The N Terminus of Orf Virus-Encoded Protein 002 Inhibits Acetylation of NF-κB p65 by Preventing Ser276 Phosphorylation

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Abstract

Orf virus-encoded protein 002 (ORFV002) inhibits NF-κB signaling pathway by decreasing the acetylation of NF-κB p65 through interference of NF-κB p65’s association with NF-κB p300. However, the precise mechanism of how ORFV002 interferes with the NF-κB p65/p300 association is still unknown. Due to similarities of the amino acid sequences of ORFV002 and the adenovirus type 12 (Ad12) E1A protein (E1A-12), we hypothesized that the N-terminal 52 amino acids of ORFV002 might play an important role in this inhibition and constructed several in-frame fusions of ORFV002 to an enhanced green fluorescent protein (EGFP) reporter, including C-terminal and N-terminal deletion mutants of ORFV002. When the N-terminus of ORFV002 was absent, the localization of ORFV002 shifted mainly from the nucleus to the cytoplasm, and it’s inhibition of NF-κB transactivation was lost. NF-κB p65 Lys310 acetylation and Ser276 phosphorylation were detected in co-transfection experiments with NF-κB p65 and ORFV002 or its mutants with, or without, the N-terminal region. The results showed that the N-terminus of ORFV002 plays a crucial role in inhibiting both the acetylation and phosphorylation of NF-κB p65. Further investigation indicated that ORFV002 and its C-terminal deletion mutants interfered with NF-κB p65 (Ser276) phosphorylation induced by mitogen- and stress-activated protein kinase-1 (MSK1) and the interaction between NF-κB p65 and MSK1. Since phosphorylated NF-κB p65 recruits transcriptional co-activators such as p300 and CBP, we concluded that the N-terminus of ORFV002 inhibits acetylation of NF-κB p65 by blocking phosphorylation of NF-κB p65 at Ser276.

Introduction

The orf virus (ORFV), the type species of the Parapoxivirus genus, is an epitheliotropic virus and causative agent of the orf disease, which mainly affects sheep, goats, wild ruminants and humans with a worldwide distribution [1-4]. The ORFV genome is 138 kb with G+C content up to 64 percent and it contains 132 putative genes [5], [6]. This parapoxivirus has evolved various strategies including encoding a protein to inhibit activation of the nuclear factors κB (NF-κB) signaling pathway [4]. Several genes of the orf virus including 121 [7], 024 [8] and 002 [4] modulate host immune function by inhibiting the activation of NF-κB. This is one of the strategies that the orf virus uses to escape the host’s immunological response and modify the disease process.

Inducible phosphorylation has been reported to occur at multiple NF-κB p65 sites, including Ser536 and Ser276, leading to the changes in NF-κB activation [9]. The human adenovirus type 12/Ad12 E1A protein (E1A-12) inhibits phosphorylation of NF-κB p65 at Ser276 and causes the loss of DNA binding and transactivation to activate major histocompatibility complex class I expression [10]. Further study found that the last 66 amino acids of the N-terminus of adenovirus type 12 E1A were as effective as the wild-type E1A protein in preventing phosphorylation of NF-κB p65 at Ser276 and inhibiting major histocompatibility complex class I expression [11]. Our previous report indicated that the ORFV002 gene inhibits the NF-κB signaling pathway by decreasing TNF-α. Furthermore, the wild-type virus induced acetylation of NF-κB p65 by interfering with the NF-κB p65/p300 association and not by affecting phosphorylation of NF-κB p65 at Ser276 [4]. The precise mechanism of how ORFV002 interferes with the NF-κB p65/p300 association is not clearly understood.

Based on the amino acid sequence analysis we found that ORFV002 shares three putative conserved protein domains with the E1A-12 protein. We hypothesized that the N-terminus of the ORFV002 has a similar function for the phosphorylation of NF-κB p65. Our results confirmed that the N-terminal 52 residues of ORFV002 could inhibit phosphorylation of NF-κB p65 at Ser276 blocking the subsequent acetylation of NF-κB p65. This unique property of ORFV002 disables NF-κB transactivation. This is the
first report of N-terminus of ORFV002 protein targeting nuclear events by regulating NF-κB transactivation.

**Materials and Methods**

**Animal and Cell Preparation**

Two 3-year old Han sheep, which were pregnant for 120 days, were obtained from the Center of Laboratorial Animals in South China Agricultural University, China. The ovine experiments were approved by the Institutional Animal Care and Use Committee at South China Agricultural University (Certification Number: CNAS BL0011). The primary ovine fetal turbinate tissues (OFTu) were prepared as previously described with the bovine turbinate cells [12], with some modifications. The sheep were anesthetized, and the fetuses were obtained. The turbinate tissue was cut into small pieces and trypsinized in 0.125% trypsin for 15 minutes at room temperature to settle the cells. Then the trypsin was decanted, and fresh trypsin was added for 30 minutes. The dispersed cells were decanted and saved; the digestive procedure was repeated on the remaining tissues. Dispersed cells collected in these serial digestions were pooled and washed twice in minimal essential medium (MEM), diluted at 5×10^5 cells/ml of complete medium and seeded at 30 ml per 150 cm^2 plastic flasks. The complete medium consisted of MEM supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml gentamicin and 2 mM L-glutamine.

**Figure 1. Amino acid sequence comparison between ORFV002 and 12 E1A and ORFV002 mutant construction.** A. Comparison of the predicted amino acid sequences of orf virus 002 protein (ORFV002; AY386263.1) and human oncogenic adenovirus type 12 E1A protein (E1A-12; CAA23400.1). These sequences were aligned by using the CLUSTAL W program (San Diego Supercomputer Center Biology Workbench: http://workbench.sdsc.edu/). Solid lines above the alignment indicate three conserved regions (CR1, CR2, and CR3) in E1A-12. B. Construction of ORFV002 wild type and mutants-GFP fusion. C. Western blotting showing the expression of wild type ORFV002GFP and mutants in OFTu cells. doi:10.1371/journal.pone.0058854.g001

ORFV002 Inhibits NF-κB p65(Ser276) Phosphorylation
Plasmid Construction

ORFV002 coding sequences were synthesized by EZBiolab, Inc. (Westfield, IN, USA), and subcloned into the expression vector pEGFP-N1 (Clontech, Mountain View, CA, USA) to generate the p002EGFP plasmid. DNA sequencing of p002EGFP confirmed the integrity of ORFV002 coding sequences and in-frame cloning with enhanced green fluorescent protein (EGFP). N-terminal fusions of EGFP to ORFV002 deletion mutants were constructed by PCR in a method similar to that described in [4]; p002EGFP was used as a template to amplify ORFV002-EGFP mutant 1 (Δ1–52, 002GM1 or M1), ORFV002-EGFP mutant 2 (Δ1–94, 002GM2 or M2), ORFV002-EGFP mutant 3 (Δ95–117, 002GM3 or M3), ORFV002-EGFP mutant 4 (Δ53–117, 002GM4 or M4). The primer sequences were synthesized as follows:

- **002GM1-Fw.** 5'-ACTATAAGCTTTGCAACATCATGCTTG-CAGCTGGAGCTAGAGC-3' 9';
- **002GM1-Rv.** 5'-ATACTGAAATTGCGCCACTAGAGAGC-GAGCCGCTGCACTCG-3' 9';
- **002GM2-Fw.** 5'-ATCATGAAATTCGCCACCATGAGAGCAGCTGCATCG-3' 9';
- **002GM2-Rv.** 5'-ATACTGAAATTGCGCCACTAGAGAGC-GAGCCGCTGCACTCG-3' 9';
- **002GM3-Fw.** 5'-GATCATGAATTCGCTAGCCACCATGACTCCTACTTCTCGAGA-3' 9';
- **002GM3-Rv.** 5'-GGTGGATCCATCTAGCAGTGGTAGTGG-3';
- **002GM4-Fw.** 5'-GATCATGAATTCGCTAGCCACCATGACTCCTACTTCTCGAGA-3' 9';
- **002GM4-Rv.** 5'-GGTGGATCCATCTAGCAGTGGTAGTGG-3'.

The PCR was carried out in a 50 μl reaction volume containing 1 μl of DNA template, 0.4 μM of each primer and 0.5 μl of Taq polymerase (Promega Co., Madison, WI, USA) in 10 μl of 5× PCR buffer (10 mM Tris-HCl, and 50 mM KCl and 25 μM MgCl2) with 200 μM final concentrations each of dATP, dTTP, dCTP and dGTP, 1 μl of DNA template, 200 μM dATP, dTTP, dCTP, dGTP, 0.4 μM of each primer, 25 μM MgCl2 and 0.5 μl of Taq polymerase (Promega Co., Madison, WI, USA). PCR was performed in a thermocycler (GeneAmp PCR 2400, PE Applied Biosystems Corp, Foster, CA, USA) for 30 cycles of denaturation (94°C for 1 minute), annealing (58°C for 30 seconds) and extension (72°C for 1.5 minutes) with a final PCR was ended.

Figure 2. The subcellular localization of ORFV002 and its deletion mutants. A. Confocal microscopy analysis of wild type ORFV002 and its mutants in OFTu cells. Wild type ORFV002, 002GM3 and 002GM4 proteins carried the GFP signal into the nucleus. The GFP signals of 002GM1 and 002GM2 were located in the cytoplasm. Bar: 10 μM. B. Western blot analysis showing ORFV002 mostly located in the nuclear fraction. Cyto: cytoplasmic fraction; Nuc: nuclear fraction. doi:10.1371/journal.pone.0058854.g002

Figure 3. Transcriptional activity by NF-κB p65 is inhibited by the N-terminal region of ORFV002. OFTu cells were co-transfected with a vector encoding a firefly luciferase gene under the control of NF-κB (pNFκB-Luc) and with a plasmid encoding sea pansy (Renilla reniformis) luciferase under the control of herpesvirus TK promoter (pRL-TK), plus a plasmid expressing GFP (negative control), wild type ORFV002-GFP fusion protein (002GFP), or a ORFV002 deletion mutant fused to GFP (002GM1, 002GM2, pEGFP-002GM3 or 002GM4). At 24 hours after transfection, cells were treated with 20 ng/ml of TNF-α (A) or 250 ng/ml of LPS (B) for 6 hours. Firefly and sea pansy luciferase activities were measured and depicted in the graph as relative fold changes in luciferase activity (**, P<0.001). Data are expressed as the mean ± SD from three separate experiments. doi:10.1371/journal.pone.0058854.g003

ORFV002 Inhibits NF-κB p65(Ser276) Phosphorylation

The PCR was carried out in a 50 μl reaction volume containing 1 μl of DNA template, 0.4 μM of each primer and 0.5 μl of Taq polymerase (Promega Co., Madison, WI, USA) in 10 μl of 5× PCR buffer (10 mM Tris-HCl, and 50 mM KCl and 25 μM MgCl2) with 200 μM final concentrations each of dATP, dTTP, dCTP and dGTP, 1 μl of DNA template, 200 μM dATP, dTTP, dCTP, dGTP, 0.4 μM of each primer, 25 μM MgCl2 and 0.5 μl of Taq polymerase (Promega Co., Madison, WI, USA). PCR was performed in a thermocycler (GeneAmp PCR 2400, PE Applied Biosystems Corp, Foster, CA, USA) for 30 cycles of denaturation (94°C for 1 minute), annealing (58°C for 30 seconds) and extension (72°C for 1.5 minutes) with a final PCR was ended.
null
prepared and 50 μg of cell lysate was loaded into each well prior to separation. Separated proteins were transferred onto nitrocellulose membranes. Membrane blots were probed with rabbit anti NF-kB-p65 (ab30623; abcam, Cambridge, UK), NF-kB-p65 (N-acetyl-Lys310) (3045; Cell Signaling Technology, Inc., Danvers, MA, USA), NF-kB-p65 (3034; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-MSK1 (ab63619; abcam, Cambridge, UK), anti-GFP (sc-8334; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-Flag (F3165; Sigma-ALDRICH, St. Louis, MO, USA) and then followed by incubation with goat-anti-rabbit HRP-conjugated IgG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and developed using chemiluminescent substrate ECL (Pierce-Thermo Scientific, Rockford, IL, USA). A densitometric analysis of the blots was performed by using Image J software, version 1.62 (National Institutes of Health, Bethesda, MD, USA). A statistical analysis of the densitometry data was performed by using student’s t test.

Confocal Microscopy

OFTu cells were grown on glass cover-slips and co-transfected with pDSRed2-Nuc (Clontech, Mountain View, CA, USA) and either plasmid pEGFP-N1 (GFP control), pEGFP-002 (wild type ORFV002-GFP fusion), or one of the ORFV002 deletion mutants fused to GFP (pEGFP-002GM1, pEGFP-002GM2, pEGFP-002GM3 or pEGFP-002GM4). At 24 hours after transfection, cells were fixed with 4% formaldehyde at room temperature for 1 hour, stained with DAPI (4’,6-diamidino-2-phenylindole) for 10 minutes and examined by laser scanning confocal microscopy (LSM700; Zeiss, Germany).

Results

Comparison of ORFV002 and E1A-12 Amino Acid Sequences and Construction of ORFV002 Mutants

Using the CLUSTAL W program (San Diego Supercomputer Center biology workbench: http://workbench.sdsc.edu/) the predicted amino acid sequences of the orf virus 002 protein (ORFV002; AY386263.1) and human oncogenic adenovirus type 12 E1A protein (E1A-12; CAA23400.1) were compared. We found that the three putative conserved protein domains, CR1, CR2 and CR3 in E1A-12 were also conserved in ORFV002 (Figure 1A). Based on the amino acid sequence alignment between E1A-12 and ORFV002 four plasmids were generated to express deletion mutants of ORFV002 tagged with EGFP designated as: 002GM1 (N-terminal deletion mutant Δ1–52, M1), expressing amino acids 53–117; 002GM2 (N-terminal deletion mutant Δ1–94, M2),

Figure 5. ORFV002 prevents NF-κB-p65 Ser276 phosphorylation. A. OFTu cells were co-transfected with plasmid pNF-κB-p65 and either pEGFP-N1 or pORFV002-EGFP. At 24 hours post transfection, cells were treated with 20 ng/ml of TNF-α for 0, 30 and 60 minutes and harvested at the indicated times. Nuclear protein fractions were extracted. Protein extracts (50 μg) were resolved by SDS-PAGE, blotted, and probed with antibodies against p-NF-κB-p65-Ser276 (top), p-NF-κB-p65-Ser536 (middle), or histone H3 (bottom). B. Densitometry of p-NF-κB-p65 Ser276 and Ser536 bands normalized to loading control histone H3 (**, P<0.005). The results are representative of three independent experiments. doi:10.1371/journal.pone.0058854.g005
expressing amino acids 95–117; 002GM3 (C-terminal deletion mutant D95–117, M3), expressing amino acids 1–94; and 002GM4 (C-terminal deletion mutant D53–117, M4), expressing amino acids 1–52 (Figure 1B). The nucleotide sequence integrity of each construct was verified by sequencing before use. The total protein levels of wild type and mutants of ORFV002 in OFTu cells were detected by Western blot (Figure 1C).

Localization of ORFV002 and Its Mutants

Confocal microscopy showed that wild type ORFV002 expressed in OFTu cells is found mainly in the nucleus, co-localized with Nuc-Red, a nuclear marker (Figure 2A). The signals of the GFP-fusions of ORFV002 C-terminal deletion mutants (002GM3 and 002GM4) were mainly located in the nucleus, while the GFP signals of ORFV002 N-terminal deletion mutants (002GM1 and 002GM2) were mainly located in the cytoplasm (Figure 2A). Western blot analysis confirmed that wild type ORFV002 was mostly located in the nuclear fraction (Figure 2B).

NF-κB p65 Transcriptional Activity is Inhibited by the N-terminal Region of ORFV002

OFTu cells were co-transfected with a vector encoding a firefly luciferase gene under the control of a NF-κB-inducible promoter (pNFκB-B-Luc) and with a plasmid encoding sea pansy (Renilla reniformis) luciferase under the control of herpesvirus TK promoter (pRL-TK), and additionally co-transfected with pEGFP002 or one of the plasmids expressing an ORFV002 deletion mutant (002GM1, 002GM2, 002GM3, or 002GM4). After transfection for 24 hours, cells were treated with 20 ng/mL of TNF-α or 250 ng/mL of lipopolysaccharide (LPS) for 6 hours. Firefly and sea pansy luciferase activities were measured and expressed as relative fold changes in luciferase activity. The fold-changes in luciferase activity indicate that ORFV002, and the intact N-terminus of ORFV002 (002GM3 and 002GM4) significantly inhibited transcriptional activity by NF-κB p65 as compared with GFP control or the intact C-Terminus of ORFV002, as demonstrated by 002GM1 and 002GM2 (Figure 3).

ORFV002 and Its N-terminus Prevent NF-κB p65 Lys310 Acetylation

We showed previously that ORFV002 inhibits the acetylation of NF-κB p65. In order to define the region of ORFV002 responsible for inhibiting the acetylation of NF-κB p65, different N- and C-terminal truncation mutants of ORFV002 were generated (Figure 1B). OFTu cells were co-transfected with a pNFκB p65 plasmid and a plasmid expressing GFP alone (negative control),
wild type ORFV002-GFP fusion protein or one of the ORFV002 deletion mutants fused to GFP. The acetylation Lys$^{310}$ was examined by Western blotting using an anti-NF-$\kappa$B p65 (N-acetyl-Lys310) antibody (Figure 4). As expected from our previous studies [4] the acetylation of NF-$\kappa$B p65 (Lys310) was dramatically reduced in the presence of wild type ORFV002 (Figure 4A and 4B, lane 3). As with wild type ORFV002, the intact N-terminus of ORFV002 (ORFV002GM3 and ORFV002GM4) also induced a significant reduction of p65 Lys310 acetylation (Figure 4B, lanes 6 and 7). In contrast, deletion of the first 52 residues of ORFV002 (D$^{1–52}$), which contains CR1, or deletion of the first 94 residues of ORFV002 (D$^{1–94}$), which contains CR1 and CR2 (see Figure 1A and 1B), abolished the ability of ORFV002 to inhibit NF-kB p65 (Lys$^{310}$) acetylation (Figure 4B, lanes 8 and 9). The total protein levels of p65 and GAPDH expressed in OFTu cells were comparable (Figure 4). These results indicate that the ORFV002 N-terminus (residues 1–52) mediates NF-$\kappa$B p65 acetylation at Lys$^{310}$ as effectively as the whole ORFV002 protein.

ORFV002 Inhibits NF-$\kappa$B p65 (Ser$^{276}$) Phosphorylation

To investigate further whether ORFV002 affects NF-$\kappa$B p65 (Ser$^{276}$) phosphorylation, OFTu cells were co-transfected with plasmid pNF-$\kappa$B p65 and either pEGFP-N1 or p002-EGFP or deletion of mutant constructs. At 24 hours post-transfection, cells were treated with 20 ng/ml TNF-$\alpha$ for 30 minutes, and cell lysates were prepared and detected by immunoblotting using an anti-NF-$\kappa$B p65 (pSer$^{276}$) phosphorylation antibody (Figure 5). The Western blotting results showed that ORFV002 significantly inhibits phosphorylation of NF-$\kappa$B.
p65 Ser\textsuperscript{276} but not NF-\kappa B p65 Ser\textsuperscript{536} (Figure 5A and 5B). As with wild type ORFV002, mutants of ORFV002 also induced significant reduction of NF-\kappa B p65 Ser\textsuperscript{276} phosphorylation (Figure 6, lanes 6 and 7). In contrast, deletion of the N-terminal 52 residues (CR1) or 94 residues (CR1+CR2) abolished the ability of ORFV002 to inhibit p65 Ser\textsuperscript{276} phosphorylation (Figure 6B, lanes 4 and 5). The total protein levels of phospho-p65 and histone-H3 expressed in OFTu cells were comparable (Figure 6). These results indicate that the N-terminus of ORFV002 (residues 1 to 52) mediates p65 phosphorylation at Ser\textsuperscript{276}.

**ORFV002 Interferes With NF-\kappa B-p65 Ser\textsuperscript{276} Phosphorylation Induced by MSK1**

To investigate further whether ORFV002 affects production of NF-\kappa B p65 (pSer\textsuperscript{276}) by MSK1, HEK293T cells were transiently transfected using expression vectors for NF-\kappa B p65, Flag-MSK1 plus GFP, 002GFP or 002GM1-4 (A and B). Activation of MSK1 was achieved by co-transfecting MKK6 and p38 kinase. At 24 hours post-transfection, cells were treated with 20 ng/ml TNF-\alpha for 30 minutes, and cell lysates were prepared and subject to immunoprecipitation with anti-Flag-MSK1 antibody. Co-precipitating p65 was detected by immunoblotting with an anti-p65 antibody. To monitor the activation status of MSK1, blots were stripped and reprobed using an anti-MSK1 antibody. doi:10.1371/journal.pone.0058854.g008

**Figure 9. Overview of the phosphorylation pathways inhibited by ORFV002.** Binding of TNF to its receptor (TNF-R) causes TNF-R to phosphorylate I\kappa B\alpha, and NF-\kappa B p65 at Ser\textsuperscript{536}, freeing NF-\kappa B p50/p65 (pSer\textsuperscript{536}) from I\kappa B\alpha, and allowing NF-\kappa B p50/p65 (pSer\textsuperscript{536}) to translocate to the nucleus. After stimulation by TNF p38 and ERK MAPK kinases were also activated, both of which can activate MSK1 independently, with maximal activation requiring phosphorylation of MSK1 by both kinases. In the nucleus, activated MSK1 phosphorylates NF-\kappa B p65 at Ser\textsuperscript{276} resulting in fully functional NF-\kappa B that can interact with other transcriptional activators, such as p300, to drive transcription of NF-\kappa B-regulated genes. Wild type ORFV002 (002GFP) and C-terminal deletion mutants of ORFV002 (002GM3 and 002GM4) can block MSK1 phosphorylation of NF-\kappa B p65 at Ser\textsuperscript{276}, while N-terminal deletion mutants of ORFV002 (002GM1 and 002GM2) cannot. SB203580, PD98059, and H89 are inhibitors of p38 activation of MSK1, MAPK activation of MSK1 and the ability of MSK1 to phosphorylate NF-\kappa B p65, respectively (See Ref [20]). doi:10.1371/journal.pone.0058854.g009

**ORFV002 Inhibits NF-\kappa B-p65 Ser\textsuperscript{276} Phosphorylation**

To investigate further whether ORFV002 affects production of NF-\kappa B p65 (pSer\textsuperscript{276}) by MSK1, HEK293T cells were transiently transfected using expression vectors for p65, Flag-MSK1 plus GFP, 002GFP, 002GM1, or 002GM3 with pcDND3.1-MKK6 and pcDNA3.1-P38. At 24 hours post-transfection, cells were treated with 20 ng/ml TNF-\alpha for 30 minutes, and cell lysates were prepared and phosphorylation of NF-\kappa B p65 at Ser\textsuperscript{276} was detected by immunoblotting using an anti-NF-\kappa B p65 (pSer\textsuperscript{276}) antibody (Figure 7). The western blotting results showed that nuclear kinase MSK1, when activated by TNF-\alpha, specifically phosphorylated NF-\kappa B p65 at the serine residue in position 276 (pSer\textsuperscript{276}) and this phosphorylation was inhibited by ORFV002, or by the intact N-terminus of ORFV002 (002GM3 and 002GM4). Expression of NF-\kappa B p65 (pSer\textsuperscript{276}) was further enhanced by TNF-\alpha treatment when cells were co-transfected with MKK6 and p38 in addition to NF-\kappa B p65 and MSK1 (Figure 7A, lane 3 and B, lane 2), whereas co-transfection of NF-\kappa B p53 and MSK1 with MKK\alpha or p38 alone resulted in relatively low levels of NF-\kappa B p65 Ser\textsuperscript{276} phosphorylation (Figure 7B, lane 6, 7 and 8). Very low level of NF-\kappa B p53 (pSer\textsuperscript{276}) phosphorylation was detected when cells were transfected with NF-\kappa B p65 alone (Figure 7A, lane 1), NF-\kappa B p53 plus MSK1 (Figure 7A, lane 2), or NF-\kappa B p65 plus MKK\alpha and p38 (Figure 7B, lane 1 and C) suggesting that MKK6 and p38 were absolutely required for MSK1 activation and NF-\kappa B-driven gene transcription in response to TNF-\alpha. GFP-fusions of wild type ORFV002 (002GFP) and the intact N-terminus of ORFV002 (002GM3 and 002GM4) significantly inhibited NF-\kappa B p65 Ser\textsuperscript{276} phosphorylation.

**Figure 8. ORFV002 inhibits the interaction between NF-\kappa B p65 and MSK1 in vivo.** HEK293T cells were transiently transfected using expression vectors for NF-\kappa B p65, Flag-MSK1 plus GFP, 002GFP or 002GM1-4 (A and B). Activation of MSK1 was achieved by co-transfecting MKK6 and p38 kinase. At 24 hours post-transfection, cells were treated with 20 ng/ml TNF-\alpha for 30 minutes, and cell lysates were prepared and subject to immunoprecipitation with anti-Flag-MSK1 antibody. Co-precipitating p65 was detected by immunoblotting with an anti-p65 antibody. To monitor the activation status of MSK1, blots were stripped and reprobed using an anti-MSK1 antibody. doi:10.1371/journal.pone.0058854.g008
phosphorylation (Figure 7A, lane 4 and 6; B, lane 3 and 9), which is consistent with previous observations [20,21]. The results indicate that p38 and ERK mitogen-activated protein kinases (MAPKs) are absolutely required for full activation of MSK1 activity and NF-κB-driven gene transcription in response to TNF-α.

ORFV002 Interferes With the Interaction Between NF-κB p65 and MSK1 in vivo

To investigate the role of ORFV002 in the interaction of NF-κB p65 and MSK1, HEK293T cells were transiently transfected using expression vectors for NF-κB p65, Flag-MSK1 plus GFP, or 002GFP or 002GM1 with expression vectors for MKK6 and p38. At 24 hours post-transfection cells were treated with 20 ng/ml TNF-α for 30 minutes and cell lysates were prepared for immunoprecipitation with an anti-Flag-MSK1 antibody. Co-precipitating NF-κB p65 was detected by immunoblotting using an anti-NF-κB p65 antibody. To monitor the activation status of MSK1 blots were stripped and re-probed using an anti-MSK1 antibody. The results showed that the MSK1 and NF-κB p65 indeed interact and that ORFV002 and the intact N-terminus of ORFV002 (002GM3 and 002GM4) interfere with the interaction between NF-κB p65 and MSK1 (Figure 8A, lane 4 and B lane 1, 4 and 5). There was no significant effect of 002GM1 and 002GM2 on the interaction between NF-κB p65 and MSK1 as compared to GFP alone, a negative control (Figure 9A, lanes 3 and 5; or B, lanes 2 and 3).

Discussion

Previous studies showed that ORFV002 inhibits the activation of NF-κB, the master transcriptional regulator of immune response during ORFV infection [4]. In this study the luciferase assay showed that ORFV002 mutants lacking the N-terminus did not inhibit activation of NF-κB, while wild type ORFV002 and deletion mutants of ORFV002 retaining an intact N-terminus did inhibit activation of NF-κB. This result confirmed that the N-terminus of ORFV002 is important for inhibiting NF-κB activation.

The post-translational modifications of NF-κB, including site-specific phosphorylation and acetylation, are important for the optimal transactivation activity of NF-κB [13,14]. In this study we revealed that the N-terminus of ORFV002 inhibits NF-κB from stimulating transcription by blocking phosphorylation of NF-κB p65 Ser276 but not Ser236. Usually NF-κB p65 is phosphorylated by the protein kinase A (PKA) upon lipopolysaccharide (LPS) triggering [19] or mitogen- and stress-activated kinase-1 (MSK1) after TNF-stimulation [20]. In our research, we transfected MKK6 and p38 kinases and used TNF-α to activate MSK1 in HEK293T cells. The HEK293T cells were co-transfected with NF-κB p65, and GFP, 002GFP, 002GM1 or 002GM4, plus plasmids expressing MKK6 and p38 to investigate the role of ORFV002 on MSK1 and status of p65 Ser276 phosphorylation. The results showed that NF-κB p65 is phosphorylated at Ser276 by MSK1 after TNF-α stimulation, which is consistent with previous studies [20,22-24] and ORFV002, and its C-terminal deletion mutants 002GM3 and 002GM4, significantly inhibited the activation of NF-κB p65 through MSK1. Our results also showed that ORFV002 interfered with the interaction between NF-κB p65 and MSK1. Based on these results, ORFV002 inhibited NF-κB p65 phosphorylation by a competitive inhibition of interactions between MSK1 and NF-κB p65 by ORFV002, which further inhibited the acetylation of NF-κB p65 at Lys310.

It has been confirmed that phosphorylated NF-κB p65 could recruit the transcriptional co-activators, such as p300 and CBP to the NF-κB-bound promoter and facilitate DNA binding ability of NF-κB p65 [15-18]. Also, through its N-terminal regions, ORFV002 inhibits phosphorylation of NF-κB p65 at Ser276 and preventing NF-κB dependent transcriptional activation. Furthermore, confocal microscopy analysis of ORFV002 mutants and the detection of low levels of NF-κB p65 (pSer276) in nuclear extracts of cells expressing ORFV002 deletion mutants retaining an intact N-terminus confirm that the N-terminus of ORFV002 is sufficient to inhibit phosphorylation of NF-κB p65 at Ser276 in the nucleus.

Many studies have shown that nuclear kinase MSK1 is a candidate for phosphorylation of NF-κB p65 at Ser276, which acts downstream of p38 and ERK MAPKs in response to TNF-α, associates with NF-κB p65 in a stimulus-dependent manner and specifically phosphorylates the serine residue at position 276, thus leading to its positioning at NF-κB containing promoter sections and selective stimulation of particular NF-κB-driven genes [20], [21], [22]. In this study we present the first data to show that ORFV002 and its N-terminus inhibit NF-κB p65 Ser276 phosphorylation by MSK1 activation pathway. Levels of NF-κB p65 phosphorylated at Ser276 was increased by TNF-α treatment when NF-κB p65 and MSK1 were co-transfected with MKK6 and p38 (Figure 7A, line 3 and B, lane 2), whereas phosphorylation of NF-κB p65 at Ser276 was only partially increased when p65 and MSK1 were co-transfected with MKK6 or p38 alone (Figure 7B, lane 6, 7 and 9), suggesting that MKK6 and p38 were required for full activation of MSK1 and NF-κB-driven gene transcription in response to TNF-α. These results are consistent with previous observations [20,23,24].

Taken together, a model of the phosphorylation pathways inhibited by ORFV002 is presented in Figure 9, in which cytoplasmic NF-κB activation is followed by nuclear phosphorylation of the p65 subunit at Ser276. Binding of TNF to its receptor (TNF-R) causes TNF-R to phosphorylate IκBα and NF-κB p65 at Ser236, freeing NF-κB p30/p65 (pSer536) from IκBα, and allowing NF-κB p30/p65 (pSer536) to translocate to the nucleus. Stimulation by TNF also activates p30 kinase and ERK MAPK kinases, both of which can activate MSK1 independently, with maximal activation requiring phosphorylation of MSK1 by both kinases. In the nucleus, activated MSK1 phosphorylates NF-κB p65 at Ser276, resulting in fully functional NF-κB that can interact with other transcriptional activators, such as p300, to drive transcription of NF-κB-regulated genes. Wild-type ORFV002 (002GFP) and C-terminal deletion mutants of ORFV002 (002GM3 and 002GM4) can block MSK1 phosphorylation of NF-κB p65 at Ser276, while N-terminal deletion mutants of ORFV002 (002GM1 and 002GM2) cannot. SB203580, PD98059, and H89 are inhibitors of p38 (Figure 7A, line 3 and B, lane 2), whereas phosphorylation of NF-κB p65 at Ser276 was only partially increased when p65 and MSK1 were co-transfected with MKK6 or p38 alone (Figure 7B, lane 6, 7 and 9), suggesting that MKK6 and p38 were required for full activation of MSK1 and NF-κB-driven gene transcription in response to TNF-α. These results are consistent with previous observations [20,23,24].

In conclusion the N-terminus of ORFV002 can inhibit phosphorylation of NF-κB p65 at Ser276, by weakening the interaction of MSK1 and NF-κB p65 and by blocking the acetylation of NF-κB p65. This is the first identification of a novel function of ORFV002 protein, in which it can inhibit the NF-κB signaling pathway through its N-terminus (residues 1 to 32).
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Author Contributions

Conceived and designed the experiments: ZN SL. Performed the experiments: ZN ZZ WL CD YW WH ML SL. Analyzed the data: ZN SL. Contributed reagents/materials/analysis tools: ZN ZZ WL. Wrote the paper: ZN SL.