Mutational Analysis of Vaccinia Virus E3 Protein: the Biological Functions Do Not Correlate with Its Biochemical Capacity To Bind Double-Stranded RNA

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ABSTRACT

Vaccinia E3 protein has the biochemical capacity of binding to double-stranded RNA (dsRNA). The best characterized biological functions of the E3 protein include its host range function, suppression of cytokine expression, and inhibition of interferon (IFN)-induced antiviral activity. Currently, the role of the dsRNA binding capacity in the biological functions of the E3 protein is not clear. To further understand the mechanism of the E3 protein biological functions, we performed alanine scanning of the entire dsRNA binding domain of the E3 protein to examine the link between its biochemical capacity of dsRNA binding and biological functions. Of the 115 mutants examined, 20 were defective in dsRNA binding. Although the majority of the mutants defective in dsRNA binding also showed defective replication in HeLa cells, nine mutants (I105A, Y125A, E138A, F148A, F159A, K171A, L182A, L183A, and I187/188A) retained the host range function to various degrees. Further examination of a set of representative E3L mutants showed that residues essential for dsRNA binding are not essential for the biological functions of E3 protein, such as inhibition of protein kinase R (PKR) activation, suppression of cytokine expression, and apoptosis. Thus, data described in this communication strongly indicate the E3 protein performs its biological functions via a novel mechanism which does not correlate with its dsRNA binding activity.

IMPORTANCE

dsRNAs produced during virus replication are important pathogen-associated molecular patterns (PAMPs) for inducing antiviral immune responses. One of the strategies used by many viruses to counteract such antiviral immune responses is achieved by producing dsRNA binding proteins, such as poxvirus E3 family proteins, influenza virus NS1, and Ebola virus V35 proteins. The most widely accepted model for the biological functions of this class of viral dsRNA binding proteins is that they bind to and sequester viral dsRNA PAMPs; thus, they suppress the related antiviral immune responses. However, no direct experimental data confirm such a model. In this study of vaccinia E3 protein, we found that the biological functions of the E3 protein are not necessarily linked to its biochemical capacity of dsRNA binding. Thus, our data strongly point to a new concept of virus modulation of cellular antiviral responses triggered by dsRNA PAMPs.

Poxviridae is a diverse family of large dsDNA viruses (130 to 300 kb) which replicate exclusively in the cytoplasm of infected cells and exhibit a varied host range (1). Some members are specific to a single host, such as variola virus, the causative agent of smallpox, while others, such as vaccinia virus, display a broad host range. Poxviruses encode a number of immunomodulatory proteins, e.g., host range proteins (2). Orthopoxviruses encode a number of host range genes, such as SPI-1, K1L, C7L, p28/N1R, B5R, K3L, and E3L, the alteration or deletion of which restricts the virus replication in specific cell lines (3). These host range genes display varied restrictions and biological functions. For example, SPI-1 is required for vaccinia virus replication in PK15 and A549 cells and in mice (4, 5). K1L, an ankyrin repeat-containing protein which inhibits I κ B- α degradation and interferon (IFN)-stimulated effectors, is required for replication in RK13 cells (6). A double deletion of C7L and K1L leads to restriction of the virus replication in PK1, RK13, and multiple human cell lines, while C7L deletion alone suppresses apoptosis and limits replication in hamster Dede cells (7). P28/N1R, an E3-RING finger ubiquitin ligase involved in protein degradation and apoptosis suppression, is required for replication in mouse macrophage (8, 9), while B5R, a membrane glycoprotein, activates Src, allowing growth in VERO, CEF, PK15, and quail (QT-6) cells (10).

The most studied vaccinia host range gene is E3L. E3 protein is

expressed early in the virus replication cycle and is known to suppress multiple innate immune pathways (3, 11, 12). E3 protein possesses biochemical capacities to bind Z-form DNA via the Z-DNA binding domain (ZBD) toward the N terminus and to bind to dsRNA through the C-terminal dsRNA binding domain (DRBD) (13, 14). Since the best characterized biological functions of vaccinia virus E3 protein, such as host range function and inhibition of innate immune responses (e.g., cytokine expression, apoptosis and interferon-induced antiviral activity), all are mediated primarily via the DRBD region of E3 protein (15–17), generally it has been believed that E3 protein functions through seques-

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tering virus-produced dsRNA; thus, it inhibits the related cellular antiviral responses. To date, however, no direct experimental data have shown that the E3 protein binds to dsRNAs in the virus-infected cells. Moreover, we have previously demonstrated that a poxvirus E3L ortholog, such as sheppox virus 034, does not supplement the biological functions of the E3 protein, although it binds dsRNA (18). It also has been reported that the ZBD plays a role in inhibition of interferon induction (19) and full pathogenesis *in vivo* in mice (14, 20, 21). Thus, it is inconclusive what role the biochemical capacity of dsRNA binding plays in the biological functions of the E3 protein.

In this study, we aim to further define the link between the biochemical capability of dsRNA binding and the biological functions of vaccinia E3 protein. Alanine scanning or valine substitution (in the case of alanine residues) mutagenesis was performed on all residues of the DRBD of vaccinia E3 protein. The mutants were screened for their dsRNA binding and host range function in HeLa cells. The biological functions of eight representative E3 mutants, such as inhibition of protein kinase R (PKR) activation, interferon resistance, suppression of cytokine expression, and apoptosis, were further examined. It was found that the dsRNA binding capacity of vaccinia E3 protein is not essential for its biological functions.

MATERIALS AND METHODS

Cell culture and viruses. HeLa, Huh7R, and BHK21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, and glutamine (Gibco Life Technologies), at 37°C in 5% CO₂. Vaccinia Copenhagen and Western Reserve (WR) strain-based E3L deletion mutant (vvΔE3L) viruses and their revertant (vvΔE3L/Rev) viruses were constructed as previously described (16, 22).

Antibodies and reagents. The antibody for total PKR was purchased from Cell Signaling Technologies. Phospho-specific PKR (Thr446) was from Epitomics. Total eIF2α and phosphorylated eIF2α were obtained from Biosource. β-Actin antibody was purchased from Sigma (clone AC-15; A5441). Polyclonal E3L antibodies were made by GenScript (New Jersey, USA) in rabbits against peptide 1 (residues 161 to 174, KADGKSKRDAKNNNA) and 2 (residues 88 to 101, VSREKSMREDHKSF), respectively. Rabbit glutathione S-transferase (GST) antibody was purchased from Sigma (G7781). All transfections were performed using Attractant transfection reagent, purchased from Qiagen.

Mutagenesis, cloning, and expression of vaccinia E3L. Every amino acid residue in the predicted dsRNA binding domain (from residues 80 to 190) of vaccinia E3 protein (based on the Copenhagen strain) were mutated either by alanine or valine (if the original residue was alanine) substitution. The mutation was performed by GenScript (New Jersey, USA). All mutants were cloned into a poxvirus expression and recombination vector, pJS5, which bears flanking regions for the vaccinia virus thymidine kinase (TK) locus to allow homologous recombination (23). The truncated E3L mutants, a dsRNA binding domain containing residues 80 to 190 (E3L/C111) and the mutant with the C-terminal 25 amino acid residues deleted (E3L/ΔC25), were described previously (16). The E3L mutants D103A, I110A, N123A, C126A, F135A, E138A, F159A, and L183A (with the authentic E3L promoter) were cloned into the pΔE3L vector between the BamHI and SmaI sites bearing a flanking region to allow homologous recombination into the E3L locus of vaccinia virus WR strain, while E3wt (with the authentic E3L promoter) was inserted back into vvΔE3L as previously described (16). N-terminal GST fusions of wild-type E3L and the mutants E3L/C111, E3L/ΔC25, I110A, N123A, C126A, F135A, and F159A were generated using the Gateway cloning system and pDEST27 destination vector (Invitrogen Life Technologies). The E3L gene and its mutants with GST fused at the N terminus were

subcloned into pJS5 for use in PKR pulldown assays and generation of recombinant viruses, respectively.

Western blotting. Cell monolayers were collected in Laemmli buffer following aspiration of media and washing with phosphate-buffered saline (PBS). The lysates were homogenized using QIAshredder columns (Qiagen). Western blotting was performed using Criterion XT 4 to 12% Bis-Tris gels and morpholinepropanesulfonic acid (MOPS) XT running buffer (Bio-Rad Laboratories Inc.), and the protein samples were transferred to Hybond-C Extra nitrocellulose membranes (Amersham Bioscience). Membranes were developed with Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore) or Western Lightning ECL (PerkinElmer Inc.).

Conjugation of poly(I-C) beads. Poly(I) (P4154; Sigma), poly(C) (P4903; Sigma), and poly(I-C) (P1530; Sigma) were bound to CNBr-activated Sepharose 4B beads (17-0430-01; GE Healthcare Life Sciences). One gram of Sepharose beads was washed 5 times in 40 ml of 1 mM HCl prior to the coupling reaction with poly(I-C). Two ml of the stock poly(I-C) solution prepared in coupling buffer (0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl) at 10 mg/ml was mixed with 1g of the Sepharose 4B beads in a total volume of 4 ml in coupling buffer and rotated end over end for 2 h at room temperature. Unconjugated poly(I-C) was removed through 5 washes in 5 volumes of coupling buffer, followed by centrifugation at 1,000 × g for 5 min. Active sites of the Sepharose beads were blocked by incubation in 0.1 M Tris-HCl, pH 8.0, for 2 h at room temperature. Three cycles of alternating low-pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0) and high-pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0) buffers were used to wash the beads. Following the final wash, beads were stored as a 50% slurry at 4°C in nondenaturing lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Triton-X, 2 mM EDTA) until use.

Poly(I-C) pulldown assays. The procedure of poly(I-C) pulldown was based on the Abcam immunoprecipitation protocol. Briefly, BHK21 cells in 6-well plates at 70 to 80% confluence were infected with vvΔE3L virus at a multiplicity of infection (MOI) of 0.2 in a 350-μl inoculum. Following 1 h of incubation at 37°C, virus was removed and 2 ml of DMEM containing 2% FBS added to each well. Monolayers then were transfected with 1 μg of pJS5 constructs bearing an individual E3L mutant to be inserted into the TK locus via homologous recombination and were incubated overnight. The cells were lysed in 300 μl of nondenaturing lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA) on ice. Pulldowns were performed following the removal of cellular debris by a 10-min centrifugation at 5,400 × g. End-over-end rotation was performed at room temperature following addition of 40 μl of poly(I-C) bead slurry to each sample. Following binding of poly(I-C), 3 washes with 350 μl nondenaturing lysis buffer were performed, and proteins were released from the poly(I-C) beads and denatured by incubation at 95°C for 10 min in Laemmli buffer. Supernatant was collected following 2 min of centrifugation at 5,000 × g and subjected to Western blotting.

Host range screening assay in HeLa cells. BHK21 cells were infected with vvΔE3L and transfected with pJS vector bearing E3L mutant genes as in the poly(I-C) pulldown assays. Virus was collected at 24 h postinfection (hpi) and passaged 3 rounds in HeLa cells. Plaque formation as determined by expression of green fluorescent protein (GFP) was visualized with fluorescence microscopy (Axiovert 200M; Carl Zeiss). If a green virus plaque was observed in the HeLa cell monolayer, the mutant E3 protein retained the host range function; otherwise, the host range function was considered defective due to the mutation and subjected to further validation and analysis.

Interferon sensitivity assays. IFN-β was purchased from PBL Bio-medical Laboratories. Assays were performed in Huh7 cells pretreated overnight with 1,000 U/ml IFN-β, infected at an MOI of 1, and collected at 5 hpi and 24 hpi as previously described (22). The virus titration was performed in BHK21 cells as described previously.

Quantitative real-time PCR. Confluent HeLa monolayers in 6-well plates were infected with virus at an MOI of 10 in a 350-μl inoculum. Following 1 h of incubation at 37°C, 2 ml of DMEM containing 2% FBS

FIG 1 Experiment outline. (A) Amino acid sequence of vaccinia E3 protein. The mutated region is in italics and underlined. (B) Initial screening of poly(I-C) binding and host range function of E3L mutants cloned into the pJ55 vector and transfected into vvΔE3L-infected BHK21 cells. The lysate was collected 24 h posttransfection/infection and subjected to poly(I-C) bead pulldown and passage in HeLa cells. (C) Validation of the E3 mutants showing phenotypic changes in the initial screening of dsRNA binding and host range in HeLa cells by construction of recombinants expressing the mutant in the E3L locus under the authentic E3L promoter.

was added to each well. Cell samples were collected at 12 hpi, and the total RNA was extracted using RNeasy minikit (Qiagen). Residual DNA contamination in the RNA sample was removed using Turbo DNase (Ambion Biosystems). Reverse transcription was performed using a QuantiTect reverse transcription kit (Qiagen). Real-time PCR was performed in 96-well PCR plates on a StepOnePlus real-time PCR system using TaqMan gene expression master mix (Applied Biosystems) in triplicate. Primers for human IFN- β , interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF- α) have been described previously (16). The data were analyzed with StepOne software, v2.0 (Applied Biosystems).

GST pulldown assays. HeLa monolayers at 80% confluence were transfected with 1,650 ng of pJ55 plasmids expressing GST, GST-E3L, GST-E3C111, GST-E3ΔC25, GST-E3I110A, GST-E3N123A, GST-E3C126A, GST-E3F135A, and GST-E3F159A 2 h prior to infection at an MOI of 2 with the vvΔE3L-Rev virus. Following 1 h of incubation at 37°C, 6 ml of DMEM containing 2% FBS was added to each dish. Cell samples were collected at 10 hpi and lysed with nondenaturing lysis buffer as described for the sample preparation for the poly(I-C) pulldown assay. Immobilized glutathione beads (Pierce) were washed 3 times by addition of 600 μ l of nondenaturing lysis buffer to 400 μ l of glutathione beads, vortexed, and collected by centrifugation at $5,000 \times g$ for 2 min. After a final wash, glutathione beads were resuspended in 700 μ l of nondenaturing lysis buffer. Forty μ l of cell lysate in nondenaturing lysis buffer was incubated with 100 μ l of glutathione beads at room temperature with end-over-end rotation for 2 h. The pellets were washed 3 times in 300 μ l lysis buffer, and final pellets were treated with 200 μ l of Laemmli buffer and subjected to Western blotting.

RESULTS

Experiment outline. Previously, we have shown that the 110-amino-acid residues at the C terminus of the E3 protein (amino acids 81 to 190; italicized and underlined in Fig. 1A), which covers the entire predicted dsRNA binding domain (amino acids 118 to 182), can fully complement the functions of the full-length E3 in inhibiting cytokine expression, apoptosis, and interferon-induced antiviral activity (16). Therefore, in this study we mutated this region of the E3 protein by alanine or valine (to replace alanine residues) substitution to define the link between the dsRNA binding capacity and the biological functions of the E3 protein. A total of 111 single-point mutations and 4 additional double-point mutations (I84A/I85A, D86A/D87A, L182A/L183A, and I187A/I188A) were made. For the initial screening of the dsRNA binding and host range function, the mutated E3L open reading frames (ORFs) were cloned into the poxvirus expression vector pJ55 under the control of the synthetic early and late promoter (23), and the proteins were transiently expressed in the context of infection with a vvΔE3L virus (22) in BHK21 cells. The same sample was divided into two aliquots: one for dsRNA binding assay and another for testing replication in HeLa cells (Fig. 1B). If a phenotypic change was observed for an E3L mutant, loss of dsRNA binding and/or no rescue of vvΔE3L replication in HeLa cells, a stable recombinant virus expressing the mutant protein driven by the

FIG 2 Initial screening E3 mutants for dsRNA binding and host range. (A) A representative set of results showing poly(I-C) bead pulldown of E3 mutant proteins. The complete set of the poly(I-C) bead pulldown results is not shown and is available upon request. The protein in the cell lysate (top) and the protein bound to the poly(I-C) beads (lower) were detected using vaccinia E3 peptide antibodies 1 and 2 (see Materials and Methods). The pulldown experiments were performed at least three times by three individuals. (B) Rescue of vvΔE3L replication in HeLa cells by E3 mutant proteins. HeLa cells were infected with the recombinant viruses expressing mutant E3 proteins collected after 3 passages in HeLa cells. The image was taken at 24 h postinfection under UV light with a Carl Zeiss Axiovert 200M at $\times 5$ magnification. The complete set of data was not shown and can be provided upon request.

authentic E3L promoter in the E3L locus will be constructed for further validation and analysis (Fig. 1C).

Amino acid residues essential for dsRNA binding. The dsRNA binding activity of vaccinia E3 protein was examined mostly using the synthetic dsRNA analog poly(I-C) (24, 25). In our laboratory, the specificity of the E3 protein binding to poly(I-C)-coated agarose beads has been demonstrated previously (18). In this study, the poly(I-C) bead pulldown assay was used to examine the dsRNA binding of vaccinia E3 mutant proteins. Thus, the dsRNA binding activity is defined as the E3 protein being able to be precipitated by the poly(I-C)-coated agarose beads. Due to the large size of the data, the complete set of dsRNA binding results was not shown and can be provided upon request. A representative set of the poly(I-C) pulldown results, as shown by Western blotting, is presented in Fig. 2A. It should be noted that the dsRNA binding assay used in this study is not quantitative. Thus, the binding phenotype was defined arbitrarily using conservative criteria. Only those mutants showing significantly reduced poly(I-C) binding (e.g., mutant E138A) (Fig. 2A) or no poly(I-C) binding (e.g., F159A) were defined as dsRNA binding defective. Other mutants which showed some reduced poly(I-C) binding (e.g., R157A) were arbitrarily defined as having normal dsRNA binding and would not be selected for further analysis, and such data should be interpreted with caution. Of the 115 E3 mutants examined, 20 (18 single-point mutants and two double-point mutants) demonstrated either no detectable or significantly decreased dsRNA binding activity, and the amino acid positions were scattered from positions 105 to 188 (Table 1). Eight of these essential residues (I105A, K109A, I110A, W113A, L183A, and

I187/I188A) for dsRNA binding are located outside the predicted dsRNA binding motif (amino acids 118 to 182). Single-point mutations of I187A and I188A had no effect on the dsRNA binding, while substitution of both Ile resulted in severely decreased dsRNA binding. With the exception of E138A, all important amino acid residues for dsRNA binding are highly conserved among the E3L orthologs from other poxvirus genera.

Amino acid residues essential for the host range function in HeLa cells. One of the characteristic phenotypes of vvΔE3L is its restricted host range, in that the replication of vvΔE3L virus is aborted in the restrictive cells (which is permissive for the wild-type virus), e.g., HeLa cells, at the intermediate protein translation step (26). For initial screening as described above (Fig. 1B), the E3L mutants were expressed using the pJ55 vector, a poxvirus expression and recombination vector for insertion into the non-essential TK locus of the vaccinia virus genome (23). To assess if E3 mutants retained the host range function, recombinant vaccinia vvΔE3L virus with an E3L mutant gene potentially inserted at the TK gene locus was selected by three rounds of passage in HeLa cells using the same materials collected for poly(I-C) binding. Replication of the viruses expressing E3L mutants in HeLa cells was monitored by visualization of green virus plaques under a UV microscope. A representative set of images for the virus replication is shown in Fig. 2B. (The complete set of the virus replication data is not shown and is available upon request.)

The E3L mutants showing changes in dsRNA binding and/or host range function in HeLa cells in the initial screening were validated further by the expression of those mutants in the E3L locus driven by an authentic E3L promoter (Fig. 1C). The expres-

TABLE 1 Amino acids of E3 protein critical for dsRNA binding and host range

Mutant	Poly(I-C) binding ^a	Host range ^b
K109A	—	—
I110A	—	—
W113A	—	—
I122A	—	—
R131A	—	—
I154A	—	—
K167A	—	—
R168A	—	—
A174V	—	—
A175V	—	—
L182/183A	—	—
I105A	—	±
Y125A	—	±
F148A	—	±
F159A	—	±
K171A	—	±
E138A	±	+
L182A	±	+
L183A	±	+
I187/188A	±	+
D103A	+	±
E124A	+	±

^a —, no E3 mutant protein was detected from poly(I-C) bead pulldown, even after extended exposure of the Western blot films; ±, only a faint band of E3 mutant protein was detected by Western blotting following poly(I-C) bead pulldown, e.g., the E138A mutant shown in Fig. 2A; +, significant amount of E3 mutant was precipitated by poly(I-C) beads.

^b —, the virus titer at 24 h postinfection is lower than the titer at 5 h postinfection; ±, the virus titer at 24 h postinfection is greater than the titer at 5 h postinfection, but the increase is not greater than 0.5 log₁₀; +, the virus titer at 24 h postinfection is at least 0.5 log₁₀ greater than the titer at 5 h postinfection.

sion of the mutant E3 protein was confirmed by Western blotting (Fig. 3A), and the mutation was confirmed by sequencing (data not shown). The replication of the recombinant virus expressing the mutant E3 protein in HeLa cells was determined using a high multiplicity of infection (MOI) of 10, and the result is shown in Fig. 3B. The results are summarized in Table 1. The host range function of the E3 mutants was arbitrarily defined as (i) the loss of the host range function (—) when the virus titer at 24 h postinfection was equal to or lower than the baseline titer as determined at 5 h postinfection; (ii) defective host range function (±) when the virus titer at 24 h postinfection was greater than the baseline virus titer at 5 h postinfection, but the increase is not greater than 0.5 log₁₀ PFU/ml; and (iii) normal host range function (+) when the virus titer at 24 h postinfection was at least 0.5 log₁₀ PFU/ml greater than the virus titer at 5 h postinfection.

Of the 16 E3 mutants, which have no detectable dsRNA binding activity, 5 mutants (I105A, Y125A, F148A, F159A, and K171A) retained certain degrees of host range function (although defective), while the other 11 have lost the host range function (Table 1). Four mutants (E138A, L182A, L183A, and I187/188A), which showed decreased dsRNA binding activity, performed normal host range functions in HeLa cells. Interestingly, the recombinant vaccinia viruses expressing the mutants D103A and E124A, which bound to dsRNA efficiently, showed defective replication in HeLa cells (±) based on the criteria defined in this study.

Since the primary objective of this study was to define the link between the dsRNA binding and the biological functions of the E3 protein, the mutants showing defective dsRNA binding but still retaining the host range functions are of particular interest. Therefore, the mutant F159A (no detectable dsRNA binding with defective host range function in a high MOI infection) and L183A (decreased dsRNA binding with normal host range function in a high MOI infection) were further examined for their replication in HeLa cells using a low MOI of 0.1. The result is shown in Fig. 3C. Compared to the wild-type control, the mutant L183A showed similar replication kinetics for the low MOI infection (Fig. 3B), in that most of the increase in the virus titer occurred in the first 24 h postinfection, and both viruses (the wild-type and L183A) grew to a comparable titer at 96 h postinfection. In contrast, the mutant F159A replicated slightly at 24 h, and the virus titer increased gradually over the time course. At 96 h postinfection, the gap in the titers of the mutant F159A and the wild type (and also the mutant L183A) became much closer than the time point of 24 h postinfection. The titers of mutant I110A and control virus vvΔE3L decreased slightly over the time course.

In summary, the E3L mutants with phenotypic changes in their dsRNA binding capacity and/or host range function in HeLa cells can be classified into four groups (Table 1): (i) complete loss of both dsRNA binding and host range function (e.g., I110A); (ii) complete loss of dsRNA binding/retained comprised host range function (e.g., F159A); (iii) defective dsRNA binding/retained normal host range function (e.g., E138A); and (iv) normal dsRNA binding/defective host range function (e.g., D103A).

Inhibition of IFN-mediated antiviral activity by E3L mutants. Based on the characterization of dsRNA binding and host range function described in the last section, a set of representative E3 mutants, including the mutant D103A (normal dsRNA binding/defective host range), I110A (loss of both dsRNA binding and host range), N123A/C126A/F135A (normal dsRNA binding and host range function), E138A/L183A (defective dsRNA binding/normal host range function), and F159A (loss of dsRNA binding/retained comprised host range function), was chosen to further examine their roles in interferon resistance, inhibition of cytokine expression, and inhibiting PKR activation and apoptosis. To facilitate the comparison of the phenotypes of the selected E3 mutants, their dsRNA binding and replication in HeLa cells (at a high MOI) are shown in Fig. 4A and B.

Previously, we reported that both type I and type II interferons can inhibit replication of vvΔE3L in Huh7 cells through inhibition of PKR (22). To investigate the effect of mutant E3 protein on interferon-induced antiviral activity, replication of the recombinant vaccinia viruses expressing the selected panel of E3 mutants was examined in IFN-β-treated and nontreated Huh7 cells. Similar to wild-type E3L, the mutants C126A (retained dsRNA binding and host range function in HeLa cells) and E138A (defective dsRNA binding and normal host range in HeLa cells) were completely resistant to IFN-β (Fig. 5). In contrast, the 2 mutants with no detectable dsRNA binding, I110A and F159A, were completely sensitive to IFN-β. The other mutants examined (D103A, W123A, and F135A, which retained normal dsRNA binding capacity) were slightly inhibited by IFN-β compared to wild-type E3 and the mutant E138A (defective dsRNA binding) recombinant viruses. Thus, the inhibition of type I IFN-induced antiviral activity by the E3 protein does not correlate with their dsRNA binding profiles.

FIG 3 Validation of host range function of the E3 mutants, which were defective in dsRNA binding and/or host range, as observed in the initial screening. The mutant E3L gene was inserted into the E3L locus as shown in Fig. 1C. (A) Expression of mutant proteins determined by Western blotting. (B) Virus replication in HeLa cells determined by infection at an MOI of 10. Virus was collected 24 h postinfection, titrated in BHK21 cells, and compared with the virus collected at 5 h postinfection. Statistical analysis of the titer difference between 5 h postinfection and 24 h postinfection is indicated as * ($P > 0.05$), ** ($0.005 \leq P \leq 0.05$), and *** ($P < 0.005$). (C) The replication kinetics of recombinant viruses expressing the selected E3 mutants determined by a low MOI of 0.1 in HeLa cells. The titer was determined in BHK21 cells.

Inhibition of cytokine responses by E3L mutants. Vaccinia E3 protein suppresses expression of a diverse array of cytokines representing members of the IFN, interleukin (IL), and tumor necrosis factor (TNF) groups (16). Here, the selected E3L mutants were examined for inhibiting expression of IFN- β , TNF- α , and IL-6 using a quantitative real-time PCR assay (Fig. 6).

The expression profiles of IFN- β (Fig. 6A) and TNF- α (Fig. 6B) induced by recombinant viruses expressing the selected E3 mutants are similar. The mutant I110A did not have any inhibition of the expression of these two cytokines. In fact, the levels of IFN- β and TNF- α mRNA were slightly higher in the cells infected with the mu-

tant I110A recombinant virus than the vvΔE3L control. Other mutants demonstrated various degrees of inhibition, in that the E138A mutant was strongest while the F159A mutant was weakest at suppressing IFN- β and TNF- α expression. Compared to the vvΔE3L control, the mutants I110A, N123A, and F159A did not show inhibition of IL-6 expression (Fig. 6C). On the contrary, expression of E3 mutants I110A and F159A enhanced the expression of IL-6. Infection with recombinant viruses expressing other mutants resulted in a lower level of IL-6 expression than that of the vvΔE3L control, in that the mutant E138A recombinant induced the least IL-6 expression among all of the selected mutants.

FIG 4 dsRNA binding and host range of the selected representative E3 mutants. (A) Poly(I·C) binding profile of the selected mutant E3 proteins expressed from transfection/infection in BHK21 cells. (B) For easier comparison, the virus titers of selected mutant recombinants (the same as those depicted in Fig. 3B), determined at a high MOI of 10, were plotted side by side. Statistical analysis of the titer difference between 5 h postinfection and 24 h postinfection is indicated by * ($P > 0.05$), ** ($0.005 \leq P \leq 0.05$), and *** ($P < 0.005$).

Suppression of PKR activation by E3 mutants. PKR plays a major role in restricting replication of vaccinia E3L deletion mutant virus in HeLa cells (27). The effect of the selected E3L mutants on activation of PKR, as measured by phosphorylation of threonine-446 and the phosphorylation of the PKR substrate, eIF2 α ,

was examined following infection in HeLa cells. As shown in Fig. 7A and B, the levels of PKR and eIF2 α phosphorylation in cells infected with the E3L wild type and the mutant C126A and F135A recombinant viruses (retaining both dsRNA binding activity and host range function) were similar to those of the cell control, while

FIG 5 IFN- β resistance of recombinant vaccinia viruses expressing selected E3 mutants in Huh7 cells. Huh7 cells were treated with 1,000 U of human IFN- β overnight and infected with the selected E3 mutant recombinant vaccinia viruses at an MOI of 5. The viruses were collected at 48 h postinfection, titrated in BHK21 cells, and compared with the viruses collected at 5 h postinfection and the viruses from non-IFN- β -treated cells. Statistical analysis of the titer difference between IFN- β -treated and nontreated controls is indicated by * ($P > 0.05$), ** ($0.005 \leq P \leq 0.05$), and *** ($P < 0.005$).

FIG 6 Modulation of cytokine expression by selected E3 mutants. HeLa cells were infected with selected E3 mutant recombinant viruses at an MOI of 10, and the total RNA was collected at 8 h postinfection. Trace DNA contamination was removed with DNase. The mRNA levels of IFN- β , TNF- α , and IL-6 were examined with quantitative real-time PCR. (A) IFN- β expression level. (B) TNF- α expression level. (C) IL-6 expression level.

FIG 7 Inhibition of PKR (A) and eIF2 α phosphorylation (B) and induction of the apoptotic markers cleaved PARP and caspase 7 (C). HeLa cells were infected with the selected E3 mutant recombinants and control viruses at an MOI of 10, and the cell lysates were collected at 6 h postinfection and subjected to Western blot analysis. To facilitate comparison, the dsRNA binding capacity of E3 mutants also was shown.

significant phosphorylation of PKR and eIF2 α was observed in the mutant N123A (which also retained dsRNA binding and host range function) virus-infected cells. The mutant E138A recombinant virus induced significantly less PKR and eIF2 α phosphorylation than the mutant L183A (both are defective in dsRNA binding and retained host range function). Similar levels of phosphorylated PKR and eIF2 α were observed following infection with recombinant viruses expressing mutants I110A (no host range function) and F159A (compromised host range function), which have no detectable dsRNA binding. Lastly, the recombinant vaccinia virus expressing the mutant D103A (dsRNA binding) also induced levels of phosphorylation of PKR and eIF2 α similar to those of the non-dsRNA binding mutants I110A and F159A. Thus, inhibition of PKR activation by the E3 protein does not correlate with its dsRNA binding capacity.

Previously, it has been reported that PKR is an important regulator of apoptosis induced by the vaccinia E3L deletion mutant (18, 27, 28). Here, induction of the apoptotic markers cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase-7 was examined by Western blotting. As shown in Fig. 7C, the level of the cleaved PARP and caspase-7 induced by the selected panel of E3L mutant recombinant viruses correlated well with the level of phosphorylated PKR (compare Fig. 7C to A).

Physical interaction of E3 mutant proteins with protein kinase R. Inhibition of PKR plays an important role in the host range function of vaccinia E3 protein (27). In this study, we have shown that the host range function of E3 protein and its inhibition of PKR activation do not correlate with its dsRNA binding capacity (Fig. 4 and 7). Previous studies reported that vaccinia E3 protein bound to and inhibited the activation of PKR in a yeast system (29, 30), and a dsRNA binding-defective E3 mutant (K167A and

FIG 8 Physical interaction of vaccinia E3 protein with PKR. The GST-fused full-length E3L wild type and mutants (both truncated and point mutants) were cloned into vaccinia expression vector pJS5 as described in Materials and Methods, the vector was transfected into HeLa cells infected with vaccinia virus, and cell lysates were collected 24 h postinfection. (A) Western blot of protein complex precipitated with glutathione beads from GST-fused truncated E3 proteins. Antibodies (Abs) used were GST Ab, E3 Ab, and PKR Ab, respectively. E3Lwt, wild-type E3 without a GST tag; E3Lwt-GST, wild-type E3 tagged with GST; E3ΔC25-GST, the C-terminal 25 amino acids deleted and fused with a GST tag; E3LC111-GST, the C-terminal 111 amino acids of the E3 protein fused with a GST tag. (B) Western blot of protein complex precipitated with glutathione beads from GST-fused E3 point mutants (I110A, N123A, C126A, F135A, and F159A). Abs used were E3 Ab and PKR Ab.

R168A) lost PKR binding activity (30). However, the link between the dsRNA binding capacity of E3 and the physical interaction between E3 and PKR in vaccinia virus-infected cells is not clear, particularly in those cells restrictive for the replication of the E3L deletion mutant virus, e.g., HeLa cells. In this study, the five selected E3L mutants were fused at their N termini with GST protein. The fusion genes were cloned into the poxvirus expression vector pJS5, and the proteins were transiently expressed in HeLa cells infected with vaccinia virus. GST pulldown assay was performed to determine the physical interaction of the selected vaccinia E3 mutant proteins with the endogenous PKR in HeLa cells. In addition, the previously described truncated E3L mutants (16), E3ΔC25 (dsRNA binding defective with C-terminal 25 amino acids deleted) and E3LC111 (the entire Z-DNA binding region of 80 amino acids truncated), were included in the analysis.

As shown in Fig. 8A, glutathione beads only precipitated GST and GST-fused full-length and truncated E3 proteins, and no wild-type E3 protein (without a GST tag) was pulled down. PKR was coprecipitated with the full-length E3 and E3LC111 (dsRNA binding domain and no Z-DNA binding domain) fused with GST (Fig. 8A, lanes 2 and 4) but was not present in the complex with GST/E3ΔC25 and GST (Fig. 8A, lanes 3 and 5). It is interesting

that the wild-type E3 protein (without a GST tag expressed from the infecting vaccinia virus) was not coprecipitated with GST-fused E3 and E3 truncated mutants (data not shown).

GST-tagged single-amino-acid mutants (I110A, N123A, C126A, F135A, and A159A) were examined for their binding of endogenous PKR in HeLa cells (Fig. 8B). Although the GST-F159A mutant displayed reduced PKR pulldown compared to other mutants, all other mutants were found to pull down PKR comparably, regardless of their dsRNA binding capability and host range function (Fig. 8B).

DISCUSSION

Vaccinia E3 protein first was identified for its biochemical capacity of binding to dsRNA using the synthetic dsRNA analog poly(I-C) as a substrate (11). Since then, many biological functions of the E3 protein have been reported, including suppression of multiple cellular antiviral responses activated by dsRNA, such as inhibition of PKR activation, apoptosis, and IFN-mediated antiviral activities (13, 22, 31). The dsRNA binding domain resides in the C-terminal 110 amino acids of the E3 protein, and this domain is sufficient for all of the major characterized biological functions of the E3 protein (15, 16, 24). Thus, the generally ac-

cepted model has been that E3 protein inhibits dsRNA-activated antiviral immune responses by binding to and sequestering virus-produced dsRNA. However, important gaps exist in understanding the mechanism of how E3 protein modulates dsRNA-dependent cellular antiviral responses. All chordopoxviruses, except molluscum contagiosum virus and avipoxviruses, express E3 ortholog proteins. Previously, our group has reported that myxoma virus and swinepox virus E3 orthologs could supplement vaccinia E3L function in cell culture. In contrast, the sheeppox virus E3 ortholog cannot, and Yaba monkey tumor virus E3L ortholog showed limited E3 supplementary function, although they both bound to dsRNA (18). Another study has found that *Escherichia coli* RNase III could supplement vaccinia E3 function. However, a mutant RNase III, which was defective in catalytic activity but has intact dsRNA binding, showed a greatly weakened supplementary function (32). In addition, E3 protein has been shown to inhibit dsDNA-induced cytokine responses (33, 34). Therefore, the mechanism of how vaccinia E3 protein functions to modulate dsRNA-activated cellular antiviral responses is undetermined.

Like most previous studies on dsRNA binding activity of vaccinia E3 protein (11, 13, 25), the synthetic dsRNA analog poly(I-C) was used in this study. It is well known that poxviruses produce dsRNA molecules during their replication cycle, mainly during the intermediate and late stages. Thus, the ideal ligand for analysis of dsRNA binding activity of E3 protein would be poxvirus-produced dsRNA. Initially, we had made several attempts to perform the dsRNA binding assay using the total RNA from vaccinia virus-infected cells, e.g., to pull down vaccinia RNA using GST-E3 fusion protein (produced from *E. coli* or from recombinant vaccinia virus) or to pull down the E3 protein using vaccinia RNA-conjugated beads. However, those attempts failed to produce meaningful data compared to negative controls. Currently, little is known about the biochemical nature of poxvirus-produced dsRNA, e.g., the proportion of the dsRNA in the total RNA, length and end structure of the virus-associated dsRNA, etc. Therefore, it is technically challenging to perform a dsRNA binding assay on the E3 protein using RNA preparations from poxvirus-infected cells. Since the majority of poxvirus dsRNAs are formed as the result of long and heterogeneous intermediate and late transcripts derived from both strands of the virus genome, the length and end structure of poxvirus-associated dsRNA species are predicted to be highly heterogeneous. Synthetic dsRNA analogs, such as poly(I-C), also contain dsRNAs of various lengths and end structures. Therefore, poly(I-C) is a suitable substitute for poxvirus dsRNA for the assay of dsRNA binding capacity of vaccinia E3 protein. For the dsRNA binding assay, poly(I-C)-coated agarose beads were used to pull down E3 proteins from the virus-infected cell lysates, and the E3 protein then was detected using Western blotting. It should be noted that this assay is not quantitative and cannot distinguish small differences in the binding affinity of different E3 mutants to the poly(I-C)-coated beads. Therefore, no conclusion can be drawn on the dsRNA binding affinity of E3 mutant proteins, although a difference in the amounts of E3 mutant proteins bound to the poly(I-C) beads can be observed. In addition, the data generated from the poly(I-C) binding assay only reflect the general biochemical property of vaccinia E3 and its mutant proteins binding to dsRNA and may not be applicable to the potential specific binding property of the viral dsRNA.

The primary objective of this study is to investigate the link between the biochemical capacity of dsRNA binding and the bio-

logical functions of the E3 protein. Previous mutational studies have identified several amino acid residues important for dsRNA binding and/or inhibition of PKR (15, 24, 25). In this study, we mutated every residue in the dsRNA binding domain by either alanine or valine substitution. The mutated E3 proteins first were screened for their biochemical activity of dsRNA binding and the biological function of the host range. Of the 115 mutants examined, 20 (18 single-point mutants and 2 double-point mutants) were defective in dsRNA binding, while 18 (17 single-point mutants and 1 double-point mutant) have defective host range function (Table 1). With the exception of the E138A mutant, all of the residues important for dsRNA binding and/or host range are those highly conserved among poxvirus E3 orthologs.

The dsRNA binding domain of the E3 protein was predicted to be between positions 118 and 182 (25, 35), which was the main focus of previous mutational studies of the E3 protein (24, 25). One study examined three single-amino-acid point mutations (G164V, K167T, and A174V) and found that these three residues were critical for dsRNA binding (24). Another study investigated 14 residues of the dsRNA binding motif region and found 6 (E124, F135, F148, K167, R168, and K171) were detrimental for dsRNA binding (25). Our study examined all of the residues in this region plus 37 upstream and 8 downstream residues (the last 8 amino acids of the C terminus) by alanine or valine substitution mutation. It was found that 13 of the 20 amino acids essential for dsRNA binding were in the predicted dsRNA binding domain, while 7 residues are outside this region. Most of the previously reported critical residues for dsRNA binding were confirmed in our investigation. However, the previous report showed that the E124A and F135A mutation rendered E3 protein defective in dsRNA binding (25). In our repeated tests, we found the E124A and F135A mutants bound poly(I-C) beads. This difference may be accounted for by the protein extraction techniques utilized and the method of dsRNA binding analysis. In the previous study, the dsRNA binding domain (residues 100 to 190) of E3 protein was expressed in *E. coli*, and the purified protein was applied to a nitrocellulose membrane and probed with radiolabeled poly(I-C) ligand (25). In our study, the full-length E3 protein was expressed in the context of vaccinia virus infection in BHK21 cells, and the total protein lysate was used for incubation with poly(I-C) beads to examine its dsRNA binding activity. Thus, the E3 protein used in this study was closer to its native form. Nonetheless, the differences in the protein sample preparation and conditions of the binding assay may account for the difference in the dsRNA binding activity of the E124A and F135A mutants. Since only alanine or valine substitution mutation was performed in the present study, it cannot be excluded that other residues (in addition to the 20 mutations identified here) are critical for dsRNA binding. For example, the G164V mutant lost dsRNA binding activity (24), while the G164A mutation cause no detectable change in the dsRNA binding (this study and reference 24). Therefore, it is likely that the actual number of critical residues involved in dsRNA binding is more than what was identified in this study.

The most characteristic biological function of E3 protein is its role in determining host range, in that deletion of the E3L gene renders the virus replication restrictive in a cell line which is permissive for the wild-type virus, e.g., HeLa cells. In parallel with testing the dsRNA binding activity, the host range function of E3 mutant proteins was examined in HeLa cells. Of the 115 E3 mutants, 18 mutants (17 single-point mutants and 1 double-point

TABLE 2 Summary of dsRNA binding and biological functions of E3 mutants

Wild type or mutant	Poly(I-C) binding	Host range ^a	IFN resistance ^b	Inhibition of PKR	PKR binding	Inhibition of apoptosis	Inhibition of cytokine:		
							IFN-β	TNF-α	IL-6
WT	+	+	+	+	+	+	+	+	+
N123A	+	+	±	—	+	—	+	+	—
C126A	+	+	+	+	+	+	+	+	+
F135A	+	+	±	+	+	+	+	+	+
I110A	—	—	—	—	+	—	—	—	—
F159A	—	±	—	—	+	—	+	+	—
E138A	±	+	+	+	ND	+	+	+	+
L183A	±	+	±	—	ND	—	+	+	+
D103A	+	±	±	—	ND	—	+	+	+

^a Host range is arbitrarily defined as described in the footnotes to Table 1.
^b IFN resistance is arbitrarily defined with the following symbols: +, no detectable difference between the virus titers from IFN-β-treated and untreated cells; ±, virus titer is lower in the IFN-β-treated sample than in the untreated sample, but it is greater than that of the 5-h postinfection sample; —, virus titer from IFN-β-treated sample is equal to or less than that of the 5-h postinfection sample.

mutant) demonstrated defective host range function in HeLa cells, in that 11 mutants completely lost and 7 retained compromised host range function (Table 1). The 11 mutants completely defective in host range function also demonstrated no detectable dsRNA binding. However, the most striking observation is that 5 mutants (I105A, Y125A, F148A, F159A, and K171A), which had no detectable dsRNA binding, still showed compromised host range function when examined after a high-MOI infection. More importantly, the recombinant virus expressing the mutant F159A grew to a level comparable to that of the wild-type E3 virus when examined at a low MOI (Fig. 3C). Three mutants with significantly compromised dsRNA binding activity (E138A, L183A, and I187/188A) demonstrated efficient host range function, while recombinant virus expressing the mutant (D103A) with normal dsRNA binding grew in HeLa cells less efficiently (Fig. 3B). Therefore, our data clearly suggest that the biochemical capacity of dsRNA binding is not essential for the host range function of vaccinia E3 protein.

The host range function of E3 protein is closely related to its suppression of PKR activation and apoptosis (27). In vaccinia virus-infected cells, we have reported that virus-produced dsRNA molecules function as pathogen-associated-molecular patterns (PAMPs) to activate PKR and induce apoptosis and cytokine expression (36). Since E3 protein has the biochemical capacity of binding to dsRNA, the mechanism of suppression of PKR and apoptosis and cytokine expression by vaccinia E3 protein has been presumed to be due to its action of sequestering vaccinia virus-produced dsRNA. Based on this model, it would be expected that mutant E3 proteins capable of dsRNA binding would display comparable inhibition on such antiviral effectors. Surprisingly, of the four mutants capable of dsRNA binding, C126A and F135A inhibited PKR phosphorylation and apoptosis signals, while the mutants D103A and N123A demonstrated the inhibition to a lesser degree than the mutant with defective dsRNA binding (E138A). Therefore, the dsRNA binding capability of the E3 protein is not essential for its activity of inhibiting PKR and apoptosis. A similar indication also applies to the modulation of cytokine expression by the E3 protein. The dsRNA binding and biological functions of the selected E3 mutants are summarized in Table 2.

It has been reported that vaccinia E3 protein inhibited PKR activation in a yeast system, and the inhibition was correlated with the physical interaction of E3 protein with PKR (29, 30). In this study, we investigated the physical interaction between E3 and PKR proteins in HeLa cells, in which the host range function of E3 is essential for the replication of vaccinia virus. To precipitate the protein complex of the E3 protein and its binding partners, GST was fused to the N terminus of wild-type E3 and E3 mutants, including previously described truncated E3 proteins E3/C111 (E3 dsRNA binding domain) and E3/ΔC25 (with the last C-terminal 25 amino acids deleted and no dsRNA binding capacity) (16), and several selected mutants (I110A, N123A, C126A, F135A, and F159A) described in this study. It was found that the dsRNA binding domain (E3/C111) is responsible for the physical interaction of E3 with PKR (Fig. 8A), while the Z-DNA binding domain (E3/ΔC25) does not mediate the binding between the E3 and PKR proteins. However, a simple physical binding between the E3 and PKR proteins does not seem to bear biological significance, as all five E3 mutant proteins, each with a single-point mutation (I110A, N123A, C126A, F135A, and F159A) in their dsRNA binding domain, bound to PKR protein comparatively well in HeLa cells regardless of the difference in their dsRNA binding and host range (Fig. 8B). Moreover, this observation also suggests that the binding of the E3 protein to PKR does not require dsRNA binding capacity, as the mutants I110A and F159A do not bind to dsRNA. Based on our current data, it is not known if all of the E3 mutants bind to the same region of PKR. Future study with truncated PKR mutants (e.g., PKR dsRNA binding domain or the kinase domain) will answer this question more conclusively. Nonetheless, caution should be taken to interpret the impact of pure physical binding on the biological function of the E3 protein. It should be noted that GST-tagged E3 and its mutant proteins were expressed from a poxvirus expression vector in HeLa cells infected with a vaccinia virus expressing wild-type E3 protein. Interestingly, no wild-type E3 protein (without a GST tag) was pulled down by the glutathione beads, indicating that E3 protein does not form an oligomer structure in HeLa cells.

In this study, we performed the systemic mutagenesis study of vaccinia E3 protein to examine the link between its biochemical

capacity of dsRNA binding and its biological functions. Our data clearly demonstrated that the biochemical capacity of dsRNA binding is not essential for biological functions of vaccinia E3 protein. On the other hand, the data also suggested that the domains of the E3 protein important for its biological functions overlap the domains critical for its biochemical capacity of dsRNA binding, as all of the mutants without detectable dsRNA binding either lost (E3/I110A) or showed compromised (E3/F159A) host range in HeLa cells. Since it has been reported that the N-terminal Z-DNA binding domain also is important for the complete biological functions of the E3 protein (14, 19–21), it would be interesting to examine the mutants (described in this report) in the system for identification of the biological functions of the Z-DNA binding domain. In addition, it will be interesting to investigate what correlation the described mutants will demonstrate with other reported functions attributed to vaccinia E3 protein, such as inhibition of cytokine in primary cells and dendritic cells (37, 38) and inhibition of ISG15 (39).

Since the E3 protein was first characterized for its biochemical capacity of dsRNA binding over 20 years ago (11), certain key issues regarding the molecular mechanism of how the E3 protein functions still remain to be answered. For example, we are still searching for experimental evidence showing that the E3 protein binds and sequesters vaccinia virus-produced dsRNA in infected cells, the cellular targets of E3 protein for its function are still elusive, and the structure of the E3 protein is not solved. We believe that data and reagents described in this communication will help to solve these important issues related to the mechanism of E3 protein function.

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