Since our data indicates an accumulation of phospho-IκBα in VV811 infected cells after TNFα stimulation, it is plausible that a vaccinia virus protein may be sequestering or redirecting the $SCF^{\beta TrCP}$ ubiquitin ligase during vaccinia virus infection, in a manner similar to vpu. This would prevent polyubiquitination of phosphorylated IκBα and subsequent IκBα degradation.

We gained insight into the possibility that $SCF^{\beta TrCP}$ was sequestered during VV811 infection when we narrowed down the list of possible NF-κB

inhibitors present in VV811. Since the mutant vaccinia virus MVA induces NF-κB activation upon infection, we determined which open reading frames were present in VV811, but absent in MVA, to produce a list of possible NF-κB inhibitors present in VV811 (Figure 3-1, Table 3-1) (4, 45, 46). Intriguingly, we identified two proteins, A55R and B4R, which have orthologs in ectromelia virus that interact with ubiquitin ligases during infection and are present in VV811 but absent in MVA (4, 46, 68, 74). The ortholog of A55R in ectromelia virus, ECTV150, is a BTB-Kelch protein that interacts with the functional cullin-3 based ubiquitin ligase complex during infection (74). The ortholog of B4R in ectromelia virus, ECTV154, contains ankyrin repeats and an F-box domain and interacts with the cullin-1 based SCF complex during infection (68). Preliminary data indicate that both ECTV150 and ECTV154 inhibit p65 nuclear translocation (K. Fagan-Garcia, Q. Wang, N. van Buuren, K. Burles, M. Barry, unpublished data). Interestingly, data in our lab by Qian Wang indicated that ECTV150 was unable to inhibit IκBα degradation (Qian Wang and M. Barry, unpublished data). Therefore, A55R is most likely not the protein in VV811 responsible for the inhibition of IκBα degradation. Significantly, ECTV154 inhibited TNFα-induced IκBα degradation, suggesting that the ortholog of ECTV154 in vaccinia virus, B4R, which is present in VV811, potentially plays a role in inhibition of IκBα degradation (N. van Buuren, K. Burles, M. Barry, unpublished data). Overall, these results imply that A55R and B4R, which are both present in VV811, inhibit TNFα-induced NF-κB activation. It remains to be determined what the substrates

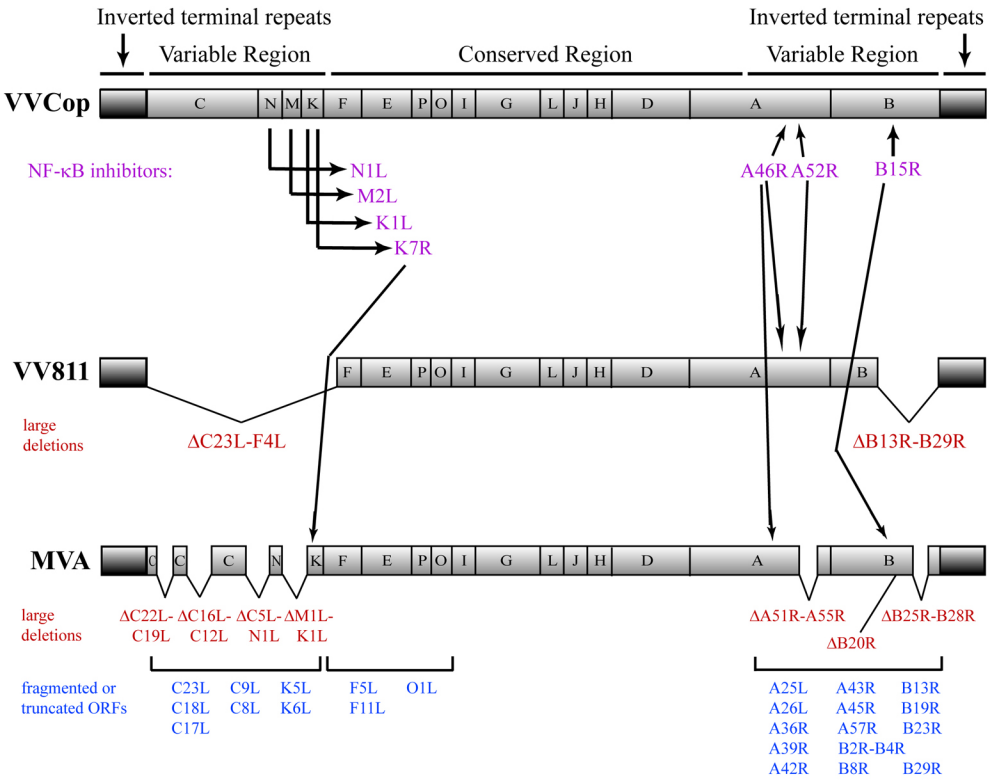

Figure 3-1. Comparison of the mutant vaccinia viruses VV811 and MVA. Schematics of wild-type vaccinia virus Copenhagen strain (VVCop), the mutant vaccinia virus VV811, and the mutant vaccinia virus modified vaccinia strain Ankara (MVA) are represented. The letters correspond to the *Hind*III restriction digest map of VVCop (DeFilippes, 1982). Locations of the known NF-κB inhibitors are indicated in purple. Large deletions in VV811 and MVA are noted in red. Fragmented and truncated open reading frames in MVA are noted in blue.

Table 3-1: Potential inhibitors of NF-κB: open reading frames present in VV811 and absent in MVA

ORF	Characteristics	Putative functions
F11L	Early gene, binds RhoA	Inhibits RhoA signaling, involved in morphogenesis and cell motility; involved in viral transcription elongation (30, 66)
F5L	Early gene, membrane protein	Major membrane protein (50)
O1L	Early gene, contains leucine zipper and nuclear target sequence	Unknown (22)
A25R	Pseudogene	Cowpox A-type inclusion protein
A26L	Late gene, present on IMV	Structural protein, involved in IMV movement within the cell, may be involved in morphogenesis and dissemination (15, 38)
A39R	Late gene, semaphorin-like	Secreted glycoprotein, induces IL-6 and IL-8 secretion, immune modulator (16)
A40R	Early gene, C-type lectin-like type II membrane protein	Unknown (72)
A42R	Late gene, profilin homolog, present in IMV	Unknown, possibly involved in phosphoinositide metabolism (11, 15, 36, 56)
A43R	Late gene, type I transmembrane protein, localizes to golgi and plasma membranes	Unknown, reduced size of skin lesions in mice infected with A43R knockout (57)
A45R	Late gene, superoxide dismutase homolog, present in IMV core	Unknown, inactive Cu-Zn superoxide dismutase (3, 56)
A51R	Unknown	Unknown
A53R	Gene fragment, secreted TNFR-like protein	Non-functional TNFR homolog (1)
A54R	Early gene	Unknown
A55R	Early gene, BTB-Kelch protein, interacts with functional cullin-3 based ubiquitin ligase	Unknown (74)
A57R	Early/late gene, guanylate kinase homolog	Unknown (56)
B2R	Early gene, schlafen homolog	Unknown, may be involved in regulation of virus virulence (24)
B4R	Late gene, ankyrin-F-box protein, interacts with functional cullin-1 based ubiquitin ligase	Unknown (68)
B8R	Early gene, interferon γ receptor homolog	Secreted interferon γ receptor (2)

RhoA, Ras homolog gene family member A; IMV, intracellular mature virus; TNFR, tumor necrosis factor receptor; BTB, Bric-a-Brac, Tramtrack, Broad complex. Additional references for all information presented in this table: Assarsson, 2008; www.poxvirus.org

of A55R and B4R are, and if A55R and B4R inhibit NF-κB signaling during vaccinia virus infection.

The question remains, what protein in VV811 is responsible for the observed suppression of IκBα degradation late during infection? The ortholog of B4R in ectromelia virus, ECTV154, inhibited IκBα degradation (N. van Buuren and M. Barry, unpublished data). B4R was reported to be a late gene product, however, in the same study, B4R transcription was not suppressed by AraC expression (5). Therefore, B4R expression may be responsible for the retained amount of IκBα observed after 12 hours of VV811 infection in the presence of AraC and treatment with TNFα (Figures 3-14, 3-15). The generation of a VV811 mutant devoid of B4R could answer this question in the future.

3.7 The evolution of NF-κB inhibition by vaccinia virus

The apparent question that arises is why does vaccinia virus express so many inhibitors of the NF-κB pathway? NF-κB signaling is a pivotal regulator of the immune response to infection (29, 67). Poxviruses most likely trigger the NF-κB response through activation of pattern recognition receptors by viral DNA and dsRNA rapidly upon infection (64). Poxviruses must therefore suppress NF-κB signaling to promote a successful infection (41). Poxviruses also possess large genomes that encode an average of approximately 200 proteins, and therefore have the ability to produce multiple proteins that target the same pathway to effectively inhibit the anti-viral response (42, 54). Due to the importance of NF-

κB signaling in the immune response and the large genomes of poxviruses, poxviruses express multiple proteins that inhibit NF-κB.

NF-κB signaling is an ancient immune response to infection. A highly conserved NF-κB pathway has been thoroughly studied in *Drosophila melanogaster*, and recently it was found that the NF-κB pathway exists in the horseshoe crab, an arthropod that has existed for approximately 550 million years (40, 69, 70). Homologs to the NF-κB transcription factor and IκBα are found in *Drosophila* as well as the horseshoe crab (40, 69, 70). This discovery indicates that the NF-κB pathway has been a key player in the immune response for millions of years, further implying that IκBα is an extremely important regulator of the NF-κB pathway. We speculate that the NF-κB pathway may have been one of the first immune responses encountered by poxviruses and thus poxviruses have developed multiple mechanisms to overcome this immune response to infect a host. In corroboration of the ancient necessity for NF-κB inhibition, all of the known inhibitors of NF-κB expressed by vaccinia virus are highly conserved among all sequenced members of the *Orthopoxviru*s genus and all orthologs share greater than 90% amino acid sequence identity (www.poxvirus.org). Exceptions, however, are N1L, M2L and K7R which do not possess orthologs in ectromelia virus (www.poxvirus.org). Additional orthologs of vaccinia virus NF-κB inhibitors are present in other poxviruses including members of the *Avipoxvirus*, *Yatapoxvirus*, *Leporipoxvirus*, *Capripoxvirus* and *Suipoxvirus* genera with amino acid sequence identities between 24 and 50% (www.poxvirus.org). The vital

importance of NF-κB signaling in the immune response combined with millions of years of co-evolution of poxviruses and the NF-κB pathway, had led to the development of multiple strategies utilized by poxviruses to effectively suppress NF-κB signaling.

3.8 Conclusions

Overall, this study has demonstrated the existence of an additional inhibitor of TNFα-induced NF-κB signaling expressed by the large deletion vaccinia virus VV811. The mechanism of NF-κB suppression by VV811 involves the inhibition of IκBα degradation and accumulation of phosphorylated IκBα and is mediated by a protein or proteins expressed late during vaccinia virus infection. The future elucidation of the identity of the responsible protein(s) will provide further insight into the complex manipulation of the immune response by vaccinia virus.

3.9 References

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