Poxvirus Host Range Protein CP77 Contains an F-Box-Like Domain That Is Necessary To Suppress NF- κ B Activation by Tumor Necrosis Factor Alpha but Is Independent of Its Host Range Function[∇]

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Tumor necrosis factor alpha (TNF- α) activates the nuclear factor κ B (NF- κ B) signaling pathway that regulates expression of many cellular factors playing important roles in innate immune responses and inflammation in infected hosts. Poxviruses employ many strategies to inhibit NF- κ B activation in cells. In this report, we describe a poxvirus host range protein, CP77, which blocked NF- κ B activation by TNF- α . Immunofluorescence analyses revealed that nuclear translocation of NF- κ B subunit p65 protein in TNF- α -treated HeLa cells was blocked by CP77. CP77 did so without blocking I κ B α phosphorylation, suggesting that upstream kinase activation was not affected by CP77. Using GST pull-down, we showed that CP77 bound to the NF- κ B subunit p65 through the N-terminal six-ankyrin-repeat region in vitro. CP77 also bound to Cullin-1 and Skp1 of the SCF complex through a C-terminal 13-amino-acid F-box-like sequence. Both regions of CP77 are required to block NF- κ B activation. We thus propose a model in which poxvirus CP77 suppresses NF- κ B activation by two interactions: the C-terminal F-box of CP77 binding to the SCF complex and the N-terminal six ankyrins binding to the NF- κ B subunit p65. In this way, CP77 attenuates innate immune response signaling in cells. Finally, we expressed CP77 or a CP77 F-box deletion protein from a vaccinia virus host range mutant (VV-hr-GFP) and showed that either protein was able to rescue the host range defect, illustrating that the F-box region, which is important for NF- κ B modulation and binding to SCF complex, is not required for CP77's host range function. Consistently, knocking down the protein level of NF- κ B did not relieve the growth restriction of VV-hr-GFP in HeLa cells.

Vaccinia virus, the prototype of the poxvirus family, infects a wide range of cells in vitro and animal species in vivo (14). Vaccinia virus has a double-stranded DNA genome that encodes 263 open reading frames (ORFs). Vaccinia virus expresses different classes of viral genes in a cascade-regulated manner and completes the virus life cycle in the cytoplasm of infected cells (11).

To replicate successfully in infected hosts, poxviruses have evolved various strategies to overcome cellular immune responses (20, 39). Viral infections activate cellular antiviral signaling and inflammatory responses (49), such as NF- κ B, which plays a critical role in inflammatory signaling and immune activation (23). NF- κ B contains five different members, NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel, all of which share a Rel homology domain for DNA binding, dimerization, and interaction with I κ B (22, 23). The most abundant activated form consists of a p50 or p52 subunit and a p65 subunit (16, 26). In the inactive state, dimerized NF- κ B (such as p65/p50) is bound by I κ B α , and the crystal structure of the I κ B α /p65/p50 complex shows multiple contact sites between the ankyrin repeats of I κ B α and NF- κ B (29). In well-characterized canonical NF- κ B signaling, such as tumor

necrosis factor alpha (TNF- α) treatment, receptor activation sends intracellular signals to activate the IKK complex (23). Activated IKK then phosphorylates I κ B α on Ser32 and Ser36 in conserved sequences DS*GXXS*, resulting in the Lys48-linked polyubiquitination of I κ B α by Skp1-Cul1-FBP (SCF) ubiquitin ligase complexes containing the F-box protein, β TrCP (43). The phosphorylation and ubiquitination of I κ B α releases the NF- κ B dimers that are then able to travel to the nucleus and bind to the promoter sequences of particular genes and enhance gene transcription through the recognition of specific κ B consensus sequences (26).

As expected, many poxvirus proteins have evolved to down-regulate NF- κ B activation through different mechanisms. Cowpox viral proteins CrmB, -C, and -D act as soluble TNF receptors to intercept ligand-receptor interaction (3, 36, 52). Vaccinia virus N1, A52R, and B14 proteins all share a Bcl-2-like fold structure that inhibits activation of the IKK complex (1, 5, 8, 12, 18, 21), while the M2L protein inhibits ERK2 (15) and K1L prevents the degradation of I κ B α (51). Molluscum contagiosum virus encodes two proteins, MC160, which targets the IKK complex (40), and MC159, a vFLIP that inhibits degradation of I κ B β (39a, 51a). Myxoma virus M150R blocks NF- κ B activity in the nucleus and interferes with inflammation (6). Finally, a 32-kDa protein encoded by A238L of African swine fever virus shares 40% homology with I κ B α (60) and binds to free NF- κ B/p65 to prevent its nuclear translocation (46, 58). Overall, these viral proteins represent the diversity of viral strategies to tame down cellular NF- κ B signaling in hosts.

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CP77 is a cowpox viral protein containing 668 amino acids, with nine predicted ankyrin repeats. CP77 is expressed in the early phase after viral infection and is necessary for vaccinia virus growth in nonpermissive cells (33, 44, 47, 50, 55). In the present study, we explored the role of CP77 in the NF- κ B signaling pathway and its relationship with host restriction regulation.

MATERIALS AND METHODS

Cell cultures, viruses, and reagents. HeLa cells and 293T cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS). BHK-21 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. VV-hr has an 18-kb deletion of the viral genome which includes two genes, designated K1L and C7L, that are known to be essential for vaccinia virus multiplication in various human cell lines as described previously (17, 41). Virus stocks of VV-hr and recombinant viruses containing green fluorescent protein (GFP), wild-type GFP-CP77, and GFP-CP77 F-box deletion ORFs were grown in BHK21 cells, and titers were determined by plaque assays as previously described (13). Antibody against GFP was purchased from Clontech, Inc. Alkaline phosphatase-conjugated goat anti-rabbit antibody and goat anti-mouse antibody were obtained from Calbiochem, Inc. Abs recognizing I κ B α , NF- κ B subunit p65, Skp1, and Cullin-1 were purchased from Santa Cruz, Inc. Anti-Flag antibody was purchased from Sigma, Inc. Antibodies recognizing a phosphorylated form of I κ B α was purchased from Cell Signaling, Inc. A goat anti-rabbit immunoglobulin G (IgG) antibody conjugated with tetramethylrhodamine (H+L) was purchased from Molecular Probes, Inc. Antibody recognizing β -actin was purchased from Abcam, Inc. A QuikChange site-directed mutagenesis kit was purchased from Stratagene, Inc. Recombinant human TNF- α was purchased from R&D Systems. The proteasome inhibitor MG132 was purchased from Sigma, Inc., and used at a concentration of 20 μ M. Glutathione-Sepharose 4B beads were from Amersham Biosciences, Inc. The Lipofectamine and Lipofectamine Plus reagents were from Invitrogen, Inc., and the dual luciferase assay system kit was purchased from Promega, Inc.

Luciferase assays. 293T cells were seeded into 24-well plates (7.5×10^4 cells per well) and rested overnight. The cells were then transfected with plasmids expressing GFP or GFP-CP77, together with NF- κ B-mediated firefly luciferase, and the control *Renilla* luciferase reporter plasmids. After overnight incubation, the transfected 293T cells were treated with recombinant human TNF- α (20 ng/ml) for various times. Cells were then harvested and lysed in 1 $\times$ PLB solution (Promega) at room temperature for 15 min. The firefly and *Renilla* luciferase activities were measured by a dual luciferase reporter assay system (Promega) according to the protocol recommended by the manufacturer. The data obtained from firefly and *Renilla* luciferase activities were processed based on the following equations: (i) normalized NF- κ B activity at each time point = firefly luciferase activity/*Renilla* luciferase activity and (ii) the fold increase in NF- κ B activity each time point = normalized NF- κ B activity at each time point/normalized NF- κ B activity at time zero.

Confocal immunofluorescence microscopy. HeLa cells were seeded on coverslips in 12-well plates. Next day, the cells were transfected with plasmids expressing GFP-CP77 or GFP, incubated for 24 h, and then treated with 20 ng of TNF- α /ml. Cells were fixed in 4% paraformaldehyde-phosphate-buffered saline (PBS), permeabilized in 0.2% Triton X-100-PBS and stained with anti-p65, tetramethylrhodamine-conjugated goat anti-rabbit IgG, and DAPI (4',6'-diamidino-2-phenylindole; 0.5 μ g/ml). Cell images were collected by confocal laser scanning microscopy (Carl Zeiss). For quantification, 100 GFP-expressing cells per experimental group were analyzed. Each experiment was repeated at least three times.

GST-pull-down assays. Glutathione S-transferase (GST), GST-CP77, GST-CP77(1-352), and GST-CP77(1-234) were overexpressed in *Escherichia coli* BL21(DE3) and purified on glutathione-Sepharose 4B beads (Amersham Bioscience) as previously described (19). To prepare cell lysates for GST pull-down, HeLa cells were treated with 20 ng of TNF- α /ml and harvested at various time points in a lysis buffer consisting of 20 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail (Roche). Lysed cells were kept at 4°C for 30 min and then centrifuged at 10,000 rpm, and the supernatant was saved. Lysates were first precleared with 30 μ g of GST bound to glutathione-Sepharose 4B beads at 4°C for 2 h and then centrifuged again. The supernatant subsequently was incubated at 4°C for 2 h with 10 μ g of each purified GST fusion proteins that were already bound to the glutathione-Sepharose 4B beads. After centrifugation, the pulled-down samples were washed five times and then sepa-

rated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-10% PAGE), with half of the samples transferred for immunoblot analyses by using anti-p65 antibody (1:1,000 dilution) and the other half used for Coomassie blue staining of GST fusion proteins.

Immunoprecipitation and immunoblot analyses. For immunoprecipitation of transfected cells, HEK293 EBNA1 cells were grown in six-well plates and transiently transfected in duplicate with 1 μ g of either pApexFlag-CPX77, pApexFlag-CPX77 Δ F, or pApexFlag using FuGENE (Roche) according to the manufacturer's instructions. CPX77 Δ F lacks the C-terminal 17 amino acids (aa) comprising its F-box-like domain. The construction of these expression vectors was performed as described previously (38, 54). Cells were harvested after 24 h and lysed in a solution of 1% NP-40, 20 mM Tris, 500 mM NaCl, and protease inhibitors (Roche) as described above. Lysates were cleared by low-speed centrifugation, and supernatants were incubated with anti-Flag-agarose (A-2220; Sigma) at 4°C. Beads were then washed extensively with lysis buffer, resuspended in SDS loading dye/ β -mercaptoethanol, and electrophoresed. Membranes were incubated with anti-Flag-horseradish peroxidase (HRP) (A-8592; Sigma), anti-Skp1 (SC-7163; Santa Cruz) and anti-rabbit-HRP (A-6154; Sigma), or anti-Cull1 (SC-11384; Santa Cruz) and anti-rabbit-HRP. For immunoprecipitation from virus-infected cells, 4×10^5 HeLa cells were seeded in 60-mm dishes, infected with individual viruses at a multiplicity of infection (MOI) of 5 PFU per cell, and harvested at 6 h postinfection (p.i.) into 0.5 ml of lysis buffer. The lysates were precleared with 30 μ l of 50% (vol/vol) protein A beads (GE Healthcare) at 4°C for 2 h and then immunoprecipitated using anti-GFP antibodies in 30 μ l of 50% (vol/vol) protein A beads at 4°C for 2 h. The immunoprecipitates were washed with lysis buffer five times, resuspended in 30 μ l of SDS sample buffer, and separated by SDS-PAGE. For immunoblots, protein samples harvested as described above were separated by SDS-PAGE gel and electroblotted onto a nitrocellulose membrane at 250 mA for 2 h at 4°C. The membrane was blocked with 0.2% I-block in PBS containing 0.1% Tween 20 (PBST) at room temperature for 1 h. The nitrocellulose membrane was then hybridized with primary antibody in I-block-PBST overnight, washed with PBST, and then hybridized with the alkaline phosphatase-conjugated antibody (1:1,000 dilution) for 1 h at room temperature. After being washed with PBST, the membrane was developed by using CDP-Star and Nitro-block II (Applied Biosystems).

SPR measurements. Surface plasmon resonance (SPR) was measured on a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden) at 25°C. The CM5 sensor chip was activated by running through a 1:1 mixture of *N*-hydroxysuccinimide (0.05 M) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.2 M) at 10 μ l/min for 10 min. Purified NF- κ B (100 μ M in 10 mM potassium acetate [pH 5.0]) was then injected for 10 min at 10 μ l/min to covalently immobilize it to the CM5 chip surface. After ethanolamine treatment (1.0 M [pH 8.5]) for 10 min, the CM5 chip surface was equilibrated in HBS-EP buffer (10 mM HEPES, 0.15 M NaCl, and 0.005% surfactant P20 [pH 7.4]). GST-CP77 was then injected for 3 min at decreasing concentrations (10, 5, 2.5, 1.25, and 0.625 μ M) in HBS-EP buffer with a flow rate of 20 μ l/min, allowing 10 min for dissociation. GST alone was injected separately by the same procedure (10, 5, and 2.5 μ M) as a control. The surface was regenerated with 10 mM glycine (pH 2.5). The data from two independent titration experiments were averaged, and a 1:1 binding model was assumed for the determination of the dissociation constant.

Construction of a mutant virus VV-hr-GFP-CP77 Δ F. (i) Plasmid construction. The plasmid expressing GFP-CP77 Δ F was constructed by using a QuikChange site-directed mutagenesis kit using the wild-type GFP-CP77 as the template. The primers used for in vitro mutagenesis (Stratagene), inserting a premature termination codon TAA after Asn655, were 5'-ACCTACATTCTAATTAATTG CCTTATACCATC-3' and 5'-GATGGTATAAGGCAATTAATTAGAAATG TAGGT-3'. The resulting amplified GFP-CP77 Δ F was sequenced and cloned into SmaI-digested pSC11 to produce pSC11-GFP-CP77 Δ F. The sequences of the PCR fragments were confirmed by DNA sequencing.

(ii) **Construction of the recombinant virus.** The recombinant virus VV-hr-GFP-CP77 Δ F was constructed according to previously established protocols (30). In brief, BHK21 cells were infected with VV-hr-GFP at an MOI of 0.1 PFU per cell and subsequently transfected with 1 μ g of pSC11-GFP-CP77 Δ F with Lipofectamine Plus reagent (Invitrogen) for 3 h, washed, and then cultured in RPMI 1640 medium supplemented with 10% FBS for 4 days. Cell lysates were prepared through three cycles of freeze-thawing in PBS containing 0.05% bovine serum albumin and 10 mM MgCl₂ (PBS-AM) and were serially diluted for isolation of individual plaques under 1% top agarose with 150 μ g of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside)/ml to stain for lacZ expression. Plaques were isolated and purified three times to 100% purity.

Preparation of cytoplasmic and nuclear extracts. Extracts were prepared as previously described (42). Briefly, the cells were collected and washed in PBS. Pellets were lysed with 3 volumes of CE buffer (10 mM HEPES, 10 mM KCl, 0.1

FIG. 1. (A to C) CP77 suppresses NF- κ B activation in HeLa (A), CHO-K1 (B), and 293T (C) cells. Each cell line was infected with WT-VV, WT-VV-CP77, VV-hr-GFP, or VV-hr-GFP-CP77 at an MOI of 5 PFU/cell and harvested at 8 h p.i., and nuclear extracts were prepared as described in Materials and Methods. Extracts were incubated with 32 P-radiolabeled oligonucleotide containing consensus NF- κ B binding sites and assayed via EMSAs. Mobility-shifted bands containing NF- κ B subunit p65 are indicated by an arrow. bg, nonspecific background bands. In some cases, additional nuclear extracts were incubated with anti-p65 antibody to "supershift" the band that was shown by an arrowhead. (D) GFP-CP77 suppressed nuclear translocation of NF- κ B/p65 in virus-infected HeLa cells. HeLa cells were either mock infected (M) or infected with VV-hr-GFP and VV-hr-GFP-CP77 at an MOI of 5 PFU per cell and harvested at 4, 8, and 12 h p.i., and the N and C fractions were separated. NF- κ B/p65 content in the N and C fractions is shown. Both N and C fractions were probed with antibodies recognizing calpain1 and hnRNPA1 that represent cytoplasmic and nuclear marker protein, respectively. (E) GFP-CP77 suppressed the NF- κ B-mediated reporter gene assay. 293T cells were transfected with plasmids expressing GFP or GFP-CP77 together with NF- κ B-mediated luciferase, and the *Renilla* luciferase reporter plasmids as described in Materials and Methods. After TNF- α (20 ng/ml) stimulation for various times, cells were then harvested and lysed in 1 $\times$ PLB solution (Promega) at room temperature for 15 min. The luciferase activity was measured by a dual luciferase assay system (Promega) and processed as described in Materials and Methods.

mM EDTA [pH 8.0], 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.05% NP-40, 2 mg of aprotinin/ml, 0.5 mg of leupeptin/ml, 20 mM β -glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4 at 4°C for 5 min. The nuclei were pelleted with a 20-s centrifugation at 14,000 rpm in a microfuge, and the supernatant was saved as the cytoplasmic extract and stored at -80°C. The nuclear pellets were washed with 5 volumes of CE buffer and then centrifuged again at 14,000 rpm for 20 s. The nuclei were resuspended in 3 volumes of NE buffer (20 mM HEPES, 25% glycerol, 0.4 M NaCl, 1 mM EDTA [pH 8.0], 1 mM EGTA, 0.5 mM PMSF, 1 mM DTT, 2 mg of aprotinin/ml, 0.5 mg of leupeptin/ml, 20 mM β -glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4) for 30 min and then centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was saved as the nuclear extract and stored at -80°C.

Electrophoretic mobility shift assays (EMSAs). The assays were performed based on previously published procedures (10). The virus-infected cells were harvested at indicated time points and separated into cytosol and nuclear fractions according to the protocol of nuclear extraction kit (Active Motif, Inc.). Nuclear extracts (20 μ g) were incubated with 32 P-end-labeled κ B-site-containing

oligonucleotides (5'-AGTTGAGGGGACTTCCAGGC-3' and 5'-GCCTGG GAAAGTCCCCTCAACT-3') (35, 53) in the gel shift buffer [20 mM HEPES (pH 7.9), 0.4 mM EDTA (pH 8.0), 1 mM DTT, 100 mM KCl, 0.2 μ g poly(dI-dC)/ μ l, 20% glycerol, 4 mM MgCl_2 , 1 mM PMSF] for 30 min at room temperature. When a supershifting experiment was performed, 2 μ g of anti-p65 antibody (sc372-G; Santa Cruz) was added into the sample described above for 30 min at room temperature. Samples were separated by gel electrophoresis through 4% acrylamide-Tris-borate-EDTA native gels, dried, and subjected to autoradiography.

Knockdown of endogenous p65 expression using siRNA. The control cyclophilin B (CypB) small interfering RNA (siRNA) were purchased from Dharmacon, Inc. NF- κ B p65 siRNA (catalog no. 6261) were obtained from Cell Signaling. First, 20 μ M concentrations of siRNA were incubated in Opti-MEM medium using Lipofectamine 2000 (Invitrogen, Inc) for 30 min at room temperature. Then, HeLa cells were transfected with the siRNA for 4 h. This process was repeated, and the amounts of CypB and NF- κ B p65 in cells were assessed by using immunoblotting.

FIG. 2. CP77 blocks NF-κB/p65 nuclear translocation induced by diverse ligands. HeLa cells were transfected with plasmids expressing GFP or GFP-CP77. After overnight incubation, these cells were either mock treated (A) or treated with 20 ng/ml of TNF-α (B), IL-1α (C), or IL-1β (D) for 20 min and then fixed and immunostained for NF-κB/p65. Arrows indicate examples of nuclei into which NF-κB/p65 has not translocated after cytokine treatment. (E) Quantification of results shown in panels A to C. The translocation of NF-κB into the nucleus was quantified from >100 GFP-expressing cells in each treatment.

RESULTS

NF-κB activation was suppressed by CP77. Wild-type vaccinia virus (WT-VV) and WT-VV expressing CP77 (WT-VV-CP77) grew well in HeLa cells and did not induce NF-κB activation at 8 h p.i., as demonstrated by EMSA analyses (Fig. 1A). In contrast, a mutant vaccinia virus, VV-hr-GFP, did not

grow in HeLa cells and induced NF-κB activation at 8 h p.i. Expression of GFP-CP77 in the mutant virus rescued virus growth in HeLa cells and suppressed NF-κB activation (Fig. 1A). As a control, anti-p65 antibody was included to produce a supershifted band demonstrating that the shifted band indeed contained NF-κB subunit p65. We then tested NF-κB activa-

tion in CHO-K1 cells in which CP77 also acts as the host range protein for WT-VV and VV-hr-GFP (Fig. 1B). Shifted bands were detected in CHO-K1 cells that were either mock infected or infected with WT-VV or VV-hr-GFP. The intensities of the p65-containing bands were reduced in CHO-K1 cells infected with WT-VV-CP77 or VV-hr-GFP-CP77, suggesting that expression of CP77 suppressed NF- κ B activation (Fig. 1B). Interestingly, when we tested 293T cells, in which all four viruses grew well, little NF- κ B activation was detected after virus infection (Fig. 1C). 293T cells are not defective in NF- κ B pathway per se since a strong p65-containing band was detected at 30 min after TNF- α treatment (Fig. 1C). These results showed that virus infection induced NF- κ B activation is cell type dependent and that expression of CP77 suppressed NF- κ B activation. Besides EMSA, we also performed biochemical fractionations to separate nuclear (N) and cytoplasmic (C) extracts harvested from HeLa cells infected with either VV-hr-GFP or VV-hr-GFP-CP77 (Fig. 1D). Both N and C fractions were probed with antibodies recognizing calpain1 and hnRNPA1 that represent cytoplasmic and nuclear marker protein, respectively (right panel in Fig. 1D). Consistently, nuclear accumulation of NF- κ B subunit p65 protein was increased in cells infected with VV-hr-GFP from 4 to 12 h p.i., and the expression of CP77 suppressed nuclear accumulation of p65 (Fig. 1D). To address whether CP77 inhibition of the NF- κ B activation required other viral proteins, we used transient expression cell culture with a luciferase reporter system in which NF- κ B p65/p50 activation was induced by the addition of TNF- α (22, 57, 59). 293T cells expressing GFP or GFP-CP77 were treated with TNF- α at 20 ng/ml for various times and harvested, and NF- κ B-regulated luciferase activity was measured. As shown in Fig. 1E, TNF- α treatment activated NF- κ B in a time-dependent manner, and expression of GFP-CP77, but not of GFP, significantly blocked luciferase activity, suggesting that CP77 alone could block NF- κ B activation induced by TNF- α .

CP77 blocked nuclear translocation of NF- κ B activated by multiple cytokines. To understand how GFP-CP77 acted to block NF- κ B activation, we transfected HeLa cells with GFP or GFP-CP77, treated these cells with TNF- α , and performed immunofluorescence analyses using antibodies recognizing NF- κ B subunit p65. Immunofluorescence staining of the endogenous NF- κ B/p65 revealed that in the mock-treated cells NF- κ B was present in the cytoplasm (Fig. 2A). GFP expression did not alter the ability of NF- κ B to translocate into the nucleus in response to TNF- α treatment (Fig. 2B). However, in cells expressing GFP-CP77, nuclear translocation of NF- κ B/p65 was significantly reduced. Quantitatively, we found that nuclear translocation of NF- κ B/p65 was detected in ca. 90% of GFP-expressing cells after TNF- α treatment, whereas only 20% GFP-CP77-expressing cells contained nuclear NF- κ B/p65 after TNF- α treatment (Fig. 2E). These results showed that CP77 blocked TNF- α -induced NF- κ B/p65 translocation into the nucleus.

Previous studies have also shown that NF- κ B could be induced by different signaling pathways through distinct receptor interactions with ligands other than TNF- α , such as interleukin-1 α (IL-1 α)/IL-1 β (9). All of these signals are transmitted through different intracellular adaptors and converged on activation of the IKK complex that phosphorylates I κ B and pro-

FIG. 3. (A) CP77 did not block phosphorylation of I κ B α induced by TNF- α . HeLa cells were transfected with plasmids expressing GFP or GFP-CP77 and treated with TNF- α as described in Materials and Methods. The cell lysates were collected at the indicated times, and the proteins were analyzed by a 10% SDS-PAGE. (B) CP77 did not suppress phosphorylation of I κ B α induced by TNF- α in virus-infected cells. HeLa cells were either mock infected or infected with VV-hr-GFP and VV-hr-GFP-CP77 at an MOI of 5 PFU/cell for 2 h with or without araC (40 μ g/ml) and then treated with TNF- α (20 ng/ml) for 15 min and harvested for immunoblot analyses with anti-phosphorylated I κ B α (1:1,000), anti-I κ B α (1:1,000), anti-E3L (1:1,000), anti-G8R (1:1,000), or anti-GFP (1:4,000) antibodies as indicated.

notes the ubiquitination of I κ B and its subsequent degradation in proteasomes (9). To test whether CP77 also suppresses NF- κ B activation by other cytokines, transfected HeLa cells were treated with IL-1 α or IL-1 β and stained for endogenous NF- κ B/p65. Immunofluorescence staining revealed that NF- κ B/p65 translocated into the nucleus in GFP-expressing cells after IL-1 α (Fig. 2C) or IL-1 β (Fig. 2D) stimulation. However, nuclear translocation of NF- κ B/p65 induced by these cytokines was blocked by CP77 (Fig. 2C and D, quantified in Fig. 2E), suggesting that CP77 suppression of NF- κ B must occur at or downstream of the activation of IKK, a converging point of both TNF- α and IL-1-mediated pathways.

CP77 influenced degradation of I κ B α but not phosphorylation. It is known that TNF- α treatment activated IKK complex to phosphorylate I κ B α , which then became ubiquitinated and degraded by proteasome in cells. We next investigated whether phosphorylation of I κ B α by IKK was inhibited by CP77 in cells treated with TNF- α . First, HeLa cells were transfected with plasmids expressing GFP or GFP-CP77, treated with TNF- α and lysates, were harvested at the indicated time points (Fig. 3A). In the presence of either GFP or GFP-CP77, phosphorylation of I κ B α was readily detected in TNF- α treated cells at 3 to 10 min after TNF- α treatment, suggesting that CP77 did not

FIG. 4. CP77 associates with Skp1 and Cullin-1 through the C-terminal F-box region. (A) Alignment of the CP77 F-box-like motif with the F-boxes of Skp2 and a typical poxvirus ankyrin repeat protein, ORF virus 008 (OV008). Conserved F-box residues are marked in blue (A, V, L, I, and M), brown (P), orange (D, E), yellow (C), green (H, K, and R), pink (W, F, and Y), and gray (S, T, N, and Q). The coordinates of the first listed amino acid are shown in brackets to the left of each sequence, the last amino acid of the F-box of Skp2 is indicated to the right, and the end of each protein is indicated with an asterisk (*). The positions of the three α -helices of the F-box of Skp2 are indicated as gray bars above. (B) CP77 contains an F-box domain and associates with endogenous SCF complexes. Cells were transfected with the plasmids indicated at the top of the figure, lysed with 1% NP-40, and then immunoprecipitated (IP) with anti-Flag-agarose and analyzed by SDS-PAGE and immunoblotting. The sample type (IP or cell lysate control) is indicated to the left. The antibodies used are indicated to the right. (C) HeLa cells were infected with VV-hr-GFP, VV-hr-GFP-CP77, or VV-hr-CP77 Δ F and harvested at 6 h p.i. Lysates were immunoprecipitated with anti-GFP and analyzed with anti-Skp1 (1:1,000) and anti-Cullin-1 (1:1,000) antibodies.

block phosphorylation of I κ B α by IKK. The level of total I κ B α in GFP-CP77-expressing cells appeared to be greater than that in GFP-expressing cells, although it was not totally stabilized. Next, we infected HeLa cells with VV-hr-GFP or VV-hr-GFP-CP77 for 2 h, treated these cells with TNF- α for 15 min, and harvested cells for immunoblot analyses (Fig. 3B). In some cases, cytosine arabinoside (araC) was added to cells to arrest virus life cycle at the early phase, as evident by blocking of

FIG. 5. (A) The C-terminal F box domain is required for CP77 to suppress NF- κ B signaling. HeLa cells were transfected with plasmids expressing GFP, GFP-CP77, or GFP-CP77 Δ F and either mock treated (left three rows) or treated with TNF- α for 20 min (right three rows) and

intermediate G8 protein expression. Treatment of mock-infected cells with TNF- α resulted in total degradation of I κ B α . Treatment of TNF- α to cells infected with VV-hr-GFP also triggered degradation of I κ B α with residual phosphorylated I κ B α detected in cells. Most importantly, phosphorylated and total I κ B α were stabilized by GFP-CP77 in the infected cells after TNF- α treatment, suggesting that CP77 did not suppress IKK activation and that CP77 blocked NF- κ B activation at the stage of degradation of phosphorylated I κ B α . Addition of araC to the cell cultures had no effect on the results, supporting a direct role of CP77 in blocking NF- κ B activation.

CP77 protein contains a C-terminal F-box motif that is truncated but mediates binding to the SCF E3 ubiquitin ligase complex. Ubiquitination has been shown to be a vital step in the course of NF- κ B activation. Phosphorylated I κ B α is recognized by the β -TrCP F-box protein and targeted to a specific cellular E3 ligase: SCF1 (Skp1-Cullin1-F box protein). I κ B α is polyubiquitinated by SCF ^{β -TrCP} and then degraded by the 26S proteasome, releasing NF- κ B (7, 9). Several poxvirus proteins have been shown to contain a C-terminal F-box-like domain functional in binding SCF (32, 38, 54, 56). When we examined the sequence of CP77, an F-box-like motif was identified at the C terminus (Fig. 4A). Although unusually short, we hypothesized that CP77 utilizes this F-box-like domain to interact with SCF E3 ubiquitin ligase. To test this hypothesis, a Flag-tagged full-length CP77 or a CP77 mutant that deleted the F-box-like region, Flag-CP77 Δ F, was transfected into 293T cells for coimmunoprecipitation experiments. Endogenous SCF components, Skp1 and Cullin-1, coprecipitated with full-length Flag-CP77, but not with Flag-CP77 Δ F or Flag only (Fig. 4B). The F-box-like motif of CP77 was therefore necessary for binding SCF.

We then generated a recombinant virus that expressed the GFP-CP77 Δ F protein in the infected cells. When lysates were prepared from HeLa cells infected with VV-hr-GFP, VV-hr-GFP-CP77, and VV-hr-GFP-CP77 Δ F for coimmunoprecipitation with anti-GFP antibodies, binding of GFP-CP77, but not GFP-CP77 Δ F or GFP alone, with Cullin-1 and Skp1 was detected (Fig. 4C). We thus conclude that CP77 binds to the SCF complex through its C-terminal F-box-like motif.

CP77 Δ F protein fails to suppress NF- κ B nuclear translocation. To address whether the F-box-like domain of CP77 was involved in NF- κ B suppression, we transfected GFP, GFP-CP77, or GFP-CP77 Δ F into HeLa cells; treated the cells with TNF- α as described above; and monitored nuclear translocation of the endogenous p65 subunit of NF- κ B by immunofluorescence analyses. The images (Fig. 5A) and quantification data (Fig. 5B) showed that the CP77 Δ F mutant protein lost the ability to suppress NF- κ B/p65 nuclear translocation, suggesting that the F-box-like region of CP77 is essential for attenuation of the NF- κ B signaling induced by TNF- α . We next tested whether the F-box-like domain of CP77 was sufficient to block

NF- κ B activation. HeLa cells expressing either mCherry protein or mCherry fused with the F-box-like sequence of CP77, named F-box-mCherry, were treated with TNF- α , and the intracellular location of NF- κ B/p65 was determined by immunofluorescence. Expression of either mCherry protein or F-box-mCherry fusion protein did not inhibit the nuclear translocation of NF- κ B/p65 after TNF- α treatment (Fig. 5C and quantified in Fig. 5D), indicating that the C-terminal F-box-like domain of CP77 alone is not sufficient to block NF- κ B activation and that other regions of CP77 also participate in blocking NF- κ B nuclear translocation.

CP77 associates with NF- κ B through the N-terminal region in vitro. One of the most notable features of CP77 is the presence of nine ankyrin repeats throughout most of the polypeptide (Fig. 6A). Since cellular I κ B protein binds to p65 subunit of NF- κ B through its six ankyrin repeats (31), we postulated that CP77 may mimic this structural feature of I κ B to bind to p65. We therefore generated GST-CP77 fusion proteins that contained different numbers of CP77 ankyrin repeats for in vitro pull-down analyses (Fig. 6A). HeLa cells were treated with TNF- α for different times and then lysed. These cell lysates were incubated with purified GST or GST-CP77 ankyrin deletion proteins for GST-pull-down analyses. The GST-CP77 protein pulled down p65, but control GST did not (Fig. 6B). Moreover, GST-CP99(1-352) pulled down p65 two to three times better than the full-length GST-CP77 did (first row in Fig. 6B) since comparable amounts of GST fusion proteins were used in the assays (second row in Fig. 6B). Further deletions from aa 1 to 352 to aa 1 to 234 eliminated almost all (~94%) of the ability of CP77 to pull down p65 in all time points. These results showed that the N-terminal region from aa 235 to 352 is necessary for binding to p65 and that CP77 (aa 1 to 352) is sufficient to bind to p65 in vitro. It is worth noting that, although CP77 can pull down p65 from mock-treated cells (M in Fig. 6B), an enhanced interaction (24 times greater) between CP77 and p65 in cell lysates was observed at 20 min after TNF- α stimulation. The timing of the enhanced binding of CP77 to p65 correlated with the kinetics of proteasomal degradation of the phosphorylated form of I κ B α by TNF- α (see Fig. 3A). To test whether I κ B α degradation modulates CP77 binding to p65, we pretreated HeLa cells with the proteasome inhibitor MG132 to prevent degradation of the phosphorylated I κ B (Fig. 6C) and prepared lysates from mock-treated or TNF- α treated cells for GST-pull-down analysis (34). Alternatively, we ectopically expressed a Flag-tagged I κ B α S32/36A mutant that is not degraded in TNF- α -treated cells (Fig. 6C) (48). The enhanced interaction between CP77 and p65 (4.8-fold) in GST-pull-down assays (Fig. 6D) was completely eliminated by I κ B α S32/36A mutant (0.5-fold) or partially reduced by MG132 treatment (2.5-fold), supporting the hypothesis that proteasomal degradation of I κ B α enhanced CP77 binding to the p65 subunit of NF- κ B. Together,

then fixed and immunostained for NF- κ B/p65. (B) Quantification charts are shown for each set of images (>100 cells per slide) in panel A. The y axis represents the percentage of cells containing stronger nuclear staining of p65 than that in cytoplasm. (C) The C-terminal F box domain of CP77 was not sufficient to suppress NF- κ B signaling. HeLa cells were transfected with plasmid expressing either mCherry or mCherry fused with the CP77 C-terminal F-box 13-aa sequence and processed as described in panel A. The white areas represent staining of p65. (D) Quantification of cell images in panel C is shown (>100 cells per slide).

FIG. 6. CP77 associates with NF-κB/p65 through the N-terminal region from aa 1 to 352 in vitro. (A) Diagram of GST-CP77 deletion constructs. The orange squares with the central numbers represent the orders of ankyrin repeats, and the top numbers represent the amino acid residues of each ankyrin repeat. (B) GST-pull-down analyses with control GST and GST-CP77 deletion proteins shown in panel A. HeLa cells were either mock treated (M) or stimulated with TNF-α and harvested at 0, 20, 40, and 60 min posttreatment, and lysates were prepared for GST-pull-down analyses. Lanes P, GST or GST fusion protein bound to glutathione beads without cell lysates. The samples were divided into two parts and separated by SDS-PAGE. One part of each sample was transferred for immunoblot analyses with anti-p65 antibody (the first and the third rows in panel B), and the other part of each sample was treated with Coomassie blue to stain GST fusion proteins (the second and fourth rows in panel B). (C) Expression of Flag-tagged IκBα^{S32/36A} in HeLa cells. HeLa cells were transfected with a construct expressing a Flag-tagged IκBα^{S32/36A}, or pretreated cells were transfected with 20 μM MG132 for 2 h to block degradation of IκBα, followed by stimulation with 20 ng of TNF-α/ml for 20 min

these results suggest that CP77 binds to p65 after NF-κB dissociates from IκBα.

The possibility of a direct interaction between p65 and CP77 was also studied by SPR. In this in vitro assay, NF-κB subunit p65 was first immobilized on the sensor chip via amine groups as described in Materials and Methods. A solution of purified GST, GST-CP77(1-352), or GST-CP77, as shown in Fig. 7A, was injected at different protein concentrations [10, 5, and 2.5 μM for GST and 10, 5, 2.5, 1.25, 0.625, and 0.3125 μM for GST-CP77(1-352) and GST-CP77] to monitor its interaction with p65. As shown in Fig. 7B, GST showed very little binding to p65. In contrast, the interaction between p65 and GST-CP77(1-352) (Fig. 7C) displayed a typical association/dissociation binding curve with simulations using a 1:1 binding model. CP77(1-352) bound to p65 much better than the full-length CP77 did, with an in vitro binding affinity constant of 1.01×10^{-7} M for CP77(1-352) and p65. For full-length CP77, a concentration-dependent association to p65 was detected under the same experimental condition (Fig. 7D), however, the binding was weaker than that of CP77(1-352), and no binding constant could be deduced from curve fitting analysis of the sensorgrams. The slow dissociation curves looked similar to what was reported for IκBα interaction with p65/p50 (4) and could be due to protein oligomerization or conformational changes (39) at the postbinding stage.

The C-terminal F-box region of CP77 protein is dispensable for the host range function in restrictive cells. CHO-K1, RK13 and HeLa cells are restrictive to the host range mutant VV-hr-GFP that needs CP77 to act as the host range protein in these three cells. Previously, we showed that CP77(1-352) supported VV-hr mutant virus growth in CHO-K1 cells (27); however, only full-length CP77, but not CP77(1-352), supported VV-hr mutant growth in RK13 and HeLa cells (Fig. 8A). These results showed that CP77 suppresses NF-κB activation in HeLa cells. We wanted to test whether NF-κB suppression contributes to the host range function of CP77 protein in RK13 and HeLa cells. To test this hypothesis, we monitored the ability of the recombinant virus VV-hr-GFP-CP77ΔF to grow in restrictive CHO-K1, RK13, and HeLa cells. Cells were infected with VV-hr-GFP, VV-hr-GFP-CP77, or VV-hr-GFP-CP77ΔF at an MOI of 5 PFU per cell and harvested at 24 h p.i. As shown in Fig. 8B, all three viruses grew well in permissive BHK21 cells, while in restrictive CHO-K1, RK13, and HeLa cells the VV-hr-GFP-CP77ΔF grew just as well as VV-hr-GFP-CP77, showing that the F-box region of CP77 is not important for its host range function in all three cell types. We then performed the reverse experiments to determine whether knocking down NF-κB subunit p65 level in HeLa cells could relieve host restriction on growth of VV-hr-GFP. As shown in

before cell harvest for immunoblot analyses with anti-p65 antibody, anti-IκBα antibody, or anti-Flag antibody. (D) Blocking degradation of IκBα reduced CP77 binding to NF-κB lysates in GST-pull-down analyses. Lysates prepared as in panel C were used in GST-pull-down analyses with GST or GST-CP77 as described in Materials and Methods, with one half of the samples separated by SDS-10% PAGE and analyzed by immunoblots with anti-p65 antibody and the other half of the samples stained with Coomassie blue to reveal the GST and GST-CP77 proteins used in the pull-down analyses.

Fig. 9A, Si-p65, but not Si-CypB, significantly reduced >90% of the p65 protein level in HeLa cells; however, growth of VV-hr-GFP remained restricted and no intermediate G8 protein was detected in p65-knockdown HeLa cells, similar to the control and Si-CypB cells (Fig. 9B). Consistently, ectopically expressed I κ B α S32/36A, known to inhibit NF- κ B activation, did not rescue growth of VV-hr-GFP in HeLa cells (data not shown). We thus concluded that CP77's ability to suppress NF- κ B activation is independent from its host range function.

DISCUSSION

NF- κ B activation leads to transcription of cellular genes important for innate immune responses (24), and many viruses, including poxviruses, encode viral proteins to suppress this signaling pathway. In the present study, we demonstrated that CP77 suppresses NF- κ B activation induced by either TNF- α or IL-1. CP77 did not appear to block IKK activation; instead, CP77 binding to the SCF complex through the C-terminal F-box-like region, and this interaction is necessary to block NF- κ B nuclear translocation. This F-box-like domain of CP77 is shorter than most other poxvirus F-box motifs, spanning only one of the three F-box α -helices present in cellular F-box proteins, demonstrating the minimum requirement for interaction with the SCF complex.

In addition to the C-terminal region of CP77, the deletion analyses revealed that the N-terminal six-ankyrin region of CP77 as the binding site of NF- κ B. First, CP77(1-352) containing the N-terminal six ankyrin repeats bound to cellular NF- κ B, as shown by GST pull-down. Furthermore, SPR analyses revealed that this N-terminal region of CP77 directly bound to NF- κ B in vitro. It is interesting that in both binding assays the CP77(1-352) bound to NF- κ B better than full-length CP77 did, suggesting that the C-terminal region of CP77 may play a regulatory role in vivo. It is also important to point out that the binding affinity of CP77(1-352) with NF- κ B/p65 ($\sim 10^{-7}$ M) is weaker than that reported of I κ B α with NF- κ B (10^{-9} to 10^{-11} M) (4, 45). This difference in binding affinity may explain why we failed to observe CP77-p65 interaction in transfected or virus-infected cells using coimmunoprecipitation analyses, despite repeated efforts (data not shown). Or it could be that CP77 tethers with SCF complex while binding to NF-

FIG. 7. (A) Purified recombinant GST fusion proteins. GST, GST-CP77(1-352), and GST-CP77 were purified as described in Materials and Methods and subjected to SDS-12% PAGE (for GST) or SDS-10% PAGE [for GST-CP77(1-352) and GST-CP77]. (B to D) SPR sensorgrams of the interaction of NF- κ B/p65 with GST protein (B), GST-CP77(1-352) (C), or GST-CP77 (D). NF- κ B/p65 was immobilized on a CM5 sensor chip surface via amine groups as described in Materials and Methods, and the binding to GST fusion proteins were analyzed. Six different concentrations (10, 5, 2.5, 1.25, 0.625, and 0.3125 μ M) of GST-CP77(1-352) and GST-CP77 fusion proteins or three concentrations (10, 5, and 2.5 μ M) of GST control protein in HSTBS-EP buffer at a flow rate of 20 μ l/min were used to determine the association/dissociation kinetic binding constants. A k_{on} of 1.91×10^3 M $^{-1}$ s $^{-1}$, a k_{off} of 1.89×10^{-4} s $^{-1}$, and a $K_A (= k_{off}/k_{on})$ of 1.01×10^7 M $^{-1}$ were determined for the NF- κ B/p65 and CP99(1-352) interaction. The binding constants were derived from a 1:1 Langmuir binding model.

FIG. 8. (A) Growth of VV-hr-GFP-CP77(1-352) in restrictive cells. CHO-K1, RK13, and HeLa cells were infected with VV-hr-GFP, VV-hr-GFP-CP77, and VV-hr-GFP-CP99(1-352) at an MOI of 5 PFU/cell and harvested at 0 and 24 h p.i. for virus titer determination in permissive BHK21 cells. Virus growth was determined by the virus titer at 24 h p.i. divided by the virus titer at 0 h p.i. (B) VV-hr-GFP-CP77ΔF grew in restricted cells. BHK21, HeLa, RK13, and CHO-K1 cells were infected with VV-hr-GFP, VV-hr-GFP-CP77, and VV-hr-GFP-CP77ΔF at an MOI of 5 FPU/cell and harvested at 0 and 24 h p.i. as described above, and titers were determined by plaque assays on permissive BHK21 cells as described previously (28).

κB, making it technically difficult to coimmunoprecipitate such a complex.

During the experiments, we noticed that CP77 pulled down more NF-κB from cells treated with TNF-α than control cells, suggesting that signal-induced degradation of IκBα allows NF-κB more accessible to CP77 binding. This conclusion is further supported by the pull-down experiments with lysates prepared from cells treated with MG132 or transfected with

FIG. 9. Knocking down NF-κB expression in HeLa cells did not relieve host restriction of VV-hr-GFP in HeLa cells. (A) Expression of NF-κB subunit p65 in siRNA treated HeLa cells. Immunoblot analyses of cell lysates from HeLa cells that were transfected with no siRNA (control), siRNA targeting CypB (Si-CypB), or siRNA targeting NF-κB p65 subunit (Si-p65) using anti-p65 or anti-CypB antibodies. (B) Viral intermediate G8 protein was not expressed in p65-knock-down cells. Control, Si-CypB, and Si-p65 HeLa cells were either mock infected or infected with VV-hr-GFP and VV-hr-GFP-CP77 at an MOI of 5 PFU/cell. The lysates were harvested at 6 h p.i. and analyzed by immunoblotting with anti-G8 antibody (1:1,000).

IκBα S32/36A mutant, i.e., interaction between CP77 and NF-κB was reduced when the latter was associated with non-degradable IκBα. Taken together, our data are most consistent with the model that CP77 functions as a bridging molecule between SCF ligase complex and free NF-κB. Therefore, when signal-induced phosphorylation and degradation of IκBα occurs in TNF-α-treated cells, the N-terminal region of CP77 serves as a “surrogate” IκB-like domain, while the C-terminal region bound to the SCF complex, preventing NF-κB from being released into cell nucleus. Alternatively, CP77 may function as a typical F-box protein that recruits NF-κB to the SCF complex for ubiquitination and subsequent degradation; however, we did not detect any increased ubiquitination or degradation of NF-κB in the presence of CP77 (data not shown). It is interesting that ASFV A238L was reported to be a viral protein that binds to free NF-κB. However, A238L has a very high homology with IκBα (~40%) and binds to free NF-κB without the need of binding to SCF complex, so the mode of CP77 action is not the same as that of the A238L protein.

Recent studies have revealed that several poxviruses contain

viral F-box proteins that interact with Skp1 and Cullin-1; the functional significance of such interactions has not been addressed (38, 54). One exception is the myxoma virus MT5 protein, which was shown to relieve cellular stress from cell cycle arrest through binding to the SCF complex in the nucleus (32). Moreover, whether these viral F-box proteins recruit SCF E3 ubiquitin ligases to degrade novel substrates is currently unknown. Our study here identified NF- κ B as the cellular substrate of viral F-box protein CP77, using the N-region ankyrins in substrate-specific binding, while the C-terminal F-box binds to the cellular SCF E3 ubiquitin ligase complex. Whether this dual binding mode is common to other viral ankyrin- and F-box containing proteins as predicted (38, 54) remains to be determined. We also cannot exclude the possibility that the F-box of CP77 differs from the majority of poxvirus F-boxes in its unusually truncated nature, and this may give rise to a variant mode of action.

Another focus of the present study was to elucidate the relationship of NF- κ B activity with CP77-regulated host restriction in HeLa cells. Using EMSA analyses, we observed that NF- κ B activation during virus infections depended not only on viruses but also on cell types. Moreover, we observed an inverse correlation of NF- κ B activity with virus growth in restrictive HeLa cells. Since only full-length CP77, but not CP77(1-352), possess the host range function in restrictive HeLa cells we tested whether the ability of CP77 binding to SCF complex is critical for its host range function in HeLa cells. The deletion protein CP77 Δ F was still effective as a viral host range protein in CHO-K1, RK13, and HeLa cells, clearly demonstrating that the ability of CP77 to bind to the SCF complex was independent of host restriction. Consistently, specific knockdown of NF- κ B in HeLa cells did not relieve host restriction of VV-hr-GFP, supporting that NF- κ B activation did not cause the suppression of virus growth in HeLa cells.

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