A Nuclear Inhibitor of NF-κB Encoded by a Poxvirus

D.G. Diel, 1,2‡, S. Luo1‡, G. Delhon3, Y. Peng1,4, E.F. Flores2, D.L. Rock1*

Department of Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL, USA1;
Programa de Pós-graduação em Medicina Veterinária, Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Centro de Ciências Rurais, Universidade Federal de Santa Maria, RS, Brazil2;
and Department of Veterinary and Biomedical Sciences, and Nebraska Center for Virology, University of Nebraska-Lincoln, Lincoln, NE, USA3; Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University, Guangdong, China4.

Running Title: ORFV002 inhibits nuclear NF-κB signaling.

Abstract word count: 176

Text word count: 5279

*Corresponding author.

Mailing Address: 2522 Veterinary Medicine Basic Science Building, University of Illinois, MC-002, 2001 S. Lincoln Avenue, Urbana 61802, IL, USA. Phone: (217) 333-2449. Fax: (217) 244-7421. E-mail: dlrock@illinois.edu.

‡These authors contributed equally to this work.
Abstract

Poxviruses have evolved various strategies to inhibit cytoplasmic events leading to activation of the Nuclear Factor-κB (NF-κB) signaling pathway, with individual viruses often encoding for multiple NF-κB inhibitors. Here, the novel orf virus (ORFV)-encoded protein ORFV002 was shown to inhibit nuclear events regulating NF-κB transcriptional activity. ORFV002 expression in cell cultures significantly decreased wild-type virus-, tumor necrosis factor alpha (TNF-α)-, and lipopolysaccharide (LPS)-induced NF-κB-mediated gene expression. Expression of ORFV002 in cells, while not affecting phosphorylation or nuclear translocation of NF-κB-p65, markedly decreased TNF-α- and wild-type virus-induced acetylation of NF-κB-p65, a p300-mediated nuclear modification of NF-κB-p65 that regulates its transactivating activity. ORFV002 was shown to colocalize and interact with NF-κB-p65, and its expression in cell cultures resulted in a reduced interaction of NF-κB-p65 with p300, suggesting that ORFV002 functions interfering with NF-κB-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-κB 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 interferon (IFN)-resistance gene (*ORFV020*), chemokine binding protein (CBP; *ORFV112*), 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 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-κB) 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-κB family of transcription factors plays a central role in integrating stress-inducing stimuli and innate immune responses in the epidermis. NF-κB 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-κB canonical pathway in keratinocytes results in enhanced inflammatory response in the skin, which indicates a complex role for NF-κB in skin immune homeostasis (36, 39).

The NF-κB family of transcription factors consists of five members in mammals, NF-κB-p65 (RelA), RelB, c-Rel, NF-κB-p50/p105, and NF-κB-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-κB dimers is initially regulated by their association with the inhibitory IκB molecules, which sequester NF-κB 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 IκB proteins by IκB kinases (IKK), resulting in proteasomal degradation of IκB and nuclear translocation of NF-κB subunits (28). Regulation of NF-κB nuclear activity is critical for NF-κB target gene selection and transcriptional activity. Various post-translational modifications as well as association with non-Rel binding partners affect NF-κB DNA binding affinity, interaction with coactivators and corepressors, and transactivating activity (16, 40, 48). For example, inducible phosphorylation by various kinases has been described at multiple NF-κB-p65 sites, leading to promoter-specific modulation of NF-κB transcriptional activity (40). Likewise, inducible NF-κB-p65 acetylation by p300/CBP or p300/CBP-associated factor (PCAF) affects NF-κB-p65 DNA binding, association with IκBα, and transcriptional activation (8, 29). Recently, inducible methylation of NF-κB-p65 by SET9 methyltransferase was shown to regulate NF-κB-p65 promoter binding and transcription 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-κB target genes (44).

Intracellular inhibitors of NF-κB 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-κB 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-κB inhibitors identified in other chordopoxvirus genera. On the other hand, parapoxvirus ORFV024 is a NF-κB inhibitor unique to this group of viruses (13).

Poxviral NF-κB inhibitors target mainly cytoplasmic events leading to activation of the NF-κB signaling pathway (31). Vaccinia virus (VACV)-encoded NF-κB inhibitors target cytoplasmic steps leading to activation of the IKK complex (A52R, A46R, B14, N1L and M2L), degradation of IκBa (K1L), or activation of the PKR-dsRNA signaling pathway (E3L) (2, 11, 14, 24, 35, 43, 46). Molluscum contagiosum virus (MOCV) protein MC159 prevents degradation of IκBβ, while MC160 induces degradation of IKKα (34, 38). ORFV-encoded protein ORFV024 decreases phosphorylation of IKKα and IKKβ, thus preventing activation of the IKK complex (13). Notably, deletion of individual genes encoding selected NF-κB inhibitors from poxviral genomes results in variable and in most cases very modest, degrees of 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-κB-p65, a transactivating NF-κB subunit, and decreases acetylation...
of NF-κB-p65, a nuclear modification required for full NF-κB transcriptional activity. This is the first description of a poxviral NF-κB inhibitor targeting nuclear events regulating NF-κB 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 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/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-IA82Δ002, and in all procedures involving infections with wild-type virus, and cloning of viral genes. OV-IA82Δ002 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-IA82Δ002), 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’-ACTACGACTAGTGCCACTATACCAGAGAGA-3’, 002LF-Rv1(SalI): 5’-ATCATCGACTGTGGAGGAGAGAGAGAGA-3’, 002RF-Fw2(NsiI):5’-AATCATATGCTCTCGAGAGAGAGAGA-3’, and 002RF-Rv2(BglII):5’-ACTACGACTAGTGCCACTATACCAGAGAGA-3’.
GTCTCT ACACCGA-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 AflII 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 RT-PCR as previously described (13). Transcription of ORFV002, ORFV055 (late control), and ORFV127 (early control) was assessed using primers 002RTFw1-5’-ACACGGTAACGGCAGTGGTA-3’, 002RTRv1-5’-AGCAGGGTGGTGAGCAAG-3’, 055LFw-5’-AATCATGGATCCGCCACCATGTTCTTCCGCCGTCG-3’, 055LRv-5’-TATCATGAGCGGGCGTGGAGGTCGCCGACC-3’, 127EintFw-5’-CTCCTCGACGACTTCAAG-3’, and 127EintRv-5’-TATGTCGAATCGTTCTGTCGCTGG-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-IA82Δ002 and revertant viruses OV-IA82Rv002 and OV-IA82Rv002GFP. OV-IA82Δ002 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 Biosience, Rockland, ME) and X-Gluc (0.5 µg/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 MOIs 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. 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-κB luciferase reporter assays.** The effect of ORFV002 expression on NF-κB-mediated transcription was assessed by using a luciferase reporter assay (13). OFTu cells were cotransfected with pNF-κBLuc (Clontech) and pRLTK (Promega), and 24 h later infected with
OVTu cells transiently transfected with plasmids pNF-κBLuc, 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 T test.

Western blots. The effect of ORFV002 on the NF-κB signaling pathway was assessed by Western immunoblots. OVTu cells were transfected with pEGFP-N1 (2 µg; control) or p002EGFP (2 µg), 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). OVTu cells were cotransfected with pT7-NFκB-p65 (0.5 µg), pHA-p300 (2 µg) and either pEGFP-N1 (1 µg; control) or p002EGFP (1 µg), treated with TNF-α for 30 or 60 min, and harvested with lysis buffer as above. OVTu cells were cotransfected with pT7-NFκB-p65 (0.5 µg), and pHAl300 (2 µg), and infected with OV-IA82, OV-IA82Δ002 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. OVTu cells were transfected with pEGFP-N1 (2 µg; control) or p002EGFP (2 µg), 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 µg of total cell lysates, and 20 µg
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-κB-p65 (Cell signaling, cat no. 3034), p-NF-κB-p65 (Ser536) (Cell signaling, cat no. 3033), acetyl-NF-κB-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 t test.

**Confocal microscopy.** OFTu cells cultured on glass coverslips were infected with OVA82RV002GFP virus (MOI = 1 or 5), fixed with 4% formaldehyde at various times post-infection (2, 3, 12 and 24 h p.i.), stained with DAPI for 10 min, and examined by laser scanning confocal microscopy (Zeis, LSM710). OFTu cells cultured on glass coverslips were transfected with either [1]. p002EGFP, [2]. pEGFP-N1 and pT7-NFκB-p65, or [3] p002EGFP and pT7-NFκB-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-κB-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 (Zeis, LSM710).

**Coimmunoprecipitation assays.** OFTu cells were cotransfected with pT7-NFκB-p65 (0.5 µg), pHA300 (2 µg), and either pEGFP-N1 (1 µg; control) or p002EGFP (1 µg), 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 µg of antibodies against GFP (sc-9996, Santa Cruz), NF-κB-p65 (Cell signaling, cat no. 3034) or p300 (Millipore, cat no. 05-257) and subsequently incubated overnight at 4°C with 50 µl 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-IA82Δ002-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 0.5 ml of a virus suspension containing $10^{7.3}$ TCID$_{50}$/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 followed 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 for 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 weight 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 times 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 times 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. 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 times p.i. (Fig. 2). Similar results were obtained when ORFV002-GFP subcellular localization was assessed during OV-IA82Rev002GFP 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-IA82Δ002), and an OV-IA82Δ002 revertant virus (OV-IA82Rev002) was investigated in vitro. No differences in OV-IA82Δ002 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-IA82Δ002 were observed. Thus, ORFV002 is nonessential for virus replication in OFTu and in OKT cells.

**OV-IA82Δ002 infection results in increased expression of NF-κB-regulated genes in primary OFTu cells.** Preliminary transcriptional profiling of OV-IA82Δ002-infected OFTu cells revealed increased expression of NF-κB-regulated genes (data not shown). Real time-PCR analysis of gene expression in OFTu cells infected with OV-IA82Δ002 demonstrated increased expression of the NF-κB-regulated genes IL-1α (30.4-fold), IL-6 (9.5-fold), IL-8 (28.3-fold), NFκBIA (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-1α, IL-6 and IL-8 was also increased in OV-IA82Δ002-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-κB-regulated gene expression, OFTu cells were transfected with a plasmid encoding a luciferase reporter gene under the control of a NF-κB responsive promoter and subsequently infected with OV-IA82, OV-IA82Δ002, OV-IA82Rv002, or mock-infected. Infection with OV-IA82Δ002 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 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-κB-regulated gene transcription during ORFV infection of OFTu cells.

**ORFV002 suppresses NF-κB-mediated gene transcription induced by TNF-α and LPS.** The ability of ORFV002 to inhibit NF-κB-mediated transcription was investigated in OFTu cells following treatment with TNF-α and LPS, two potent inducers of the NF-κB signaling pathway. Expression of ORFV002-GFP in TNF-α- and LPS-treated cells significantly decreased NF-κB-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 control GFP-expressing cells (Fig. 4C and D). Thus, ORFV002 inhibits NF-κB-mediated gene transcription following TNF-α- and LPS-stimulation.

**ORFV002 expression does not affect phosphorylation or nuclear translocation of NF-κB-p65.** The effect of ORFV002 on cytoplasmic events of the NF-κB signaling pathway was investigated by examining phosphorylation and nuclear translocation of NF-κB-p65 in ORFV002-expressing cells. OFTu cells transfected with plasmids encoding for GFP (control) or ORFV002-GFP were treated with TNF-α and harvested at various times post treatment. Similar levels of phosphor-NF-κB-p65 were detected in GFP- and ORFV002-GFP-expressing cells, indicating that ORFV002 had no significant effect on TNF-α-induced phosphorylation of NF-κB-p65.
κB-p65S536 (Fig. 5A). Additionally, as evidenced by similar levels of nuclear NF-κB-p65 in GFP- or ORFV002-GFP-expressing cells, ORFV002 did not affect TNF-α-induced nuclear translocation of NF-κB-p65 (Fig. 5B). Nuclear levels of NF-κB-p65 were not due to leakage from the cytoplasmic fraction, since NF-κB-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-κB-p65, and further suggest that ORFV002 interferes with nuclear events of the NF-κB signaling pathway.

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

The effect of ORFV002 on acetylation of NF-κB-p65 was also assessed during ORFV infection. OFTu cells transiently transfected with plasmids pT7-NF-κB-p65 and pHA-p300 were infected with OV-IA82, OV-IA82Δ002, or OV-IA82Rv002 and harvested at various times p.i.. While infection with the wild-type virus resulted in low levels of NF-κB-p65<sup>K310</sup> acetylation (Fig. 6C and D), OV-IA82Δ002-infection markedly increased acetylation of NF-κB-p65<sup>K310</sup> at 30 and 60 min p.i. (p ≤ 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-κB-p65\(^{K310}\).

**ORFV002 interacts with NF-κB-p65.** Interaction of ORFV002 with NF-κB-p65 was investigated as a potential mechanism for ORFV002 inhibitory effect on NF-κB-p65 acetylation. OFTu cells transiently transfected with either [1]. p002EGFP, [2]. pEGFP and pT7-NFκB-p65, or [3] p002EGFP and pT7-NFκB-p65 were treated with TNF-α, probed with an antibody against NF-κB-p65, and subsequently examined by confocal microscopy. Both ORFV002 and NF-κB-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-κB-p65 was further investigated by using co-immunoprecipitation assays. OFTu cells transiently transfected with plasmids pT7-NFκB-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-κB-p65 (Fig. 7D) antibodies demonstrated that ORFV002 coprecipitates with NF-κB-p65. No interaction between ORFV002 and p300 was detected (data not shown). Together, these results indicate that ORFV002 physically interacts with NF-κB-p65.

**ORFV002 interferes with association of p300 and NF-κB-p65.** Acetylation of NF-κB-p65 is dependent on the interaction of p300 and NF-κB-p65 (9). To investigate the effect of ORFV002 expression on association of p300 and NF-κB-p65, OFTu cells transiently transfected with plasmids pT7-NFκB-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-κB-p65 when compared to control GFP-expressing cells (Fig. 8). The decreased association between p300 and NF-κB-p65 correlated with reduced levels of acetyl-NF-κB-p65 detected in cell lysates of ORFV002-expressing cells (Fig. 8). Together, these results suggest that by binding to NF-κB-p65, ORFV002 interferes with association of p300 and NF-κB-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-IA82Δ002, 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$^{\text{K310}}$. 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$^{\text{K310}}$ 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 IκKα degradation by competitively interacting with the cellular heat shock protein 90 (HSP90) which is necessary for IκKα 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 punctuate 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-κB-p65 activity and seven acetylation sites have been identified in NF-κB-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-κB-p65 (7). For example, acetylation of lysine 221 increases NF-κB-p65 DNA binding affinity and, in combination with acetylation of lysine 218, prevents association of NF-κB-p65 with newly synthesized IκBα, thus regulating the duration of NF-κB-mediated responses (8). Acetylation of lysine 310 recruits the coactivator Brd4 to the transcriptional complex enhancing the transcriptional activity of NF-κB-p65 (8, 25). The decreased acetylation of NF-κB-p65K310 in TNF-α-stimulated ORFV002-expressing cells or wild-type virus-infected cells suggests that ORFV002 prevents full transcriptional activity of NF-κB-p65. However, the possibility that ORFV002 affects other acetylation sites on NF-κB-p65 interfering with additional regulatory mechanisms of NF-κB in the nucleus cannot be formally excluded.

P300-mediated acetylation of NF-κB-p65 requires interaction between the proteins, and previous phosphorylation of NF-κB-p65 (9). Phosphorylation of NF-κB-p65 at serines 276 and 536 enhances NF-κB-p65/p300 interaction, and consequently stimulates acetylation of lysine 310 (9). Although, ORFV002 did not affect phosphorylation of NF-κB-p65S536, its expression in cells was associated with decreased interaction between p300 and NF-κB-p65. Given that ORFV002 physically interacts with NF-κB-p65, it is tempting to speculate that ORFV002 decreases acetylation of NF-κB-p65 by competitively disrupting the interaction between p300 and NF-κB-p65. A similar mechanism has been described for African swine fever virus (ASFV) A238L.
protein, which binds to and inhibits nuclear acetylation of NF-κB-p65 presumably by disrupting p300/NF-κB-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 times 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-κB signaling pathway. However, the increased levels of ORFV002 transcribed/expressed at late times p.i. and the rapid kinetics by which ORFV002 inhibits activation of NF-κB signaling following infection, suggest that ORFV002 may potentially be a virion component to function early in subsequent rounds of ORFV replication.

The role of poxviral NF-κB inhibitors in virus virulence and pathogenesis remains poorly understood. Deletion of selected poxviral NF-κB 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 N1L 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-κB may exert complementary or redundant
functions during poxvirus infections in vivo. Multiple poxviral NF-κB inhibitors may exert a fine level of regulation of distinct branches of the NF-κB pathway, which may be temporally regulated during virus infection. Therefore, deletion of single poxviral NF-κB 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-κB 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-κB signaling pathway plays complex, sometimes paradoxical roles in keratinocyte survival, differentiation, and immune homeostasis (39), making the impact of poxviral NF-κB inhibitors in virus virulence and pathogenesis difficult to predict. The multiple and often novel NF-κB 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-κB. Elucidation of the molecular mechanisms employed by parapoxviruses to modulate the NF-κB signaling pathway may contribute to improve understanding of poxvirus infection biology and disease.
Acknowledgments

We thank Dr J. Shisler (Department of Microbiology, University of Illinois), Dr L-F. Chen (Department of Biochemistry University of Illinois), Dr T. Shors (Department of Biology and Microbiology, University of Wisconsin-Oshkosh), Dr M. Varela and Dr P.R. Murcia (Institute of Comparative Medicine, University of Glasgow Veterinary School, UK), Dr D. Refojo (Max Planck Institute of Psychiatry, Munich, Germany), for providing plasmids used in this study, and Dr F.A. Osorio for laboratory support.
References


Figure Legends

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 times post-infection (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 times post-infection (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) virus 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-κB-regulated gene transcription. (A) OFTu cells were infected with OV-IA82, OV-IA82Δ002 (MOI = 10), or mock-infected and expression of NF-κB-regulated genes was determined by real-time PCR analysis at 2 h p.i.. (B) OFTu cells were cotransfected with plasmids pNF-κBLuc and pRL-TK and subsequently infected with OV-IA82, OV-IA82Δ002, 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-κBLuc, 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-κB-p65 phosphorylation and nuclear translocation. (A) OFTu cells transiently transfected with plasmids encoding for GFP (control) or ORFV002-GFP were treated with TNF-α (20 ng/ml) and harvested at the indicated times (UN, untreated controls). Protein extracts (50 µg) 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 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). The results (A and B) are representative of two independent experiments.

FIG. 6. Effect of ORFV002 on NF-κB-p65 acetylation. (A) 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 the indicated times (UN, untreated controls). Protein extracts (50 µg) were resolved by SDS-PAGE, blotted, and probed with antibodies directed against proteins indicated on the right. (B) Relative densitometry of
Acetyl NF-κB-p65 bands normalized to loading control GAPDH (*, p < 0.01). (C) OFTu cells were cotransfected with plasmids pT7-NF-κB-p65 and pHA-p300, subsequently infected with OV-IA82, OV-IA82Δ002 or OV-IA82Rv002 (MOI = 10) and harvested at the indicated times (UN, uninfected controls). Protein extracts (50 µg) were resolved by SDS-PAGE, blotted, and probed with antibodies directed against proteins indicated on the right. (D) Relative densitometry of Acetyl NF-κB-p65 bands normalized to loading control GAPDH (*, p ≤ 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-κB-p65. 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-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).