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PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA**

**CARACTERIZAÇÃO DE GENES DO VÍRUS DO
ECTIMA CONTAGIOSO ENVOLVIDOS NA
REGULAÇÃO DA VIA DE SINALIZAÇÃO DO NF- κ B**

TESE DE DOUTORADO

Diego Gustavo Diel

**Santa Maria, RS, Brasil
2010**

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CONTAGIOSO ENVOLVIDOS NA REGULAÇÃO DA VIA DE
SINALIZAÇÃO DO NF- κ B**

Diego Gustavo Diel

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Elaborada por
Diego Gustavo Diel

Como requisito parcial para obtenção do grau de
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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

CARACTERIZAÇÃO DE GENES DO VÍRUS DO ECTIMA CONTAGIOSO ENVOLVIDOS NA REGULAÇÃO DA VIA DE SINALIZAÇÃO DO NF- κ B

AUTOR: DIEGO GUSTAVO DIEL
ORIENTADOR: EDUARDO FURTADO FLORES
Santa Maria, 15 de dezembro de 2010.

O vírus da orf (ORFV), protótipo do gênero *Parapoxvirus* da família *Poxviridae*, é o agente etiológico da orf ou ectima contagioso, uma enfermidade contagiosa de distribuição mundial que afeta primariamente ovinos e caprinos. O genoma do ORFV consiste de uma molécula de DNA de fita dupla com aproximadamente 138 Kb, que contém presumidamente 131 genes. Dentre estes, 15 são genes novos, identificados apenas nos parapoxvírus e que não possuem homologia com outros genes de origem viral ou celular. O presente estudo descreve a caracterização funcional de três destes genes, *ORFV024*, *ORFV002* e *ORFV121*. Os resultados apresentados no presente estudo demonstram que as proteínas codificadas pelos genes *ORFV024*, *ORFV002* e *ORFV121* inibem a ativação da via de sinalização do fator de transcrição nuclear-kappa B (NF- κ B). O produto da *ORFV024* bloqueia a ativação da via do NF- κ B no citoplasma celular, inibindo a fosforilação das quinases I κ B (IKK), IKK α e IKK β e, consequentemente inibindo a ativação do complexo IKK. A deleção do gene *ORFV024* do genoma do ORFV não alterou a severidade, a progressão, ou o tempo de resolução das lesões produzidas pelo ORFV em ovinos, indicando que o produto deste gene não contribui para a virulência do vírus. O gene *ORFV002* codifica um inibidor do NF- κ B que atua no núcleo das células. O produto do *ORFV002* interage com a subunidade NF- κ B-p65 do NF- κ B, inibindo a sua acetilação, uma modificação pós-traducional do NF- κ B-p65 mediada pela acetiltransferase p300 que regula a sua atividade transcricional. Semelhante ao *ORFV024*, a deleção do gene *ORFV002* do genoma do ORFV não afetou a virulência do vírus nem alterou a patogenia da enfermidade em ovinos. O produto do gene *ORFV121* atua no citoplasma das células, onde esta proteína viral interage com o NF- κ B-p65 inibindo sua fosforilação e translocação nuclear. A deleção do gene *ORFV121* do genoma do ORFV reduziu significativamente a severidade, a progressão e o tempo de resolução da doença em ovinos, indicando que este produto viral constitui-se em um fator de virulência para o ORFV em seu hospedeiro natural. Estes resultados demonstram que, assim como outros poxvírus, o ORFV

também desenvolveu múltiplas estratégias para modular a via de sinalização do NF- κ B, codificando proteínas que atuam em diferentes eventos desta complexa via de sinalização intracelular. Os resultados obtidos nos estudos de patogenia sugerem que os inibidores do NF- κ B codificados pelo ORFV desempenham funções complementares e/ou redundantes, provavelmente, para promover um bloqueio eficiente dos processos biológicos regulados pelo NF- κ B. Além disso, estes produtos virais podem modular diferentes processos biológicos controlados pelo NF- κ B *in vivo*. Um melhor entendimento das interações do ORFV com o seu hospedeiro pode favorecer o desenvolvimento de vacinas mais eficazes para o ectima contagioso, ou ainda, promover o desenvolvimento de vacinas vetoriais ou imunoterápicos, baseados no ORFV, mais eficazes e com uma maior espectro de aplicações.

Palavras-chave: Orf, ORFV, *Parapoxvirus*, Poxvírus, NF- κ B.

ABSTRACT

Doctorate's Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

CHARACTERIZATION OF ORF VIRUS-ENCODED GENES INVOLVED IN THE REGULATION OF THE NF- κ B SIGNALING PATHWAY

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Santa Maria, December, 15th, 2010.

Orf virus (ORFV), the type member of the genus *Parapoxvirus* of the family *Poxviridae*, is the etiologic agent of orf or contagious ecthyma, a contagious and ubiquitous disease of sheep and goats. ORFV genome consists of a double stranded DNA molecule with approximately 138 Kb, and contains 131 putative genes. Among those, 15 are novel genes, unique to parapoxviruses, which lack homology to other known viral or cellular genes. In the present study we describe the functional characterization of three of these genes, *ORFV024*, *ORFV002*, and *ORFV121*. Results presented here demonstrate that the proteins encoded by these genes inhibit the activation of the nuclear factor-kappa B (NF- κ B) signaling pathway. ORFV-encoded *ORFV024* inhibits activation of the NF- κ B signaling pathway in the cell cytoplasm by inhibiting phosphorylation of the I κ B kinases, IKK α and IKK β , consequently inhibiting the activation of the IKK complex. Deletion of *ORFV024* from the ORFV genome had no significant effect on disease severity, progression or time to resolution in sheep, indicating that *ORFV024* does not contribute to ORFV virulence. ORFV-encoded *ORFV002* functions in the cell nucleus, where it interacts with the NF- κ B subunit NF- κ B-p65, inhibiting its acetylation, a p300-mediated modification of NF- κ B-p65 which modulates its transcriptional activity. Similarly to *ORFV024*, deletion of *ORFV002* from the ORFV genome had no significant effect on ORFV virulence and disease pathogenesis in sheep. ORFV-encoded *ORFV121* functions in the cell cytoplasm, where it binds to and inhibits phosphorylation and nuclear translocation of NF- κ B-p65. Deletion of *ORFV121* from the ORFV genome resulted in a marked attenuated disease phenotype in sheep, indicating that *ORFV121* is a determinant of virulence of ORFV in the natural host. These results indicate that ORFV, like other poxviruses, has evolved multiple strategies to modulate NF- κ B, targeting different steps of the signaling pathway. Results obtained in the pathogenesis studies performed here suggest that multiple NF- κ B inhibitors encoded by ORFV may exert complementary and/or redundant functions to effectively block host cell responses regulated

by the NF- κ B signaling pathway. Additionally, it is possible that ORFV-encoded NF- κ B inhibitors modulate distinct cellular processes regulated by NF- κ B *in vivo*. A better understanding of ORFV-host interactions may provide valuable insights for the development of improved vaccines against orf, or yet for the development of novel ORFV-based therapeutic agents and vaccine vectors with enhanced safety and efficacy, and a broader applicability.

Key words: Orf, ORFV, *Parapoxvirus*, Poxvirus, NF- κ B.

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LISTA DE ABREVIATURAS

AKT	Proteína quinase B
ASFV	Vírus da peste suína africana
BAFF	Fator de ativação de células B
Bcl-2	Linfoma de células B 2
BPSV	Vírus da estomatite papular bovina
Brd4	Proteína condendo bromo domínios 4
CARDIF	Fator inibidor do CARD
CBP	Proteína de ligação a quemoquinas
CD4	Cluster de diferenciação 4
CCL20	Ligante de quemoquinas CC 20
CD8	Cluster de diferenciação 8
CD40	Cluster de diferenciação 40
CKII	Caseína quinase II
CPE	Efeito citopático
CPXV	Vírus do <i>cowpox</i>
CXCL3	Ligante de quemoquinas CXC 3
DEPC	Dietilpirocarbonato
DMEM	Meio essencial mínimo modificaco por Dulbecco
DNA	Ácido desoxirribonucleico
cDNA	Ácido desorribonucleico complementar
dsRNA	Ácido ribonucleico de cadeia dupla
ERK	Proteína quinase regulada por sinal extracelular
FBS	Soro fetal bovino
FADD	Domínio de morte associado ao FAS
GAPDH	Gliceraldeido-3-fostato desidrogenase
GFP	<i>Green fluorescent protein</i>
GIF	Fator inibidor do GMC-SF e IL-2
GMC-SF	Fator estimulador de colônia granulocítica e macrofágica
GSK-3 β	Proteína quinase glicogênio sintetase 3 beta
HDAC3	Histona deacetilase 3
HSP90	Proteína de choque térmico 90
ICAM-1	Molécula de adesão intercelular 1
IFN- γ	Interferon gama
IgG2	Imunoglobulina G tipo 2
I κ B α	Inibidor kappa B alfa
I κ B β	Inibidor kappa B beta
I κ B ϵ	Inibidor kappa B epsilon
IKK	Complexo de quinases da molécula inibitória kappa B
IKK α	Proteína quinase da molécula inibitória kappa B alfa
IKK β	Proteína quinase da molécula inibitória kappa B beta
IL-1 α	Interleucina 1 alfa
IL-1 β	Interleucina 1 beta
IL-2	Interleucina 2
IL-6	Interleucina 6
IL-8	Interleucina 8
IL-10	Interleucina 10
IL-18	Interleucina 18
IRF-1	Fator regulador de interferon 1

ITR	Regiões repetidas invertidas
LPS	Lipopolissacarídeo
LT β	Linfotoxina beta
MEM	Meio essencial mínimo
MEK	Proteína quinase ativada por mitógeno
MCP-1	Proteína quimiotática de monócitos 1
MIP-1 α	Proteína macrofágica inflamatória 1 alfa
MIP-1 β	Proteína macrofágica inflamatória 1 beta
MOCV	Vírus do molusco contagioso
MOI	Multiplicidade de infecção
MSK1	Proteína quinase ativada por mitógenos e stress 1
mRNA	Ácido ribonucléico mensageiro
mTOR	Alvo da rapamicina de mamíferos
NAK	Proteína quinase ativadora do fator de transcrição nuclear kappa B
NEMO	Modulador essencial do fator de transcrição nuclear kappa B
NF- κ B	Fator de transcrição nuclear kappa B
NF- κ B-p50	Fator de transcrição nuclear kappa B subunidade p50
NF- κ B-p52	Fator de transcrição nuclear kappa B subunidade p52
NF- κ B-p65	Fator de transcrição nuclear kappa B subunidade p65
NF- κ B-p100	Fator de transcrição nuclear kappa B subunidade p100
NF- κ B-p105	Fator de transcrição nuclear kappa B subunidade p105
NP-1	Neutrofilina 1
OFTu	Turbinado fetal ovino
OVINFR	Proteína de resistência ao interferon do vírus da orf
OV-IL-10	Interleucina 10 do vírus da orf
ORFV	Vírus da orf ou vírus do ectima contagioso
ORF	Região aberta de leitura
PCPV	Vírus da Pseudovariola
PCR	Reação em cadeia da polimerase
PDK1	Proteína quinase dependente de fosfoinosítido 1
PI-3K	Proteína quinase fosfatidilinositol 3
PKAc	Proteína quinase A
PKC	Proteína quinase C
PKR	Proteína quinase dependente de ácido ribonucléico
PIGF	Fator de crescimento placentar
PPV	Parapoxvírus
PTGS2	Prostaglandina endoperóxido sintetase 2
RANTES	Regulado após ativação, expressão normal por células T, e Secretado
RNA	Ácido ribonucléico
RIP	Proteína de interação com o receptor
RSK1	Proteína quinase ribossomal S6 1
SDS-PAGE	Dodecil sulfato de sódio-eletroforese em gel de poliacrilamida
TCID ₅₀	Dose infectante para cultivo celular 50
TLR	Receptor do tipo Toll
TNF- α	Fator de necrose tumoral alfa
TNFr	Receptor para o fator de necrose tumoral
TRADD	Domínio de morte associada ao receptor do fator de necrose tumoral
TRAF2	Fator associado ao receptor fator de necrose tumoral 2
USDA	Departamento de agricultura dos Estados Unidos
VACV	Vírus vaccinia

VEGF	Fator de crescimento do endotélio vascular
VEGF-A	Fator de crescimento do endotélio vascular A
VEGF-B	Fator de crescimento do endotélio vascular B
VEGF-C	Fator de crescimento do endotélio vascular C
VEGF-E	Fator de crescimento do endotélio vascular E
VEGF-F	Fator de crescimento do endotélio vascular F
VEGFR-1	Receptor do fator de crescimento do endotélio vascular 1
VEGFR-2	Receptor do fator de crescimento do endotélio vascular 2
VEGFR-3	Receptor do fator de crescimento do endotélio vascular 3

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1. INTRODUÇÃO

O vírus da Orf (ORFV), protótipo do gênero *Parapoxvirus* da família *Poxviridae*, é o agente etiológico do ectima contagioso, uma enfermidade contagiosa que afeta principalmente ovinos e caprinos (HAIG & MERCER, 1998; CANAL, 2007). A enfermidade é caracterizada por lesões muco-cutâneas que afetam a pele ao redor da boca, focinho, tetas e a mucosa oral (HAIG, 2006; CANAL, 2007; FLEMING & MERCER, 2007). A infecção pelo ORFV ocorre através de abrasões na pele ou mucosa oral. As lesões progridem pelos estágios de eritema, vesícula, pústula, pápula, e crostas e são restritas às áreas ao redor dos sítios de entrada do vírus (HAIG & MERCER, 1998).

O ORFV afeta primariamente animais com menos de um ano de idade. A mortalidade resultante da infecção é baixa, mas a morbidade é alta podendo atingir até 90% dos animais do rebanho. A disseminação do vírus é rápida e ocorre pelo contato com animais infectados ou com crostas eliminadas no ambiente (FLEMING & MERCER, 2007). O ORFV tem potencial zoonótico, e pode afetar pessoas que entram em contato com animais infectados (ERICKSON et al., 1975; MOURTADA et al., 2000).

O genoma do ORFV consiste de uma molécula de DNA linear de fita dupla com aproximadamente 138 Kb que contém presumidamente 131 genes (DELHON et al., 2004; MERCER et al., 2006). O genoma do ORFV é organizado em uma região central, conservada em outros poxvírus, flanqueada por regiões terminais variáveis (MOSS, 2007; DELHON et al., 2004; MERCER et al., 2006). O ORFV codifica 15 genes, localizados principalmente nas regiões terminais variáveis do genoma, que não possuem homologia com outros genes de origem celular ou viral (DELHON et al., 2004). A função da maioria desses genes, únicos dos *Parapoxvirus*, ainda não foi determinada. Recentemente, o produto de um destes genes, a ORFV125 foi caracterizada como um fator anti-apoptótico semelhante ao Bcl-2 (WESTPHAL et al., 2007; WESTPHAL et al., 2009).

Os poxvírus são conhecidos por modular vários aspectos das defesas antivirais do hospedeiro. Estes vírus codificam diversos fatores imunomodulatórios que agem em diferentes vias de sinalização celular. As vias de sinalização celular alvo de regulação pelos poxvírus incluem a via dos interferons, interleucina-1 β (IL-1 β), IL-18, IL-10, fator de necrose tumoral (TNF), quemoquinas, serpinas, complemento, semaforinas, apoptose e a via de sinalização do fator de transcrição nuclear-kappa B (NF- κ B) (ALCAMI, 2003).

A via de sinalização do NF- κ B é composta por uma família de fatores de transcrição que participam da regulação de diversos processos biológicos, incluindo resposta imune inata

e inflamatória, ciclo celular, proliferação e diferenciação celular e apoptose (LI & VERMA, 2002). A função do NF- κ B é regulada por uma família de proteínas inibidoras, conhecidas como inibidores-kappa B (I κ B) (BALDWIN, 1996), que atuam bloqueando a translocação nuclear dos NF- κ Bs e a sua ligação ao DNA (SUN & XIAO, 2003). A via do NF- κ B pode ser ativada por uma grande variedade de estímulos, incluindo citocinas inflamatórias, fatores de crescimento, agentes que causam dano ao DNA celular, componentes bacterianos e proteínas virais (PAHL, 1999). Um passo fundamental na ativação do NF- κ B é a fosforilação e degradação do I κ B, resultando na liberação do NF- κ B no citoplasma e sua subsequente translocação para o núcleo, onde irá ativar a transcrição dos genes alvo (SUN & XIAO, 2003).

Os poxvírus desenvolveram várias estratégias para regular a via do NF- κ B (MOHAMED & McFADDEN, 2009) e inibidores do NF- κ B já foram identificados em poxvírus dos generos *Orthopoxvirus*, *Leporipoxvirus*, *Yatapoxvirus*, e *Molluscipoxvirus*, com vírus de uma espécie geralmente codificando múltiplos inibidores (MOHAMED & McFADDEN, 2009). Por exemplo, o vírus vaccinia (VACV), protótipo da família *Poxviridae*, codifica pelo menos sete inibidores do NF- κ B (A52R, A46R, B14, N1L, M2L, K1L e E3L) (BOWIE et al., 2000; DiPERNA et al., 2004; SHISLER & JIN, 2004; GEDEY et al., 2006; CHEN et al., 2008; MYSKIW et al., 2009), o que sugere que a inibição desta via é importante para o estabelecimento das infecções pelos poxvírus. Com a exceção de um homólogo do gene *E3L* do VACV, os parapoxvírus não codificam nenhum inibidor do NF- κ B identificado em outros poxvírus, sugerindo que estes vírus desenvolveram novas estratégias (proteínas e/ou mecanismos de ação) para modular a via de sinalização do NF- κ B. No presente estudo, a análise da expressão gênica de células primárias de ovino em resposta a infecção com o ORFV cepa IA82 ou com seis vírus mutantes contendo deleções de genes únicos dos parapoxvírus, utilizando-se a técnica de *Microarray*, permitiu a identificação de três genes, *ORFV024*, *ORFV002* e *ORFV121*, que inibem a transcrição mediada pelo NF- κ B.

2. REVISÃO BIBLIOGRÁFICA

2.1 Orf: características gerais do vírus (ORFV) e da infecção

Orf ou ectima contagioso é uma enfermidade localizada e debilitante que acomete principalmente ovinos e caprinos. Eventualmente, pequenos ruminantes silvestres e humanos também podem ser afetados (HAIG & MERCER, 1998; CANAL, 2007). O ectima contagioso é causado pelo *Parapoxvirus* (PPV) vírus da Orf (ORFV), pertencente à subfamília *Chordopoxvirinae* da família *Poxviridae*. Outros PPVs de importância veterinária incluem o vírus da pseudovariola (PCPV) e o vírus da estomatite papular bovina (BPSV) que produzem lesões cutâneas benignas em bovinos (HAIG, 2006). Dentre os membros do gênero *Parapoxvirus*, o ORFV é responsável pelos problemas mais graves em animais de interesse econômico, principalmente em casos complicados por infecções bacterianas secundárias (BUTTNER & RZIHA, 2002).

O ORFV possui distribuição mundial, e é endêmico na maioria dos países que possuem rebanhos ovinos e caprinos (HOSAMANI et al., 2009). Estudos sorológicos sobre a prevalência da infecção pelo ORFV são escassos. Entretanto, alguns estudos realizados na Arábia Saudita e na Turquia revelaram uma prevalência de 60 e 52,8%, respectivamente (HOUSAWI et al., 1992; GÖKCE et al., 2005). No Brasil, surtos da infecção acompanhados de isolamento do vírus têm sido frequentemente descritos (MAZUR & MACHADO, 1990; NÓBREGA et al., 2008; MACÊDO et al., 2008; ABRAHÃO et al., 2009), indicando a circulação do vírus nos rebanhos brasileiros.

A infecção pelo ORFV ocorre principalmente em ovinos e caprinos, e localiza-se na região ao redor da boca e das narinas (HAIG, 2006). A enfermidade afeta primariamente animais com menos de um ano de idade, sendo mais frequentemente observada logo após o nascimento e entre os três e quatro meses de idade, causando uma condição debilitante por diminuir a ingestão de alimentos. No entanto, animais adultos também podem ser acometidos (FLEMING & MERCER, 2007).

A mortalidade resultante da infecção é relativamente baixa, mas a morbidade é alta, podendo atingir mais de 90% dos animais do rebanho. A disseminação do vírus entre os animais do rebanho é rápida e pode ocorrer pelo contato direto com animais infectados, pelo contato com as crostas das lesões eliminadas no solo ou pelo contato com alimentos e pasto contaminados (FLEMING & MERCER, 2007). Animais lactentes também podem transmitir o vírus para as tetas e úbere das mães durante a amamentação, acarretando no

desenvolvimento de lesões e, conseqüentemente, na transmissão do vírus para outros animais (FLEMING & MERCER, 2007).

As lesões causadas pelo ORFV são geralmente restritas aos sítios de entrada do vírus, incluindo o epitélio dos lábios, narinas e mucosa oral. Após penetrar por abrasões na pele, na junção mucocutânea dos lábios ou na mucosa oral (FLEMING & MERCER, 2007), o vírus replica em queratinócitos da epiderme (HAIG et al., 1997). O período de incubação varia entre dois e seis dias, e as lesões progridem através dos estágios de eritema, vesícula, pústula, pápula e crostas (HAIG, 2006; CANAL, 2007; FLEMING & MERCER, 2007). O curso clínico inicia com hiperemia e edema em torno do sítio de infecção e desenvolvimento de pequenas vesículas em aproximadamente 48-72 horas. As vesículas progridem para os estágios de pústulas e pápulas. Lesões adjacentes podem coalescer e com a progressão da doença culminam com a formação de crostas (BUTTNER & RZIHA, 2002; CANAL, 2007; FLEMING & MERCER 2007). Em infecções primárias, as lesões resolvem em quatro a seis semanas, enquanto que em reinfecções o período de resolução normalmente é mais curto, ocorrendo em duas ou três semanas (HAIG et al., 1997; HAIG, 2006; FLEMING & MERCER, 2007). Até o presente momento, não existe evidência de disseminação sistêmica do ORFV (HAIG, 2006).

O exame histológico das lesões em casos de infecção natural e experimental com o ORFV, revela uma lesão marcadamente proliferativa na epiderme, caracterizada por proliferação, vacuolização e edema de queratinócitos do estrato espinhoso, degeneração reticular, acúmulo de crostas e presença de micro abscessos (HAIG et al., 1997). A derme apresenta lesões como edema, dilatação capilar pronunciada e infiltração de células inflamatórias. Além disso, lesões papilomatosas que consistem de hiperplasia pseudo-epiteliomatosa e formação de granulomas são frequentemente observadas e, em alguns casos, podem ser extensas (FLEMING & MERCER, 2007).

2.2 O genoma do ORFV

O genoma do ORFV consiste de uma molécula de DNA linear de fita dupla com aproximadamente 138 Kb (MOSS, 2007; DELHON et al., 2004; HAIG, 2006), conteúdo médio de G + C de 64%, que contém presumidamente 131 genes (DELHON et al., 2004; MERCER et al., 2006). As duas cadeias de DNA que compõe o genoma são unidas por *loops* em *hairpin* (MOSS, 2007). Semelhante aos outros poxvírus, o genoma do ORFV é composto por uma região codificante central flanqueada por duas regiões de sequências repetidas invertidas (*ITRs*), que são idênticas mas opostamente orientadas nas duas extremidades do

genoma (MOSS, 2007; DELHON et al., 2004). As *ITRs* do genoma dos poxvírus incluem: as regiões em *loop*, ricas em A + T e não complementares entre si; uma região de 100 pb altamente conservada que contém sequências requeridas para a resolução das formas concateméricas de replicação do genoma; variável número de sequências repetidas em *tandem*; e até sete regiões abertas de leitura (ORFs) (MOSS, 2007). As *ITRs* do ORFV contém 3,9 Kb e apenas um gene (*ORFV001*) é completamente localizado nas *ITRs*, enquanto que o gene *ORFV002* inicia na região central única e termina nas *ITRs* (DELHON et al, 2004).

O genoma dos poxvírus, incluindo o do ORFV, é organizado em uma região central conservada, flanqueada por regiões terminais variáveis (MOSS, 2007; DELHON et al., 2004; MERCER et al., 2006). A região central do genoma contém genes homólogos e conservados entre os poxvírus (*ORFV009* à *ORFV111*), e os produtos desses genes participam de mecanismos básicos de replicação, estrutura e morfogênese (MOSS, 2007; DELHON et al., 2004; MERCER et al., 2006). Em contrapartida, as regiões terminais variáveis do genoma (*ORF001* à *ORFV008* e *ORFV112* à *ORFV134*) representam aproximadamente 20% do genoma e codificam produtos potencialmente envolvidos na patogenia. Estes, incluem genes com potencial papel na determinação do espectro de hospedeiros (proteínas com sequências repetidas de ankirina: *ORF008*, *ORFV123*, *ORFV126*, *ORFV128* e *ORFV129*), evasão (*ORFV127*) e modulação da resposta imune (*ORFV117*), resposta anti-apoptótica (*ORFV125*) e virulência (*ORFV132*) (FLEMING et al., 1997; DEANE et al., 2000; DELHON et al., 2004; MERCER et al., 2006; WESTPHAL et al., 2007).

O genoma do ORFV contém 15 genes, localizados principalmente nas regiões terminais variáveis do genoma, que não possuem homologia com outros genes de origem celular ou viral. Dentre estes, 13 genes (*ORFV001*, *ORFV005*, *ORFV012*, *ORFV013*, *ORFV024*, *ORFV073*, *ORFV113*, *ORFV115*, *ORFV116*, *ORFV119*, *ORFV120*, *ORFV121*, e *ORFV124*) são também encontrados no genoma de outro membro do gênero *Parapoxvirus*, o BPSV, com identidade de aminoácidos variando de 29 a 64%, enquanto que os outros dois genes (*ORF002* e *ORFV118*) estão presentes apenas no genoma do ORFV (DELHON et al., 2004). A função desses genes, únicos dos *Parapoxvirus*, ainda não foi determinada.

2.3 O ciclo replicativo

Os poxvírus constituem-se em uma exceção entre os vírus DNA, uma vez que o processo replicativo ocorre no citoplasma das células infectadas (MOSS, 2007; SCHRAMM & LOCKER, 2005). Os poxvírus codificam todas as enzimas necessárias para a transcrição e

replicação do genoma viral, e empacotam nos vírions as enzimas essenciais para a produção e modificação dos RNAs mensageiros (mRNA) para a síntese de suas proteínas no início do ciclo replicativo (CANAL, 2007).

O processo de expressão gênica dos poxvírus é caracterizado, classicamente, pela transcrição temporal de três classes de genes: *early*, *intermediate* e *late* (MOSS, 2007; CANAL, 2007). A transcrição de cada classe de genes requer a presença de fatores de transcrição específicos, que são produzidos pela expressão dos genes da classe precedente (CANAL, 2007).

Após a fusão do envelope do vírion com a membrana plasmática ou após a endocitose, o núcleo viral é liberado no citoplasma e a expressão gênica é iniciada com a transcrição dos genes da classe *early* ainda no interior do núcleo viral (MOSS, 2007). Os mRNAs transcritos no interior do núcleo viral são liberados no citoplasma para serem traduzidos. A maioria dos genes transcritos na fase *early* participa dos processos de desnudamento e replicação do DNA, virulência, evasão do sistema imune e na transcrição dos genes das classes *intermediate* e *late* (SCHRAMM & LOCKER, 2005; ASSARSSON et al., 2008). Em contrapartida, grande parte dos genes pertencentes às classes *intermediate* e *late* codificam proteínas que serão empacotados no vírion como componentes do núcleo ou do envelope viral (ASSARSSON et al., 2008).

A replicação do genoma do vírus vaccinia (VACV) é iniciada aproximadamente uma a duas horas após a infecção, enquanto que nos *Parapoxvirus* o início da replicação é mais tardio, ocorrendo em torno de 4-6 horas após a infecção (MOSS, 2007; CANAL, 2007). O mecanismo de replicação do genoma viral ainda não foi completamente elucidado. No entanto, o modelo do *auto-priming* é o mais aceito, e se baseia na observação de junções concatêmicas no DNA em replicação e na ausência de uma origem de replicação (MOSS, 2007). Este modelo sugere que o início da replicação ocorre pela clivagem do DNA em uma ou nas duas extremidades, resultando em uma extremidade 3' livre que atua como *primer* para a DNA polimerase iniciar a replicação. A molécula de DNA que está sendo replicada assume a conformação original e o genoma é copiado por deslocamento da cadeia complementar (MOSS, 2007). Ao final do processo replicativo longos concatâmeros (moléculas do DNA genômico unidos pelas extremidades) são formados. Estas formas concatêmicas serão separadas por clivagem, após o início da transcrição dos genes *late*, dando origem a cópias únicas do genoma, nas quais, as extremidades em *loop* são regeneradas (MOSS, 2007).

A replicação do genoma dos poxvírus ocorre em locais específicos do citoplasma, denominados viroplasmas ou fábricas virais, que são acopladas ao retículo endoplasmático rugoso (MOSS, 2007; CANAL, 2007; KATSAFANAS & MOSS, 2007), e com a progressão do ciclo tornam-se dispersas pelo citoplasma (TOLONEN et al., 2001). Além de servirem como sítio de replicação do genoma e montagem dos vírions, as fábricas virais também suportam a transcrição de genes das classes *intermediate* e *late* e ainda atuam como local de tradução de proteínas virais (KATSAFANAS & MOSS, 2007).

Após o início da replicação, ocorre uma alteração na expressão gênica, quando os produtos dos genes *early* atuam como fatores de transcrição para os genes *intermediate* e *late*. Por sua vez, esses serão transcritos e traduzidos originando as proteínas que irão participar da morfogênese, bem como os fatores de transcrição dos genes *early* que serão empacotados com o vírion para atuarem no próximo ciclo de infecção (MOSS, 2007; CANAL, 2007).

2.4 A resposta imune à infecção pelo ORFV

A resposta imune à infecção e à reinfecção pelo ORFV tem sido extensivamente estudada. No entanto, muitos aspectos dessa complexa interação vírus-hospedeiro ainda não foram completamente elucidados. O entendimento dos mecanismos imunes que protegem da infecção pelo ORFV é dificultado pelo fato da imunidade gerada ser de curta duração e pela habilidade do vírus em reinfetar seus hospedeiros (FLEMING & MERCER, 2007). O papel da imunidade humoral em prevenir ou reduzir a severidade das lesões na reinfecção tem sido constantemente debatido (FLEMING & MERCER, 2007). Vários relatos sugerem que os anticorpos não desempenham papel importante na proteção contra a infecção (BUDDLE & PULFORD, 1984; McKEEVER et al., 1988; MERCER et al., 1994). Entretanto, outros estudos demonstraram correlação entre níveis de IgG2 e resolução das lesões, sugerindo que anticorpos do isotipo IgG2 podem desempenhar um papel importante na defesa contra o vírus (LLOYD, 1996). Em um estudo no qual foi realizada a depleção de linfócitos CD4⁺ e CD8⁺ em ovinos, observou-se uma correlação entre os títulos de anticorpos e a resolução das lesões. Neste estudo, os autores concluíram que os anticorpos conferem uma proteção parcial contra a infecção pelo ORFV (LOYD et al., 2000). No entanto, anticorpos neutralizantes na infecção pelo ORFV são raros ou ausentes (CZERNY et al., 1997) e a transferência de imunidade passiva não protege cordeiros da infecção (BUDDLE & PULFORD, 1984; MERCER et al., 1994).

A imunidade celular desempenha, potencialmente, um papel mais importante na proteção contra a infecção pelo ORFV (FLEMING & MERCER, 2007). Estudos imuno-

histológicos realizados em biópsias de pele coletadas após a infecção primária ou a reinfecção demonstraram que há um acúmulo de neutrófilos, linfócitos T ($CD4^+$ e $CD8^+$), linfócitos B e células dendríticas nas áreas adjacentes às células da epiderme infectadas pelo vírus (JENKINSON et al., 1991; 1992; LLOYD et al., 2000; ANDERSON et al., 2001). Linfócitos T $CD4^+$ e células dendríticas são predominantes nas lesões (JEKINSON et al., 1992; ANDERSON et al., 2001). Em infecções primárias, linfócitos T $CD8^+$ e linfócitos B acumulam mais lentamente quando comparados com os linfócitos T $CD4^+$. As células T $\gamma\delta$ são mais numerosas em infecções primárias do que nas reinfecções (HAIG & McINNES, 2002). A infecção primária é marcada pela replicação viral, espessamento da epiderme e neovascularização. Na reinfecção, a replicação viral não é tão pronunciada e as lesões resolvem mais rapidamente (HAIG & McINNES, 2002). A dinâmica das células imunes nas regiões adjacentes às lesões ocorre em dependência da presença de antígenos virais nas células da epiderme (HAIG & McINNES, 2002), entretanto, a função destas células na resolução das lesões ainda não foi elucidada (HAIG et al., 1996a). Um estudo utilizando hibridização *in situ* demonstrou a presença de células expressando o IFN- γ na reinfecção e um aumento no número e na variedade de células expressando TNF- α , sugerindo um papel importante para o IFN- γ na proteção parcial à infecção pelo ORFV (ANDERSON et al., 2001).

A dinâmica da resposta imune à infecção pelo ORFV também tem sido estudada pela detecção de células e mediadores na linfa que drena os sítios de infecção (FLEMING & MERCER, 2007). Estes estudos envolvem a canulação dos ductos linfáticos aferentes e eferentes que drenam o sítio de infecção e constituem-se em uma excelente ferramenta para o estudo da cinética da resposta imune *in vivo* (HAIG et al., 1996a). Uma série de estudos (HAIG et al., 1996b, c) demonstraram que na reinfecção pelo ORFV ocorre uma resposta celular bifásica envolvendo células T $CD4^+$, T $CD8^+$, B e células dendríticas. Assim como a análise da resposta imune no sítio de infecção, estes estudos também demonstraram que os linfócitos T $CD4^+$ constituem a população celular mais abundante na linfa que drena a região da infecção, com picos ocorrendo nos dias 4 e 12 após a infecção primária e a reinfecção, respectivamente (HAIG et al., 1996a). Um padrão semelhante também foi observado na produção do fator estimulador de colônia granulocítica e macrófaga (GM-CSF), interleucina-1 (IL-1), IL-8, IL-2 e IFN- γ por células linfáticas cultivadas a partir da linfa aferente, coletada em diferentes intervalos após a infecção (FLEMING & MERCER, 2007). Estes resultados indicam que linfócitos T $CD4^+$, IFN- γ e, em menor proporção, linfócitos T $CD8^+$ desempenham um papel importante na proteção contra a infecção pelo ORFV.

Embora a resposta imune à infecção pelo ORFV seja do tipo Th1, tipicamente antiviral, um fato marcante é a habilidade do vírus em reinfetar e replicar repetidamente no seu hospedeiro (HAIG & FLEMING, 1999). Dentre as possíveis explicações para este fato, convém destacar que o vírus pode ter desenvolvido mecanismos para subverter ou interferir com componentes do sistema imune, conforme já demonstrado para outros poxvírus (HAIG et al., 1996a).

2.5 Fatores de virulência e imunomodulatórios codificados pelo ORFV

Os poxvírus são conhecidos por modular vários aspectos das defesas antivirais do hospedeiro. Estes vírus codificam diversos fatores imunomodulatórios que agem em diferentes vias de sinalização celular. As vias de sinalização celular alvo de regulação pelos poxvírus incluem a via dos interferons, interleucina-1 β (IL-1 β), IL-18, IL-10, fator de necrose tumoral (TNF), quemoquinas, serpinas, complemento, semaforinas, apoptose e a via de sinalização do fator de transcrição nuclear-kappa B (NF- κ B) (ALCAMI, 2003). O conhecimento existente sobre fatores imunomodulatórios codificados pelos poxvírus provém, em grande parte, de estudos realizados com os *Orthopoxvirus*. Em contrapartida, pouco se sabe a respeito destes fatores codificados pelos *Parapoxvirus*, que possuem o ORFV como protótipo. Dentre o arsenal de genes codificados pelo ORFV vários exercem, presumidamente, um importante papel na determinação do espectro de hospedeiros, ou como fatores imunomodulatórios (DELHON et al., 2004).

Alguns fatores imunomodulatórios, potencialmente envolvidos na virulência, têm sido identificados no genoma do ORFV, incluindo genes que codificam um homólogo da IL-10 (OV-IL-10), proteína de ligação a quemoquinas (CBP), inibidor do fator de crescimento monocítico e fagocitário (GM-CSF) e interleucina 2 (IL-2) (GIF), fator de crescimento do endotélio vascular (VEGF), proteína de resistência ao interferon (OVIFNR) e inibidor da apoptose (*ORFV125*) (FLEMING & MERCER, 2007).

2.5.1 Interleucina 10 (OV-IL-10)

Um gene homólogo da interleucina-10 (IL-10) de mamíferos foi identificado no genoma do ORFV (*ORFV127*; OV-IL-10). A OV-IL-10 possui grande identidade com seus homólogos em mamíferos, incluindo a IL-10 ovina (80%), bovina (75%), humana (67%) e murina (64%), bem como, com a IL-10 codificada pelo vírus do Epstein-Barr (63%) e pelo herpesvírus equino (67%) (FLEMING, et al., 1997).

A IL-10 é uma citocina multifuncional que possui propriedades imunossupressivas e imunoestimulatórias. A sua função biológica mais importante parece ser a regulação de macrófagos ativados por patógenos. Esta citocina age reduzindo a produção de mediadores pró-inflamatórios por macrófagos e suprimindo a produção de IL-2 e IFN- γ por células T (MOORE et al., 2001). Além disso, pode estimular a proliferação de timócitos, mastócitos e linfócitos B (MOORE et al., 2001), ou inibir a proliferação de monócitos (O'FARRELL et al., 1998; 2000).

Aparentemente, a OV-IL-10 exibe todas as atividades dos seus homólogos de mamíferos, incluindo inibição da produção de TNF- α e IL-18 por macrófagos, INF γ e GM-CSF por monócitos, e maturação e apresentação de antígenos por células dendríticas (DCs), sugerindo que a OV-IL-10 suprime a resposta inflamatória e o desenvolvimento da imunidade inata e adquirida durante a infecção pelo ORFV (FLEMING & MERCER, 2007). A deleção do gene OV-IL-10 do genoma do ORFV, revelou que esta proteína viral constitui-se em um fator de virulência para o ORFV no seu hospedeiro natural (FLEMING et al., 2007).

2.5.2 Proteína de ligação a quemoquinas (CBP)

O produto da *ORFV112* do ORFV foi identificado como a proteína de ligação a quemoquinas (CBP). Esta proteína possui similaridades estruturais e funcionais com as proteínas de ligação a quemoquinas do tipo II (CBP-II) encontradas nos *Orthopoxvirus* e *Leporipoxvirus* (SEET et al., 2003). As quemoquinas compreendem uma família de proteínas que atuam na atração e ativação de leucócitos durante o processo inflamatório ou infeccioso (GOH et al., 2000). Semelhante as CBP-II, a CBP do ORFV (ORFV-CBP) liga-se a diversas quemoquinas inflamatórias da classe CC, como o MCP-1, MIP-1 α e RANTES, que controlam o recrutamento de monócitos, macrófagos e linfócitos T ao sítio de infecção (SEET et al., 2003). Além de ligar-se a quemoquinas da classe CC, a ORFV-CBP também interage com a quemoquina linfotactina pertencente à classe C, que tem sido implicada na quimiotaxia de linfócitos T, linfócitos B e neutrófilos (SEET et al., 2003).

As quemoquinas MIP-1 α , MIP-1 β , RANTES e linfotactina podem atuar em conjunto com o IFN- γ , agindo como citocinas da resposta T *helper* 1 (Th1), coativando macrófagos e ativando células *natural killer* (NK) e T CD8⁺ (DORNER et al., 2002). A ação da ORFV-CBP sobre essas quemoquinas sugere que, além de inibir a quimiotaxia, esta proteína viral também pode atuar na redução da resposta antiviral do tipo Th1 (SEET et al., 2003).

2.5.3 Fator inibidor do GM-CSF e IL-2 (GIF)

O fator inibidor do GM-CSF e IL-2 (GIF) codificado pelo gene *ORFV117* do ORFV é encontrado exclusivamente nos *Parapoxvirus* (DEANE et al., 2000; DELHON et al., 2004). O GIF é uma proteína secretada que forma dímeros ou tetrâmeros biologicamente ativos. A forma ativa do GIF liga-se ao GM-CSF e a IL-2 *in vitro* e inibe a atividade hematopoiética do GM-CSF em ensaios de estimulação de colônia. A atividade do GIF também foi detectada na linfa que drena o sítio de infecção em ovinos infectados com o ORFV, com a atividade máxima do produto viral correspondendo ao período de maior replicação viral (DEANE et al., 2000).

2.5.4 Fator de crescimento do endotélio vascular (VEGF)

A família VEGF compreende sete glicoproteínas: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E (ORFV-VEGF), VEGF-F e fator de crescimento placentar (PlGF) (OTROCK et al., 2007). Os fatores de crescimento do endotélio vascular medeiam a proliferação de células endoteliais, permeabilidade vascular, angiogênese e linfangiogênese através da ligação com receptores para tirosina quinase (VEGFR-1, VEGFR-2, VEGFR-3 e neutrofilinas NP-1 e -2) (FLEMING & MERCER, 2007).

O VEGF-E foi identificado inicialmente no ORFV (*ORFV132*) (LYTTLE et al., 1994) e constitui-se em um dos membros da família VEGF menos estudados até o presente (OTROCK et al., 2007). O VEGF-E possui identidade de 20 a 25% com o VEGF-A. Este fator viral liga-se especificamente e com alta afinidade ao receptor VEGFR-2, resultando na ativação do receptor por autofosforilação e aumento na concentração intracelular de Ca^{++} (OGAWA et al., 1998).

O VEGF-E do ORFV apresenta um alto grau de variabilidade entre diferentes cepas do vírus (41% de identidade entre as cepas NZ2 e NZ7 e 40% entre as cepas SA-00 e IA-82) (WISE et al., 1999; DELHON et al., 2004), no entanto, o significado biológico desta variabilidade ainda não foi elucidado. Comparações realizadas *in vitro*, entre VEGF recombinantes das cepas NZ2 e NZ7, revelaram diferenças na habilidade destas variantes em induzir permeabilidade vascular e na ligação com o VEGFR-2 e NP-1 (WISE et al., 2003). Entretanto, as duas cepas do vírus (NZ2 e NZ7) induziram níveis similares de vascularização, edema e formação de crostas *in vivo* (WISE et al., 2007). ORFV recombinantes, nos quais os variantes VEGF-E_{NZ2} e VEGF-E_{NZ7} foram deletados, demonstraram marcada redução nas alterações vasculares, indicando que o VEGF é um fator de virulência para o ORFV em ovinos (WISE et al., 2007).

2.5.5 Proteína de resistência ao interferon (OVIFNR)

O ORFV codifica um produto de resistência ao interferon (OVINFR, *ORFV020*), homólogo ao gene *E3L* do VACV (HAIG et al., 1998; McINNES et al., 1998). A OVINFR liga-se a moléculas de RNA de cadeia dupla (dsRNA), produzidas em consequência da infecção. Esta ligação previne a ativação da proteína quinase RNA (PKR), sugerindo que, semelhante ao seu homólogo E3L do VACV, a OVINFR do ORFV bloqueia a inibição da tradução induzida pelo interferon (HAIG et al., 2002). Em consequência da expressão do OVIFNR, a tradução de proteínas celulares e virais progride e o efeito antiviral do interferon é bloqueado (HAIG et al., 1998).

2.5.6 Inibidor da apoptose semelhante ao Bcl-2 (ORFV125)

A infecção pelo ORFV inibe a apoptose em células de cultivo (WESTPHAL et al., 2007). Utilizando-se uma biblioteca de vírus recombinantes (VACV-ORFV), um gene relacionado com a inibição da apoptose foi identificado no genoma do ORFV (*ORFV125*). A expressão da ORFV125 em células de cultivo inibiu a fragmentação do DNA, a ativação de caspases e a liberação de citocromo *c* da mitocôndria (WESTPHAL et al., 2007). O mecanismo pelo qual a ORFV125 exerce sua ação anti-apoptótica é semelhante ao da proteína celular anti-apoptótica Bcl-2 (WESTPHAL et al., 2009).

2.6 A via de sinalização do NF- κ B

A via de sinalização do NF- κ B participa da regulação de diversos processos biológicos, incluindo a resposta imune e inflamatória, ciclo celular, proliferação e diferenciação celular e apoptose (LI & VERMA, 2002). A família do NF- κ B em mamíferos é composta por cinco subunidades: NF- κ B-p50/p105, NF- κ B-p52/p100, NF- κ B-p65 (RelA), RelB e c-Rel (CHEN & GOSH, 1999). Os diferentes componentes da família NF- κ B possuem grande homologia na região N-terminal, denominada domínio de homologia Rel (RHD), que é responsável pela dimerização, interação com o inibidor do NF- κ B (I κ B), translocação nuclear e ligação ao DNA (SUN & XIAO, 2003). Além do domínio de homologia Rel, as subunidades NF- κ B-p65, c-Rel e RelB também possuem um domínio de transativação (TAD) na região C-terminal, que é necessário para induzir a transcrição dos genes alvo. Os outros dois componentes da família, NF- κ B-p105 e NF- κ B-p100, são precursores das subunidades NF- κ B-p50 e NF- κ B-p52, respectivamente (LIN et al., 1998).

Os componentes da família NF- κ B formam homo e heterodímeros que se ligam a sequências específicas de DNA na região promotora dos genes alvo. Até o presente, 12 heterodímeros diferentes do NF- κ B foram descritos. No entanto, o dímero mais abundante nas células é formado pelas subunidades NF- κ B-p50 e NF- κ B-p65. O RelB não forma homodímeros, mas pode dimerizar com o NF- κ B-p50 e o NF- κ B-p52. Os homodímeros formados por NF- κ B-p50 e NF- κ B-p52 não induzem transcrição gênica, pois estas subunidades não possuem o domínio de transativação (TAD) (SCHMITZ & BAEUERLE, 1991).

A atividade transcripcional do NF- κ B pode ser ativada por meio de duas vias de sinalização: a via clássica ou canônica e a via alternativa ou não canônica (BONIZZI & KARIN, 2004). A via clássica é induzida por citocinas pró-inflamatórias como o fator de necrose tumoral (TNF- α) e interleucina 1 β (IL-1 β), bem como por infecções virais e produtos virais e bacterianos (PAHL, 1999). Os eventos da via clássica são os principais alvos de inibição por produtos virais. A via alternativa é ativada principalmente por linfotoxina- β (LT β), fator de ativação de células B (BAFF), e CD40 (VALLABHAPURAPU & KARIN, 2009).

Os dímeros do NF- κ B contendo o NF- κ B-p65 ou o c-Rel situam-se no citoplasma de células não estimuladas, ligados aos inibidores I κ B, em uma forma inativa (HAYDEN & GOSH, 2008). A família I κ B é composta por cinco membros, I κ B α , I κ B β , I κ B ϵ , NF- κ B-p100 e NF- κ B-p105 (BALDWIN, 1996). As proteínas da família I κ B possuem domínios com repetições de anquirina, que mediam a sua associação com diferentes membros da família NF- κ B (SUN & XIAO, 2003). O protótipo da família I κ B é o I κ B α , que se liga preferencialmente aos heterodímeros NF- κ B-p50/NF- κ B-p65. Esta ligação oculta os sinais de localização nuclear presentes no NF- κ B-p65, resultando no sequestro destes fatores de transcrição no citoplasma (VALLABHAPURAPU & KARIN, 2009).

Quando a célula é estimulada, o complexo de quinases I κ B (IKK) é ativado, induzindo a fosforilação dos inibidores I κ B (aminoácidos S32 e S36). A fosforilação do I κ B leva a ubiquitinação e subsequente degradação dos I κ Bs pelo proteossoma celular 26S, resultando na exposição dos sinais de localização nucleares no NF- κ B-p65 e induzindo a sua translocação para o núcleo. Uma vez no núcleo, as subunidades do NF- κ B ligam-se à sequências específicas de DNA nas regiões promotoras ou no *enhancer* dos genes alvo, promovendo a sua transcrição (Figura 1) (VALLABHAPURAPU & KARIN, 2009).

A via do NF- κ B pode ser ativada por uma grande variedade de estímulos, incluindo citocinas inflamatórias, fatores de crescimento, agentes que causam dano ao DNA celular, componentes bacterianos e proteínas virais (PAHL, 1999). Uma etapa fundamental na ativação da via do NF- κ B é a degradação do I κ B, acarretando na liberação do NF- κ B no citoplasma e subsequente translocação ao núcleo, onde irá ativar a transcrição de diversos genes (SUN & XIAO, 2003). O NF- κ B pode promover a expressão de mais de 150 genes que participam de diversos processos celulares incluindo resposta imune, ciclo celular, diferenciação e proliferação celular e apoptose (PAHL, 1999). A interferência com a atividade transcripcional do NF- κ B, exercida por diversos vírus, incluindo os poxvírus, permite a modulação de processos celulares essenciais para o estabelecimento das infecções virais (HISCOTT et al., 2006).

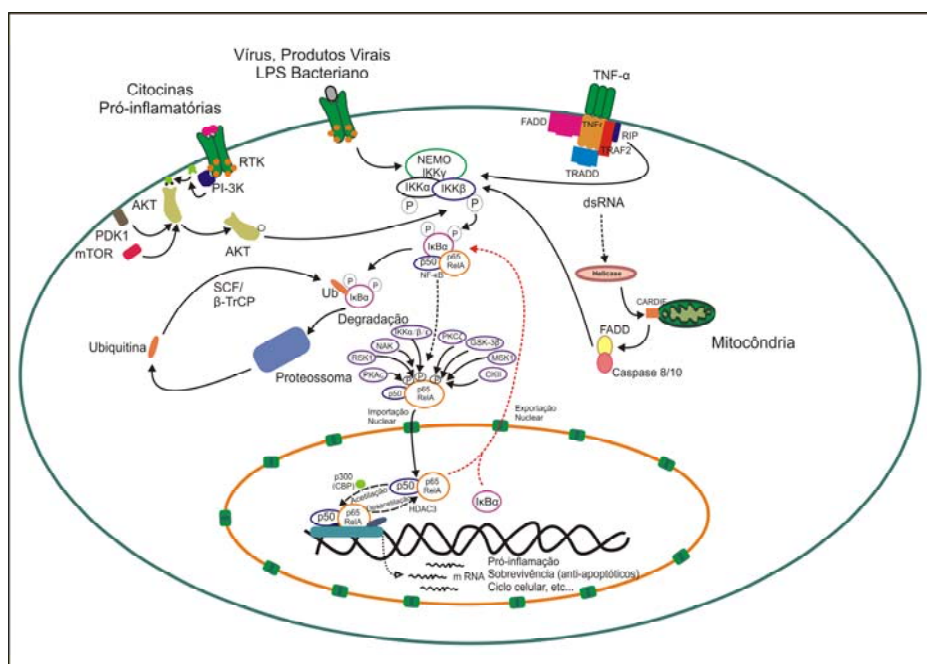


Figura 1 – Representação esquemática da via clássica de ativação do NF- κ B.

2.6.1 Regulação da via do NF- κ B

Os eventos envolvidos na ativação e ação do NF- κ B podem ser divididos em duas fases que ocorrem no citoplasma ou no núcleo das células (CHEN & GREENE, 2004). Os eventos da fase inicial de ativação ocorrem no citoplasma e envolvem a ativação do complexo IKK, degradação da molécula inibitória I κ B α , e modificações pós-traducionais ou processamento das subunidades do NF- κ B, acarretando em translocação destas subunidades para o núcleo (CHEN & GREENE, 2004). Os eventos envolvidos na segunda fase de ação do

NF- κ B ocorrem no núcleo celular e envolvem modificações pós-traducionais (fosforilação, acetilação e metilação) e associação das subunidades do NF- κ B com proteínas celulares que atuam como co-ativadores ou co-repressores da sua atividade transcripcional (Figura 1) (CHEN & GREENE, 2004). A ação dos mecanismos regulatórios das duas fases afeta a afinidade do NF- κ B pelas sequências de DNA, é crítica para a seleção dos genes a serem transcritos e é responsável pela intensidade e duração da atividade transcripcional do NF- κ B (CHEN & GREENE, 2004; WAN & LENARDO, 2009).

As modificações pós-traducionais das subunidades do NF- κ B desempenham um papel importante na regulação de sua função (PERKINS, 2006). Por exemplo, a fosforilação do NF- κ B-p65 modula a sua atividade no núcleo (PERKINS et al., 2006; WAN & LENARDO, 2009). Dez sítios de fosforilação foram identificados no NF- κ B-p65 (serinas 205, 276, 281, 311, 468, 529 e 536, e treoninas 254, 435 e 505), muitos dos quais desempenham efeito modulatório na sua atividade transcripcional (PERKINS, 2006). A fosforilação da serina 536 afeta negativamente a interação do NF- κ B-p65 com o I κ B α , o que pode acelerar a translocação nuclear do NF- κ B-p65 (BOHUSLAV et al., 2004), ou reduzir a exportação nuclear pelo I κ B α sintetizado *de novo*, regulando assim a duração da transcrição mediada pelo NF- κ B (WAN & LENARDO, 2009). Além disso, a fosforilação das serinas 276 e 536 é necessária para o recrutamento do co-ativador p300 para o complexo de transcrição, o que promove a atividade transcripcional do NF- κ B-p65 (PERKINS, 2006; CHEN et al., 2005).

A acetilação também desempenha um importante papel na regulação da atividade transcripcional do NF- κ B-p65 no núcleo (CHEN & GREENE, 2004). Sete sítios de acetilação foram identificados no NF- κ B-p65 (lisinas 122, 123, 218, 310, 314 e 315) e a modificação de um ou de múltiplos sítios regula diferentes funções biológicas do NF- κ B-p65 (CHEN & GREENE, 2004). Por exemplo, a acetilação da lisina 221 aumenta a afinidade do NF- κ B-p65 pelo DNA e, em combinação com a acetilação da lisina 218, previne a associação do NF- κ B-p65 com o I κ B α sintetizado *de novo*, regulando assim a duração das respostas mediadas pelo NF- κ B (CHEN et al., 2002). A acetilação da lisina 310 recruta o co-ativador Brd4 para o complexo de transcrição aumentando a atividade transcripcional do NF- κ B-p65 (CHEN et al., 2002; HUANG et al., 2009). Devido ao papel central exercido pelo NF- κ B na regulação de diversos processos que são vitais para a célula, a via de sinalização do NF- κ B é precisamente regulada (CHEN & GREENE, 2004).

2.7 Inibição da via do NF- κ B pelos poxvírus

Os poxvírus desenvolveram várias estratégias para regular a via do NF- κ B (MOHAMED & McFADDEN, 2009). Inibidores do NF- κ B já foram identificados em poxvírus dos generos *Orthopoxvirus*, *Leporipoxvirus*, *Yatapoxvirus*, e *Molluscipoxvirus*, com vírus de uma espécie geralmente codificando múltiplos inibidores (MOHAMED & McFADDEN, 2009). Por exemplo, o VACV, o protótipo da família *Poxviridae*, codifica pelo menos sete inibidores do NF- κ B (A52R, A46R, B14, N1L, M2L, K1L e E3L) (BOWIE et al., 2000; DiPERNA et al., 2004; SHISLER & JIN, 2004; GEDEY et al., 2006; CHEN et al., 2008; MYSKIW et al., 2009), o que sugere que a inibição desta via é importante para o estabelecimento das infecções pelos poxvírus.

Os inibidores do NF- κ B codificados pelos poxvírus exibem uma ampla gama de mecanismos de ação, agindo em interações ligante-receptor na superfície da célula ou em eventos intra-citoplasmáticos que levam a ativação da via de sinalização (BOWIE et al., 2000; DiPERNA et al., 2004; SHISLER & JIN, 2004; GEDEY et al., 2006; CHEN et al., 2008; MOHAMED & McFADDEN, 2009; MYSKIW et al., 2009). Por exemplo, os inibidores K1L, B14 e N1L codificados pelo VACV atuam diretamente em componentes da via do NF- κ B. O gene K1L atua prevenindo a degradação do I κ B (SHISLER & JIN, 2004), enquanto que as proteínas B14 e N1L inibem o complexo IKK (DiPERNA et al., 2004; CHEN et al., 2008). Em contrapartida, outros genes como A46R, A52R e M2L atuam em eventos anteriores a cascata do NF- κ B, inibindo a ativação ou bloqueando a fosforilação de proteínas quinases celulares que irão ativar os membros da família NF- κ B ou I κ B (HARTE et al., 2003; STACK et al., 2005; GEDEY et al., 2006). O vírus do molusco contagioso (MCV) possui dois genes cujos produtos interferem com a ativação do NF- κ B. O gene MC160 atua inibindo a formação do complexo IKK induzida pelo TNF- α (NICHOLS & SHISLER, 2006) e a proteína MC159 bloqueia os eventos iniciais resultantes da ativação do NF- κ B pelo TNF α (MURAO & SHISLER, 2005). No presente estudo, três novos inibidores do NF- κ B codificados pelo *Parapoxvirus* ORFV foram identificados e caracterizados. Estes inibidores são codificados pelos genes *ORFV024*, *ORFV002* e *ORFV121* e são únicos dos PPVs. O mecanismo de ação destes inibidores (*ORFV024*, *ORFV002* e *ORFV121*) foi caracterizado *in vitro* e o papel destes genes para a virulência e patogenia do ORFV foram investigados *in vivo*. A identificação e caracterização destes inibidores serão apresentados sob a forma de três capítulos que seguem a presente revisão bibliográfica.

- 3. CAPÍTULO 1: A Novel Inhibitor of the NF- κ B Signaling Pathway Encoded by the Parapoxvirus Orf Virus.
- 4. CAPÍTULO 2: A Nuclear Inhibitor of NF- κ B Encoded by a Poxvirus.
- 5. CAPÍTULO 3: Orf Virus *ORFV121* Encodes for a Novel Inhibitor of NF- κ B that contributes to virus virulence

3. CAPÍTULO 1

A Novel Inhibitor of the NF- κ B Signaling Pathway Encoded by the Parapoxvirus Orf Virus

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Abstract

The parapoxvirus orf virus (ORFV) is a pathogen of sheep and goats, which has been used as a preventive and therapeutic immunomodulatory agent in several animal species. However, the functions (genes/proteins/mechanisms of action) evolved by ORFV to modulate and manipulate immune responses are poorly understood. Here, the novel ORFV protein ORFV024 was shown to inhibit activation of the NF- κ B signaling pathway, an important modulator of early immune responses against viral infections. Infection of primary ovine cells with an *ORFV024*-deletion mutant virus resulted in a marked increase in expression of NF- κ B-regulated chemokines and other proinflammatory host genes. Expression of ORFV024 in cell cultures significantly decreased LPS- and TNF- α -induced NF- κ B-responsive reporter gene expression. Further, ORFV024 expression decreased TNF- α -induced phosphorylation and nuclear translocation of NF- κ B-p65, phosphorylation and degradation of I κ B α , and phosphorylation of IKK subunits IKK α and IKK β , indicating that ORFV024 functions by inhibiting activation of IKKs, the bottleneck for most NF- κ B activating stimuli. Although ORFV024 interferes with activation of the NF- κ B signaling pathway, its deletion from the OV-IA82 genome had no significant effect on disease severity, progression, and time to resolution in sheep, indicating that ORFV024 is non essential for virus virulence in the natural host. This represents the first description of a NF- κ B inhibitor encoded by a parapoxvirus.

Introduction

Orf virus (ORFV) is the type member of *Parapoxvirus*, one of the eight genera of the subfamily *Chordopoxvirinae* of the *Poxviridae* (43). Other members of the genus include bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV), and parapoxvirus of red deer in New Zealand (PVNZ) (43). Parapoxviruses are characterized by the ovoid shape of virions, the presence of criss-cross-arranged surface tubule-like structures, and the high G+C content of their genomes (21).

ORFV is the causative agent of orf (contagious ecthyma, contagious pustular dermatitis, or scabby mouth), a nonsystemic, highly contagious, ubiquitous disease of sheep and goats which is characterized by maculopapular and proliferative lesions affecting the skin around the mouth, nostrils and teats, and the oral mucosa (29). Lesions are largely confined to areas surrounding the virus entry sites, evolving through stages of erythema, vesicles, pustules, and scabs (21). In the absence of secondary infections, lesions are usually resolved in 6 to 8 weeks; however, persistent infections have been reported (13, 27, 48). Orf primarily affects animals less than one year old, and morbidity in a susceptible flock may reach 90%. Mortality is usually low; however, lesions in lips and udders may prevent affected animals from suckling, which can result in rapid emaciation (39). In spite of a vigorous and typical anti-viral T-helper type 1 (Th1) immune response, immunity elicited by ORFV is short-lived, and animals can be repeatedly infected, albeit lesions are smaller and resolve sooner than in primary infections (28, 40, 69). Orf is a zoonotic disease, affecting humans in close contact with infected animals (17, 44, 55).

The ORFV genome is approximately 138 kilobase pairs, with a G+C content of 64%, and contains 131 putative genes, 89 of which are conserved in all characterized chordopoxviruses (14, 61). Based on sequence homology to host and viral genes, several immunomodulatory genes with putative virulence functions have been identified in ORFV, including a homologue of IL-10 (*ORFV127*), a gene encoding a chemokine binding protein (CBP; *ORFV112*), an inhibitor of granulocyte/macrophage-colony stimulating factor and IL-2 (GIF; *ORFV117*), a vascular endothelial growth factor (VEGF; *ORFV132*), and a interferon (IFN)-resistance gene (*ORFV020*) [reviewed in (21)]. Recently, screening of vaccinia virus-orf virus recombinants led to identification of a Bcl-2-like inhibitor of apoptosis (*ORFV125*) (66). Notably, ORFV encodes 15 mostly terminally located genes, which lack similarity to other poxvirus or cellular proteins and with putative virulence and host range functions (14).

Historically, ORFV has been used in Veterinary Medicine as a preventive and

therapeutic immunomodulatory agent [reviewed in (64)]. Live or inactivated ORFV preparations exhibit dose-dependent immunomodulatory effects, with therapeutic efficacy and favorable side effect profile demonstrated for various diseases, including infectious diseases of companion and farm animals (5, 7, 18, 19, 38, 70). Inactivated ORFV has been shown to induce an autoregulatory cytokine response in mice, involving up-regulation of Th1-type cytokines (IL-12, IL-18, and IFN- γ) and their subsequent down-regulation, which is accompanied by induction of IL-10 and IL-4 (64). Additionally, inactivated ORFV promotes activation of monocytes and dendritic cells via Toll-like receptor (TLR)-dependent and independent pathways, leading to the synthesis and release of early proinflammatory cytokines (22, 60). Currently, ORFV functions (genes, proteins, and mechanisms of action) associated with modulation and manipulation of early host immune responses are poorly understood.

Many viruses, including Hepatitis C virus, paramyxoviruses, influenza virus, African Swine Fever Virus, and poxviruses encode for proteins that disrupt or modulate immune responses by targeting specific aspects of the Nuclear Factor kappa B (NF- κ B) signaling pathway [reviewed in (31, 63)]. By binding to specific promoter sequences, NF- κ B mediate expression of a wide variety of cellular genes, including many involved in innate immunity, inflammation, and apoptosis, which are critical for early antiviral responses (31, 52, 57, 63). The activity of NF- κ B is regulated by its association with the inhibitory I κ B molecules, which sequester NF- κ B in the cytoplasm (33). Various stimuli, including the proinflammatory cytokines tumor necrosis factor α (TNF- α) and IL-1, bacterial lipopolysaccharide (LPS), viruses, and viral products, lead to phosphorylation of I κ B proteins by I κ B kinases, resulting in proteasomal degradation of I κ B and nuclear translocation of NF- κ B subunits (33). NF- κ B subunits are subject to extensive post-translational modifications, which may facilitate their nuclear translocation or define their transcriptional functions in the nucleus (9, 53). The critical I κ B kinase (IKK) complex is the bottleneck for most NF- κ B-activating pathways (33, 57), including those initiated at TLRs and TNF receptors (8), and several viruses have evolved strategies to inhibit IKK complex activation [reviewed by (56)].

Poxviral proteins involved in inhibition of the NF- κ B pathway have been identified in members of the genera *Orthopoxvirus*, *Leporipoxvirus*, *Yatapoxvirus*, and *Molluscipoxvirus*, with selected viruses encoding for multiple inhibitors [for a review see (41)]. For example, the type member of the *Poxviridae* vaccinia virus (VACV) encodes at least seven NF- κ B inhibitors (A52R, A46R, B14, K1L, N1L, M2L, and E3L), which target different steps leading to NF- κ B activation, most often by preventing IKK complex activation (11, 15, 24,

30, 46, 59, 62). Notably, deletion of individual genes encoding selected VACV NF- κ B inhibitors from the viral genome resulted in various degrees of attenuation in mice. However no individual gene-deletion rendered complete virus attenuation (2, 10, 30), suggesting that encoded proteins exhibit, to some extent, complementary functions during VACV infection (41).

Homologues of known poxviral NF- κ B inhibitors are absent in parapoxviruses, suggesting either they encode novel NF- κ B inhibitors or employ alternative immune evasion strategies. Here we present data demonstrating that *ORFV024*, a gene unique to parapoxviruses, profoundly affects chemokine and other proinflammatory gene transcription in infected cells. Expression of ORFV024 in cell cultures decreased phosphorylation and nuclear translocation of NF- κ B-p65, phosphorylation and degradation of I κ B α , and phosphorylation of IKK α and IKK β , indicating that ORFV024 expression suppresses early cellular responses to ORFV infection by inhibiting activation of the NF- κ B pathway. Notably, *ORFV024* is non essential for viral virulence as its deletion did not affect ORFV pathogenesis in sheep.

Materials and Methods

Cells and virus. Primary ovine fetal turbinate (OFTu) cells were kindly provided by Dr. Howard D. Lehmkuhl (USDA) and were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 50 μ g/mL of gentamicin, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin. HeLa cells (American Type Culture Collection) were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% FBS, 2 mM of L-glutamine, 50 μ g/mL of gentamicin, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin. ORFV strain OV-IA82 was isolated from sheep nasal secretions at the Iowa Ram test Station during an orf outbreak in 1982 and its genome has been fully sequenced (14). Low passage parental OV-IA82 virus was used to construct the deletion mutant virus OV-IA82 Δ 024, and in all procedures involving infections with wild type virus, and PCR amplification and cloning of viral genes.

Plasmids. *ORFV024* coding sequence was PCR amplified from the OV-IA82 genome and cloned into the expression vectors pCMVTag4A (Stratagene, La Jolla, CA) and pEGFP-N1 (Clontech, Mountain View, CA). PCR primers were 024Flag-Fw(HindIII)-5'-TAAGGCCTCTAAGCTTATGGCCTCCTACGTCAGCGGCGCT-3'; 024Flag-Rv(XhoI)-5'-CAGAATTCGCCTCGAGCACGTAGACCGTGTGCGTGCGC-3'; 024EGFP-Fw(XhoI)-5'-ATCTTAGCTCGAGCTAAGATGGCCTCCTAC-3'; and 024EGFP-Rv(HindIII)-5'-

CAGA ATAAGCTTCACGTAGACCGTGTGCGTG-3'. The restriction enzymes used for cloning are indicated on each primer. DNA sequencing of constructs confirmed the integrity of *ORFV024* sequence and in frame cloning of FLAG (pCMV024-Flag) and EGFP (p024EGFP) tag proteins.

For mutant virus generation a recombination cassette was constructed by PCR amplification of *ORFV024* left (LF; 856 bp) and right (RF; 866 bp) flanking regions from the OV-IA82 genome followed by cloning of amplicons into vector pZippy-Neo/Gus (16). Primers used for amplification were: 024LF-Fw(HindIII)-5'-TAAGGCCTCTAAGCTTA ACCAGCAGACCTTCTTCACCAA-3'; 024LF-Rv(SalI)-5'-CAGAATTCGCGTCGACCTT AGCTCTGTCTGAACTGAAGCA-3'; 024RF-Fw(NotI)-5'-ATTCTTATGCGGCCGCGCC GGCTTCATCCGCCGCAGCATA-3'; and 024RF-Rv(BglII)-5'-CAGAATTCGCAGATCT TACGGCGACACCGACTCCGTGTTC-3'. Restriction enzymes used for cloning are indicated on each primer. The recombination cassette pZ024LF-Neo/Gus-024RF was constructed by cloning *ORFV024* LF and RF flanking regions on each side of a reporter construct consisting of neomycin (Neo) resistance and β -glucuronidase (Gus) genes preceded by VACV VV7.5 and modified H5 promoters, respectively.

ORFV024 sequence alignment. Alignment of *ORFV024* was performed using CLUSTALW (Biology Workbench). Accession numbers of the aligned proteins are AAR98119.1 (OV-IA82), ABA00541.1 (OV-NZ2), AAP76384.1 (OV-Orf11), AAR98249.1 (OV-SA00), AAR98381.1 (BPSV-AR02), and GQ911582 (BPSV-TX-09).

Reverse transcription-PCR. Transcription kinetics of *ORFV024* was assessed during *ORFV* infection *in vitro* by RT-PCR. OFTu cells were cultured in 35 mm dishes for 16 hours and inoculated with the wild type virus OV-IA82 (multiplicity of infection [m.o.i] = 10) in presence or absence of cytosine arabinoside (AraC, 40 μ g/mL; Sigma Aldrich, Saint Louis, MO) an inhibitor of DNA replication and, consequently, of late poxviral transcription (12). Cells were harvested at 0, 1, 2, 3, 6, 12 and 24 hours post inoculation (pi) and total RNA was extracted using Trizol reagent (Invitrogen, San Diego, CA). Samples were treated with DNaseI (New England Biolabs, Ipswich, MA) for 10 min at 37°C and RNA was purified using the RNeasy mini kit (QIAGEN, Valencia, CA). Two μ g of total RNA was reverse transcribed using the Superscript III kit (Invitrogen) following the manufacturer's protocol. The oligo dT used in reverse transcription reactions was oligo-dT-5'-ATGGATGCCTACAGCGCTCTTTTTTTTTTTTTTTTTTTT-3'. *ORFV024*, *ORFV127* (early gene control), and *ORFV055* (late gene control) transcription was assessed using 1 μ l of cDNA and the following primers 024intFw-5'-GCGGACACAGCCACAACCACAGTC-

3'; 024intRv-5'-CTAGCACGCGCTTTCGGTACCGCC-3'; 127EintFw-5'-CTCCTCGA CCACTTCAAAGG-3'; 127EintRv-5'-TATGT CGAACTCGCTCATGG-3'; 055LFw-5'-AATCATGGATCCGCCACCATGTTCTTCGCGCGTCGC-3'; and 055LRv-5'-TATC ATC TCGAGCGGGCGTGGAGGTCGCCGACC-3'. Negative controls and controls for DNA contamination (no reverse transcriptase) were included in all reactions.

Construction and characterization of *ORFV024*-deletion mutant virus OV-IA82 Δ 024.

OV-IA82 Δ 024 was obtained by homologous recombination between the parental ORFV strain OV-IA82 and the recombination cassette pZ024LF-Neo/Gus-024RF. OFTu cells cultured in 25 cm² flasks were infected with OV-IA82 (m.o.i = 1) and three hours later transfected with pZ024LF-Neo/Gus-024RF (10 μ g) using Lipofectamine 2000 according to manufacturer's recommendations. Forty-eight hours after infection-transfection, cultures were harvested and cell lysates used for selection of recombinant viruses by plaque assay. OFTu cells cultured in 6-well plates were infected with serial 10-fold dilutions of cell lysates from the infection-transfection and overlaid with culture media containing 0.5% SeaKem GTC agarose (Cambrex Bioscience, Rockland, ME) and 5-bromo-4-chloro-3-indolyl beta-D-glucuronic acid (X-Gluc, 0.5 μ g/mL, Gold Biotechnologies, Saint Louis, MO). Blue plaques indicative of recombination, were harvested and subjected to additional rounds of plaque purification. The absence of *ORFV024* sequence and presence of Neo/Gus sequences in the purified recombinant virus were confirmed by PCR and Southern Blot. The primers used for PCR amplification of *ORFV024* and Neo/Gus reporter gene were 024intFw-5'-GCGGACACAGCCACAACCACAGTC-3'; 024intRv-5'-CTAGCACGCGCTTTCGG TAC CGCC-3'; NG2Fw-5'-ATCAGGACATAGCGTTGGCTACC-3' and NG2 Rv-5'-TGCCG TAATGAGTGACCGCATCGA-3', respectively. To assess the integrity of regions involved in recombination, 824 bp and 874 bp on the left and right flanking regions of *ORFV024* were sequenced using an Applied Biosystems PRISM 3730 X1 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Growth characteristics of deletion mutant virus OV-IA82 Δ 024 and wild type virus OV-IA82 were analyzed and compared in primary OFTu cells and in HeLa cells. Multiple step growth curve was performed in OFTu cells inoculated with mutant or wild type virus (m.o.i = 0.1), and at 12, 24, 48 and 72 h pi viral yields were quantitated in cultures of OFTu cells by the Spearman-Kärber's tissue culture infectious dose 50 method (TCID₅₀/mL). One step growth curves were performed in OFTu and HeLa cells inoculated with mutant or wild type virus (m.o.i = 10), and at 6, 12, 24 and 48 h pi viral yields were quantitated in cultures of OFTu cells as described above. To compare the cytopathic effect (CPE) induced by OV-

IA82Δ024 and OV-IA82, cells were inoculated with each virus (m.o.i = 10) and 48 hours pi evaluated under an inverted light microscope (Leica DMI 4000 B; 100X). To analyze plaque sizes and morphology, OFTu cells were cultured in 6-well plates inoculated with serial 10-fold dilutions of each virus (10^{-4} to 10^{-6}) and fixed with 3.7% formaldehyde, 72 hours pi. Cells were stained with 1% crystal violet for 15 min at room temperature and plaques were examined under an inverted microscope (Leica DMI 4000 B; 100X).

Microarray and real-time PCR analysis. Gene expression profile of virus-infected OFTu cells was initially analyzed by microarray and then confirmed by real-time PCR. OFTu cells grown in 35 mm dishes for 16 hours at 37°C with 5% of CO₂ were inoculated with wild type or mutant virus (m.o.i = 10) and harvested for total RNA extraction with Trizol reagent (Invitrogen, San Diego, CA) at 2 and 4 hours pi.

For microarray analysis, RNA samples were processed and prepared according to standard Affymetrix protocols (Santa Clara, CA) at the Biotechnology Center of the University of Illinois at Urbana-Champaign. The gene expression profile at each time point was measured using the Affymetrix GeneChip® Bovine Genome Array which contains 24,128 probe sets that measure over 23,000 bovine transcripts. After the data passed the Affymetrix's recommended quality control, arrays were pre-processed using the GCRMA algorithm (68) as part of the Affymetrix (23) and GCRMA (68) packages from the Bioconductor project (25). Fold changes in gene expression were calculated for each time point by comparing expression in cells infected with the mutant virus with those infected by wild type virus.

Real-time PCR was performed to validate the microarray results. One microgram of total RNA, obtained from samples of three independent experiments, was used for cDNA synthesis as described above. Expression of genes *CCL20*, *CXCL3*, *IL-1 alpha*, *IL-6*, *IL-8*, *ICAM-1*, *IRF-1*, *NFκBIA*, and *PTGS2* was analyzed in an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA). Primers and probes used to detect the expression of these genes were synthesized by Applied Biosystems (TaqMan® Gene Expression Custom Assays), based on ovine gene sequences in GenBank. Real-time PCR amplifications were performed in 10 µl reactions, with 4.5 µl of cDNA (1:80 dilution in DEPC-H₂O), 5 µl of TaqMan® Master mix (Applied Biosystems) and 0.5 µl of the mixture of primers and probe (TaqMan® Gene Expression Custom Assays, Applied Biosystems). PCR conditions consisted of initial denaturation and DNA polymerase activation (AmpliTaq Gold®, Applied Biosystems) at 95°C for 15 s, followed by annealing-extension at 60°C for 1 min.

Each sample was tested in triplicate and the average of the three replicates was used to calculate fold changes. Expression levels of the genes tested were normalized to the housekeeping gene GAPDH. The measurement of gene expression was performed using the relative quantitation method (67). Standard curves were prepared from a pool of cDNA (2-fold dilutions; 1:20 to 1:2560) for GAPDH and all tested genes. Samples from mock infected cells collected at 2 and 4 hours pi were used as calibrators to calculate the fold changes. Negative controls (no template) and controls for DNA contamination (no RT control) were included in all reactions. Statistical analysis of the real-time PCR data was performed using Student's T-test.

Establishment of cell lines stably expressing ORFV024. HeLa cells constitutively expressing GFP or ORFV024-GFP fusion protein were obtained using a retroviral expression system (pLNCX2; Clontech). DNA sequences encoding GFP or ORFV024-GFP were cleaved from vectors pEGFP-N1 and p024EGFP with *BglIII* and *NotI* and sub-cloned into plasmid pLNCX2. Plasmid constructs were transfected into the packaging cell lines GP2-293 using Lipofectamine 2000. Forty eight hours after transfection, the supernatant containing GFP- or ORFV024-GFP- encoding recombinant retrovirus particles was harvested and used to infect HeLa cells. Individual cell clones were selected, amplified and maintained in presence of G418 (500 µg/ml; Gibco). Expression of GFP and ORFV024-GFP was monitored by fluorescence microscopy and by western immunoblot using an anti-GFP antibody (sc-8334, Santa Cruz Biotechnology, Santa Cruz, CA).

NF-κB luciferase reporter assays. The ability of ORFV024 to inhibit NF-κB-transcriptional activity was investigated *in vitro* using a luciferase reporter assay. OFTu cells were cultured in 12-well plates (1.2×10^5 cells per well), and co-transfected the next day with the vectors pNF-κB Luc (450 ng; Clontech, Mountain View, CA), and pRLTK (50 ng; Promega, Madison, WI) which encode firefly luciferase gene under control of κB-responsive elements and sea pansy (*Renilla reniformis*) luciferase, respectively, using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were mock-infected or infected with wild type (OV-IA82) or mutant virus (OV-IA82Δ024) (m.o.i = 10). Cells were harvested with passive lysis buffer at 2, 4 and 6 hours pi (PLB; Promega) and luciferase activities were determined using the Dual Luciferase Reporter Assay (Promega) and a luminometer (Victor²; Perkin-Elmer, Waltham, MA).

OFTu cells transiently expressing or HeLa cells stably expressing ORFV024 were co-transfected with the reporter plasmids, treated with LPS or TNF-α, respectively, and assayed for firefly and sea pansy luciferase activities. OFTu cells cultured in 12-well plates (1.2×10^5

cells per well) were co-transfected with pNF- κ B*Luc* (450 ng), pRLTK (50 ng), and pCMV024-Flag (500 ng), or control empty vector pCMVTag4A (500 ng), using Lipofectamine 2000. Twenty-four hours after transfection, cells were exposed to control media or media containing LPS (250 ng/ml; Invivogen, San Diego, CA) for 6 hours. LPS-treated and untreated cells were harvested with PLB, and luciferase activity was determined as above. HeLa cells stably expressing GFP or ORFV024-GFP fusion protein were cultured in 12-well plates (1.1×10^5 cells per well) and co-transfected with pNF- κ B*Luc* (450 ng) and pRLTK (50 ng) as above. Twenty-four hours after transfection, cells were exposed to control media or media containing 20 or 50 ng/ml of TNF- α (Cell Signaling, Danvers, MA) for 6 hours and assayed for firefly and sea pansy luciferase activities as described above. All transfections were performed in triplicate. Luciferase activities were measured as relative light units (RLU), and firefly luciferase was normalized to the sea pansy luciferase activity to correct for transfection efficiencies. Resultant ratios were used to calculate fold changes in the luciferase activity between control and treated cells. Statistical analysis of the data was performed using Student's T-test.

Western blots. Western immunoblots were used to assess the effect of ORFV024 expression on the NF- κ B pathway. HeLa cells stably expressing GFP (GFP/HeLa) or ORFV024-GFP (024GFP/HeLa) were cultured in 6-well plates for 48 hours, treated with TNF- α (20 ng/ml, Cell Signaling), harvested at various times post TNF- α -treatment (5, 10 and 20 min; or 10 and 20 min), and lysed with ProteoJET™ mammalian lysis buffer (Fermentas, Glen Burnie, MD) containing protease and phosphatase inhibitors (Sigma-Aldrich, St Louis, MO). OFTu cells cultured in 6-well plates were transfected with pCMVTag4A (2 μ g; control) or with pCMV024-Flag (2 μ g) and, 24 hours after transfection, treated with TNF- α (20 ng/ml, Cell Signaling), and harvested at 10 and 20 min after TNF- α treatment as described above. Untreated cells were used as controls in all experiments. Approximately 50 μ g of total protein lysate were resolved by SDS-PAGE in 10% acrylamide gels followed by blotting to nitrocellulose membranes (Bio-Rad). Blots were incubated overnight at 4°C with antibodies against IKK α (sc-52932, Santa Cruz), IKK β (#2370, Cell Signaling), phospho-IKK α / β (#2681, Cell Signaling), I κ B α (sc-371, Santa Cruz), phospho-I κ B α (Ser32/36) (#9246, Cell Signaling), NF- κ B-p65 (sc-7151, Santa Cruz), phospho-NF- κ B-p65 (Ser536) (#3033, Cell Signaling), G β (sc-378, Santa Cruz), GAPDH (sc-25778, Santa Cruz), Flag-M2 (200471, Stratagene) or GFP (sc-8334, Santa Cruz). After washing to remove unbound antibodies (3X for 10 min with TBS-Tween 20 0.1%), blots were incubated with secondary goat anti-rabbit IgG-(HRP) (sc-2004, Santa Cruz) or goat anti-mouse IgG-(HRP) (#7076, Cell Signaling) for

1.5 h at room temperature and developed using chemiluminescent reagents (ECL; Pierce-Thermo Scientific, Rockford, IL). Densitometric analysis of the blots was performed using the software Image J, version 1.62 (National Institute of Health, Bethesda, MD). Statistical analysis of the densitometry data was performed using Student's T-test.

Nuclear translocation of NF- κ B-p65. Nuclear translocation of NF- κ B-p65 was investigated in cells stably expressing GFP (GFP/HeLa) or ORFV024-GFP (024GFP/HeLa). Cells were cultured in 6-well plates for 48 hours (5×10^5 cells/well), treated with TNF- α (20 ng/mL) for 45 min, and harvested in PBS (0.5 mL/well). Cytoplasmic and nuclear protein fractions were extracted using the ProteoJetTM cytoplasmic and nuclear protein extraction kit (Fermentas, Glen Burnie, MD) following the manufacturer's protocol. Levels of translocated NF- κ B-p65 were determined by western immunoblots as described above. Twenty micrograms of cytoplasmic and nuclear protein extracts were resolved by SDS-PAGE and blotted to nitrocellulose membranes. Blots were incubated with antibodies against NF- κ B-p65 (sc-7151, Santa Cruz), GAPDH (sc-25778, Santa Cruz) or Histone H3 (sc-10809, Santa Cruz) and detected with a secondary goat anti-rabbit-IgG-(HRP) antibody followed by incubation with ECL substrate.

Animal inoculation. Four to five months-old lambs (70-80 lbs) were obtained from an orf-free flock and randomly allocated to three groups, consisting of mock-infected (n=3), OV-IA82 infected (n=3), and OV-IA82 Δ 024-infected lambs (n=3). Lambs were tranquilized with xylazine (Rompun[®], Bayer) and sites of inoculation were cleaned with sterile water. Inoculation was performed by scarification of the mucocutaneous junction of the inferior lip, and the left axillary skin, followed by local application of 0.5 ml virus suspension containing $10^{7.5}$ TCID₅₀/mL in MEM. Animals were monitored daily for 19 days for changes in body temperature and presence of mucocutaneous lesions including, erythema, papules, vesicles, pustules and scabs. Skin biopsies were collected from the axilla on days 3, 5, 7, and 19 pi, and processed for histological examination following standard procedures. All animal procedures were reviewed and approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee, IACUC, protocol # 08-01-001D of 01/22/08.

Results

Parapoxvirus *ORF024* encodes for a novel protein with no homology to other viral or cellular proteins. ORFV and BPSV ORF024 are 288-292 and 227 amino acids in length, respectively, with predicted molecular masses ranging from 26.2 to 32.7 kDa. ORFV strain OV-IA82 ORFV024 shares 93-98%, 92% and 62% amino acid identity with homologues from sheep isolated ORFV strains OV-NZ2 and OV-Orf11, goat isolated ORFV strain OV-SA00, and BPSV strains BV-AR02 and BV-TX09, respectively. No homologues of ORFV024 were detected outside the parapoxviruses. Notably, while retaining residues 1 to 4 and 12 to 19, BPSV ORF024 lacks 64 residues present in the amino terminus of all ORFV strains. Although ORF024 carboxyl-terminus is highly conserved among ORFV and BPSV strains, some degree of inter-species variability was observed at amino acid residues 116 to 132, 193 to 201 and 274 to 283. No motifs indicative of protein function were detected in ORFV024.

***ORFV024* is transcribed as an early gene and the protein localizes to the cell cytoplasm.**

To determine the kinetics of *ORFV024* transcription, primary OFTu cells were inoculated with OV-IA82 in the presence or absence of AraC and cells were harvested at various time points p.i. Transcription of *ORFV024* was detected by RT-PCR as early as one hour p.i., and the levels of expression increased thereafter during the infection cycle (up to 24 hours p.i.) either in the presence or absence of AraC (Fig. 1A). A similar transcription pattern was observed for the early ORFV gene *ORFV127* (Fig. 1A) (20). In contrast, transcription of *ORFV055*, which encodes for a late virion membrane protein (14) was detected at late time points p.i. but was markedly decreased in AraC-treated cells (Fig. 1A). These results indicate that *ORFV024* belongs to the early class of poxviral genes, which is known to encode for proteins involved in immune evasion and immunomodulation (1).

The subcellular localization of ORFV024 was investigated in HeLa cells stably expressing ORFV024-GFP fusion protein. ORFV024 localized to the cell cytoplasm, exhibiting a punctate distribution pattern in this compartment (Fig. 1B). A similar distribution pattern of ORFV024-GFP was observed in stable cell lines infected with OV-IA82 Δ 024 (data not shown). No co-localization was detected when 024GFP/HeLa cells were transfected with plasmids encoding markers for the nucleus (pDSRed-Nuc) (Fig. 1B), endoplasmic reticulum (pDSRed-ER), mitochondria (pDSRed-Mito) or peroxisomes (pDSRed-Peroxi) (data not shown). ORFV024 was similarly distributed in OFTu cells transiently expressing ORFV024-GFP fusion protein (data not shown).

Construction and characterization of *ORFV024*-deletion mutant virus OV-IA82 Δ 024.

Using homologous recombination, *ORFV024* was deleted from the OV-IA82 genome and recombinant viruses were selected and plaque purified in the presence of X-Gluc. Deleted gene sequences were not detected in samples from cells infected with recombinant virus. In contrast, β -glucuronidase sequences were detected in DNA samples from cells infected with recombinant but not wild type virus (data not shown). DNA sequencing of regions flanking the deleted gene confirmed the integrity of parental virus sequences in the mutant virus (data not shown).

***ORFV024* is non essential for ORFV replication *in vitro*, but affects cytopathic effect and plaque morphology.**

Replication properties of OV-IA82 Δ 024 and OV-IA82 were compared after infection of primary OFTu cells and HeLa cells. No significant differences in replication kinetics and viral yields were detected between wild type and mutant virus either in OFTu or in HeLa cells, indicating that *ORFV024* is non essential for ORFV replication in these cells (Fig. 2A, B, C). Cytopathic effect (CPE) and plaque morphology induced by wild type and mutant virus differed in OFTu infected cells. While CPE induced by OV-IA82 was characterized by enlargement and flattening of infected cells followed by cell rounding (Fig. 2D), CPE induced by OV-IA82 Δ 024 was characterized by cell rounding, followed by arrangement of cells in grape-like clusters, and absence of enlarged flattened cells (Fig. 2D). Although the plaques produced by wild type and mutant virus were of similar size, OV-IA82 Δ 024 plaques exhibited a distinct morphology (Fig. 2E). OV-IA82 plaques consisted of large flat cells around a small cell-free central area, while OV-IA82 Δ 024 plaques were characterized by a large cell-free central area and absence of flattened cells.

OV-IA82 Δ 024 infection results in an increased expression of NF- κ B-regulated genes in primary OFTu cells.

To investigate potential function(s) for *ORFV024* during ORFV infection, microarray analysis was performed to determine the profile of gene expression in OFTu cells infected with wild type or *ORFV024*-deletion mutant virus. Cells infected with OV-IA82 Δ 024 exhibited a marked increase in expression of some chemokines and other pro-inflammatory genes (Table 1), most of which are known to be regulated by the NF- κ B family of transcription factors (51). Notably, genes *CCL20*, *CXCL1*, *CXCL2*, *IL-6*, *IL-8*, *NF κ BIA* and *PTGS2*, were markedly up-regulated (fold-increases up to 205.1, 73.7, 69.9, 8.9, 11.4, 9.1 and 9.3, respectively) in OV-IA82 Δ 024-infected cells at 2 and 4 hours p.i. (Table 1). The microarray data set was deposited in the Gene Expression Omnibus (GEO) database under the accession number [GSE19415](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19415).

Microarray data were confirmed with real-time PCR, with all genes tested exhibiting increased expression in OV-IA82Δ024-infected cells (Table 1). No significant differences were observed between mock and OV-IA82-infected cells (Table 1). These results indicate that expression of ORFV024 inhibits transcription of NF-κB-regulated genes in wild type virus- infected cells, likely by inhibiting the NF-κB signaling pathway.

Deletion of *ORFV024* from the OV-IA82 genome results in increased NF-κB-mediated gene transcription in infected cells. To investigate the effect of ORFV024 on NF-κB transcriptional activity, primary OFTu cells were transfected with a plasmid encoding a luciferase reporter gene under the control of a NF-κB-responsive promoter, and subsequently inoculated with OV-IA82, or OV-IA82Δ024, or mock-infected. Luciferase activity levels as determined at 2, 4 and 6 hours p.i. were similarly low in mock-infected and in wild type virus-infected cells. In contrast, infection with OV-IA82Δ024 resulted in a marked and significant increase of up to 5.8-fold ($P < 0.005$) in luciferase activity relative to the levels for mock-infected cells (Fig. 3A). These results indicate that expression of ORFV024 inhibits NF-κB-mediated gene transcription in ORFV-infected cells.

ORFV024 suppresses NF-κB-regulated gene transcription following treatment of cells with LPS and TNF-α. The ability of ORFV024 to inhibit NF-κB transcriptional activity was further investigated in primary OFTu and in HeLa cells. OFTu cells were co-transfected with a vector encoding ORFV024-Flag fusion protein and with a plasmid encoding a firefly luciferase reporter gene. Expression of ORFV024-Flag in LPS-treated OFTu cells significantly decreased NF-κB-regulated luciferase activity (~2-fold; $P < 0.01$) when compared to the levels for control cells (Fig. 3B). Additionally, HeLa cells stably expressing GFP or ORFV024-GFP were transfected with the vector encoding the firefly luciferase reporter gene and treated with TNF-α for 6 hours. NF-κB-mediated transcription of the luciferase gene was decreased approximately 4- to 5-fold ($P < 0.05$) in ORFV024-GFP-expressing cells when compared to the levels for GFP-expressing controls (Fig. 3D). Together, these results indicate that ORFV024 inhibits activation of the NF-κB signaling pathway by two potent inducers, LPS and TNF-α.

ORFV024 decreases phosphorylation and nuclear translocation of NF-κB-p65 induced by TNF-α. Given that post-translational modifications define transcriptional functions of NF-κB-p65 in the nucleus (4, 36, 54), the effect of ORFV024 expression on NF-κB-p65 phosphorylation was investigated. HeLa cells stably expressing GFP (GFP/HeLa) or ORFV024-GFP (024GFP/HeLa) and OFTu cells transfected with pCMVTag4A (empty) or pCMV024-Flag were treated with TNF-α and harvested at various time points post-treatment.

Expression of ORFV024 markedly decreased phosphorylation of NF- κ B-p65 at Ser536 induced by TNF- α in both HeLa and OFTu cells (Fig. 4A, and C). Densitometric analysis of blots probed with phosphor-NF- κ B-p65 (Ser536) antibody revealed a reduction of 51 and 66% in phosphorylation of NF- κ B-p65 in HeLa and OFTu cells, respectively (Fig. 4B, [$P < 0.005$], and D [$P < 0.05$]). The reduced levels of phosphor-NF- κ B-p65 are not due to protein degradation since levels of pan-NF- κ B-p65 and G β were constant among all samples examined (Fig. 4A and C).

Additionally, nuclear translocation of NF- κ B-p65 in response to TNF- α treatment was assessed in cells stably expressing GFP (GFP/HeLa) or ORFV024-GFP (024GFP/HeLa). Expression of ORFV024-GFP markedly decreased nuclear translocation of NF- κ B-p65 in response to TNF- α -treatment when compared to the levels for control GFP-expressing cells (Fig. 4E). The decreased levels of NF- κ B-p65 in the nucleus of 024GFP/HeLa cells were not due to protein degradation or to differences in protein loading, since levels of NF- κ B-p65 in cell cytoplasm extracts were constant and no differences in the levels of the nuclear protein histone H3 were detected among GFP and ORFV024-GFP samples. Together, the decreased levels of phosphor and nuclear translocated NF- κ B-p65 demonstrate an inhibitory effect for ORFV024 on the NF- κ B signaling pathway and further indicate the protein interferes with the pathway upstream of NF- κ B-p65 phosphorylation.

ORFV024 decreases phosphorylation of IKK α and IKK β in TNF- α -treated cells. The crucial step on the activation of NF- κ B signaling pathway is the phosphorylation and subsequent degradation of I κ B α , which leads to release, post-translational modification, and nuclear translocation of NF- κ B-p65 (33, 53). To investigate whether the decreased levels of phosphor and nuclear translocated NF- κ B-p65 detected in ORFV024 expressing cells were due to an interference with I κ B α phosphorylation and/or degradation, GFP/HeLa and 024GFP/HeLa cells were examined for pan and phosphor levels of I κ B α . ORFV024 expression was accompanied by a decrease in TNF- α -induced I κ B α phosphorylation and, consequently, its degradation (Fig. 5A). Densitometric analysis of blots probed with antibody against phosphor I κ B α demonstrated a reduction of approximately 50% in I κ B α phosphorylation in 024GFP/HeLa cells (Fig. 5B, [$P < 0.005$]).

I κ B phosphorylation is mediated by upstream I κ B kinases (33, 53). Given the importance of IKKs in triggering NF- κ B activation and the decreased levels of phosphor I κ B α detected in ORFV024 expressing cells, the potential role of ORFV024 as an inhibitor of IKK activation was investigated. ORFV024 expression markedly reduced phosphorylation of IKK α and IKK β in 024GFP/HeLa cells (Fig. 6). These results indicate a role for ORFV024 in

inhibiting I κ B α phosphorylation and degradation by decreasing phosphorylation of IKK subunits IKK α/β .

ORFV024 is non essential for ORFV virulence in the natural host. To investigate whether ORFV024 affects ORFV virulence, 4-5 months old, crossbreed lambs were inoculated with OV-IA82, OV-IA82 Δ 024, or MEM (control group). Following local scarification, lambs were inoculated topically at the mucocutaneous junction of the lower lip and at the axillary skin, and examined for the duration of the 19-days experiment. Characteristic clinical orf was observed in all virus-inoculated animals. Lesions developed by day 3 p.i., and consisted of erythema and small papules, which evolved into vesicles and pustules at later time points p.i. (Fig. 7). Local tissue proliferation and scab formation were first observed by days 5 and 7 p.i., respectively. Lesions started to subside on day 14 p.i., and by day 19 p.i. only limited scabs remained at lesion margins. No significant differences were observed in lesion severity, progression, or time to resolution between lambs inoculated with OV-IA82 or OV-IA82 Δ 024. Lambs from the control group did not exhibit changes other than mild scab formation due to skin scarification, which was completely resolved by day 4 p.i. (Fig. 7).

Histopathological changes in axillary skin from virus-inoculated lambs consisted of marked epidermal hyperplasia, ballooning degeneration of keratinocytes in the stratum spinosum, hyperkeratosis, dyskeratosis, dermal leukocyte infiltration, occasional intraepithelial microabscesses, and accumulation of scale-crust (data not shown). No significant differences in the severity or time course of histological changes were observed between lambs inoculated with OV-IA82 or OV-IA82 Δ 024. Samples from mock-inoculated lambs were indistinguishable from normal skin. These results indicate that ORFV024 does not significantly affect ORFV virulence in the natural host.

Discussion

Microarray and real-time PCR analysis of cells infected with deletion mutant virus OV-IA82 Δ 024 indicated that ORFV024 suppresses expression of NF- κ B regulated proinflammatory genes, suggesting that ORFV024 inhibits activation of the NF- κ B signaling pathway. Consistent with this hypothesis, we found a significant increase in expression of a NF- κ B-responsive reporter gene in cells infected with OV-IA82 Δ 024 relative to those infected with wild type virus. ORFV024 expression was shown here to suppress NF- κ B-mediated transcription following treatment of cells with LPS or TNF- α . ORFV024 expression decreased TNF- α -induced phosphorylation and nuclear translocation of NF- κ B-p65, phosphorylation and degradation of I κ B α , and phosphorylation of IKK complex subunits

IKK α and IKK β . Together, these results indicate that ORFV024 prevents critical IKK complex activation, thus providing a mechanism for inhibition of NF- κ B-mediated gene expression in infected cells.

Many poxviral proteins have been shown to inhibit activation of the I κ B kinases, the bottleneck for most NF- κ B-activating stimuli (11, 15, 24, 30, 45, 50, 62). For example, VACV proteins A46R, A52R, B14, and M2L interfere with activation of the IKK complex by antagonizing TLR signaling pathway (A46R and A52R), inhibiting activation of the IKK complex by the MEK/ERK signaling pathway (M2L), or directly binding to the IKK complex (B14L) (3, 11, 15, 24, 30, 62). Additionally, VACV N1L presumably inhibits activation of the IKK complex by functioning upstream of IKKs, either at the level of adaptor protein TRAF6, or on downstream proteins that precede the IKK complex in the signaling cascade (26).

ORFV024 was shown here to inhibit activation of the IKK complex by decreasing phosphorylation of IKK α and IKK β . Interaction of ORFV024 with IKK α , IKK β , and IKK γ was investigated as a potential mechanism to prevent phosphorylation of the I κ B kinases. However, no association between ORFV024 and IKK α , IKK β , or IKK γ was detected (data not shown), suggesting that ORFV024 targets steps upstream of the IKK complex. Given that ORFV024 decreased expression of a NF- κ B-responsive reporter gene following stimulation by both LPS and TNF- α , it is likely that this protein has evolved to interfere with activation of both, TLRs and TNFR signaling pathways (34, 65). The lack of homology between ORFV024 and other poxviral NF- κ B inhibitors or any other molecule involved in the NF- κ B signaling pathway, precludes assumptions on the precise mechanism by which ORFV024 interferes with IKK activation.

Like many poxviral genes encoding immune evasion and immunomodulatory proteins (1), including NF- κ B inhibitors (42), transcription of *ORFV024* in cultured cells was detected throughout ORFV infection cycle, indicating that it belongs to the early class of viral genes (1). ORFV024-GFP fusion protein exhibited a punctate distribution in the cytoplasm of stably expressing cells, and no co-localization of ORFV024-GFP with markers for the nucleus and specific cellular organelles was observed. This is likely of biological significance given that NF- κ B activation pathways affected by ORFV024 take place in the cell cytoplasm [reviewed by (33)].

Replication characteristics of OV-IA82 Δ 024 in cultures of OFTu and HeLa cells were similar to those of OV-IA82, indicating that *ORFV024* is non essential for ORFV replication in these cells, and that like other poxviral NF- κ B inhibitors (2, 11, 30, 41, 62),

ORFV024 is dispensable for virus replication *in vitro*. Notably, OV-IA82 Δ 024 exhibited a distinct cytopathic effect (cell rounding and arrangement of cells in grape-like clusters) and plaque morphology (large cell-free central area and absence of flattened cells) in OFTu cells. These differences are likely explained by the increased expression of NF- κ B-regulated genes induced by OV-IA82 Δ 024 infection, since activation of the NF- κ B signaling pathway, and consequent gene expression, have been implicated in morphological and cytoskeletal changes in microglial cells after exposure to dsRNA (47) and in human endothelial cells expressing a viral activator of the NF- κ B pathway (K13 protein from Human herpesvirus-8) (37). The mechanisms by which ORFV024 influences cell morphology and cytoskeleton organization remain to be determined.

Given that the NF- κ B signaling pathway integrates many aspects of the host innate and adaptive immunity (56), it was surprising that deletion of *ORFV024* from the OV-IA82 genome had no significant effect on disease severity, progression and resolution in sheep, indicating that ORFV024 is non essential for virus virulence in the natural host. Although selected poxviral NF- κ B inhibitors have been shown to affect virus virulence and pathogenesis to some extent (2, 11, 30, 41, 62), the degree of attenuation after single viral gene deletions has been modest and variable. For example, VACV *B14R* was shown to contribute to virus virulence in an intranasal but not intradermal murine model of infection (10), while deletion of VACV *A46R*, *A52R* or *NIL*, rendered the virus partially attenuated in a mouse intranasal model (2, 30). In contrast, deletion of ankyrin repeat-encoding myxoma virus *M150R* and cowpox virus *CPXV006* genes resulted in marked virus attenuation in rabbit intradermal and mouse intratracheal models of infection, respectively (6, 42). These observations indicate that contribution of poxviral NF- κ B inhibitors to virus infection and disease pathogenesis is complex, and further suggest that multiple inhibitors encoded by individual poxviruses may exert complementary, perhaps partially overlapping functions to effectively suppress NF- κ B-mediated host cell responses.

Results here, showing that *ORFV024* deletion had no significant effect on ORFV pathogenesis in sheep further support the possibility for complementary functions by poxviral NF- κ B inhibitors during infections *in vivo*. Indeed, we have identified two additional ORFV-encoded genes that interfere with the NF- κ B signaling pathway (unpublished data), suggesting that ORFV NF- κ B inhibitors may function in a coordinated and complementary fashion on distinct branches of the NF- κ B signaling cascade to effectively suppress NF- κ B-mediated host responses *in vivo*. Given the potential complementary actions of multiple

poxviral NF- κ B inhibitors, single viral gene deletions may not result in significant effects on virus virulence and pathogenesis.

Alternatively, ORFV024 may function in other, less understood aspects of host-ORFV interactions. For example, persistent/subclinical infections have been described for ORFV and BPSV (32, 49, 58). The high prevalence of antibodies against parapoxviruses in herds and flocks with no history of clinical infections [(35) unpublished data], and the ability of ORFV to persist in a flock in the absence of clinical manifestation of infection (49), suggest that persistent/subclinical infections likely play a role in maintenance of parapoxviruses in nature. It is tempting to speculate that ORFV NF- κ B inhibitors such as ORFV024 may contribute to aspects of virus persistence and transmission in the absence of overt viral infection.

The present study demonstrates that the novel ORFV protein ORFV024 interferes with activation of the NF- κ B signaling pathway, while not significantly affecting virus virulence in the natural host. This represents the first description of an NF- κ B inhibitor for ORFV or any other member of the *Parapoxvirus* genus. An improved understanding of ORFV functions associated with modulation and manipulation of host cell responses may contribute to increase current knowledge about the molecular aspects of ORFV biology and host-virus interactions.

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TABLE 1 – Summary of NF- κ B-regulated genes detected by microarray and real-time PCR in primary OFTu cells infected with OV-IA82 or *ORFV024*-deletion mutant virus OV-IA82A024.

Gene	Description	GenBank	Fold increases in mRNA expression detected by microarray analysis of OFTu cells infected with OV-IA82A024 ^a		Fold changes in mRNA expression detected by real-time PCR in OFTu cells infected with OV-IA82 or OV-IA82A024 ^b			
					OV-IA82		OV-IA82A024	
			2 hpi	4 h pi	2 hpi	4 hpi	2 hpi	4 hpi
CCL20	Chemokine ligant 20	NM_174263	80.02	205.16	1.02 ± 0.11 ^c	42.68 ± 6.17*	0.43 ± 0.03	38.21 ± 4.05*
CXCL1	Chemokine ligant 1	NM_175700	73.72	1.75	- ^d	-	-	-
CXCL2	Chemokine ligant 2	NM_174299	69.95	7.10	-	-	-	-
CXCL3	Chemokine ligant 3	NM_001046513	7.27	12.34	0.71 ± 0.07	49.5 ± 6.34*	1.49 ± 0.13	20.14 ± 4.85*
ICAM1	Intercellular adhesion molecule (CD54)	NM_174348	3.29	3.29	0.92 ± 0.08	3.98 ± 0.07**	0.73 ± 0.08	2.66 ± 0.30*
IL-1 α	Interleukin 1 alpha	NM_174092	70.09	1.05	0.87 ± 0.06	27.79 ± 4.48*	0.75 ± 0.03	10.31 ± 1.52**
IL-6	Interleukin 6	NM_173923	3.09	8.97	1.27 ± 0.37	7.60 ± 1.53*	0.70 ± 0.11	8.03 ± 0.12**
IL-8	Interleukin 8	NM_173925	10.98	11.46	0.94 ± 0.03	26.65 ± 3.62*	0.61 ± 0.11	20.56 ± 1.69**
IRF1/LOC789216	Interferon regulatory factor 1	NM_177432	17.01	1.00	0.89 ± 0.23	5.80 ± 0.38*	0.83 ± 0.1	1.14 ± 0.12
LOC614198	Similar to T cell activation NF- κ B like protein (TA-NF- κ B)	XM_865597	7.23	1.00	-	-	-	-
MAIL	Molecule possessing ankyrin repeats induced by LPS	NM_174726	15.09	2.05	-	-	-	-
MGC142340	Similar to pentraxin related protein PTX3 precursor	NM_001076259	5.98	7.52	-	-	-	-
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	NM_174112	3.01	4.91	-	-	-	-
MMP13	Matrix metalloproteinase 13 (collagenase 3)	NM_174398	1.70	2.88	-	-	-	-
MMP3	Matrix metalloproteinase 3 (progelatinase)	XM_586521	2.08	2.92	-	-	-	-
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	NM_001045868	9.12	4.40	0.92 ± 0.07	7.32 ± 0.93*	0.95 ± 0.08	3.4 ± 0.7*
PTGS2	Prostaglandin-endoperoxidase synthase 2	NM_17445	9.36	3.73	0.74 ± 0.10	8.44 ± 1.02**	0.59 ± 0.08	2.35 ± 0.64*

^a Fold changes are relative to OFTu cells infected with the wild type virus OV-IA82.

^b Fold changes are relative to mock infected OFTu cells, and expression levels on those cells were equaled to 1.

^c Standard deviation calculated based on the mean expression levels of three independent experiments.

^d Expression levels were not tested by real time-PCR.

*: $p < 0.05$; **: $p < 0.01$.

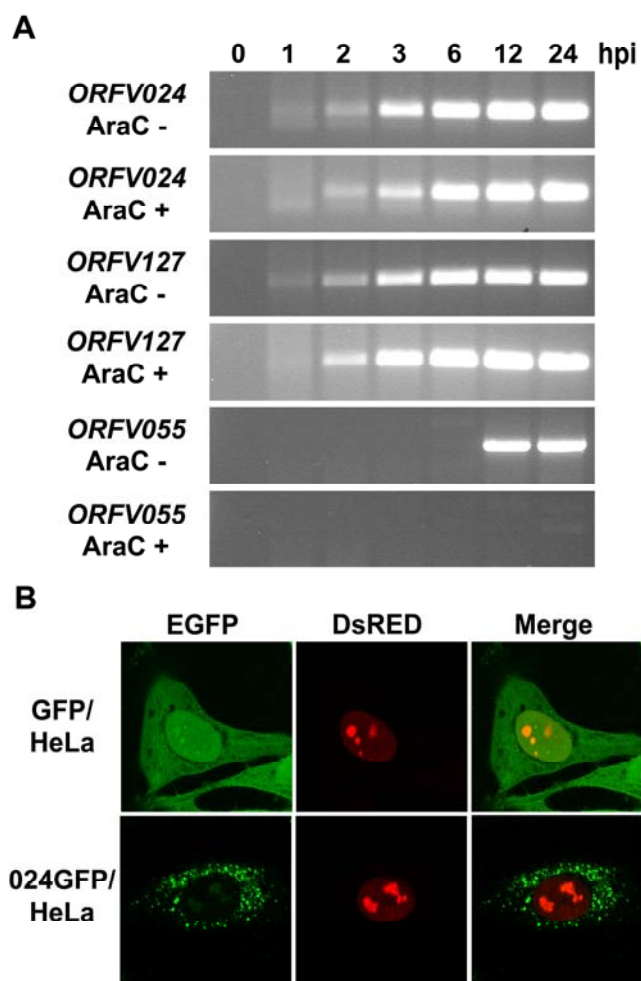


FIG. 1. Transcription kinetics and subcellular localization of ORFV024. (A) Transcription kinetics of *ORFV024*, *ORFV127* and *ORFV055* during ORFV infection cycle in OFTu cells in the presence or absence of AraC as determined by RT-PCR. (B) ORFV024 punctate localization in the cytoplasm of 024GFP/HeLa stable cell lines (60X).

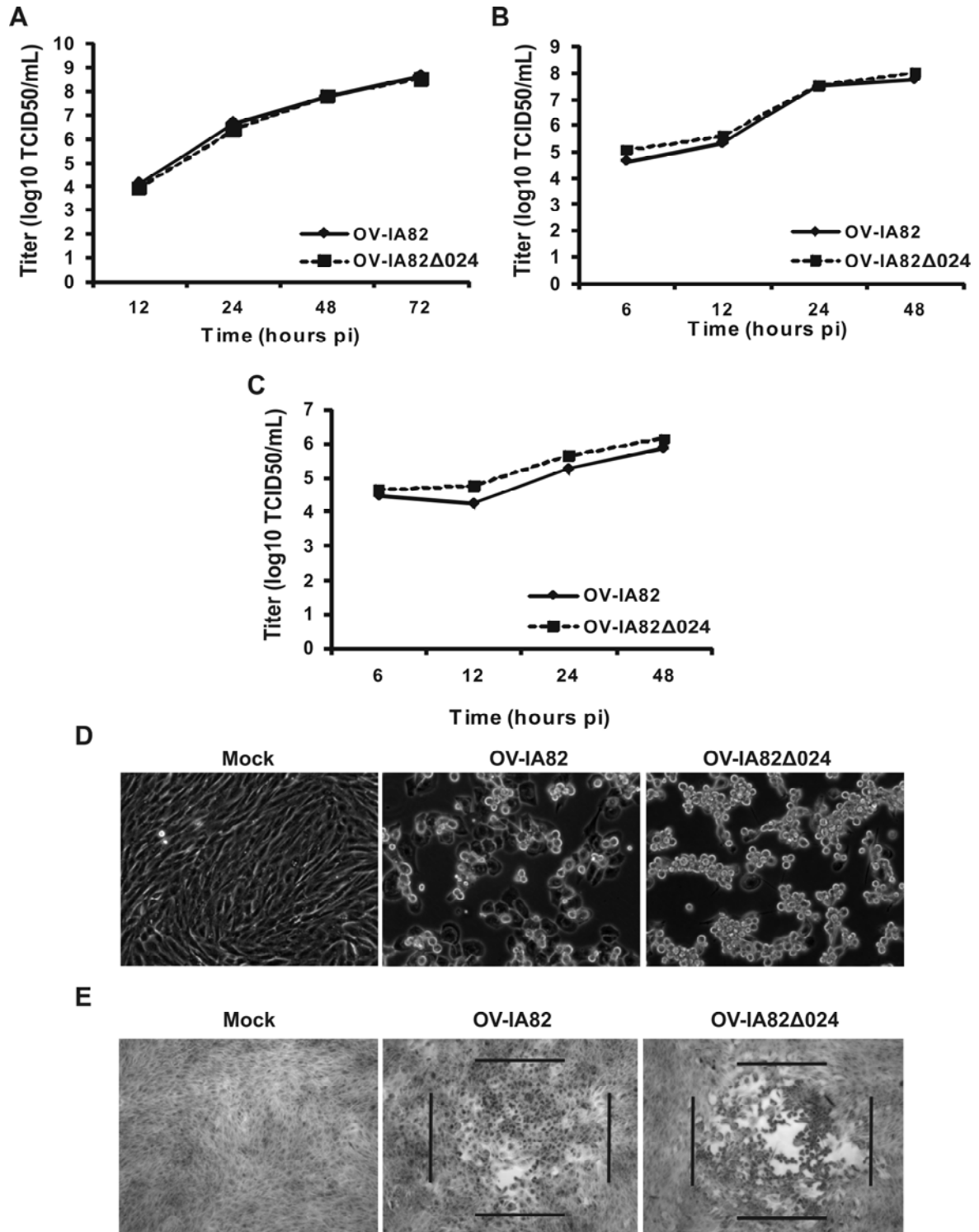


FIG. 2. Growth characteristics of *ORFV024*-deletion mutant virus OV-IA82Δ024. (A) Multiple step growth curves of wild type and mutant virus in primary OFTu cells. One step growth curves of wild type and mutant virus in primary OFTu (B) and in HeLa cells (C). (D) Cytopathic effect of wild type and mutant virus in primary OFTu cells 48 hours after infection (MOI = 10) (100X). (E) Plaque morphology of wild type and mutant viruses in OFTu cells. Seventy-two hours after infection cells were fixed with formaldehyde (3.7%) and stained with 1% crystal violet (100X). Black bars demarcate plaques produced by OV-IA82 and OV-IA82Δ024.

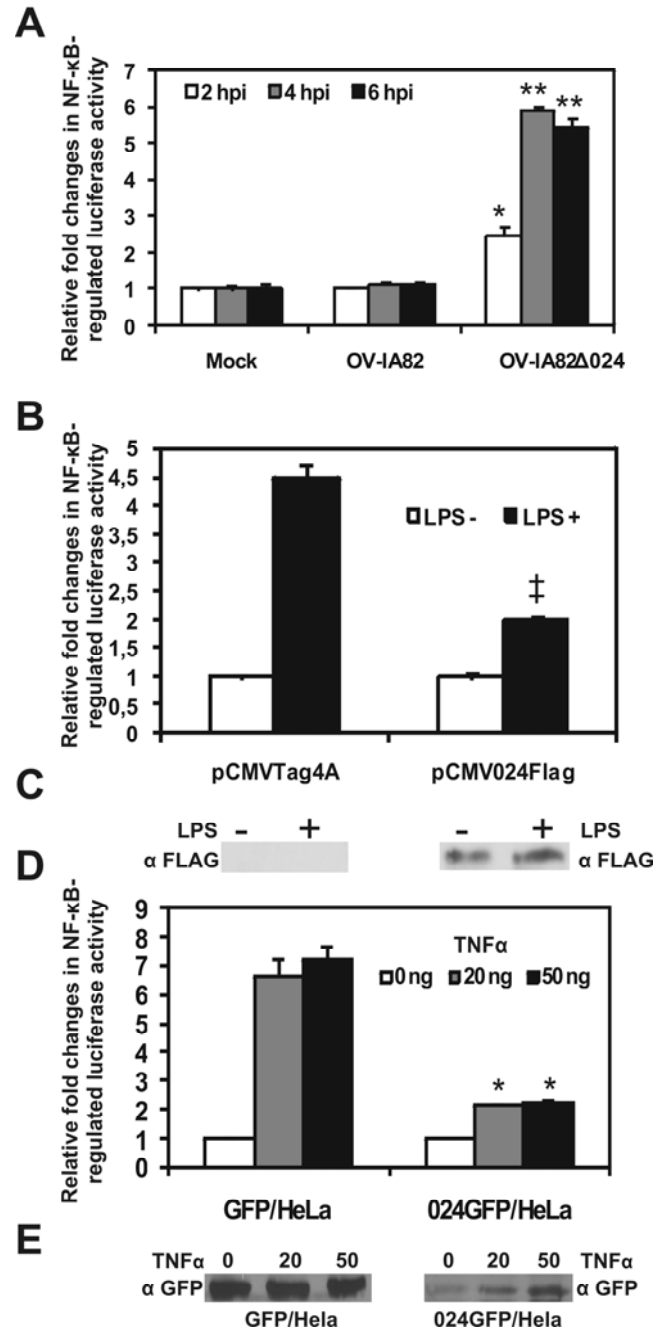


FIG. 3. Effect of ORFV024 on NF- κ B-mediated transcription. (A) Primary OFTu cells were co-transfected with a vector encoding a firefly luciferase gene under the control of NF- κ B (pNF κ B-*Luc*) and with a plasmid encoding sea pansy (*Renilla reniformis*) luciferase under the control of herpesvirus TK promoter (pRL-TK). At 24 h after transfection cells were mock-infected or infected with OV-IA82, or OV-IA82 Δ 024. Firefly and sea pansy luciferase activities were measured at 2, 4 and 6 hours p.i. and expressed as relative fold changes in luciferase activity (*: $P < 0.05$; **: $P < 0.005$). (B) OFTu cells were co-transfected with pNF κ B-*Luc*, pRL-TK and pCMV024-Flag. Twenty four hours after transfection cells were exposed to MEM containing 2% FBS and 0 or 250 ng/ml of LPS for 6 hours. Cells were harvested, and firefly and sea pansy luciferase activities were measured and expressed as fold changes in luciferase activity (‡: $P < 0.01$). (C) Western blot analysis of cell lysates from samples tested in B (anti-Flag M2 antibody). (D) HeLa cells stably expressing GFP or ORFV024-GFP were co-transfected with pNF κ B-*Luc* and pRL-TK. At 24 h after transfection, cells were exposed to MEM containing 2% FBS and 0, 20 or 50 ng/ml of TNF- α for 6 hours. Cells were harvested and firefly and sea pansy luciferase activities were determined. Firefly luciferase activity was normalized to sea pansy luciferase activity and fold changes were calculated (*: $P < 0.05$). (E) Western blot analysis of cell lysates representative of samples tested in D (anti-GFP antibody). Results are representative of two (B and D) or three (A) independent experiments.

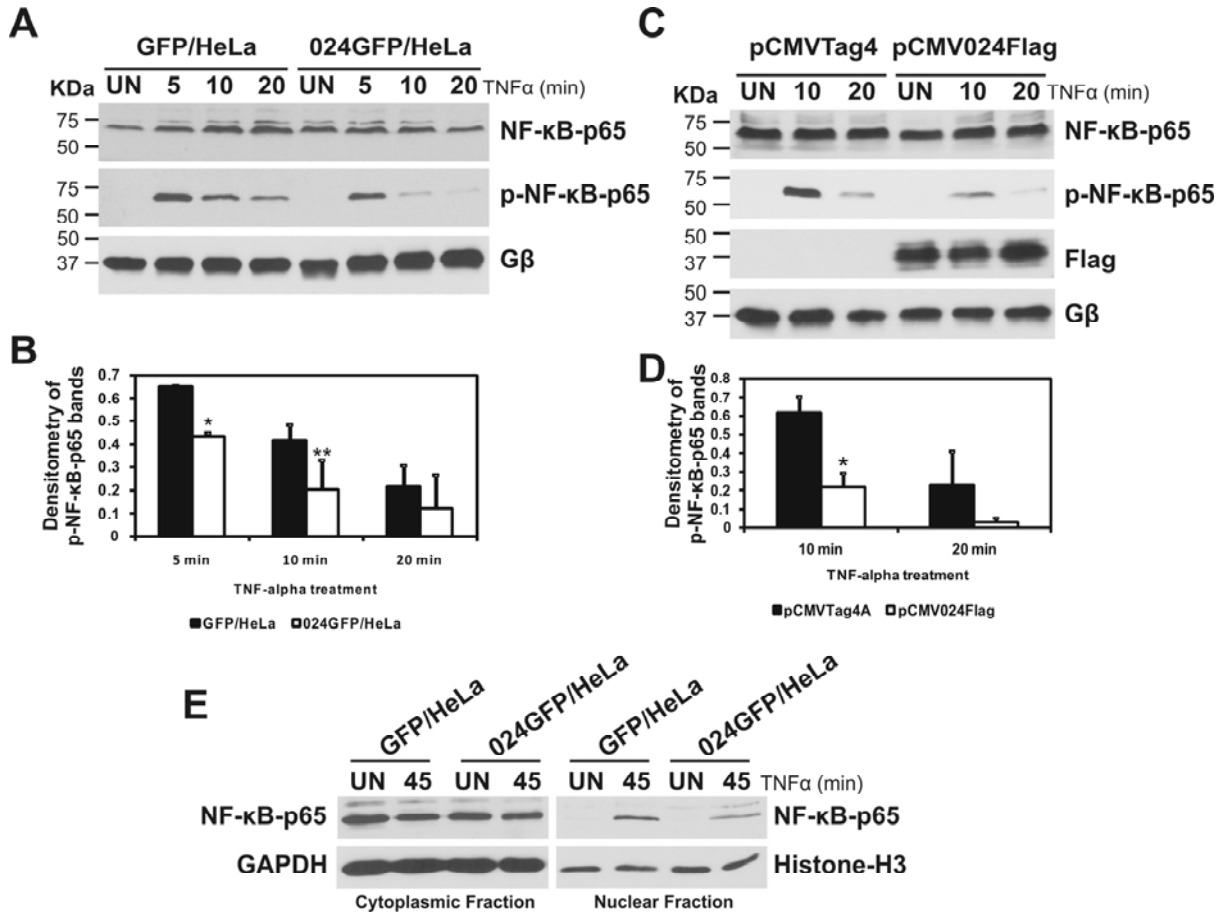


FIG. 4. Effect of ORFV024 expression on NF- κ B-p65 phosphorylation and nuclear translocation. (A) HeLa cells stably expressing GFP (GFP/HeLa) or ORFV024-GFP (024GFP/HeLa) were treated with TNF α (20 ng/mL) and harvested at the indicated time points (UN: untreated controls). Fifty micrograms of total protein extracts were resolved by SDS-PAGE, blotted, and probed with antibodies against proteins indicated on the right. (B) Densitometry of phosphor NF- κ B-p65 bands normalized to the levels for loading control G β (*: $P < 0.05$; **: $P < 0.005$). (C) OFTu cells were transfected with vectors pCMVTag4A (empty) or pCMV024-Flag, treated with TNF- α (20 ng/mL) and harvested at the indicated time points (UN: untreated controls). Fifty micrograms of total protein extracts were resolved by SDS-PAGE, blotted, and probed with the antibodies directed against the proteins indicated on the right. (D) Densitometry of phosphor NF- κ B-p65 bands normalized to the levels for loading control G β (*: $P < 0.05$). (E) HeLa cells stably expressing GFP (GFP/HeLa) or ORFV024-GFP (024GFP/HeLa) were treated with TNF- α (20 ng/mL) for 45 min, and cytoplasmic and nuclear protein fractions were extracted (UN: untreated controls). Twenty micrograms of total protein extracts were resolved by SDS-PAGE, blotted, and probed with antibodies against NF- κ B-p65 (top panels), GAPDH (bottom left panel) or Histone-H3 (bottom right panel). Results are representative of two (E) or three independent experiments (A, B, C and D).

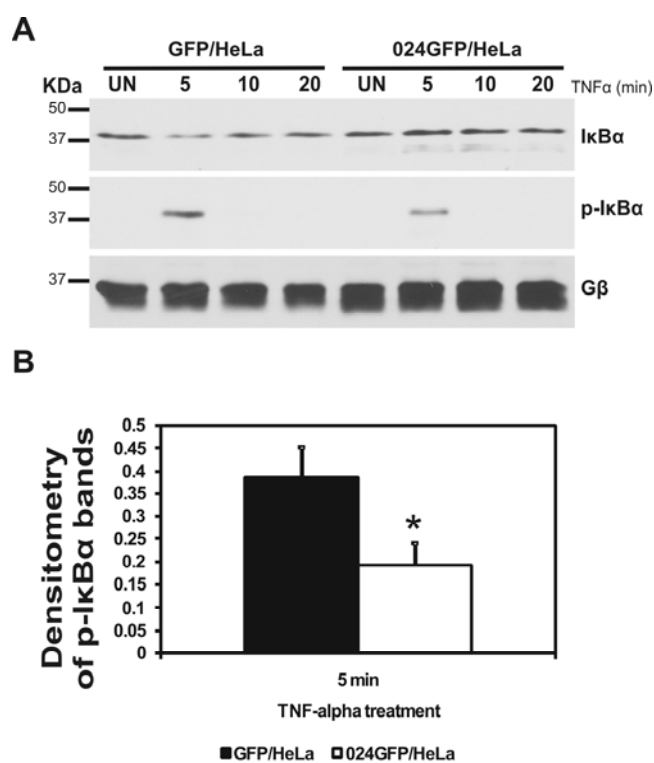


FIG. 5. Effect of ORFV024 expression on I κ B α phosphorylation and degradation. (A) HeLa cells stably expressing GFP (GFP/HeLa) or ORFV024-GFP (024GFP/HeLa) fusion protein were treated with TNF- α (20 ng/ml) and harvested at the indicated time points (UN: untreated controls). Fifty micrograms of total protein extracts were resolved SDS-PAGE, blotted, and probed with the antibodies directed against the proteins indicated on the right. (B) Densitometry of phosphor I κ B α bands normalized to the levels of G β (*: $P < 0.005$). Results are representative of five independent experiments.

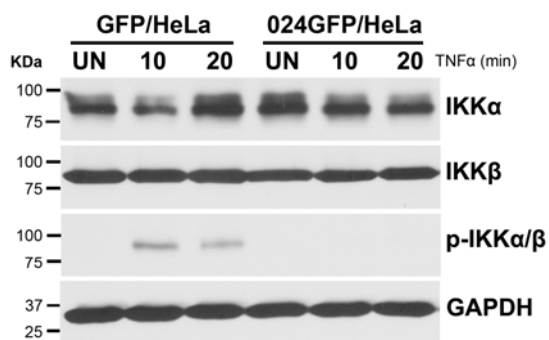


FIG. 6. Effect of ORFV024 expression on IKK α and IKK β phosphorylation. HeLa cells stably expressing GFP (GFP/HeLa) or ORFV024-GFP (024GFP/HeLa) fusion protein were treated with TNF- α (20 ng/ml) and harvested at the indicated time points (UN: untreated controls). Fifty micrograms of total protein extracts were resolved by SDS-PAGE, blotted, and probed with the antibodies against the proteins indicated on the right. Results are representative of three independent experiments.

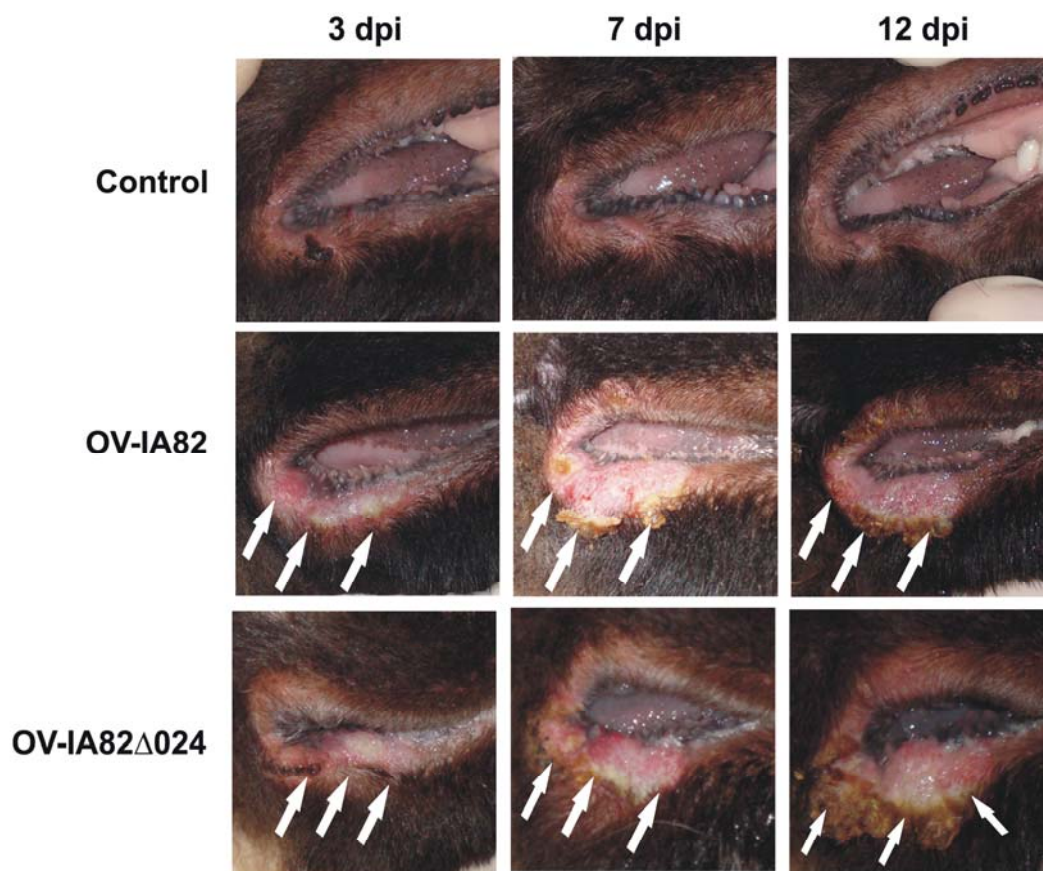


FIG. 7. Clinical course of orf in sheep. Groups of four to five months-old lambs were inoculated with MEM, OV-IA82 or OV-IA82 Δ 024 at the mucocutaneous border of the lower lip following scarification. Characteristic orf lesions (arrows) were observed in both OV-IA82 and OV-IA82 Δ 024 inoculated lambs (days 3, 7 and 12 p.i.).

4. CAPÍTULO 2

A Nuclear Inhibitor of NF- κ B Encoded by a Poxvirus

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Abstract

Poxviruses have evolved various strategies to inhibit cytoplasmic events leading to activation of the Nuclear Factor- κ B (NF- κ B) signaling pathway, with individual viruses often encoding for multiple NF- κ B inhibitors. Here, the novel orf virus (ORFV)-encoded protein ORFV002 was shown to inhibit nuclear events regulating NF- κ B transcriptional activity. ORFV002 expression in cell cultures significantly decreased wild-type virus-, tumor necrosis factor alpha (TNF- α -), and lipopolysaccharide (LPS)-induced NF- κ B-mediated gene expression. Expression of ORFV002 in cells, while not affecting phosphorylation or nuclear translocation of NF- κ B-p65, markedly decreased TNF- α - and wild-type virus-induced acetylation of NF- κ B-p65, a p300-mediated nuclear modification of NF- κ B-p65 that regulates its transactivating activity. ORFV002 was shown to colocalize and interact with NF- κ B-p65, and expression of ORFV002 in cell cultures resulted in a reduced interaction of NF- κ B-p65 with p300, suggesting that ORFV002 interferes with NF- κ B-p65/p300 association. Deletion of *ORFV002* from the OV-IA82 genome had no significant effect on ORFV pathogenesis in sheep, indicating that *ORFV002* is non essential for virus virulence in the natural host. This represents the first description of a nuclear inhibitor of NF- κ B encoded by a poxvirus.

Introduction

Orf virus (ORFV), the type member of the genus *Parapoxvirus* of the *Poxviridae*, is the causative agent of orf or contagious ecthyma, a ubiquitous disease of sheep and goats (33). Orf is characterized by proliferative lesions affecting muco-cutaneous tissues, that evolve through stages of erythema, papules, vesicles, pustules, and scabs (19, 23). Orf is a zoonotic disease affecting humans in close contact with infected animals (17, 30, 42).

The ORFV genome is approximately 138 kilobase pairs in length, and contains 131 putative genes, 89 of which are conserved in all characterized chordopoxviruses (12, 45). Several immunomodulatory genes with putative virulence functions have been identified in the ORFV genome, including an interferon (IFN)-resistance gene (*ORFV020*), a gene encoding a chemokine binding protein (CBP; *ORFV112*), an inhibitor of granulocyte/macrophage-colony stimulating factor and IL-2 (GIF; *ORFV117*), a Bcl-2-like inhibitor of apoptosis (*ORFV125*), a homologue of IL-10 (*ORFV127*), and a vascular endothelial growth factor (VEGF; *ORFV132*) (19, 49). Notably, ORFV encodes 15 mostly terminally located genes, with no similarity to other poxvirus or cellular proteins and with putative virulence and host range functions (12). Recently one of these, ORFV ORFV024 was shown to inhibit activation of the nuclear factor-kappa B (NF- κ B) signaling pathway, while not significantly affecting ORFV pathogenesis in sheep (13).

ORFV is a highly epitheliotropic virus and keratinocytes and their counterparts in the oral mucosa are the most important if not the only cell type to support ORFV replication *in vivo* (27). Keratinocytes produce the protective stratum corneum of the epidermis, and function as immune sentinels and instigators of inflammatory responses in the skin (39). The NF- κ B family of transcription factors plays a central role in integrating stress-inducing stimuli and innate immune responses in the epidermis. NF- κ B also plays roles in keratinocyte proliferation and differentiation, although the mechanisms involved may be indirect (41). Remarkably, continuous activation or continuous inhibition of the NF- κ B canonical pathway in keratinocytes results in enhanced inflammatory response in the skin, which indicates a complex role for NF- κ B in skin immune homeostasis (36, 39).

The NF- κ B family of transcription factors consists of five members in mammals, NF- κ B-p65 (RelA), RelB, c-Rel, NF- κ B-p50/p105, and NF- κ B-p52/p100, which contain an N-terminal Rel homology domain (RHD) responsible for homo- and heterodimerization and for sequence specific DNA binding (47). The activity of NF- κ B dimers is initially regulated by their association with the inhibitory I κ B molecules, which sequester NF- κ B in the cytoplasm

(28). Various stimuli, including the proinflammatory cytokines tumor necrosis factor α (TNF- α) and IL-1, bacterial lipopolysaccharide (LPS), viruses, and viral products, lead to phosphorylation of I κ B proteins by I κ B kinases (IKK), resulting in proteasomal degradation of I κ B and nuclear translocation of NF- κ B subunits (28). Regulation of NF- κ B nuclear activity is critical for NF- κ B target gene selection and transcriptional activity. Various post-translational modifications as well as association with non-Rel binding partners affect NF- κ B DNA binding affinity, interaction with coactivators and corepressors, and transactivating activity (16, 40, 48). For example, inducible phosphorylation by various kinases has been described to occur at multiple NF- κ B-p65 sites, leading to promoter-specific modulation of NF- κ B transcriptional activity (40). Likewise, inducible NF- κ B-p65 acetylation by p300/CBP or p300/CBP-associated factor (PCAF) affects NF- κ B-p65 DNA binding, association with I κ B α , and transcriptional activation (8, 29). Recently, inducible methylation of NF- κ B-p65 by SET9 methyltransferase was shown to regulate NF- κ B-p65 promoter binding and transcriptional activation of selected genes (16). Functional interplay between the various post-translational modifications has been reported (9). An additional level of regulation is represented by the requirement of nucleosome remodeling for activation of selected NF- κ B target genes (44).

Intracellular inhibitors of NF- κ B have been identified in viruses of the genera *Orthopoxvirus*, *Leporipoxvirus*, *Yatapoxvirus*, *Molluscipoxvirus*, and *Parapoxvirus*, with selected viruses encoding for multiple inhibitors (13, 31). While orthologs of some NF- κ B inhibitors are found in viruses belonging to multiple poxvirus genera (e.g. VACV A52R, VACV E3L), others are restricted to a particular genera (e.g. VACV A46R and VACV B14R in *Orthopoxvirus*). Notably, with the exception of the E3L homologue (ORFV020), parapoxviruses lack homologues of NF- κ B inhibitors identified in other chordopoxvirus genera. On the other hand, parapoxvirus ORFV024 is a NF- κ B inhibitor unique to this group of viruses (13).

Poxviral NF- κ B inhibitors target mainly cytoplasmic events leading to activation of the NF- κ B signaling pathway (31). Vaccinia virus (VACV)-encoded NF- κ B inhibitors target cytoplasmic steps leading to activation of the IKK complex (A52R, A46R, B14, N1L and M2L), degradation of I κ B α (K1L), or activation of the protein kinase RNA (PKR) signaling pathway (E3L) (2, 11, 14, 24, 35, 43, 46). Molluscum contagiosum virus (MOCV)-encoded protein MC159 prevents degradation of I κ B β , while MC160 induces degradation of IKK α (34, 38). ORFV-encoded protein ORFV024 decreases phosphorylation of IKK α and IKK β , thus preventing activation of the IKK complex (13). Notably, deletion of individual genes

encoding selected NF- κ B inhibitors from poxviral genomes results in variable and in most cases very modest, degrees of virus attenuation *in vivo* (1, 10, 13, 24). With a few exceptions (myxoma virus MXV150, cowpoxvirus CPXV006) no single gene-deletion rendered complete virus attenuation (1, 4, 10, 24, 32).

Here, we present data demonstrating that the novel ORFV protein ORFV002 localizes to the cell nucleus, binds to NF- κ B-p65, a transactivating NF- κ B subunit, and decreases acetylation of NF- κ B-p65, a nuclear modification required for full NF- κ B transcriptional activity. This is the first description of a poxviral NF- κ B inhibitor targeting nuclear events regulating NF- κ B transactivating activity.

Materials and Methods

Cells and viruses. Primary ovine fetal cells (Ovine fetal turbinate [OFTu]) were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), containing L-glutamine (2 mM), gentamicin (50 μ g/ml), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Primary ovine keratinocytes (OKTs) were obtained by treating inguinal skin strips with dispase (1.2 UI/ml; Invitrogen) in RPMI1640 medium containing 10% FBS and antibiotics overnight at 4°C. Epidermal sheets were mechanically separated, washed in PBS, and digested with trypsin (TrypLE; Invitrogen) at room temperature for 1 hour. OKT suspensions were washed with PBS, resuspended and maintained in CnT-8 medium (CELLnTEC Advanced Cell Systems, Switzerland). ORFV strain OV-IA82 (12) was used to generate the *ORFV002*-deletion mutant virus OV-IA82 Δ 002, and in all procedures involving infections with wild-type virus, and cloning of viral genes. OV-IA82 Δ 002 was used to generate the *ORFV002*-revertant viruses OV-IA82Rv002 and OV-IA82Rv002GFP.

Plasmids. *ORFV002* coding sequences were synthesized by EZBiolab Inc. (Westfield, IN) and subcloned into the expression vector pEGFP-N1 to generate p002EGFP plasmid (Clontech, Mountain View, CA). DNA sequencing of p002EGFP confirmed the integrity of *ORFV002* coding sequences and in frame cloning with EGFP.

To generate *ORFV002* deletion mutant virus (OV-IA82 Δ 002), left (LF; 1012 bp) and right (RF; 1085 bp) *ORFV002* flanking regions were PCR amplified from the OV-IA82 genome and cloned into the vector pZippy-Neo/Gus (15). Primers used for amplification were: 002LF-Fw1(SpeI):5'-ACTACGACTAGTGCCACTATACCAGCCAGAG-3', 002LF-Rv1(SalI): 5'-ATCATCGTTCGACTGTGACGACAAGGAGAGAC-3', 002RF-Fw2(NsiI):5'-

AATCATATGCATAGCCCTTCGCCTGCGGAGGA-3', and 002RF-Rv2(BglII):5'-ACCA GTAGATCTCTCGTCTCTACACCGA-3'. Restriction enzymes used for cloning are indicated in parenthesis for each primer. The recombination cassette pZippy-002LF-Neo/Gus-002RF was constructed as previously described (13).

To generate an ORFV002 revertant virus (OV-IA82Rv002), a 2.5 Kb DNA fragment containing *ORFV002* coding sequence and its left and right flanking regions was excised from the OV-IA82 genome by using restriction enzymes AflIII and BamHI (nucleotide position 1186 and 2573, respectively), treated with large fragment DNA polymerase I (Invitrogen, San Diego, CA), and cloned into the EcoRV restriction site of plasmid pcDNA3.1 (Invitrogen), resulting in the recombination cassette pcDNA-Rv002.

ORFV002-GFP was PCR amplified from the p002EGFP vector and cloned into plasmid pZippy-002LF-Neo/Gus-002RF lacking the Neo/Gus reporter cassettes. The resulting recombination vector pZippy-002LF-002GFP-002RF was used to generate an ORFV002-GFP-tagged revertant virus (OV-IA82Rv002GFP).

RT-PCR. Transcription kinetics of *ORFV002* was investigated during ORFV infection in OFTu cells by reverse transcription PCR (RT-PCR) as previously described (13). Transcription of *ORFV002*, *ORFV055* (late gene control), and *ORFV127* (early gene control) was assessed using primers 002RTFw1-5'-ACA CGGTAACGGCAGTGGTA-3', 002RTRv1-5'-AGCAGGGTGGTGAGCAAG-3', 055LFw-5'-AATCATGGATCCGCCACC ATGTTCTTCCGCCGTCGC-3'; 055LRv-5'-TATCATCTCGAGCGGGCGTGGAGGTC GCCGACC-3', 127EintFw-5'-CTCCTCGACGACTTCAAAGG-3', and 127EintRv-5'-TATGTCTGAAGTTCGCTCATGG-3', respectively. Negative controls and controls for DNA contamination (no reverse transcriptase) were included in all reactions.

Construction and characterization of *ORFV002* deletion mutant virus OV-IA82 Δ 002 and revertant viruses OV-IA82Rv002 and OV-IA82Rv002GFP. OV-IA82 Δ 002 was obtained by homologous recombination between the parental ORFV strain OV-IA82 and the recombination cassette pZippy002LF-Neo/Gus-002RF as previously described (13). OFTu cells cultured in 6-well plates were infected with serial 10-fold dilutions of cell lysates and overlaid with culture media containing 0.5% SeaKem GTC Agarose (Cambrex Bioscience, Rockland, ME) and X-Gluc (0.5 μ g/mL, Gold Biotechnologies, Saint Louis, MO). Blue plaques were harvested and subjected to additional rounds of plaque purification. The absence of *ORFV002* sequence and presence of Neo/Gus sequences in the purified recombinant virus were confirmed by PCR and Southern blot analysis.

OV-IA82Rv002 was obtained by homologous recombination between the OV-IA82Δ002 deletion mutant virus and the recombination cassette pcDNARv002 as described for OV-IA82Δ002. OV-IA82Rv002 virus was purified from cell lysates by limiting dilution followed by plaque purification. OFTu cells cultured in 96-well plates were infected with 10-fold dilutions of cell lysates from the infection/transfection (10^{-3} to 10^{-8}) and incubated at 37°C for 72 h. Supernatants were transferred to a new 96-well plate and frozen at -80°C, and cells were fixed with 3.7% formaldehyde and stained (staining solution: 50 mM NaPO₄, pH 7.2; 0.5 mM K₃Fe[CN]₆; 0.5 mM K₄Fe[CN]₆; and 10 mM X-Gluc) for 3 h at 37°C. Unstained cytopathic effect (CPE)-positive wells, indicative of recombination, were selected and the supernatant subjected to additional rounds of limiting dilution followed by plaque purification. The presence of *ORFV002* sequence and absence of Neo/Gus sequences in the purified recombinant virus were confirmed by PCR and Southern blot analysis.

OV-IA82Rv002GFP was generated by homologous recombination between the OV-IA82Δ002 deletion mutant virus and the recombination cassette pZippy-002LF-002GFP-002RF as described for OV-IA82Δ002. OV-IA82Rv002GFP virus was purified from cell lysates by limiting dilution followed by plaque purification. OFTu cells cultured in 96-well plates were infected with 10-fold dilutions of cell lysates from the infection/transfection (10^{-3} to 10^{-8}), incubated at 37°C for 24-48 h and screened under a fluorescence microscope for GFP signal. Supernatants of GFP-positive wells were subjected to additional rounds of limiting dilution followed by plaque purification. The integrity of regions involved in recombination was assessed by DNA sequencing.

Cytopathic effect and plaque morphology of OV-IA82, OV-IA82Δ002 and OV-IA82Rv002 were examined and compared using primary OFTu cells as previously described (13). One step and multi step growth curves were performed using multiplicities of infection (MOI) of 10 and 0.1, respectively.

Real-time PCR analysis. The expression of NF-κB-regulated genes was investigated in ORFV-infected OFTu cells by real-time PCR (13). OFTu cells were mock-infected or infected with OV-IA82 or OV-IA82Δ002 (MOI = 10) and harvested at 2 and 4 h p.i. for total RNA extraction and reverse transcription (13). Expression of genes *CCL20*, *CXCL3*, *IL-1 alpha*, *IL-6*, *IL-8*, *ICAM-1*, *IRF-1*, *NFκBIA*, and *PTGS2* was examined using primers and probes synthesized by Applied Biosystems (TaqMan[®] Gene Expression Custom Assays), based on ovine gene sequences in GenBank. Reaction conditions and data analysis were performed as previously described (13).

NF- κ B luciferase reporter assays. The effect of ORFV002 expression on NF- κ B-mediated transcription was assessed by using a luciferase reporter assay (13). OFTu cells were cotransfected with pNF- κ B*Luc* (Clontech) and pRLTK (Promega), and 24 h later infected with OV-IA82, OV-IA82 Δ 002, or OV-IA82Rv002 (MOI = 10) or mock infected. Cells were harvested with passive lysis buffer (PLB; Promega) at 4, 6, 12 and 24 hours p.i. and luciferase activities were determined using the Dual Luciferase Reporter Assay kit (Promega) and a luminometer (Victor²; Perkin-Elmer, Waltham, MA).

OFTu cells transiently transfected with plasmids pNF- κ B*Luc*, pRLTK and either pEGFP-N1 or p002EGFP were treated with TNF- α (20 ng/ml) or LPS (250 ng/ml) for 6 h and assayed for luciferase activities as described above. Statistical analysis of the data was performed by using Student's T test.

Western blots. The effect of ORFV002 on the NF- κ B signaling pathway was assessed by Western immunoblots. OFTu cells were transfected with pEGFP-N1 (2 μ g; control) or p002EGFP (2 μ g), treated with TNF- α (20 ng/ml), and harvested at 5 and 15 min post-treatment with ProteoJet mammalian lysis buffer (Fermentas, Glen Burnie, MD) containing protease and phosphatase inhibitors (Sigma-Aldrich, St Louis, MO). OFTu cells were cotransfected with pT7-NF κ B-p65 (0.5 μ g), pHA-p300 (2 μ g) and either pEGFP-N1 (1 μ g; control) or p002EGFP (1 μ g), treated with TNF- α for 30 or 60 min, and harvested with lysis buffer as above. OFTu cells were cotransfected with pT7-NF κ B-p65 (0.5 μ g), and pHA300 (2 μ g), and infected with OV-IA82, OV-IA82 Δ 002 or OV-IA82Rv002 at 24 h after transfection. Cells were harvested at 15, 30 and 60 min p.i. with ProteoJet lysis buffer as described above. OFTu cells were transfected with pEGFP-N1 (2 μ g; control) or p002EGFP (2 μ g), treated with TNF- α (20 ng/ml), and harvested in phosphate buffered saline (PBS; 0.5 ml) at 60 min post treatment. Cytoplasmic and nuclear protein fractions were extracted using ProteoJet cytoplasmic and nuclear protein extraction kit (Fermentas) according to the manufacturer's protocol. Untreated or uninfected cells were used as controls in the corresponding experiments. Protein extracts (50 μ g of total cell lysates, and 20 μ g of cytoplasmic and nuclear fractions) were resolved by SDS-PAGE in 10% gels followed by blotting to nitrocellulose membranes. Blots were incubated with antibodies against NF- κ B-p65 (Cell signaling, cat no. 3034), p-NF- κ B-p65 (Ser536) (Cell signaling, cat no. 3033), acetyl-NF- κ B-p65 (Lys310) (Cell Signaling, cat no. 3045), GAPDH (sc-25778, Santa Cruz), Histone H3 (sc-10809, Santa Cruz), or GFP (sc-8334, Santa Cruz) and developed by using a chemiluminescent substrate (ECL, Pierce-Thermo Scientific). Densitometric analysis of the blots was performed by using ImageJ software, version 1.62 (National Institute of Health,

Bethesda, MD). Statistical analysis of the densitometry data was performed by using Student's T test.

Confocal microscopy. OFTu cells cultured on glass coverslips were infected with OV-IA82Rv002GFP virus (MOI = 1 or 5), fixed with 4% formaldehyde at various time points post-infection (2, 3, 12 and 24 h p.i.), stained with DAPI for 10 min, and examined by laser scanning confocal microscopy (Zeiss, LSM710). OFTu cells cultured on glass coverslips were transfected with either (i) p002EGFP, (ii) pEGFP-N1 and pT7-NF κ B-p65, or (iii) p002EGFP and pT7-NF κ B-p65, treated with TNF- α at 24 h post transfection (60 min), fixed with 4% formaldehyde, and permeabilized with 0.25% Triton X-100 for 10 min at room temperature. After blocking with 1% BSA-PBS, cells were incubated with antibody against NF- κ B-p65 (Cell Signaling, cat no. 3034), for 1 h at room temperature. Unbound antibodies were washed and samples incubated with secondary antibodies (goat anti-rabbit or anti-mouse-Alexa Fluor 594) for 1 h at room temperature, stained with DAPI for 10 min, and examined by confocal microscopy (Zeiss, LSM710).

Coimmunoprecipitation assays. OFTu cells were cotransfected with pT7-NF κ B-p65 (0.5 μ g), pHA300 (2 μ g), and either pEGFP-N1 (1 μ g; control) or p002EGFP (1 μ g), treated with TNF- α for 30 or 60 min, harvested in 1 ml of PBS, and incubated with lysis buffer (25 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, and protease and phosphatase inhibitors) for 20 min on ice. Protein extracts were immunoprecipitated with 5 μ g of antibodies against GFP (sc-9996, Santa Cruz), NF- κ B-p65 (Cell signaling, cat no. 3034) or p300 (Millipore, cat no. 05-257) and subsequently incubated overnight at 4°C with 50 μ l of protein G agarose beads (Upstate). Samples were washed three times with lysis buffer, and immunoprecipitated proteins resolved in SDS-PAGE gels (10%), blotted to nitrocellulose membranes, and developed as described above.

Animal inoculations. Three to four months-old lambs were randomly allocated to three experimental groups consisting of OV-IA82-infected ($n = 3$), OV-IA82 Δ 002-infected ($n = 3$) and OV-IA82Rv002-infected lambs ($n = 2$). The inoculation sites (inferior lips or inner side of the hind limbs) were cleaned with water and scarified with a needle or a razor blade, respectively. A 0.25 ml of a virus suspension containing $10^{7.3}$ TCID₅₀/ml was applied topically on each inoculation site. Animals were monitored during 19 days for characteristic orf lesions including erythema, vesicles, pustules and scabs. Skin biopsies were collected at days 1, 2, 3, 5 and 19 p.i. and processed for histological examination using standard procedures. All animal procedures received ethical approval from the University of Nebraska-Lincoln Institutional Animal Care and Use Committee (IACUC, protocol # 214 as

of 01/23/08), and were performed according the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Results

Parapoxvirus *ORFV002* localizes to the cell nucleus during ORFV infection.

Parapoxvirus *ORF002* encodes a novel protein with homologues in ORFV and pseudocowpox virus (PCPV). Notably, the bovine papular stomatitis virus (BPSV strain AR02) genome lacks an *ORF002* (12). ORFV strain OV-IA82 *ORFV002* (AAR98100) is 117 amino acids in length, with a predicted molecular mass of 11.7 kDa. It is most similar to homologues in the sheep ORFV isolate NZ2 (ABA00521) and the goat ORFV isolate OV-SA00 (NP_957782) (98% and 90% amino acid identity, respectively), and less similar to PCPV homologues (*ORF132.5*; 84.6% amino acid identity [ADC53898 and YP_003457305]). Although *ORF002* carboxyl terminus is highly conserved among ORFV and PCPV strains, some degree of inter-species variability is observed at amino acid residues 83 to 87, with a two amino acid deletion and a two amino acid insertion in ORFV strain OV-SA00 and PCPV strains F00.120R and VR634, respectively. No motifs indicative of putative protein function were identified in *ORFV002*.

Transcription kinetics of *ORFV002* was assessed during ORFV replication in OFTu cells by RT-PCR. Low levels of *ORFV002* transcription was reproducibly detected at early time points p.i. (1, 2, 3 and 6 h p.i.), and was markedly increased at 12 and 24 h p.i. (Fig. 1). *ORFV002* transcription was markedly decreased at late time points p.i. in the presence of AraC, an inhibitor of DNA replication and of late poxviral gene transcription (Fig. 1). Similar *ORFV002* transcription kinetics was observed by using a real-time-PCR (data not shown). These results indicate that *ORFV002* is an early-late poxviral gene.

To assess *ORFV002* subcellular localization, OFTu cells were infected with the OV-IA82Rv002GFP virus and examined at various times p.i. by confocal microscopy and Western immunoblots. *ORFV002* localized mainly to the cell nucleus exhibiting a diffuse distribution pattern first detectable as early as 2 h p.i. (Fig. 2). In addition to the nuclear localization, a cytoplasmic distribution of *ORFV002* was also observed at late time points p.i. (Fig. 2). Similar results were obtained when *ORFV002*-GFP subcellular localization was assessed during OV-IA82Rv002GFP infection by using Western immunoblots (data not shown). Transient expression of *ORFV002* in OFTu cells resulted in a similar subcellular localization and distribution pattern (data not shown).

ORFV002 is nonessential for ORFV replication *in vitro*. Replication of the wild-type (OV-IA82), deletion mutant (OV-IA82 Δ 002), and an OV-IA82 Δ 002 revertant virus (OV-IA82Rv002) was investigated *in vitro*. No differences in OV-IA82 Δ 002 replication kinetics or viral yields were observed when multiple-step (Fig. 3A) or one-step (Fig. 3B) growth curves were compared to those of the revertant or wild-type viruses in OFTu cells. Similarly, deletion of ORFV002 did not affect ORFV ability to replicate in primary ovine keratinocyte cultures (OKT; Fig. 3C). No significant differences in cytopathic effect (CPE) and plaque morphology for OV-IA82 Δ 002 were observed. Thus, ORFV002 is non essential for virus replication in OFTu and in OKT cells.

OV-IA82 Δ 002 infection results in increased expression of NF- κ B-regulated genes in primary OFTu cells. Preliminary transcriptional profiling of OV-IA82 Δ 002-infected OFTu cells revealed increased expression of NF- κ B-regulated genes (data not shown). Real time-PCR analysis of gene expression in OFTu cells infected with OV-IA82 Δ 002 demonstrated increased expression of the NF- κ B-regulated genes *IL-1 α* (30.4-fold), *IL-6* (9.5-fold), *IL-8* (28.3-fold), *NF κ BIA* (7.4-fold), *CCL20* (45.2-fold), *CXCL3* (50.1-fold), *IRF-1* (5.4-fold), *ICAM-1* (4.1-fold) and *PTGS2* (9.0-fold) at 2 h p.i. (Fig. 4A) and 4 h p.i. (data not shown). Expression of *IL-1 α* , *IL-6* and *IL-8* was also increased in OV-IA82 Δ 002-infected OFTu cells at 12 and 24 h p.i. (data not shown). No significant differences in gene expression were observed between mock and wild-type virus-infected cells (Fig 4A).

To further examine the effect of ORFV002 on NF- κ B-regulated gene expression, OFTu cells were transfected with a plasmid encoding a luciferase reporter gene under the control of a NF- κ B responsive promoter and subsequently infected with OV-IA82, OV-IA82 Δ 002, OV-IA82Rv002, or mock-infected. Infection with OV-IA82 Δ 002 virus resulted in a significant increase of up to 3.5-, 8.5-, 4.1-, and 2.5-fold in luciferase activity ($P < 0.01$) at 4, 6, 12 and 24 h p.i., respectively when compared to the levels for mock-infected and wild-type virus-infected cells (Fig. 4B). Restoration of *ORFV002* in the revertant virus rescued the wild-type virus phenotype (Fig. 4B). These results indicate that ORFV002 affects NF- κ B-regulated gene transcription during ORFV infection of OFTu cells.

ORFV002 suppresses NF- κ B-mediated gene transcription induced by TNF- α and LPS. The ability of ORFV002 to inhibit NF- κ B-mediated transcription was investigated in OFTu cells following treatment with TNF- α and LPS, two potent inducers of the NF- κ B signaling pathway. Expression of ORFV002-GFP in TNF- α - and LPS-treated cells significantly decreased NF- κ B-regulated luciferase activity (~4.5-fold post TNF- α -treatment [$P < 0.01$], and ~6-fold post LPS-treatment [$P < 0.01$]) when compared to the levels for control GFP-

expressing cells (Fig. 4C and D). Thus, ORFV002 inhibits NF- κ B-mediated gene transcription following TNF- α - and LPS-stimulation.

ORFV002 expression does not affect phosphorylation or nuclear translocation of NF- κ B-p65. The effect of ORFV002 on cytoplasmic events of the NF- κ B signaling pathway was investigated by examining phosphorylation and nuclear translocation of NF- κ B-p65 in ORFV002-expressing cells. OFTu cells transfected with plasmids encoding GFP (control) or ORFV002-GFP were treated with TNF- α and harvested at various time points post treatment. Similar levels of phosphor-NF- κ B-p65 were detected in GFP- and ORFV002-GFP-expressing cells, indicating that ORFV002 had no significant effect on TNF- α -induced phosphorylation of NF- κ B-p65^{S536} (Fig. 5A). Additionally, as evidenced by similar levels of nuclear NF- κ B-p65 in GFP- or ORFV002-GFP-expressing cells, ORFV002 did not affect TNF- α -induced nuclear translocation of NF- κ B-p65 (Fig. 5B). Nuclear levels of NF- κ B-p65 were not due to leakage from the cytoplasmic fraction, since NF- κ B-p65 was not detected in the nuclear fraction of untreated control cells (Fig. 5B). These results indicate that ORFV002 does not affect phosphorylation (serine 536) or nuclear translocation of NF- κ B-p65, and further suggest that ORFV002 interferes with nuclear events of the NF- κ B signaling pathway.

Expression of ORFV002 results in decreased acetylation of NF- κ B-p65. Nuclear acetylation plays an important role in modulating NF- κ B-p65 transactivating activity (6, 8). To investigate the effects of ORFV002 expression on NF- κ B-p65^{K310} acetylation, OFTu cells transiently transfected with plasmids encoding for NF- κ B-p65, coactivator p300 (acetyltransferase) and either GFP or ORFV002-GFP were treated with TNF- α and harvested at various time points post-treatment. Expression of ORFV002 significantly decreased acetylation of NF- κ B-p65^{K310} by ~57% at 60 min ($P < 0.01$) post TNF- α treatment (Fig. 6A and B). The reduced levels of acetyl-NF- κ B-p65 were not due to protein degradation since levels of pan-NF- κ B-p65 and GAPDH were constant among all samples (Fig 6A).

The effect of ORFV002 on acetylation of NF- κ B-p65 was also assessed during ORFV infection. OFTu cells transiently transfected with plasmids pT7-NF- κ B-p65 and pHA-p300 were infected with OV-IA82, OV-IA82 Δ 002, or OV-IA82Rv002 and harvested at various time points p.i. While infection with the wild-type virus resulted in low levels of NF- κ B-p65^{K310} acetylation (Fig. 6C and D), OV-IA82 Δ 002-infection markedly increased acetylation of NF- κ B-p65^{K310} at 30 and 60 min p.i. ($P \leq 0.019$) (Fig. 6C and D). Restoration of *ORFV002* in the revertant virus rescued the wild-type virus phenotype (Fig. 6C). These results indicate that expression of ORFV002 results in decreased acetylation of NF- κ B-p65^{K310}.

ORFV002 interacts with NF- κ B-p65. Interaction of ORFV002 with NF- κ B-p65 was investigated as a potential mechanism for ORFV002 inhibitory effect on NF- κ B-p65 acetylation. OFTu cells transiently transfected with either (i) p002EGFP, (ii) pEGFP and pT7-NF κ B-p65, or (iii) p002EGFP and pT7-NF κ B-p65 were treated with TNF- α , probed with an antibody against NF- κ B-p65, and subsequently examined by confocal microscopy. Both ORFV002 and NF- κ B-p65, when expressed individually, exhibited a homogeneous and diffuse distribution in the nucleus (Fig. 7A and B). Notably, coexpression of these proteins resulted in an altered distribution pattern, which was characterized by a punctate colocalized nuclear staining (Fig. 7B).

Specific interaction of ORFV002 with NF- κ B-p65 was further investigated by using co-immunoprecipitation assays. OFTu cells transiently transfected with plasmids pT7-NF- κ B-p65, pHA-p300 and either pEGFP-N1 or p002EGFP were treated with TNF- α and harvested at 60 min post-treatment. Reciprocal co-immunoprecipitation assays with either anti-GFP (Fig. 7C) or anti-NF- κ B-p65 (Fig. 7D) antibodies demonstrated that ORFV002 coprecipitates with NF- κ B-p65. No interaction between ORFV002 and p300 was detected (data not shown). Together, these results indicate that ORFV002 physically interacts with NF- κ B-p65.

ORFV002 interferes with association of p300 and NF- κ B-p65. Acetylation of NF- κ B-p65 is dependent on the interaction of p300 and NF- κ B-p65 (9). To investigate the effect of ORFV002 expression on association of p300 and NF- κ B-p65, OFTu cells transiently transfected with plasmids pT7-NF- κ B-p65, pHA-p300, and either pEGFP-N1 or p002EGFP, were treated with TNF- α and harvested at 60 min post-treatment. Co-immunoprecipitation assays demonstrated that expression of ORFV002 resulted in reduced association between p300 and NF- κ B-p65 when compared to the levels for control GFP-expressing cells (Fig. 8). The decreased association between p300 and NF- κ B-p65 correlated with reduced levels of acetyl-NF- κ B-p65 detected in cell lysates of ORFV002-expressing cells (Fig. 8). Together, these results suggest that by binding to NF- κ B-p65, ORFV002 interferes with association of p300 and NF- κ B-p65.

ORFV002 does not affect ORFV virulence in the natural host. The role of ORFV002 in ORFV pathogenesis was investigated in sheep, the natural host of the virus. All inoculated lambs (OV-IA82, [$n = 3$], OV-IA82 Δ 002, [$n = 3$], and OV-IA82Rv002, [$n = 2$]) developed characteristic clinical orf. Erythema and small papules were first observed by day 2 p.i. and evolved into vesicles, pustules and scabs at later times p.i. (Fig. 9). Lesions started to subside by day 15 p.i., and by day 19 p.i. only a few scabs were observed at lesion margins. No

significant differences were observed in disease onset, severity, progression and time to resolution between animals inoculated with OV-IA82, OV-IA82Δ002 or OV-IA82Rv002 (Fig. 9).

Histological examination of skin lesions revealed characteristic pathological changes of orf consisting of hyperplasia and ballooning degeneration of keratinocytes, hyperkeratosis, dyskeratosis, and dermal and epidermal inflammatory infiltration. No significant differences in the severity or time course of histological changes were observed between animals inoculated with OV-IA82, OV-IA82Δ002 or OV-IA82Rv002 (data not shown). These results indicate that ORFV002 does not significantly affect ORFV pathogenesis or virulence in the natural host.

Discussion

In the present study, we show that ORFV002 expression, while not affecting phosphorylation or nuclear translocation of NF-κB-p65, decreases TNFα- and ORFV-induced acetylation of NF-κB-p65^{K310}. ORFV002 colocalizes and interacts with NF-κB-p65 in the nucleus, and interferes with NF-κB-p65/p300 interaction, thus providing a mechanism for inhibition of NF-κB-p65^{K310} acetylation and transactivating activity.

Poxviruses have evolved various strategies to modulate cytoplasmic events leading to activation of the NF-κB signaling pathway (31). VACV proteins A46R, A52R, B14, M2L and N1L counteract pathways upstream of the IKK complex, inhibiting activation of the IκB kinases (2, 11, 14, 20, 24). CPXV protein CPXV077 was shown to associate with NF-κB-p65, inhibiting its translocation to the nucleus (5). MOCV MC159 prevents TNF-α-induced degradation of IκBβ, presumably by preventing MEKK2-IKK complex formation while MC160 was shown to induce IKKα degradation by competitively interacting with the cellular heat shock protein 90 (HSP90) which is necessary for IKKα stabilization (34, 38). ORFV ORFV024 was shown to counteract activation of the IKK complex by preventing phosphorylation of the IκB kinases (13). ORFV ORFV002 represents the first identified poxviral protein that functions as a nuclear inhibitor of the NF-κB signaling pathway. Interestingly, the myxoma virus virulence factor M150R has been shown to colocalize with NF-κB-p65 in the nucleus of TNF-α-treated cells, exhibiting a punctate pattern reminiscent of ORFV002/NF-κB-p65 colocalization (4). Whether this observation reflects the ability of M150R to inhibit NF-κB-mediated transcription remains to be determined.

Regulation of NF-κB activity in the nucleus involves primarily post-translational modifications of NF-κB subunits or the histones in proximity of NF-κB target genes (6, 7). In

particular, acetylation plays a critical role in the nuclear regulation of NF- κ B-p65 activity and seven acetylation sites have been identified in NF- κ B-p65, lysines 122, 123, 218, 221, 310, 314 and 315 (3, 8, 29). Modification of single or multiple acetylation sites modulates distinct biological actions of NF- κ B-p65 (7). For example, acetylation of lysine 221 increases NF- κ B-p65 DNA binding affinity and, in combination with acetylation of lysine 218, prevents association of NF- κ B-p65 with newly synthesized I κ B α , thus regulating the duration of NF- κ B-mediated responses (8). Acetylation of lysine 310 recruits the coactivator Brd4 to the transcriptional complex enhancing the transcriptional activity of NF- κ B-p65 (8, 25). The decreased acetylation of NF- κ B-p65^{K310} in TNF- α -stimulated ORFV002-expressing cells or wild-type virus-infected cells suggests that ORFV002 prevents full transcriptional activity of NF- κ B-p65. However, the possibility that ORFV002 affects other acetylation sites on NF- κ B-p65, interfering with additional regulatory mechanisms of NF- κ B in the nucleus cannot be formally excluded.

P300-mediated acetylation of NF- κ B-p65 requires interaction between the proteins, and previous phosphorylation of NF- κ B-p65 (9). Phosphorylation of NF- κ B-p65 at serines 276 and 536 enhances NF- κ B-p65/p300 interaction, and consequently stimulates acetylation of lysine 310 (9). Although, ORFV002 did not affect phosphorylation of NF- κ B-p65^{S536}, its expression in cells was associated with decreased interaction between p300 and NF- κ B-p65. Given that ORFV002 physically interacts with NF- κ B-p65, it is tempting to speculate that ORFV002 decreases acetylation of NF- κ B-p65 by competitively disrupting the interaction between p300 and NF- κ B-p65. A similar mechanism has been described for African swine fever virus (ASFV) A238L protein, which binds to and inhibits nuclear acetylation of NF- κ B-p65 presumably by disrupting p300/NF- κ B-p65 complex formation (21, 22).

Transcription kinetics studies here have shown that *ORFV002* is reproducibly transcribed/expressed at very low levels at early times post ORFV infection with increasing amounts of the transcript/protein being detected at late time points p.i. (12 to 24 h p.i.) (Fig. 1 and 2). These observations explain the early-late inhibitory effects of ORFV002 on the NF- κ B signaling pathway. However, the increased levels of ORFV002 transcribed/expressed at late time points p.i. and the rapid kinetics by which ORFV002 inhibits activation of NF- κ B signaling following infection, suggest that ORFV002 may potentially be a virion component functioning early in subsequent rounds of ORFV replication.

The role of poxviral NF- κ B inhibitors in virus virulence and pathogenesis remains poorly understood. Deletion of selected poxviral NF- κ B inhibitors resulted in variable and, in most cases, very modest effects on virus virulence and pathogenesis (1, 4, 10, 13, 24, 32, 46).

For example, deletion of VACV *A46R*, *A52R* or *NIL* rendered the virus partially attenuated in a mouse model of infection (1, 24, 46), while deletion of VACV *B14R* was shown to affect virus virulence in an intranasal but not intradermal murine model of infection (10). Deletion of ORFV *ORFV024* had no significant effect on ORFV virulence and pathogenesis in sheep (13). In contrast, deletion of cowpoxvirus *CPXV006*, and myxoma virus *MYXV150* resulted in marked virus attenuation in a murine, or rabbit model of infection, respectively (4, 32).

Here, deletion of *ORFV002* from the ORFV genome did not affect disease severity, progression, or time to resolution in sheep, indicating that *ORFV002* is not essential for virus virulence in the natural host. This result, consistent with observations discussed above, supports the hypothesis that poxviral inhibitors of NF- κ B may exert complementary or redundant functions during poxvirus infections *in vivo*. Multiple poxviral NF- κ B inhibitors may exert a fine level of regulation of distinct branches of the NF- κ B pathway, which may be temporally regulated during virus infection. Therefore, deletion of single poxviral NF- κ B inhibitors may be complemented to some extent by the action(s) of other inhibitors, thus masking potential effects on virus virulence and pathogenesis. Alternatively, ORFV NF- κ B inhibitors may play roles in less understood aspects of ORFV biology, such as subclinical/persistent infections (26, 37), favoring virus replication and transmission in the absence of overt viral infection.

Regardless of particular clinical outcomes, most if not all chordopoxviruses replicate in keratinocytes at some stage during infection of the host. The NF- κ B signaling pathway plays complex, sometimes paradoxical roles in keratinocyte survival, differentiation, and immune homeostasis (39), making the impact of poxviral NF- κ B inhibitors in virus virulence and pathogenesis difficult to predict. The multiple and often novel NF- κ B inhibitors encoded by poxviruses further complicate this matter.

The results presented here demonstrate that the parapoxvirus ORFV evolved a novel mechanism to modulate nuclear function of NF- κ B. Elucidation of the molecular mechanisms employed by parapoxviruses to modulate the NF- κ B signaling pathway may contribute to improve understanding of poxvirus infection biology and disease.

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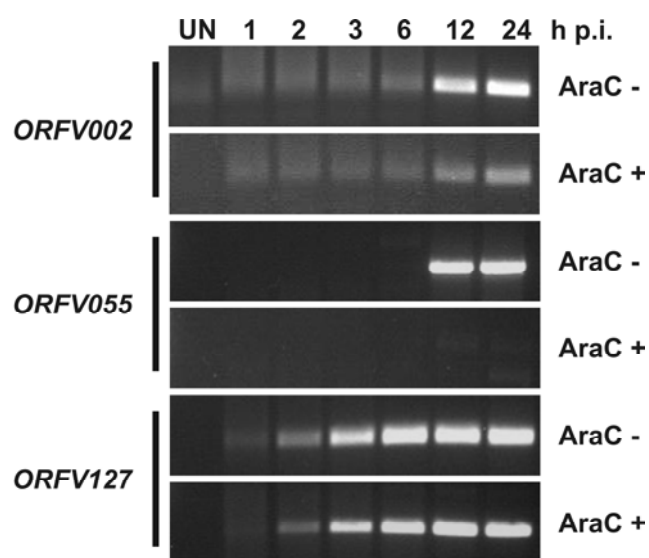


FIG. 1. Transcription kinetics of *ORFV002*. Transcription kinetics of *ORFV002*, *ORFV055*, and *ORFV127* were assessed during ORFV infection in OFTu cells in the presence (+) or absence (-) of AraC. Cells were infected with wild-type virus OV-IA82 (MOI = 10), harvested at various time points postinfection (p.i.) and transcription levels of *ORFV002*, *ORFV055* (late gene control), and *ORFV127* (early gene control) were assessed by RT-PCR. The results are representative of three independent experiments.

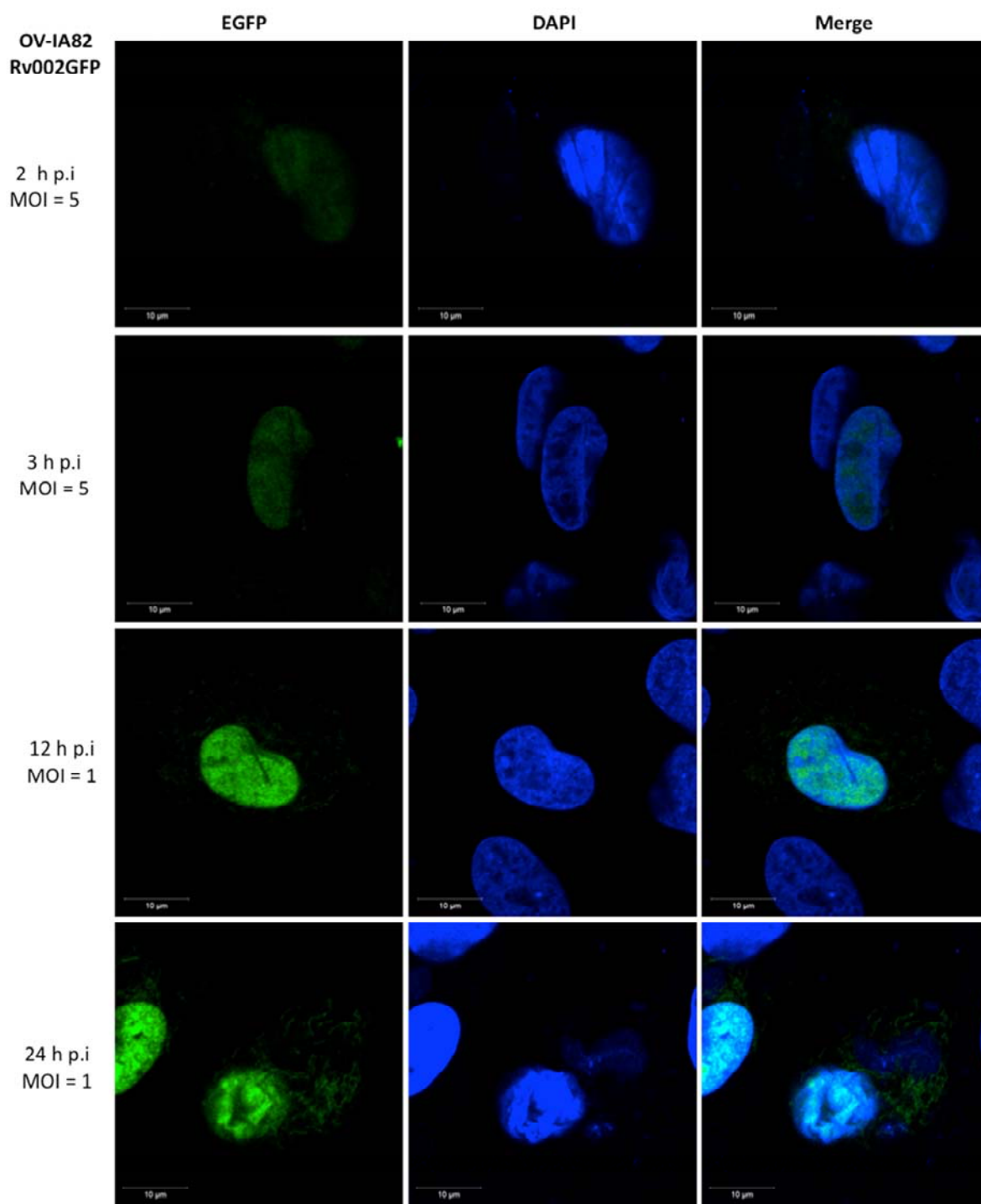


FIG. 2. Subcellular localization of ORFV002. OFTu cells were infected with OV-IA82Rv002GFP virus (MOI = 1 or 5), fixed with 4% formaldehyde at various time points postinfection (2, 3, 12 and 24 h p.i.), stained with DAPI and examined by confocal microscopy.

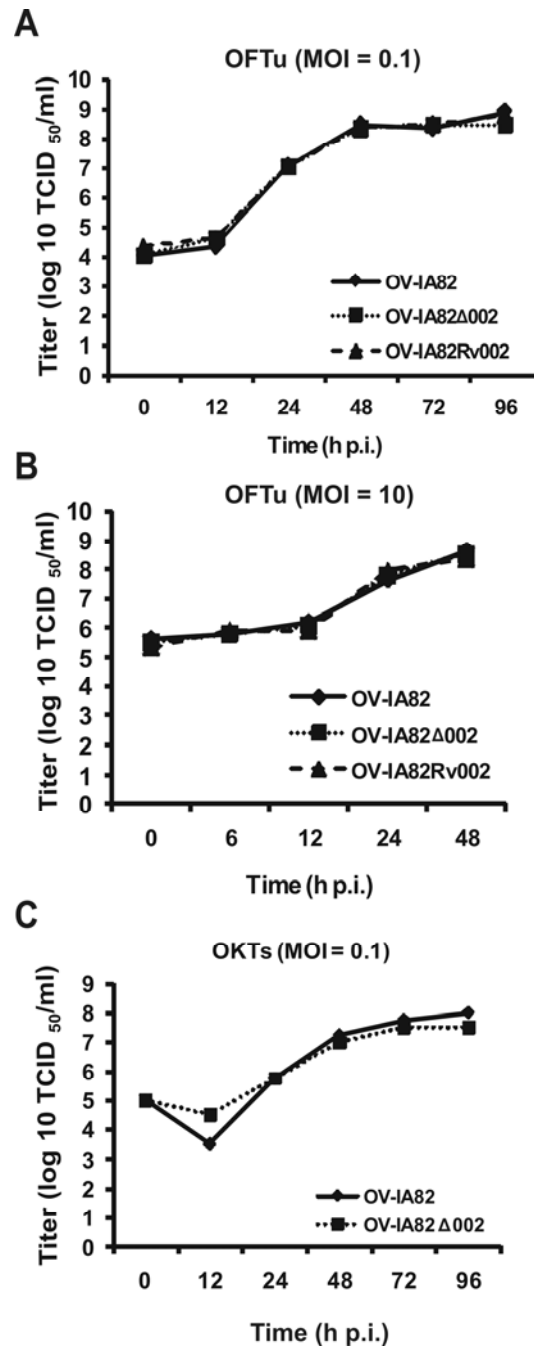


FIG. 3. Replication characteristics of *ORFV002* deletion mutant virus OV-IA82Δ002. (A) Multiple step growth curves of wild-type (OV-IA82), deletion mutant (OV-IA82Δ002) and revertant (OV-IA82Rv002) viruses in primary OFTu cells (MOI = 0.1). (B) One step growth curves of OV-IA82, OV-IA82Δ002 and OV-IA82Rv002 viruses in primary OFTu cells (MOI = 10). (C) Multiple step growth curves of OV-IA82 and OV-IA82Δ002 in primary ovine keratinocytes (OKTs) (MOI = 0.1).

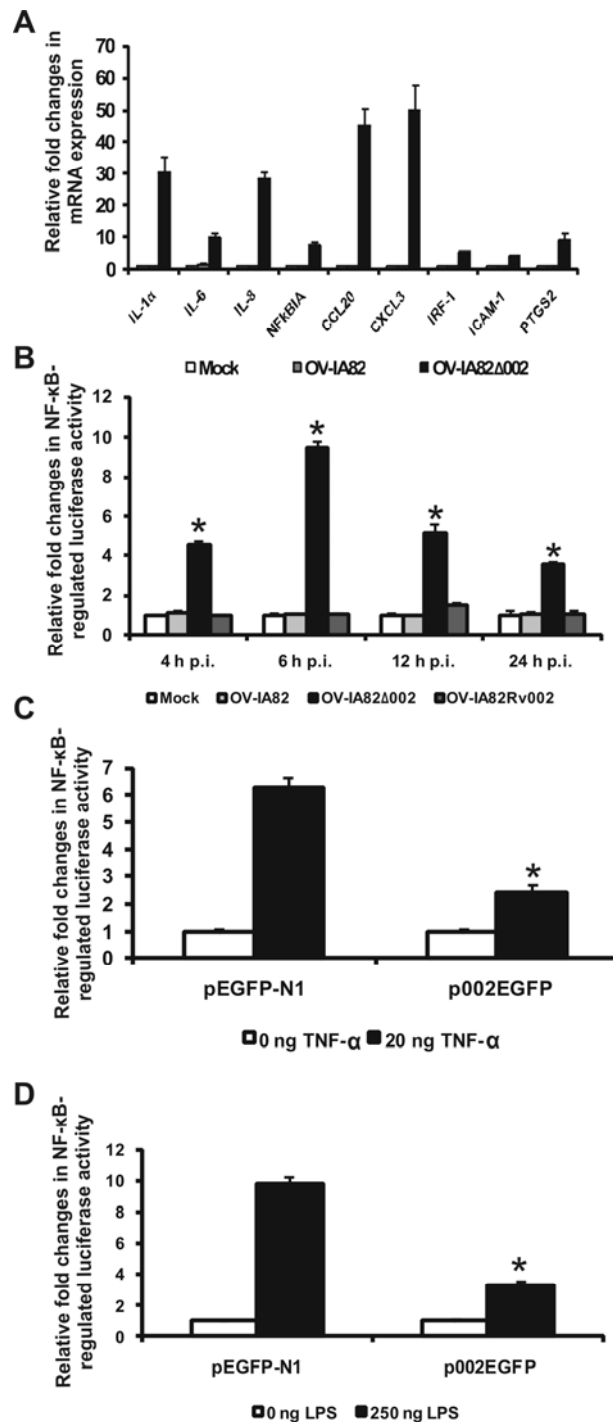


FIG. 4. Effect of ORFV002 on NF- κ B-regulated gene transcription. (A) OFTu cells were infected with OV-IA82, OV-IA82 Δ 002 (MOI = 10), or mock-infected and expression of NF- κ B-regulated genes was determined by real-time PCR analysis at 2 h p.i.. (B) OFTu cells were cotransfected with plasmids pNF- κ BLuc and pRL-TK and subsequently infected with OV-IA82, OV-IA82 Δ 002, OV-IA82Rv002 (MOI = 10), or mock-infected. Firefly and sea pansy luciferase activities were measured at 4, 6, 12 and 24 h p.i. and expressed as relative fold changes in luciferase activity (*, $P < 0.01$). (C) OFTu cells were cotransfected with plasmids pNF- κ BLuc, pRL-TK and either pEGFP-N1 or p002EGFP, and subsequently treated with TNF- α (20 ng/ml) for 6 h (*, $P < 0.01$). Luciferase activities were determined as in B. (D) OFTu cells were cotransfected as in C and subsequently treated with LPS (250 ng/ml) for 6 h (*, $P < 0.01$). Luciferase activities were determined as in B. The results (A-D) are representative of three independent experiments.

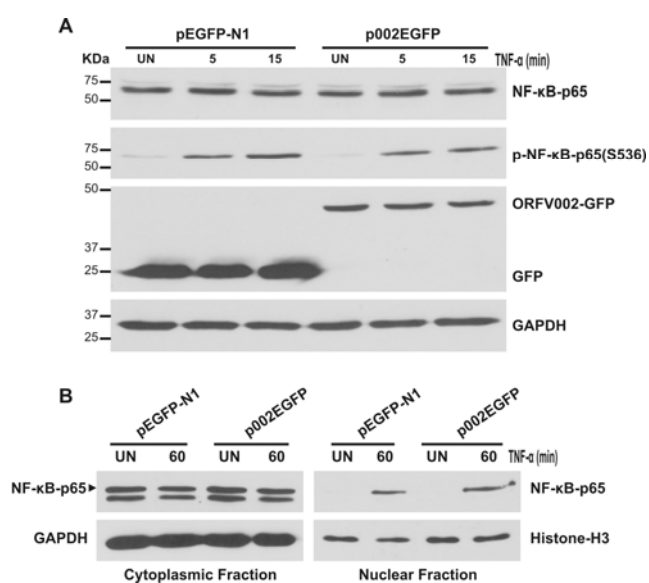


FIG. 5. Effect of ORFV002 on NF- κ B-p65 phosphorylation and nuclear translocation. (A) OFTu cells transiently transfected with plasmids encoding GFP (control) or ORFV002-GFP were treated with TNF- α (20 ng/ml) and harvested at the indicated time points (UN, untreated controls). Protein extracts (50 μ g) were resolved by SDS-PAGE, blotted, and probed with antibodies directed against proteins indicated on the right. (B) OFTu cells transiently transfected with plasmids encoding for GFP (pEGFP-N1, control) or ORFV002-GFP (p002EGFP) were treated with TNF- α (20 ng/ml) for 60 min and cytoplasmic and nuclear protein fractions were extracted (UN, untreated controls). Protein extracts (20 μ g) were resolved by SDS-PAGE, blotted, and probed with antibodies against NF- κ B-p65 (top panels), GAPDH (bottom left panel), or Histone H3 (bottom right panel). The results (A and B) are representative of two independent experiments.

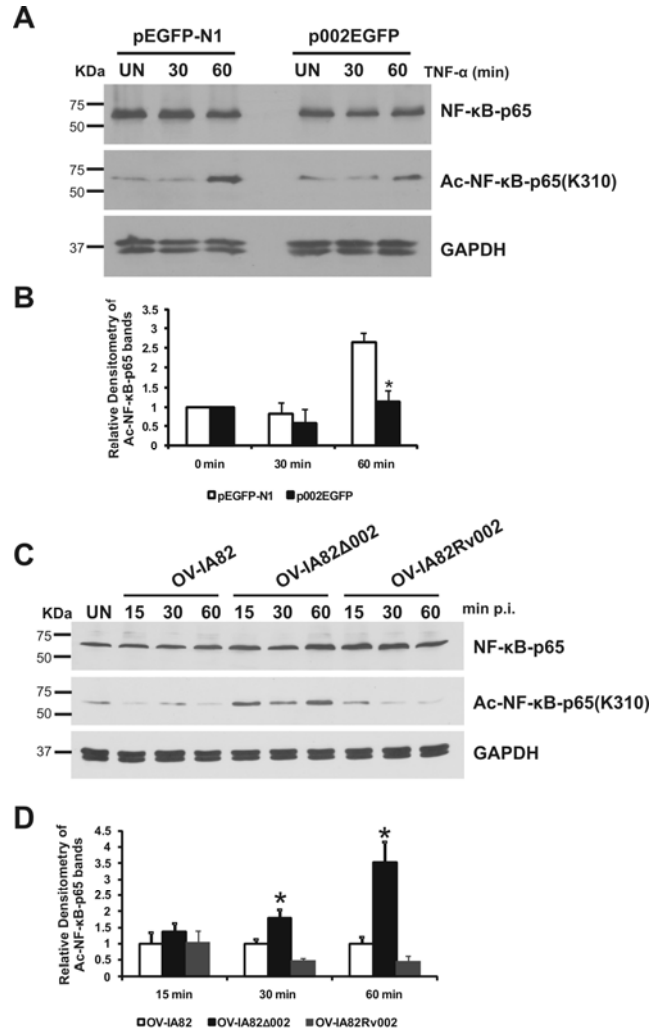


FIG. 6. Effect of ORFV002 on NF- κ B-p65 acetylation. (A) OFTu cells were cotransfected with plasmids pT7-NF- κ B-p65, pHA-p300 and either pEGFP-N1 (GFP, control) or p002EGFP (ORFV002-GFP), treated with TNF- α (20 ng/ml) and harvested at the indicated time points (UN, untreated controls). Protein extracts (50 μ g) were resolved by SDS-PAGE, blotted, and probed with antibodies directed against proteins indicated on the right. (B) Relative densitometry of Acetyl NF- κ B-p65 bands normalized to loading control GAPDH (*, $P < 0.01$). (C) OFTu cells were cotransfected with plasmids pT7-NF- κ B-p65 and pHA-p300, subsequently infected with OV-IA82, OV-IA82 Δ 002 or OV-IA82Rv002 (MOI = 10) and harvested at the indicated time points (UN, uninfected controls). Protein extracts (50 μ g) were resolved by SDS-PAGE, blotted, and probed with antibodies directed against proteins indicated on the right. (D) Relative densitometry of Acetyl NF- κ B-p65 bands normalized to loading control GAPDH (*, $P \leq 0.019$). The results are representative of three (A and B) or four to six (C and D) independent experiments.

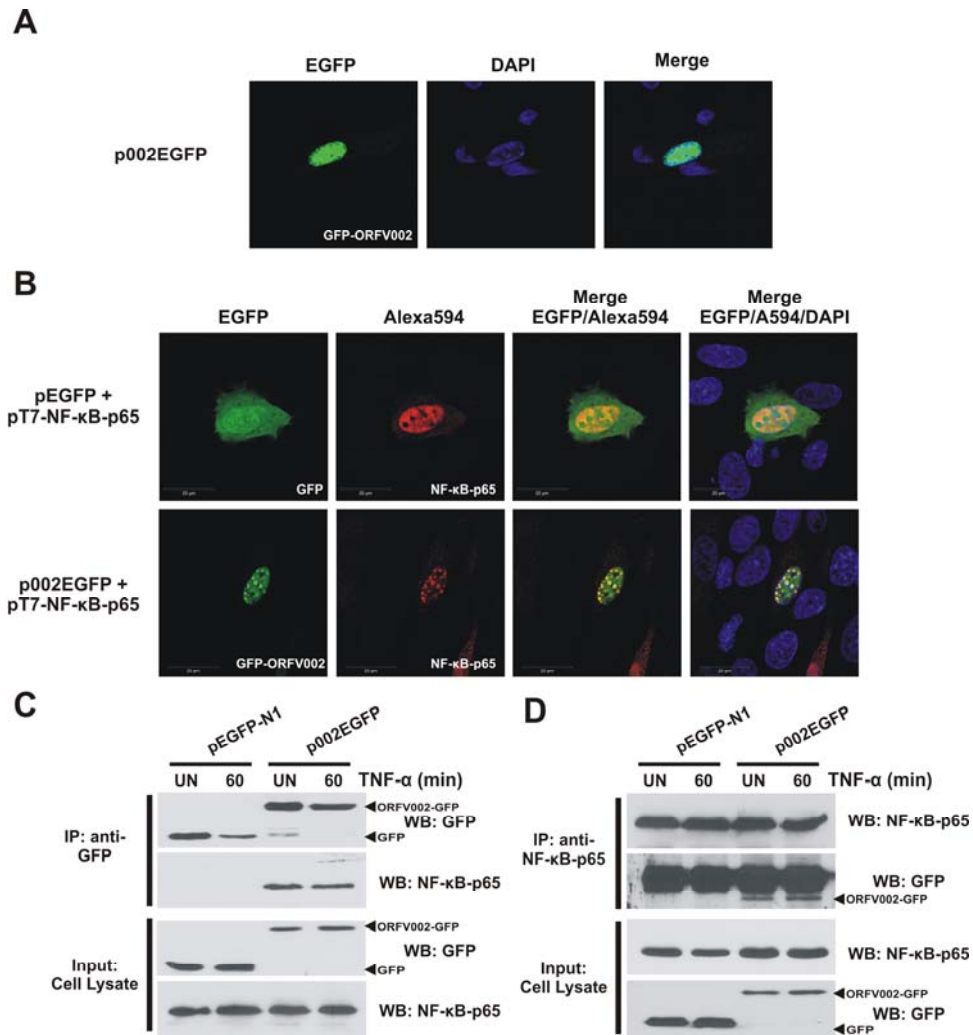


FIG. 7. ORFV002 colocalizes and interacts with NF-κB-p65. (A) OFTu cells were transfected with the plasmids indicated on the left. At 24 h post-transfection cells were treated with TNF- α (20 ng/ml for 60 min) fixed, stained with DAPI, and examined by confocal microscopy. (B) OFTu cells were transfected with the plasmids indicated on the left. At 24h post-transfection cells were treated with TNF- α (20 ng/ml for 60 min), fixed, probed with an antibody against NF-κB-p65, stained with DAPI, and examined by confocal microscopy. (C) OFTu cells were cotransfected with plasmids pT7-NF-κB-p65, PHA-p300 and either pEGFP-N1 (GFP, control) or p002EGFP (ORFV002-GFP), treated with TNF- α (20 ng/ml) and harvested at 60 min post TNF- α -treatment (UN, untreated controls). Protein extracts were immunoprecipitated with anti-GFP antibody coupled to protein G agarose beads and examined by SDS-PAGE/Western blot (upper panels) with antibodies directed against proteins indicated on the right. Cell lysates were examined by SDS-PAGE/Western blot (bottom panels) with antibodies directed against proteins indicated on the right. (D) OFTu cells were cotransfected and treated with TNF- α as in C (UN, untreated controls). Protein extracts were immunoprecipitated with anti-NF-κB-p65 antibody coupled to protein G agarose beads and analyzed as described for C. The immunoprecipitation results shown in C and D are representative of four independent experiments.

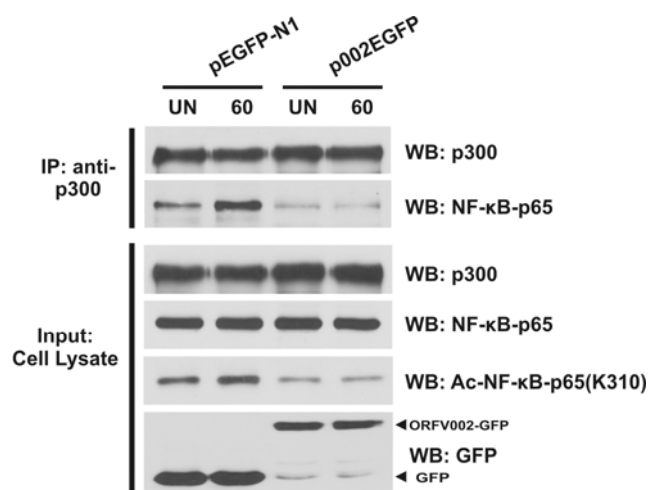


FIG. 8. ORFV002 interferes with association of p300 and NF- κ B-p65. OFTu cells were cotransfected with plasmids pT7-NF- κ B-p65, pHA-p300 and either pEGFP-N1 (GFP, control) or p002EGFP (ORFV002-GFP), treated with TNF- α (20 ng/ml) and harvested at 60 min post TNF- α -treatment (UN, untreated controls). Protein extracts were immunoprecipitated with anti-p300 antibody coupled to protein G agarose beads and analyzed by SDS-PAGE/Western blot (upper panels) with antibodies directed against proteins indicated on the right. Cell lysates were analyzed by SDS-PAGE/Western blot (bottom panels) with antibodies directed against proteins indicated on the right. The results are representative of three independent experiments.

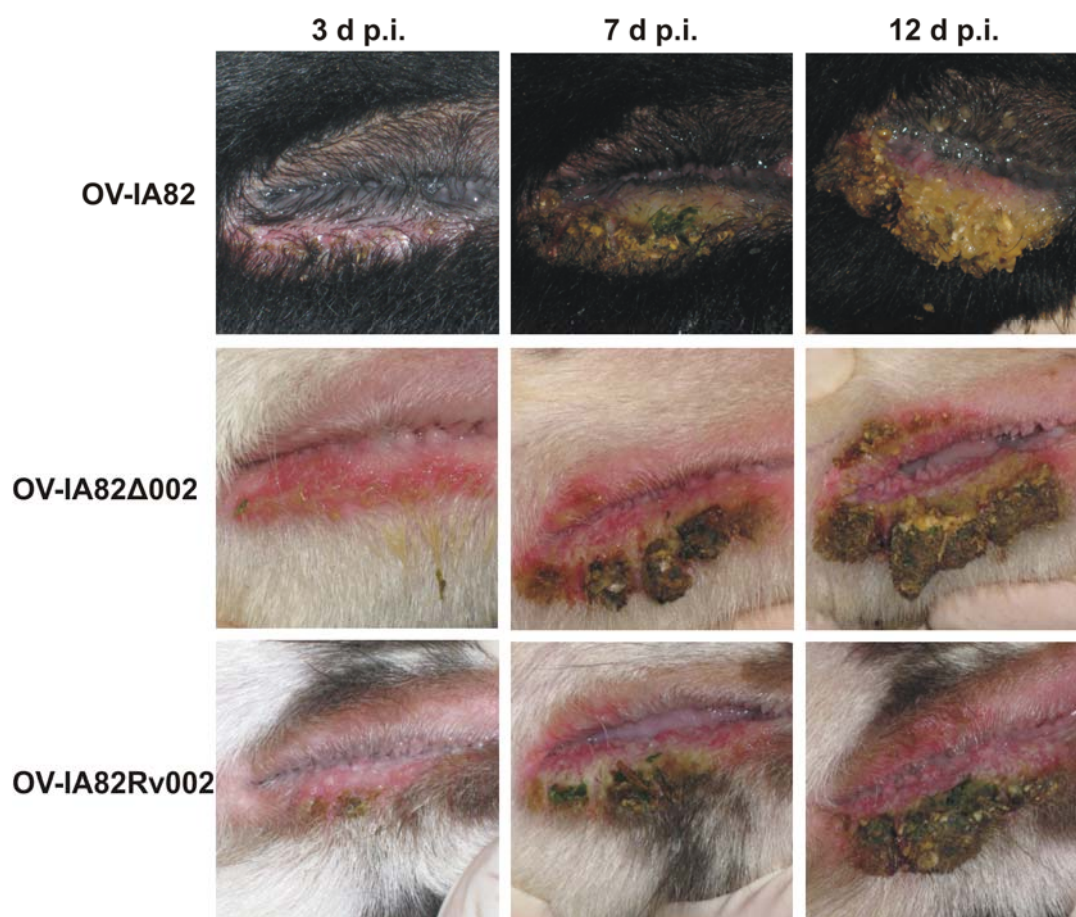


FIG. 9. ORFV002 does not affect ORFV virulence in the natural host. Clinical course of orf in lambs inoculated with wild-type (OV-IA82), ORFV002 deletion mutant (OV-IA82Δ002), or revertant (OV-IA82Rv002) viruses at the mucocutaneous junction of the lower lip (d p.i., days post infection).

5. CAPÍTULO 3

Orf Virus *ORFV121* Encodes a Novel Inhibitor of NF- κ B that Contributes to Virus Virulence

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Abstract

Orf virus (ORFV), the type member of the genus *Parapoxvirus* of the *Poxviridae*, has evolved novel strategies (proteins and/or mechanisms of action) to modulate host cell responses regulated by the Nuclear Factor- κ B (NF- κ B) signaling pathway. Here, we present data indicating that ORFV *ORFV121*, a gene unique to parapoxviruses, encodes for a novel viral NF- κ B inhibitor that binds to and inhibits phosphorylation and nuclear translocation of NF- κ B-p65. Infection of cells with an *ORFV121*-deletion mutant virus (OV-IA82 Δ 121) resulted in increased NF- κ B-mediated gene transcription, and expression of ORFV121 in cell cultures significantly suppressed NF- κ B-regulated reporter gene expression. ORFV ORFV121 physically interacts with NF- κ B-p65 in the cell cytoplasm, thus providing a mechanism for inhibition of NF- κ B-p65 phosphorylation and nuclear translocation. Notably, deletion of ORFV121 from the viral genome markedly decreased ORFV virulence and disease pathogenesis in sheep, indicating that ORFV121 is a virulence determinant for ORFV in the natural host.

Introduction

Orf, also known as contagious ecthyma or scabby mouth, is a nonsystemic, ubiquitous disease of sheep and goats caused by orf virus (ORFV), the type member of the genus *Parapoxvirus* of the *Poxviridae* (31). The disease is characterized by inflammatory, often proliferative lesions affecting the skin of the lips, muzzle, nostrils, teats, and the oral mucosa (20). Lesions, usually limited to areas surrounding the virus entry sites, evolve through stages of erythema, vesicles, pustules, and scabs (16). In the absence of secondary infections, lesions are usually resolved in 6 to 8 weeks; however, persistent infections have been reported (9, 18, 35). In spite of a vigorous and typical anti-viral T-helper type 1 (Th1) immune response, immunity elicited by ORFV is short-lived, and animals can be repeatedly infected, albeit lesions are smaller and resolve sooner than in primary infections (19, 52). Orf is a zoonotic disease, affecting humans in close contact with infected animals (15, 32, 43).

ORFV is an epitheliotropic virus and no cell type other than keratinocytes, or their counterparts in the oral mucosa, has been shown to support ORFV replication *in vivo* (23, 27). Keratinocytes produce the protective stratum corneum of the epidermis, and function as immune sentinels and instigators of cutaneous inflammation (39). These cells express several Toll-like receptors (TLRs) and receptors encoded by the nucleotide-binding domain leucine-rich repeat-containing (NLR) family members (reviewed by [36]), which recognize pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs), leading to activation of pro-inflammatory signaling pathways, including the Nuclear Factor-kappa B (NF- κ B) signaling pathway (36).

The NF- κ B family of transcription factors NF- κ B-p65 (RelA), RelB, c-Rel, NF- κ B-p50/p105, and NF- κ B-p52/p100 form homo- or heterodimers and bind to specific promoter DNA sequences to mediate expression of a wide variety of cellular genes, including many involved in innate immunity, inflammation, cell proliferation and differentiation, and apoptosis (41, 44, 49). Various stimuli, including the proinflammatory cytokines tumor necrosis factor α (TNF α) and interleukin 1 (IL-1), bacterial lipopolysaccharide (LPS), viruses, and viral products, lead to phosphorylation of inhibitor-kappa B (I κ B) proteins by I κ B kinases (IKK complex), resulting in proteasomal degradation of I κ B and nuclear translocation of NF- κ B subunits (25). NF- κ B subunits are subject to extensive post-translational modifications, including phosphorylation, acetylation and methylation, which may facilitate their nuclear translocation or define their transcriptional functions (6, 40).

Recent studies using genetic mouse models to assess the function(s) of NF- κ B signaling *in vivo* revealed a critical role for NF- κ B in the maintenance of skin immune

homeostasis (reviewed by [39]). Notably, either continuous activation or continuous inhibition of the NF- κ B signaling pathway in the epidermis resulted in enhanced skin inflammation (39). However, the mechanisms underlying these paradoxical effects of NF- κ B in the skin remain poorly understood.

In addition to its role in regulation of innate immune responses, the NF- κ B signaling pathway also plays key functions in the maintenance of skin homeostasis by regulating keratinocyte proliferation, differentiation and apoptosis (51). Inhibition of the NF- κ B signaling pathway in transgenic murine and human epidermis has been shown to result in epidermal hyperplasia *in vivo*, while overexpression of NF- κ B-p50 and NF- κ B-p65 subunits induced epidermal hypoplasia and growth inhibition (45). Deletion of the *IKK α* gene in transgenic murine epidermis led to epidermal hyperplasia, and terminal differentiation defects in keratinocytes, indicating that IKK α functions as a switch for keratinocyte proliferation and differentiation (26). Although the importance of NF- κ B signaling in skin homeostasis is well defined, the mechanisms underlying such regulation and the interplay among different components of the pathway remains elusive and awaits further experimentation.

Given the central role of NF- κ B in regulation of skin immune homeostasis and keratinocyte proliferation, differentiation and apoptosis, it is not surprising that poxviruses have evolved various strategies to inhibit the NF- κ B signaling pathway. Inhibitors of NF- κ B have been identified in members of the genera *Orthopoxvirus*, *Leporipoxvirus*, *Yatapoxvirus*, *Molluscipoxvirus* and *Parapoxvirus*, with selected viruses encoding for multiple inhibitors (for a review see [28]). The type member of the *Poxviridae* vaccinia virus (VACV) encodes at least seven NF- κ B inhibitors (A52R, A46R, B14, K1L, N1L, M2L, and E3L), which target different steps leading to NF- κ B activation, most often by preventing IKK complex activation (8, 13, 17, 21, 34, 46, 48). VACV proteins A46R and A52R, for example, associate with myeloid differentiation factor 88 (MyD88) and IRAK2 and TRAF6, respectively, disrupting activation of the pathway through multiple TLRs (3, 21, 48). VACV protein B14 associates with IKK complex inhibiting phosphorylation of IKK β (8). Molluscum contagiosum virus (MOCV) protein MC159 prevents degradation of I κ B β and MC160 induces degradation of IKK α (33, 38).

The role of poxviral inhibitors of NF- κ B during infection *in vivo* remains poorly understood. Pathogenesis studies using mutant viruses, with deletion of selected NF- κ B inhibitors, have shown a wide range of phenotypes *in vivo*, with individual gene-deletions

resulting in phenotypes varying from no effect (1, 8, 21, 48) to marked virus attenuation (4, 24, 30, 42).

With the exception of VACV E3L (ORFV020), the parapoxviruses lack homologues of NF- κ B inhibitors encoded by other poxviruses, indicating that these viruses have evolved novel strategies to counteract the NF- κ B signaling pathway. Recently, two novel inhibitors of NF- κ B have been identified in the parapoxvirus ORFV, ORFV024 and ORFV002 (11, 12). ORFV024 was shown to inhibit phosphorylation of I κ B kinases thus preventing activation of the IKK complex (11), while ORFV002 encodes for a nuclear NF- κ B inhibitor which prevents p300-mediated acetylation of the transactivating NF- κ B subunit NF- κ B-p65 (12).

Here, we present data indicating that ORFV *ORFV121*, a gene unique to parapoxviruses, encodes for a novel viral NF- κ B inhibitor that binds to and inhibits phosphorylation and nuclear translocation of NF- κ B-p65, a mechanism that is distinct from that of ORFV024- or ORFV002-mediated NF- κ B inhibition. Deletion of ORFV121 from the viral genome markedly decreased ORFV virulence and disease severity in sheep, indicating that ORFV121 is a virulence determinant for ORFV in the natural host.

Materials and Methods

Cells and viruses. Primary ovine cells (OFTu – Ovine fetal turbinate) were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and containing gentamicin (50 μ g/ml), penicillin (100 μ g/ml) and streptomycin (100 μ g/ml). HeLa cells stably expressing GFP (GFP/HeLa) or ORFV121-GFP (121GFP/HeLa) fusion protein were established as previously described (11), and maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, and containing gentamicin (50 μ g/ml), penicillin (100 μ g/ml) streptomycin (100 μ g/ml) and neomycin (G418, Gibco – 500 μ g/ml). ORFV strain OV-IA82 (10) was used to construct an *ORFV121*-deletion mutant virus (OV-IA82 Δ 121) and in all procedures involving wild-type virus infection and cloning of viral genes. OV-IA82 Δ 121 was used to construct an *ORFV121* revertant virus (OV-IA82Rv121).

Plasmids. *ORFV121* coding sequence was PCR amplified from the OV-IA82 genome with primers 121Flag-Fw(HindIII)-5'-TAAGGCCTCTAAGCTTATGGCTGGCTTCCTAGGC GCGTTC-3'; 121Flag-Rv(XhoI)-5'-CAGAATTCGCCTCGAGCAGAACTTCCTCCACT TTGCAGCA-3'; 121EGFP-Fw(XhoI)-5'-ACTTACACTCGAGCAACCATGGCTGGCTTC-3'; and 121EGFP-Rv(HindIII)-5'-CGTCGCAAGCTTCAGAACTTCCTCCACTTTG-3', and cloned into plasmids pCMVTag4A (Stratagene, La Jolla, CA) and pEGFP-N1 (Clontech,

Mountain View, CA) to obtain the expression vectors pCMV121Flag and p121EGFP, respectively.

ORFV121 left (LF; 1016 bp) and right (RF; 853 bp) flanking regions were PCR amplified from the OV-IA82 genome and cloned into plasmid pZippy-Neo/Gus (14) to obtain the recombination vector pZippy-121LF-Neo/Gus-121RF. PCR primers were 121LF-Fw(SpeI)-5'-TAAGGCCTCTACTAGTCTGCGACCGACATCGCACACATGA-3'; 121LF-Rv(HindIII)-5'-CAGAATTCGCAAGCTTGGTTGTGTGGGCCACAGAGTTGAG-3'; 121RF-Fw(NotI)-5'-AT TCTTATGCGGCCGCGCAGCACTGCTCGGAGGAGTGCTC-3'; and 121RF-Rv(BglII)-5'-CAGAATTCGCAAGCTTGGTTGTGTGGGCCACAGAGTTGAG-3'.

ORFV121 and its left and right flanking regions were PCR amplified from the OV-IA82 genome and cloned into the recombination vector pZippy (14) lacking the Neo/Gus reporter genes. The resultant recombination plasmid pZippy-LF/ORFV121/RF was used to generate the revertant OV-IA82Rv121 virus. PCR primers used for amplification were described above (121LF-Fw[HindIII] and 121RF-Rv[BglII]). DNA sequencing of plasmid constructs confirmed the integrity of OV-IA82 sequences in the recombination plasmids, and in-frame cloning of ORFV121 with Flag and GFP fusion proteins.

RT-PCR. Transcription kinetics of *ORFV121* was examined during ORFV replication in OFTu cells by reverse transcription-PCR (RT-PCR). Cells were inoculated with OV-IA82 (multiplicity of infection [MOI] = 10) in the presence or absence of cytosine arabinoside (AraC; 40 µg/ml) and harvested at 0, 1, 2, 3, 6, 12 and 24 h post inoculation (p.i.). RNA samples were processed and reverse transcribed as previously described (11). Transcription of *ORFV121*, *ORFV024* (early control gene) and *ORFV059* (late control gene), was assessed by PCR using primers 121Fw-5'-TCCAGCTGCCCCGCGACGACGCGC-3', 121Rv-5'-GGAGGTTGGGTCTGCCGGCGCCGCA-3', 024Fw-5'-GCGGACACAGCCACAACCACAGTC-3', 024Rv-5'-CTAGCACGCGCTTTCGGTACCGCC-3', 059Fw-5'-ATGGATCCACCCGAAATCAC-3' and 059Rv-5'-TCACACGATGGCCGTGACCAGC-3', respectively.

Confocal microscopy. OFTu cells cultured on glass coverslips were transiently transfected with plasmids pEGFP-N1 or p121EGFP and subsequently infected with the OV-IA82Δ121 (24 h post transfection). Cells were fixed, stained with DAPI and examined by confocal microscopy at 6 h p.i.. To investigate a potential colocalization of ORFV121 and NF-κB-p65, OFTu cells were transiently transfected with either [1]. pEGFP-N1 (control vector) and pT7NF-κB-p65, or [2]. p121EGFP and pT7NF-κB-p65, and treated with TNF-α for 60 min. After fixation and permeabilization, cells were probed with an anti-NF-κB-p65 antibody (Catalog no. 3034, Cell Signaling, Danvers, MA), incubated with a secondary goat anti-rabbit

antibody (Alexa Fluor 594, catalog no. A31631, Invitrogen) and examined by confocal microscopy.

Construction and characterization of *ORFV121*-deletion mutant virus OV-IA82 Δ 121 and *ORFV121*-revertant virus OV-IA82Rv121. *ORFV121*-deletion mutant OV-IA82 Δ 121 virus was generated by homologous recombination between OV-IA82 and the recombination vector pZippy-121LF-Neo/Gus-121RF (11). The absence of *ORFV121* sequence and the presence of Neo/Gus sequences in the purified recombinant virus were determined by PCR and Southern Blotting.

ORFV121-revertant virus OV-IA82Rv121 was generated by homologous recombination between the deletion mutant virus OV-IA82 Δ 121 and the recombination vector pZippy-LF/*ORFV121*/RF. Construction, selection and purification of the OV-IA82Rv121 were performed as previously described (12). The presence of *ORFV121* sequence and the absence of Neo/Gus sequences in the purified revertant virus were determined by PCR and Southern blotting. The integrity and identity of the OV-IA82 sequences flanking the recombination regions were confirmed by DNA sequencing.

Real-time PCR analysis. Gene expression of OFTu cells in response to ORFV infection was examined by real-time PCR. OFTu cells cultured in 35-mm dishes for 16 h were mock-infected or infected with OV-IA82 or OV-IA82 Δ 121 (MOI = 10). Cells were harvested at 2 and 4 h p.i. and subjected to RNA extraction and reverse transcription as previously described (11). Expression of genes IL-1 α , IL-6, IL-8, NF κ B, CCL20, CXCL3, IRF-3, ICAM-1 and PTGS2 was assessed using primers and probes synthesized based on ovine gene sequences in GenBank (Applied Biosystems, TaqMan[®] Gene Expression Custom Assays). Real-time PCR conditions and data analysis were performed as previously described (11).

NF- κ B luciferase assays. The effect of *ORFV121* on NF- κ B-mediated transcription was investigated during ORFV infection in cell cultures. OFTu cells cultured in 12-well plates (1.2×10^5 cells per well) were co-transfected with the vectors pNF- κ B-Luc (450 ng; Clontech, Mountain View, CA), and pRL-TK (50 ng; Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen). Cells were mock-infected (MEM) or infected with OV-IA82, OV-IA82 Δ 121 or OV-IA82Rv121 (MOI = 10) at 24 h post transfection. Luciferase activities were determined at 2, 4 and 6 h p.i. using the Dual Luciferase Reporter Assay (Promega) and a luminometer.

The ability of *ORFV121* to inhibit NF- κ B-transcriptional activity induced by LPS and TNF- α was investigated. OFTu cells were co-transfected with pNF- κ B-Luc (450 ng; Clontech), pRL-TK (50 ng; Promega), and pCMV121Flag (500 ng), or control empty vector

pCMVTag4A (500 ng), using Lipofectamine 2000 (Invitrogen). Cells were exposed to control media (MEM, 2% FBS) or media containing LPS (250 ng/ml; Invivogen, San Diego, CA) for 6 h at 24 h post transfection. Luciferase activities were determined as described above.

Stable cell lines expressing GFP (GFP/HeLa) or ORFV121-GFP (121GFP/HeLa) were co-transfected with pNF- κ B-Luc (450 ng; Clontech), pRL-TK (50 ng; Promega) and exposed to control media (DMEM, 2%FBS) or media containing TNF- α (20 ng/ml or 50 ng/ml, Cell Signaling, Danvers, MA) for 6 h. Cells were harvested and luciferase activities determined as above. Firefly luciferase activity was normalized to the sea pansy luciferase activity and resultant ratios were used to calculate fold changes in luciferase activity. All transfections were performed in triplicate and the mean of the three replicates was used to calculate fold changes. Statistical analysis of the data was performed by using Student's *t* test.

Western immunoblots. The effect of ORFV121 on the NF- κ B signaling pathway was investigated by Western immunoblots. Cells stably expressing GFP (GFP/HeLa) or ORFV121-GFP (121GFP/HeLa) were cultured in 6-well plates for 48 h, exposed to control media (DMEM, 2% FBS) or media containing TNF- α (20 ng/ml) for 5 and 15 min, and lysed as above. OFTu cells cultured in 6-well plates were transfected with pCMV121Flag (2 μ g) or with control empty vector pCMVTag4A (2 μ g), using Lipofectamine 2000. Cells were exposed to control media (MEM, 2% FBS) or media containing TNF- α (20 ng/ml) for 5 and 15 min, and lysed as above at 24 h post transfection. OFTu cells cultured in 6-well plates were inoculated with OV-IA82, OV-IA82 Δ 121, or OV-IA82Rv121 (MOI =10), and harvested at various times p.i. (15, 30 and 60 min). Uninfected cells were used as controls.

In the experiments above, fifty micrograms of protein extracts were resolved by SDS-PAGE (11). Antibodies used include: I κ B α (sc-371, Santa Cruz), phospho-I κ B α (Ser32/36) (Catalog no. 9246, Cell Signaling), NF- κ B-p65 (Catalog no. 3034, Cell Signaling), phospho-NF- κ B-p65 (Ser536) (Catalog no. 3033, Cell Signaling), GAPDH (sc-25778, Santa Cruz), Flag-M2 (Catalog no. 200471, Stratagene) or GFP (sc-8334, Santa Cruz). Densitometric analysis of the blots was performed by using ImageJ software, version 1.62 (National Institute of Health, Bethesda, MD). Statistical analysis of the data was performed by using Student's *t* test.

Cytoplasmic and nuclear cell fractionation. GFP/HeLa and 121GFP/HeLa stable cell lines cultured in 6-well plates for 48 h were treated with TNF- α (20 ng/ml) for 1 h and harvested in phosphate buffered saline (PBS). Untreated cells were used as controls. OFTu cells cultured in 6-well plates were mock-infected or infected with OV-IA82, OV-IA82 Δ 121, or OV-

IA82Rv121 and harvested at 60 min. Cytoplasmic and nuclear protein fractions were extracted with ProteoJet cytoplasmic and nuclear protein extraction kit (Fermentas, Glen Burnie, MD) following the manufacturer's protocol. Approximately 20 µg of cytoplasmic and nuclear protein extracts were resolved by SDS-PAGE, blotted to nitrocellulose membranes, and blots were probed with antibodies against NF-κB-p65 (Catalog no. 3034, Cell Signaling), GAPDH (sc-25778, Santa Cruz), or Histone-H3 (sc-10809, Santa Cruz).

Immunoprecipitation. Interaction of ORFV121 with NF-κB-p65 was assessed by using co-immunoprecipitation assays. OFTu cells were cotransfected with either [1]. pCMVTag4A and pT7NF-κB-p65 or [2]. pCMV121Flag and pT7NF-κB-p65 and exposed to control media or media containing TNF-α (20 ng/ml) for 15 min at 24 h post transfection. Cells were lysed with 200 µl of lysis buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% TRITON X-100) and incubated at room temperature for 15 min. Proteins were immunoprecipitated by using the FLAG immunoprecipitation kit (Sigma, St Louis, MO) following the manufacturer's protocol or an anti-NF-κB-p65 antibody coupled to protein G agarose beads (Upstate) as previously described (12). Immunoprecipitated proteins were resolved by SDS-PAGE and blots were probed with antibodies against Flag-M2 (Catalog no. 200471, Stratagene) and NF-κB-p65 (Catalog no. 3034, Cell Signaling) as described above.

Animal inoculations. The effect of *ORFV121* on ORFV virulence and pathogenesis was investigated in lambs. Three to five months-old crossbreed lambs acquired from an orf-free flock, were allocated in 5 groups distributed in two independent experiments: experiment I (exp I) consisted of mock-infected ($n = 2$), OV-IA82-infected ($n = 2$) and OV-IA82Δ121-infected lambs ($n = 2$); and experiment II (exp II) consisted of OV-IA82-infected ($n = 3$), OV-IA82Δ121-infected ($n = 3$), and OV-IA82Rv121-infected lambs ($n = 3$). Animals were tranquilized with Xilazine (Rompun[®], Bayer) and sites of inoculation were cleaned with water. After scarification of the muco-cutaneous junction of the inferior lips and the skin of the axillary (exp I) or the inner side of the thighs (exp II), 0.25 ml of virus suspension containing $10^{7.3}$ TCID₅₀/ml was inoculated on each site by using a cotton swab. Animals were monitored during 19 days for characteristic orf lesions, including erythema, vesicles, pustules and scabs. Skin biopsies were collected from the thighs (exp II) at days 1, 2, 3, 5 and 19 p.i, fixed in 10% buffered formalin, embedded in paraffin, sectioned (4 µm), and stained with hematoxylin-eosin using standard methods. Histological sections were examined for the presence of epidermal hyperplasia, ballooning degeneration of keratinocytes, hyper- and parakeratosis, intraepidermal microabscesses, rete ridges formation, keratinocyte intracytoplasmic inclusion bodies, and infiltration of epidermis and underlying dermis with

neutrophils and mononuclear cells. All animal procedures have been revised and approved approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee, (IACUC, protocol no. 214 as of 01/23/08).

Results

ORFV *ORFV121* is transcribed early-late during ORFV infection and the protein localizes to the cell cytoplasm. ORFV *ORFV121* encodes for a protein of 300-306 amino acids in length with a predicted molecular weight of 34 kDa. ORFV strain IA82 ORFV121 (AAR98216.1) is highly conserved among ORFV isolates, sharing 98%, 96%, and 88% amino acid identity with its homologues in the sheep ORFV isolates Orf11 (AAO91831.1) and NZ2 (ABA00639.1), and the goat ORFV isolate SA00 (NP_957898.1), respectively. Notably, the pseudocowpoxvirus (PCPV) reference strain VR634 ORF121 homologue (ADC54021; 70% amino acid identity) lacks most of the amino terminal half of the protein (ORFV IA82 ORF121 positions 4 to 145), while the reindeer PCPV isolate F00.120R lacks an *ORF121* gene (22). The most divergent parapoxvirus (PPV) ORF121 homolog, BPSV strain AR02 BPSV121 (NP_958029.1; 39% amino acid identity), contains three unique insertions in the amino-terminal region of the protein (ORFV IA82 ORF121 positions 24-27, 42-46, and 87-92), a 27-amino acid deletion in the middle of the protein (positions 132-158), and two additional deletions in the carboxyl terminal of the protein (positions 204-208 and 285-300). A sixty-amino acid region at the carboxyl termini of PPV ORF121 proteins exhibits the highest sequence conservation (ORFV IA82 ORF121 position 218-278; $\geq 75\%$). PPV ORF121 lacks homology to other known proteins of viral or cellular origin and no motifs or domains suggestive of putative protein function were identified.

ORFV121 transcription kinetics was investigated during ORFV replication in OFTu cells by RT-PCR. *ORFV121* transcription was detected throughout ORFV infection cycle (1 to 24 h p.i.), and was decreased at 12 and 24 h p.i. in the presence of AraC, an inhibitor of DNA replication and of late poxviral gene transcription (Fig. 1A). These results indicate that *ORFV121* is an early-late poxviral gene.

Subcellular localization of ORFV121 was investigated in OFTu cells transiently expressing ORFV121-GFP and subsequently infected with the *ORFV121*-deletion mutant virus OV-IA82 Δ 121. ORFV121-GFP localized to the cell cytoplasm, exhibiting a punctate distribution pattern (Fig. 1B). No colocalization of ORFV121 with markers for endoplasmic reticulum and mitochondria was observed (data not shown).

***ORFV121* is nonessential for ORFV replication *in vitro*.** Replication properties of OV-IA82, OV-IA82 Δ 121 and OV-IA82Rv121 were examined *in vitro*. No differences in replication kinetics and viral yields were observed when multiple-step or one-step growth curves of OV-IA82 Δ 121 were compared to those of the revertant OV-IA82Rv121 or wild-type OV-IA82 virus in OFTu (Fig. 2A and B) or in HeLa cell cultures (data not shown). Additionally, no differences in cytopathic effect, and plaque size and morphology were observed for OV-IA82, OV-IA82 Δ 121 and OV-IA82Rv121 in OFTu cells (data not shown). Thus, *ORFV121* is nonessential for ORFV replication in OFTu and in HeLa cell cultures.

OV-IA82 Δ 121 infection results in increased expression of NF- κ B-regulated genes in primary OFTu cells. Preliminary microarray screening of OV-IA82 Δ 121-infected OFTu cells indicated increased expression of genes regulated by the NF- κ B family of transcription factors (data not shown). Real-time PCR analysis of OV-IA82 Δ 121-infected OFTu cells at 2 h p.i. confirmed a marked increase in expression of NF- κ B-regulated genes IL1- α (24.4-fold), IL6 (14.7-fold), IL8 (28.2-fold), NF κ BIA (6.8-fold), CCL20 (51.1-fold), CXCL3 (49.1-fold), IRF-1 (4.3-fold), ICAM-1 (4.5-fold) and PTGS2 (8.4-fold), when compared to mock-infected or to wild-type virus-infected cells (Fig. 3A). Expression of these genes was similarly increased in OV-IA82 Δ 121-infected cells at 4 h p.i. (data not shown). No significant differences in expression levels were observed between mock- and wild-type virus-infected cells (Fig. 3A).

The ability of ORFV121 to inhibit NF- κ B-mediated transcription was further investigated by using a NF- κ B-luciferase reporter assay. Infection with OV-IA82 Δ 121 virus resulted in a marked and significant increase in luciferase activity of up to 4.8 ($P < 0.01$), 11.0 ($P < 0.005$) and 7.6-fold ($P < 0.005$) at 2, 4, and 6 h p.i., respectively (Fig. 3B). Restoration of *ORFV121* in the revertant virus (OV-IA82Rv121) rescued the wild type virus phenotype (Fig. 3B). Together, these data indicate that ORFV121 inhibits NF- κ B-mediated transcription during ORFV infection in OFTu cells.

Expression of ORFV121 decreases NF- κ B-mediated transcription induced by LPS and TNF- α . OFTu cells transiently expressing or HeLa cells stably expressing ORFV121 were stimulated with LPS and TNF- α , respectively and assayed for NF- κ B-mediated luciferase activity. Transient expression of ORFV121-Flag in OFTu cells significantly decreased LPS-induced NF- κ B-luciferase activity by \sim 1.7-fold ($P = 0.01$) when compared to control cells transfected with the empty vector (Fig. 3C and E). Additionally, stable expression of ORFV121-GFP in HeLa cells markedly decreased TNF- α -induced NF- κ B-mediated luciferase activity by \sim 3.5-fold ($P < 0.05$) when compared to control GFP-expressing cells

(Fig. 3D and F). These results indicate that ORFV121 inhibits NF- κ B-mediated transcription following stimulation of cells with LPS and TNF- α , two potent NF- κ B inducers.

ORFV121 decreases phosphorylation and nuclear translocation of NF- κ B-p65 but does not affect upstream phosphorylation of I κ B α . The effect of ORFV121 on phosphorylation and nuclear translocation of NF- κ B-p65 and on phosphorylation of I κ B α was investigated. HeLa cells stably expressing ORFV121-GFP, OFTu cells transiently expressing ORFV121-Flag, and control cells were treated with TNF- α and harvested at various times post-treatment. ORFV121 expression markedly decreased TNF- α -induced phosphorylation of NF- κ B-p65 in both ORFV121-stably and -transiently expressing cells (Fig. 4A and B, and Fig. 4C and D). To assess whether the decreased levels of phosphor-NF- κ B-p65 detected in ORFV121-expressing cells were due to its effects on events upstream of NF- κ B-p65, phosphorylation levels of I κ B α were investigated. ORFV121 expression did not affect TNF- α -induced phosphorylation of I κ B α (Fig. 4A).

The effect of ORFV121 expression on translocation of NF- κ B-p65 to the nucleus was investigated by using Western immunoblots and confocal microscopy. HeLa cells stably expressing ORFV121-GFP and control cells expressing GFP alone were stimulated with TNF- α for 60 min and cytoplasmic and nuclear cell fractions were obtained. ORFV121 expression markedly decreased nuclear translocation of NF- κ B-p65 when compared to control cells (Fig. 4E and F). Reduced levels of phosphor- or nuclear translocated NF- κ B-p65 in ORFV121-expressing cells were not due to protein degradation, since levels of pan-NF- κ B-p65 and GAPDH (Fig. 4A and C) or Histone-H3 (Fig. 4E) were constant in all samples. Levels of nuclear translocated NF- κ B-p65 in GFP/HeLa cells were not due to leakage from the cytoplasmic fraction, since NF- κ B-p65 was not detected in the nuclear fraction of control untreated cells (Fig. 4E). Similarly, transient expression of ORFV121 in TNF- α -treated OFTu cells markedly reduced nuclear translocation of NF- κ B-p65 (Fig. 6A). Together, these results indicate that ORFV121 functions downstream of I κ B α by inhibiting phosphorylation and nuclear translocation of NF- κ B-p65.

ORFV121 decreases phosphorylation and nuclear translocation of NF- κ B-p65 during ORFV infection in OFTu cells. The ability of ORFV121 to suppress phosphorylation and nuclear translocation of NF- κ B-p65 during ORFV infection in OFTu cells was further investigated. OFTu cells were mock-infected or infected with OV-IA82, OV-IA82 Δ 121 or OV-IA82R ν 121 and harvested at various times p.i.. Infection with the wild-type virus OV-IA82 resulted in low levels of NF- κ B-p65 phosphorylation (Fig. 5A and B). OV-IA82 Δ 121-infection induced a marked increase in NF- κ B-p65 phosphorylation as detected at 60 min p.i.,

(Fig. 5A and B). Restoration of *ORFV121* in the revertant virus (OV-IA82Rv121) rescued the wild-type virus phenotype (Fig. 5A and B). To assess nuclear translocation of NF- κ B-p65 during ORFV infection, OFTu cells were mock-infected or infected with OV-IA82, OV-IA82 Δ 121 or OV-IA82Rv121, fractionated, and cytoplasmic and nuclear cell extracts analyzed by Western immunoblots. Infection with wild-type and revertant viruses resulted in similar low levels of NF- κ B-p65 in the nucleus, whereas OV-IA82 Δ 121-infection markedly increased nuclear translocation of NF- κ B-p65 (Fig. 5C and D). Together, these results indicate that *ORFV121* suppress phosphorylation and nuclear translocation of NF- κ B-p65 during ORFV infection.

ORFV121 interacts with NF- κ B-p65. Interaction of ORFV121 and NF- κ B-p65 was investigated as a potential mechanism for ORFV121 inhibitory effect on NF- κ B signaling pathway. OFTu cells were co-transfected with expression vectors p121EGFP and pT7-NF κ Bp65, treated with TNF- α for 60 min, and the localization of these proteins was assessed by confocal microscopy. ORFV121 co-localized with NF- κ B-p65 in the cytoplasm of transfected cells, suggesting an interaction between ORFV121 and NF- κ B-p65 (Fig. 6A). Specific interaction of ORFV121 and NF- κ B-p65 was further investigated by using co-immunoprecipitation assays. OFTu cells co-transfected with pT7-NF κ Bp65 and either pCMVTag4A or pCMV121Flag were treated with TNF- α and harvested at 15 min post treatment. Reciprocal immunoprecipitation assays with either anti-Flag (Fig. 6B) or anti-NF- κ B-p65 (Fig. 6C) antibodies resulted in co-precipitation of ORFV121-Flag and NF- κ B-p65. A potential interaction of ORFV121-Flag with NF- κ B-p50 was also investigated by using co-immunoprecipitation assays. No association of ORFV121-Flag with NF- κ B-p50 was detected (data not shown). These results indicate that ORFV121 physically interacts with NF- κ B-p65.

ORFV121 contributes to ORFV virulence in sheep. To investigate the effect of ORFV121 on ORFV virulence and pathogenesis, 3 to 5-months old lambs were inoculated with OV-IA82, OV-IA82 Δ 121, OV-IA82Rv121 or MEM (control group; exp I), and monitored for clinical orf. Characteristic orf lesions were observed in all wild-type and revertant virus-inoculated lambs. Lesions were initially observed by day 3 p.i. and consisted of erythema, pustules (day 5 p.i.) and scabs at later times p.i. (6-7 days p.i.; Fig. 7). Local scabby tissue deposition was first observed by day 5 p.i. and continued until day 10 p.i.. Lesions began to resolve by day 15 p.i. and by day 19 p.i. only the lesion margins were partially covered with scabs. In contrast, lesions induced by the OV-IA82 Δ 121 virus were less severe and resolved sooner than those induced by wild-type and revertant viruses (exp I, 2 of 2; exp II, 3 of 3). Lesions were characterized by erythema, small vesicles and limited scab formation (Fig. 8A).

Erythema was initially observed at day 3 p.i., evolving into pustules (5 d p.i.) which persisted until day 8 p.i. Scabby tissue formation was markedly reduced, with no scabs observed beyond day 10 p.i.. A similar low severity and short duration disease was observed in lambs inoculated with OV-IA82 Δ 121 in the axillary region (exp I) or in the inner side of the thighs (exp II) (data not shown). Control group animals did not exhibit significant changes, and minor scabs induced by skin scarification were resolved by day 4 p.i. (data not shown). These results indicate that *ORFV121* contributes to ORFV virulence and pathogenesis in the natural host.

Deletion of *ORFV121* from the ORFV genome results in a reduced cellular inflammatory response in the skin. To compare histological changes induced by OV-IA82, OV-IA82 Δ 121 and OV-IA82Rv121 viruses, lambs were inoculated in the inner side of the thighs, and skin biopsies were collected at 1, 2, 3, 5 and 19 days p.i.. No changes other than partial loss of epidermis and neutrophil infiltration associated with scarification were observed in mock-infected control lambs on days 1 and 2 p.i. (data not shown). Typical histological changes associated with ORFV infection, including epidermal hyperplasia, ballooning degeneration of keratinocytes, skin infiltration with inflammatory cells, pustules, and accumulation of scabby tissue were observed in samples from lambs inoculated with OV-IA82 (data not shown), OV-IA82 Δ 121 (Fig. 8, panels a, b, e and f) and OV-IA82Rv121 (Fig. 8, panels c, d, g and h). However, significant differences were observed in time to appearance and magnitude of changes between OV-IA82 Δ 121- and OV-IA82Rv121-inoculated groups. By day 3 p.i., all lambs (3/3) inoculated with OV-IA82Rv121 virus exhibited marked ballooning degeneration of the stratum spinosum of the epidermis (and, occasionally, the external root sheath of hair follicles), infiltration of dermis and epidermis with neutrophils and mononuclear cells, and pustules (Fig. 8, panels c and d). In contrast, ballooning degeneration of keratinocytes and marked skin infiltration with inflammatory cells was observed in only one of three lambs inoculated with OV-IA82 Δ 121, while pustules were absent (Fig. 8, panels a and b). By day 5 p.i., micropustules were observed in samples from two of three OV-IA82 Δ 121-infected lambs, while infiltration with inflammatory cells (mostly mononuclear cells) was limited to areas exhibiting ballooning degeneration of keratinocytes (Fig. 8, panels e and f). Accumulation of scabby tissue in OV-IA82 Δ 121-inoculated lambs was limited or absent. In contrast, lambs infected with OV-IA82Rv121 exhibited massive inflammatory cell infiltration of the dermis and epidermis, pustule formation, epidermal pathology, and accumulation of scabby tissue (Fig. 8, panels g and h). These results indicate

that deletion of ORFV121 from the viral genome results in a reduced epidermal pathology and cellular inflammatory response in the skin.

Discussion

ORFV infection markedly suppresses NF- κ B-mediated transcription in primary OFTu cells (11, 12) indicating that this virus efficiently blocks activation of the NF- κ B signaling pathway. Here, the novel ORFV *ORFV121*, a gene unique to parapoxviruses, was shown to encode for an inhibitor of NF- κ B that contributes to ORFV virulence and disease pathogenesis in sheep. ORFV121 colocalizes and interacts with NF- κ B-p65 in the cell cytoplasm, thus providing a mechanism for inhibition of NF- κ B-p65 phosphorylation and nuclear translocation. Deletion of ORFV121 from the ORFV genome resulted in a marked attenuated disease phenotype in sheep, indicating that ORFV121 is a bona fide virulence determinant for ORFV in the natural host.

ORFV *ORFV121*, while not affecting phosphorylation of I κ B α , inhibits phosphorylation (Ser536) and nuclear translocation of NF- κ B-p65. Normal levels of phosphor-I κ B α in ORFV121-expressing cells indicated that upstream activation of I κ B kinases was not affected by ORFV121 and further suggested that ORFV121 functions downstream in the pathway. Consistent with this, ORFV121 co-localized and co-precipitated with NF- κ B-p65, indicating that physical interaction of ORFV121 with NF- κ B-p65 is a likely mechanism by which ORFV121 inhibits phosphorylation, nuclear translocation and, consequently, transcriptional activity of NF- κ B-p65.

Phosphorylation is a critical post-translational modification that regulates the activity of key components of the NF- κ B signaling pathway, including the transactivating NF- κ B subunit NF- κ B-p65 (40, 50). Ten phosphorylation sites have been identified in NF- κ B-p65, serines 205, 276, 281, 311, 468, 529 and 536, and threonines 254, 435, and 505, many of which have a modulatory role on NF- κ B transcriptional activity (40). For example, phosphorylation of NF- κ B-p65 at Ser536 was shown to impair NF- κ B-p65-I κ B α interaction, which may accelerate nuclear translocation of NF- κ B-p65 (2), or decrease its nuclear export by newly synthesized I κ B α , thus regulating the duration of NF- κ B-mediated transcription (50). Additionally, phosphorylation of NF- κ B-p65 at serines 276 and 536 is required for recruitment of the coactivator p300 to the transcriptional complex, thus promoting full transcriptional activity of NF- κ B-p65 (7, 40). The inhibitory effects of ORFV121 on NF- κ B-mediated gene transcription observed here are likely due to the decreased phosphorylation

and nuclear translocation of NF- κ B-p65. However, given the physical interaction between ORFV121 and NF- κ B-p65, the possibility that ORFV121 affects additional events regulating transcriptional activity of NF- κ B-p65 cannot be formally excluded.

Most poxviral NF- κ B inhibitors identified to date function in the cell cytoplasm mainly by preventing activation of the IKK complex (reviewed by [28]), the bottleneck for most NF- κ B activating stimuli (25). However, selected inhibitors encoded by these viruses have been shown to function downstream in the pathway directly on NF- κ B subunits (5, 29, 30). For example, MYXV M013, variola virus (VARV) G1R and its orthologs in monkeypox virus (MPXV MPXV003), ectromelia virus (ECTV ECTV002) and CPXV (CPXV006) were shown to interact with the NF- κ B subunit NF- κ B-p105, consequently inhibiting nuclear translocation of NF- κ B-p50/NF- κ B-p65 (29, 30). Additionally, CPXV-encoded CPXV077 was shown to interact with and inhibit nuclear translocation of the NF- κ B transactivating subunit NF- κ B-p65 (5). Here, we have shown that a novel poxviral NF- κ B inhibitor encoded by ORFV *ORFV121* targets NF- κ B-p65 in the cell cytoplasm. ORFV121 binds to and inhibits phosphorylation and nuclear translocation of NF- κ B-p65. Therefore, targeting NF- κ B subunits in the cell cytoplasm and preventing their translocation to the nucleus represents an efficient strategy that selected poxviruses use to inhibit NF- κ B transactivating activity.

The NF- κ B signaling pathway integrates several aspects of skin homeostasis, including keratinocyte proliferation, differentiation, apoptosis, and innate immune responses (39, 51). This broad spectrum of significant biological activities makes predictions on the role of poxviral NF- κ B inhibitors on viral host range and pathogenesis difficult. Multiple NF- κ B inhibitors encoded by individual poxviruses, including ORFV (e.g. ORFV002, ORFV024 and ORFV121), further complicate this matter. Some poxviral NF- κ B inhibitors have been shown to affect virus virulence and disease pathogenesis to some extent; however, the degree of virus attenuation resultant from single gene deletions has been variable and, in most cases, very modest (1, 8, 10, 11, 21, 48). With a few exceptions (CPXV *CPXV006*, MYXV *M013* and *M150R*), no single gene deletion rendered marked virus attenuation *in vivo*, suggesting that at the host level multiple NF- κ B inhibitors encoded by individual poxviruses may exert complementary functions to effectively suppress the NF- κ B signaling pathway during virus infection (4, 24, 30, 42).

Deletion of *ORFV121* from the ORFV genome resulted in a marked attenuated disease phenotype in sheep, indicating that ORFV121 is a virulence determinant for ORFV in the natural host. Histological changes and inflammatory responses induced by OV-IA82 Δ 121

were less severe than those induced by the wild-type or revertant viruses. These results contrast with our findings for ORFV-encoded NF- κ B inhibitors ORFV002 and ORFV024, where viruses containing individual gene deletions had no significant effect on ORFV virulence and disease pathogenesis in sheep (11, 12). These observations suggest that, in addition to possible complementary functions, individual NF- κ B inhibitors encoded by ORFV may modulate distinct cellular processes regulated by the NF- κ B signaling pathway during infection *in vivo* (e.g. immune responses, cell proliferation, cell differentiation and/or apoptosis). While regulation of some of these processes is essential for virus virulence and disease pathogenesis, modulation of others may play roles in less understood and perhaps subtle aspects of ORFV host range and infection biology, such as subclinical and/or persistent infections (37).

Results here demonstrating that OV-IA82 Δ 121 infection induces a reduced inflammatory response in the skin when compared to wild-type or revertant virus infection contrast with observations for CPXV- and MYXV-encoded NF- κ B inhibitors, CPXV006 and M150R, respectively, for which deletion from the viral genomes resulted in increased inflammatory response at sites of virus replication (4, 30). These findings can be reconciled by the apparently paradoxical roles of the NF- κ B signaling pathway in the skin (39). Remarkably, either continuous activation or continuous inhibition of the NF- κ B signaling pathway in the epidermis, have been shown to result in enhanced skin inflammatory responses (39). Therefore, it is possible that by inhibiting activation of the NF- κ B signaling pathway, ORFV121 stimulates local inflammatory responses following ORFV infection, thus contributing to disease development and pathogenesis. Alternatively, ORFV121 may function in other cellular processes regulated by NF- κ B in the skin, such as keratinocyte proliferation, differentiation and/or apoptosis, potentially contributing to the keratinocyte-restricted host range of ORFV.

Notably, poxviral NF- κ B inhibitors that markedly affect virus virulence and disease pathogenesis *in vivo* have been shown to target and/or function directly on NF- κ B subunits. For example, CPXV006, a virulence determinant for CPXV in mice, binds to and prevents degradation of the NF- κ B subunit NF- κ B-p105 (30). Similarly, MYXV M013, a significant virulence determinant for MYXV in rabbits, has been shown to interact with NF- κ B-p105 (24, 42). And MYXV M150R, an additional virulence determinant for MYXV in rabbits, was shown to colocalize with NF- κ B-p65 in the cell nucleus, although its actual function is unknown (4). ORFV *ORFV121* was shown here to encode for a virulence determinant for

ORFV in sheep, which binds to and inhibits phosphorylation and nuclear translocation of NF- κ B-p65. These findings suggest that by functioning directly on NF- κ B family members selected poxviral NF- κ B inhibitors may effectively suppress critical NF- κ B-regulated responses without the need for complementary action of other NF- κ B inhibitors encoded by these viruses. An exception here is ORFV-encoded ORFV002, which binds to and decreases acetylation of NF- κ B-p65 in the nucleus without significantly affecting virus virulence in the natural host (12). It would be interesting to determine if other poxviral encoded proteins that target NF- κ B subunits (28, 29) have a similar effect on virus virulence and disease pathogenesis in natural hosts *in vivo*.

Poxviruses have evolved a striking variety of mechanisms to inhibit the NF- κ B signaling pathway, targeting ligand-receptor interactions on the cell surface or intracellular cytoplasmic events of the pathway (28). Notably, while lacking homologues of most poxviral NF- κ B inhibitors (exception being *ORFV020* a homologue of VACV *E3L* gene), ORFV has evolved novel proteins to modulate NF- κ B transactivating activity by targeting distinct aspects of the pathway, including both cytoplasmic and nuclear events (11, 12). ORFV-encoded ORFV024 functions in the cell cytoplasm upstream of the IKK complex preventing activation of IKK by inhibiting phosphorylation of the I κ B kinases, IKK α and IKK β (11). ORFV ORFV121 functions in the cell cytoplasm, immediately downstream of I κ B α , by binding to and inhibiting phosphorylation and nuclear translocation of NF- κ B-p65. While ORFV-encoded ORFV002 functions in the cell nucleus, where it binds to and inhibits acetylation of NF- κ B-p65 (Fig. 9) (12). Given the complexity of the NF- κ B signaling pathway and the broad spectrum of cellular processes regulated by this pathway, it is not surprising that ORFV, like other poxviruses, has evolved an array of mechanisms to interfere with NF- κ B-regulated responses. Indeed, it is possible that ORFV encodes for additional NF- κ B inhibitors, either novel ones or those conserved in other chordopoxviruses such as the ankyrin repeat-containing family of proteins (10, 47) to effectively suppress NF- κ B-regulated host cell responses.

In summary, we have described a third novel NF- κ B inhibitor encoded by ORFV. ORFV121 binds to NF- κ B-p65 in the cell cytoplasm suppressing its phosphorylation, nuclear translocation and consequently transactivating activity. Deletion of *ORFV121* from the ORFV genome resulted in an attenuated disease phenotype in sheep, indicating that ORFV121 represents a bona fide virulence determinant for ORFV in the natural host.

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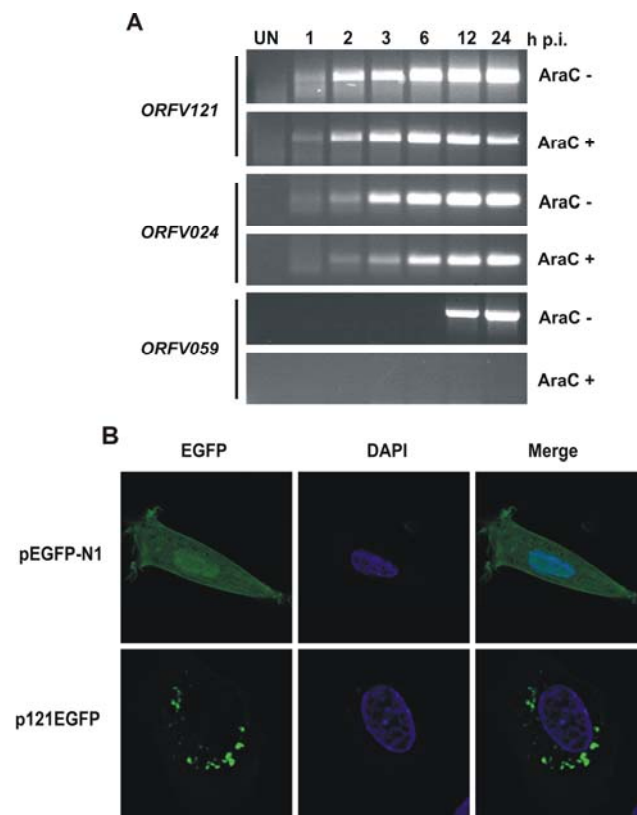


FIG 1. Transcription kinetics, and subcellular localization of ORFV121. (A) Transcription kinetics of *ORFV121*, *ORFV024* (early gene control), and *ORFV059* (late gene control) during ORFV infection in OFTu cells in the presence (+) or absence (-) of AraC as determined by RT-PCR. (B) Subcellular localization of ORFV121 in OFTu cells transfected with plasmids encoding for GFP (pEGFP-N1) or ORFV121-GFP (pEGFP121) and subsequently infected with OV-IA82 Δ 121 (MOI = 10). Cells were stained with DAPI and examined by confocal microscopy (6 h p.i.).

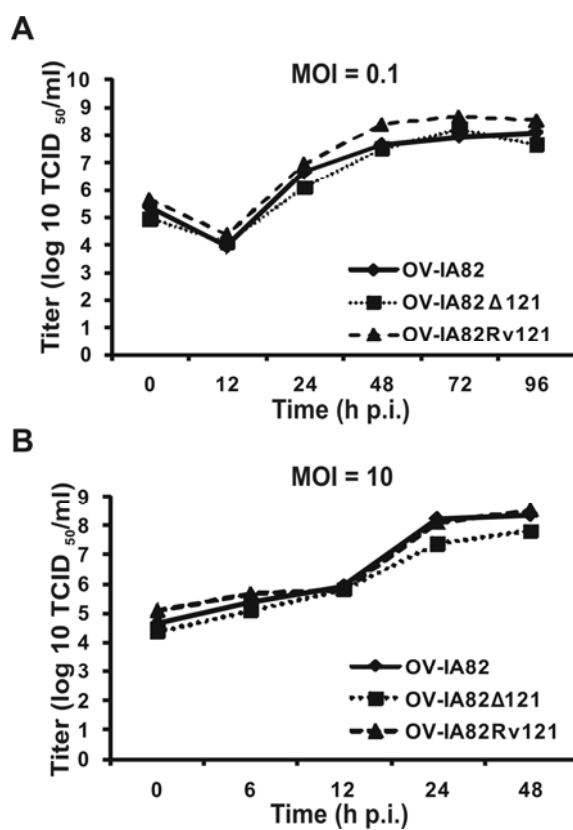


FIG 2. Replication characteristics of *ORFV121* deletion mutant virus OV-IA82Δ121. (A) Multiple step (A) and one step (B) growth curves of wild-type (OV-IA82), deletion mutant (OV-IA82Δ121) and revertant (OV-IA82Rv121) virus in primary OFTu cells (MOI = 0.1 and 10, respectively).

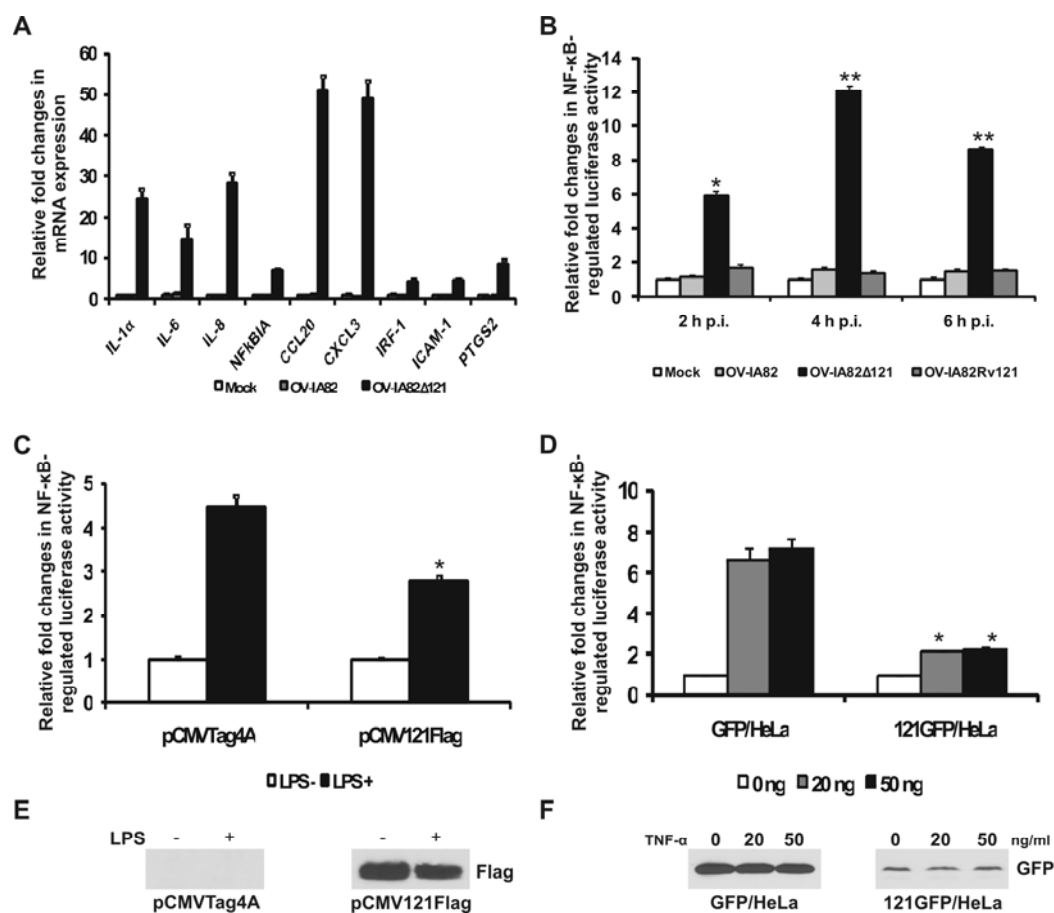


FIG 3. Effect of ORFV121 on NF- κ B-regulated gene transcription. (A) OFTu cells were infected with OV-IA82, OV-IA82 Δ 121 (MOI = 10), or mock-infected, and expression of selected NF- κ B-regulated genes was determined by real-time PCR at 2 h p.i. (B) OFTu cells were cotransfected with plasmids pNF- κ B Luc and pRL-TK and subsequently infected with OV-IA82, OV-IA82 Δ 121, OV-IA82Rv121 (MOI = 10), or mock-infected. Firefly and sea pansy luciferase activities were measured at 2, 4 and 6 h p.i. and expressed as relative fold changes in luciferase activity (*, $P < 0.01$; **, $P < 0.005$). (C) OFTu cells were cotransfected with plasmids pNF- κ B Luc , pRL-TK and either pCMVTag4A or pCMV121Flag, and subsequently treated with LPS (250 ng/ml) for 6 h. Luciferase activities were determined as in B (*, $P = 0.01$). (D) HeLa cells stably expressing GFP (GFP/HeLa) or ORFV121-GFP (GFP121/HeLa) were cotransfected with plasmids pNF- κ B Luc , pRL-TK and subsequently treated with TNF- α (20 ng/ml) for 6 h. Luciferase activities were determined as in B (*, $P < 0.05$). (E) Expression of ORFV121-Flag in samples examined in C, as determined by Western blot. (F) Expression of GFP and ORFV121-GFP in samples examined in D, as determined by Western blot. The results are representative of two (C) or three (A, B and D) independent experiments.

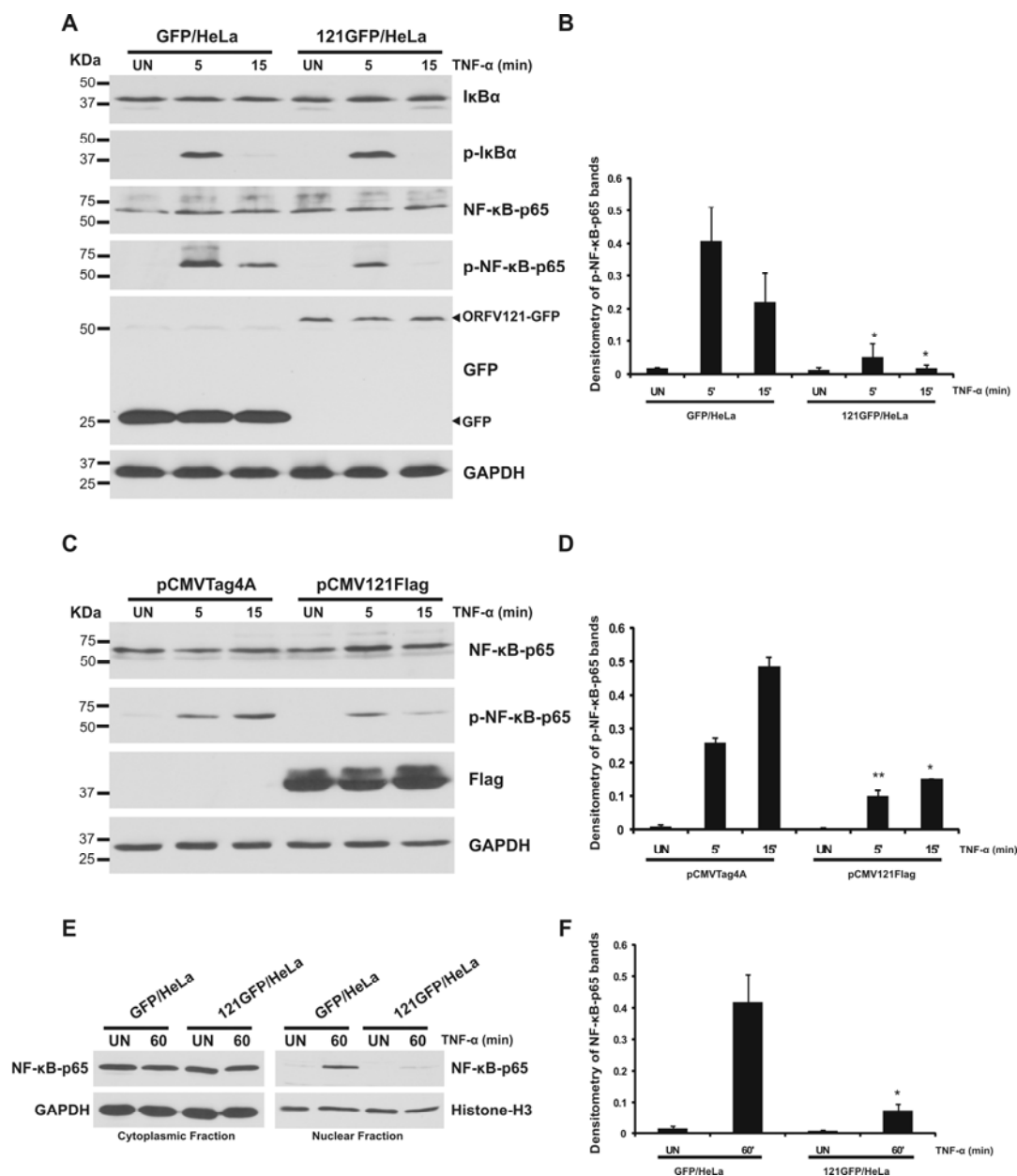


FIG 4. Effect of ORFV121 on activation of the NF- κ B signaling pathway, and on nuclear translocation of NF- κ B-p65. (A) HeLa cells stably expressing GFP (GFP/HeLa) or ORFV121-GFP (GFP121/HeLa) were treated with TNF- α (20 ng/ml) and harvested at the indicated times (UN, untreated controls). Protein extracts (50 μ g) were resolved by SDS-PAGE, blotted, and probed with antibodies directed against proteins indicated on the right. (B) Densitometry of phosphor NF- κ B-p65 bands normalized to the levels for the control GAPDH (*, $P < 0.05$). (C) OFTu cells transiently transfected with plasmids pCMVTag4A or pCMV121Flag were treated with TNF- α (20 ng/ml) and harvested at the indicated times (UN, untreated controls). Protein extracts (50 μ g) were resolved by SDS-PAGE, blotted, and probed with antibodies directed against proteins indicated on the right. (D) Densitometry of phosphor NF- κ B-p65 bands normalized to the levels for the control GAPDH (*, $P < 0.05$; **, $P < 0.005$). (E) OFTu cells transiently transfected with plasmids pCMVTag4A or pCMV121Flag were treated with TNF- α (20 ng/ml) for 60 min and cytoplasmic and nuclear protein fractions were extracted (UN, untreated controls). Protein extracts (20 μ g) were resolved by SDS-PAGE, blotted, and probed with antibodies against NF- κ B-p65 (top panels), GAPDH (bottom left panel), or Histone H3 (bottom right panel). (F) Densitometry of NF- κ B-p65 bands normalized to loading control GAPDH (*, $P < 0.05$). The results (A-F) are representative of three independent experiments.

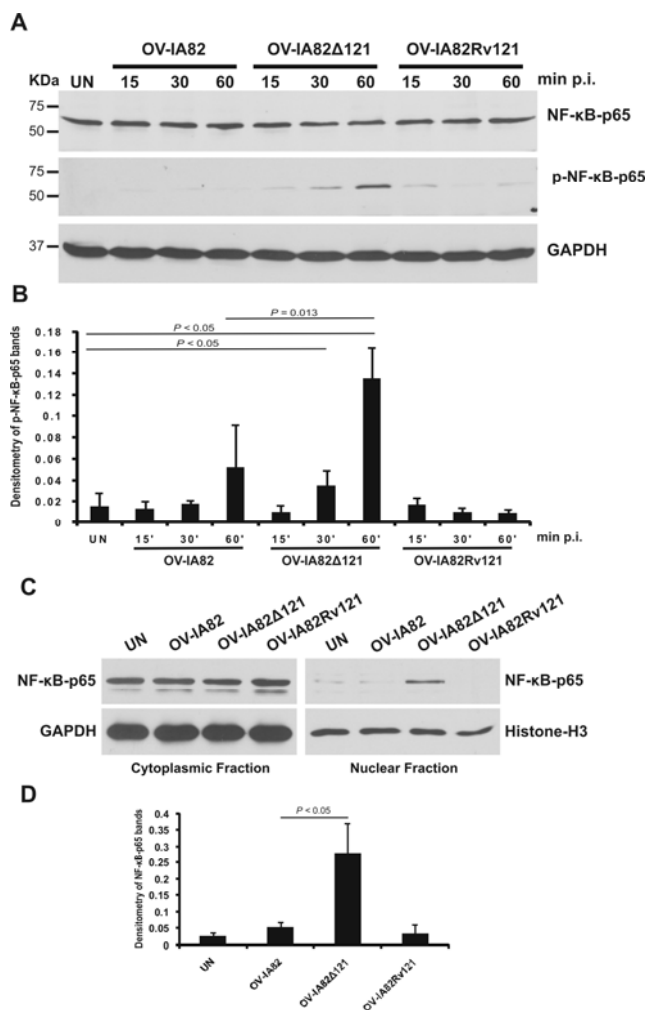


FIG 5. Effect of ORFV121 on phosphorylation and nuclear translocation of NF-κB-p65 during ORFV infection. (A) OFTu cells were infected with wild-type (OV-IA82), deletion mutant (OV-IA82Δ121) or revertant (OV-IA82Rv121) virus (MOI = 10), and harvested at the indicated times p.i. (UN, uninfected controls). Protein extracts (50 μg) were resolved by SDS-PAGE, blotted, and probed with antibodies directed against proteins indicated on the right. (B) Densitometry of phosphor NF-κB-p65 bands normalized to the levels for the control GAPDH. (C) OFTu cells were infected with wild-type (OV-IA82), deletion mutant (OV-IA82Δ121) and revertant (OV-IA82Rv121) virus (MOI = 10), harvested at 60 min p.i. and cytoplasmic and nuclear protein fractions were extracted (UN, uninfected controls). Protein extracts (20 μg) were resolved by SDS-PAGE, blotted, and probed with antibodies against NF-κB-p65 (top panels), GAPDH (bottom left panel), or Histone H3 (bottom right panel). (D) Densitometry of NF-κB-p65 bands normalized to the levels for the control GAPDH. The results (A-D) are representative of three independent experiments.

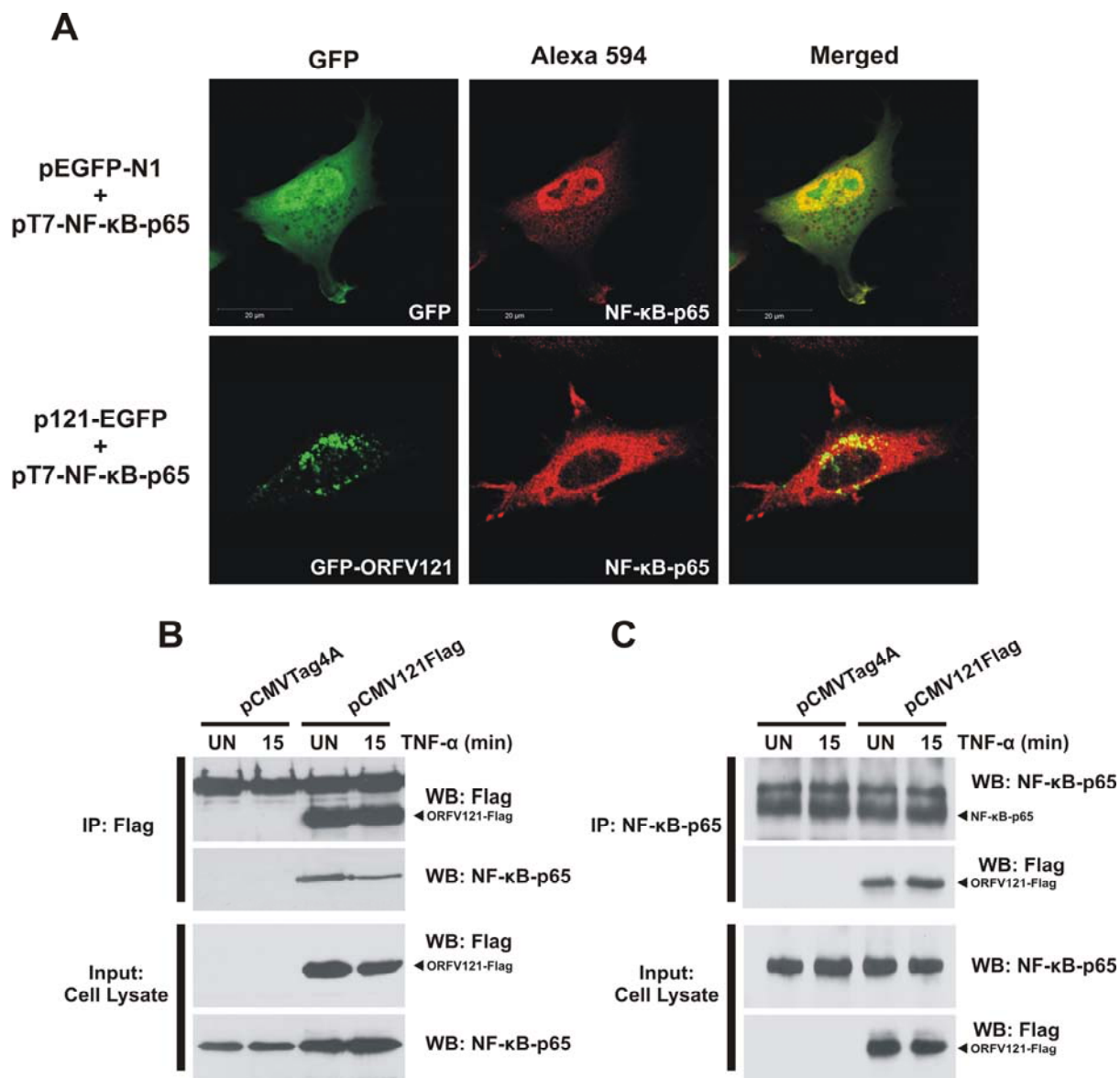


FIG 6. ORFV121 colocalizes and interacts with NF- κ B-p65. (A) OFTu cells were cotransfected with plasmid pNF- κ B-p65 and either pEGFP-N1 or p121EGFP. At 24 h post-transfection cells were treated with TNF- α (20 ng/ml for 60 min) fixed, and examined by confocal microscopy. (B) OFTu cells were cotransfected with plasmids pT7-NF- κ B-p65, and either pCMVTag4A (control) or pCMV121Flag (ORFV121-Flag), treated with TNF- α (20 ng/ml) and harvested at 15 min post TNF- α -treatment (UN, untreated controls). Protein extracts were immunoprecipitated with anti-Flag antibody coupled to agarose resin and examined by SDS-PAGE/Western blot (upper panels) with antibodies directed against proteins indicated on the right. Cell lysates were examined by SDS-PAGE/Western blot (bottom panels) with antibodies directed against proteins indicated on the right. (C) OFTu cells were cotransfected with plasmids pT7-NF- κ B-p65, pCMVTag4A (control) or pCMV121Flag (ORFV121-Flag), treated with TNF- α (20 ng/ml) and harvested at 15 min post TNF- α -treatment (UN, untreated controls). Protein extracts were immunoprecipitated with anti-NF- κ B-p65 antibody coupled to protein G agarose beads and examined by SDS-PAGE/Western blot (upper panels) with antibodies directed against proteins indicated on the right. Cell lysates were examined by SDS-PAGE/Western blot (bottom panels) with antibodies directed against proteins indicated on the right. The immunoprecipitation results shown in B and C are representative of three independent experiments.

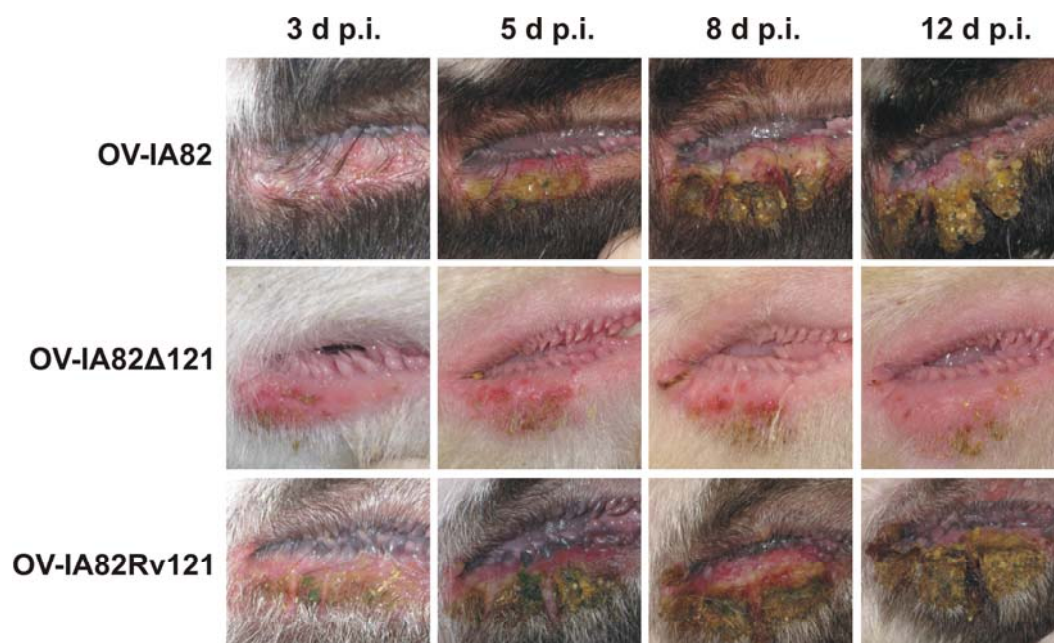
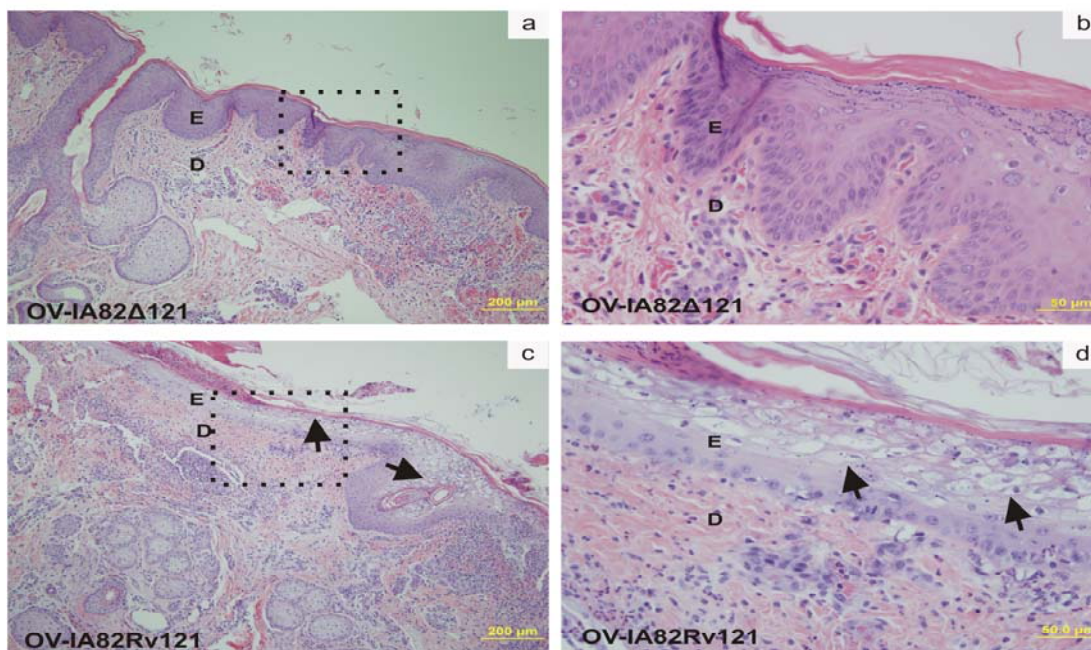


FIG 7. ORFV121 contributes to virus virulence and pathogenesis in the natural host. (A) Clinical course of orf in lambs inoculated with wild-type (OV-IA82), ORFV121-deletion mutant (OV-IA82Δ121), or revertant (OV-IA82Rv121) viruses at the mucocutaneous junction of the lower lip (d p.i. days post infection).

3 d p.i.



5 d p.i.

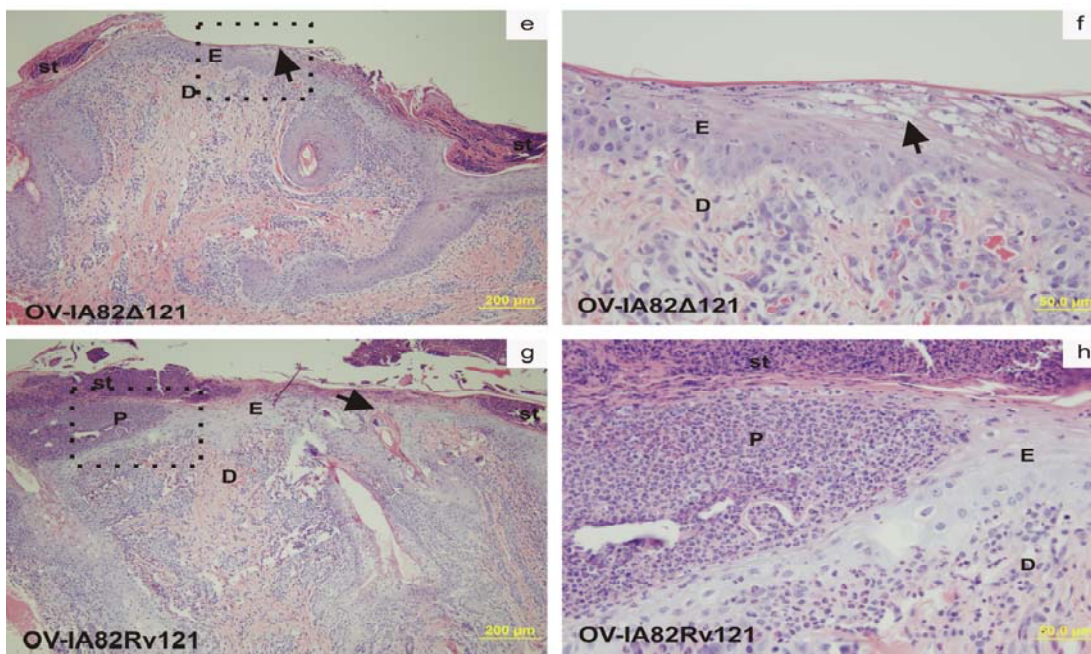


FIG 8. Histopathological changes in the skin of lambs following infection with OV-IA82 Δ 121 and OV-IA82Rv121 viruses. Lambs were infected with OV-IA82 Δ 121 or OV-IA82Rv121 viruses at sites on the inner side of the thighs, and skin biopsies were collected at various days post-inoculation and processed for histology. Images are representative of changes at 3 (top panels a-d) and 5 d p.i. (bottom panels e-h). Panels on the right (b, d, f, and h) represent a higher magnification of dotted rectangles highlighting the differences in the histological changes observed between OV-IA82 Δ 121 and OV-IA82Rv121-inoculated lambs (d: increased ballooning degeneration in keratinocytes and increased inflammatory cell infiltration of the dermis when compared to b; h: increased scabby tissue deposition, pustule formation and inflammatory cell infiltration when compared to f). E: epidermis; D: dermis; P: pustules; st: scabby tissue; arrows: ballooning degeneration; H&E.

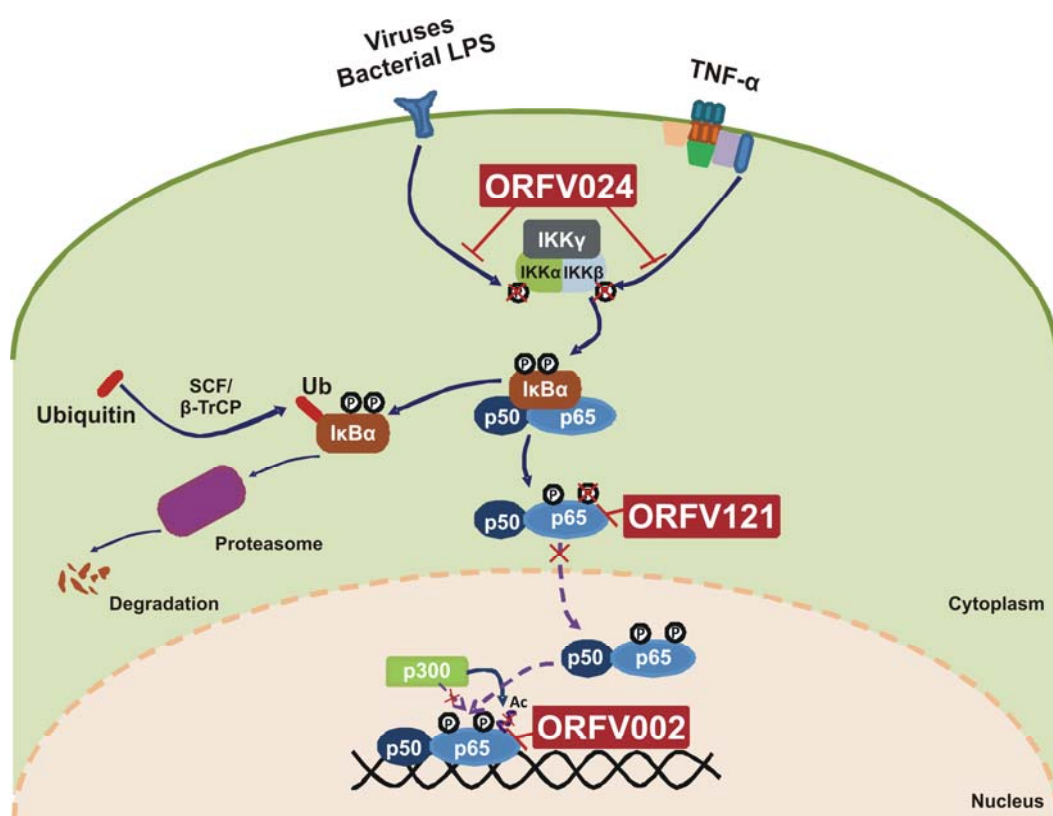


FIG 9. NF- κ B signaling pathway and its regulation by ORFV-encoded proteins ORFV024, ORFV121 and ORFV002. ORFV024 inhibits phosphorylation of I κ B kinases, IKK α and IKK β , by targeting steps upstream of the IKK complex. ORFV121 binds to and inhibits phosphorylation and nuclear translocation of NF- κ B-p65. ORFV002 binds to and inhibits p300-mediated acetylation of NF- κ B-p65 likely by disrupting association of p300 and NF- κ B-p65.

6. CONCLUSÃO

Os resultados do presente estudo indicam que o parapoxvírus vírus do ectima contagioso (ORFV), assim como outros poxvírus, desenvolveu várias estratégias para modular a atividade transcripcional do NF- κ B. O ORFV desenvolveu novos mecanismos que atuam em diferentes pontos da via de sinalização do NF- κ B, incluindo proteínas que atuam nos eventos iniciais de ativação do NF- κ B no citoplasma das células, ou ainda, que modulam a atividade transcripcional do NF- κ B no núcleo. O produto da *ORFV024* inibe a ativação do complexo de proteína quinases IKK no citoplasma celular, por inibir a fosforilação das quinases IKK α e IKK β . Já o produto da *ORFV121* atua *downstream* na via do NF- κ B, ligando-se a subunidade NF- κ B-p65 do NF- κ B e consequentemente inibindo a sua fosforilação e translocação nuclear. Já o produto da *ORFV002* atua no núcleo da célula, onde esta proteína se liga ao NF- κ B-p65 inibindo a sua acetilação, uma modificação pós-traducional que aumenta a atividade transcripcional do NF- κ B-p65.

O papel exercido pelos inibidores do NF- κ B dos poxvirus durante infecções *in vivo* ainda não é completamente entendido. Os resultados obtidos no presente estudo demonstram que os produtos dos genes *ORFV024* e *ORFV002* não afetam a virulência do ORFV, enquanto que o produto do gene *ORFV121* constitui-se em um fator de virulência que contribui para a patogenia do ectima contagioso, sugerem que estas proteínas possam exercer funções complementares e/ou redundantes durante as infecções *in vivo*. Além disso, é possível que os múltiplos mecanismos adotados pelo ORFV atuem na modulação de diferentes processos celulares controlados pelo NF- κ B, incluindo proliferação e diferenciação celular, apoptose e/ou resposta inflamatória. Enquanto que a modulação de alguns destes processos não afeta o desenvolvimento da doença (*ORFV002* e *ORFV024*), a regulação de outros (*ORFV121*) é essencial para a virulência do vírus e patogenia da enfermidade.

Um melhor entendimento das interações do ORFV com o seu hospedeiro podem favorecer o desenvolvimento de vacinas mais eficazes contra o ectima contagioso e também promover o desenvolvimento de imunomoduladores ou vacinas vetoriais com base no ORFV, mais eficazes e com um maior espectro de aplicações.

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