Identification and functional characterization of the Varicella zoster virus ORF11 gene product

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ABSTRACT

The deletion of ORF11 severely impaired VZV infection of human skin xenografts. Here, we investigate the characteristics and functions of the ORF11 gene product. ORF11 is expressed as a 118 kDa polypeptide in VZV-infected cells; the protein is present in the nucleus and cytoplasm and is incorporated into VZ virions. Although ORF11 had little effect in transactivating VZV gene promoters in transfection assays, deleting ORF11 from the virus was associated with reduced expression of immediate early proteins IE4, IE62 and IE63, and the major glycoprotein, gE. ORF11 was identified as an RNA binding protein and its RNA binding domain was defined. However, disrupting the ORF11 RNA binding domain did not affect skin infection, indicating that RNA binding capacity, conserved among the alphaherpesviruses homologues, is not essential while the contribution of ORF11 to the expression of the IE proteins and gE may be required for VZV pathogenesis in skin in vivo.

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Introduction

Varicella zoster virus (VZV) open reading frame 11 (ORF11) is one of a conserved gene cluster found in the alphaherpesviruses that includes VZV ORF9-ORF12 (Che et al., 2008; Cohen et al., 2007; Davison, 1991; Davison and Scott, 1986). The protein encoded by VZV ORF11 is presumed to be a tegument component but its gene product and functional characteristics have not been described. We reported recently that a VZV recombinant lacking ORF11 (POKAΔ11) replicates much more slowly than POKA in cultured cells, as did mutants from which ORF10 and ORF12 had been deleted (Che et al., 2008; Zhang et al., 2010). These studies suggested that ORF11 and its flanking genes are not critical in vitro. However, a hierarchy of effects was observed when these mutants were evaluated for virulence in human skin xenografts in SCID mice. Deleting ORF12 did not alter VZV pathogenesis in skin whereas deleting ORF10 was associated with a 10-fold decrease in infectious virus yields and the ORF11-null virus had even more severely impaired infectivity, replicating 500-fold less efficiently than POKA (Che et al., 2006, 2008; Zhang et al., 2010). Furthermore, the effect of dual deletions of both ORF10 and ORF11 (POKAΔ10/11) or ORF11 and ORF12 (POKAΔ11/12) was similar to the consequences of removing only ORF11. Thus, although ORF11 functions were not defined in these experiments, ORF11 was identified as a major VZV virulence determinant in differentiated epidermal cells in their tissue microenvironment in vivo.

VZV ORF11 is homologous to the UL47 of other alphaherpesviruses (Kinchington and Cohen, 2000; Roizman and Campadelli-Fiume, 2007). Removing UL47 from herpes simplex virus type 1 (HSV-1) is associated with diminished expression of IE proteins and the HSV-1 UL47-null mutant replicated more slowly than wild-type virus in cultured cells, indicating a regulatory role for UL47 in gene expression early in the infectious cycle (Zhang et al., 1991; Zhang and Mcknight, 1993). Moreover, replication of pseudorabies virus (PRV) and Marek's disease virus (MDV) mutants that lack UL47 was also delayed compared to wild-type viruses in cell culture (Dorange et al., 2002; Kopp et al., 2002). Recent studies revealed that HSV-1 UL47 binds to RNA and has the capacity to shuttle between the nucleus and the cytoplasm during infection, suggesting that the UL47 gene product may be involved in RNA biogenesis during viral infection (Donnelly and Elliott, 2001a; Donnelly et al., 2007; Verhagen et al., 2006a). Subsequently, studies of the bovine herpesvirus type 1 (BHV-1) homolog demonstrated that it also binds to RNA and shuttles between the nucleus and the cytoplasm (Carpenter and Misa, 1991; Donnelly and Elliott, 2001a; Verhagen et al., 2006b). Most recently, Dobrikova and coworkers have shown that HSV-1 UL47, via interaction with ICP27, another HSV-1 RNA binding protein, exists in a complex with cytoplasmic polyadenylate-binding protein (PABP) (Dobrikova et al., 2010). Further investigation showed that ICP27 and UL47 did not alter the translation efficiency of transfected reporter RNAs but modulated transcript abundance of reporter cDNAs in transfected cells, implying that ICP27 and UL47 may regulate viral or host gene expression in the steps of mRNA processing and/or export rather than translation initiation.

While these ORF11 homologues have been identified as RNA binding proteins and are implicated in functions related to viral or cellular RNA processes, it has not been determined whether their RNA
binding property is an essential function based on identifying a specific RNA binding motif in the protein and examining effects of disrupting the coding sequence for the motif in the viral genome on replication. The present study was designed to investigate the characteristics and functions of the ORF11 gene product and when ORF11 was identified as an RNA binding protein, to investigate the effects of mutations that disrupted this function in vitro and in human skin xenografts in SCID mice in vivo.

Results

Identification of the ORF11 gene product

ORF11 expressed as a GST fusion protein was used to generate a rabbit polyclonal antiserum, designated Ab11. POKA-infected cell lysates were prepared 48 h after infection. When this antiserum was used for Western blot to test infected cell lysates, a single 118 kDa protein was detected in lysates from POKA-infected cells but not in lysates from mock-infected cells or those from cells infected with four ORF11 deletion mutants, POKAΔ11, POKAΔ10/11, POKAΔ11/12, and POKAΔ10/11/12. The IE4 protein, used as a positive control, was detected at similar levels in lysates of cells infected with POKA and the ORF11 deletion mutants (Fig. 1A).

To investigate the products of ORF11 expressed in the absence of other viral proteins, cell lysates were extracted from melanoma cells after transfection with the ORF11 expression vector pCMV11 and analyzed by Western blot using Ab11. One product migrating at 118 kDa was identical to the species detected in VZV-infected cell lysates, whereas a second species of 114 kDa was produced with transient expression of ORF11 (Fig. 1B).

ORF11 protein distributes to the nuclei and cytoplasm of infected cells

To determine the distribution of ORF11 during viral infection, the infected cells were fractionated to make nuclear and cytoplasmic extracts. When cell fractions were analyzed by Western blot with Ab11, ORF11 was present in both the nuclear and cytoplasmic fractions of infected cells but not in uninfected cell fractions (Fig. 2A). To ensure the purity of the fractionations, the same membrane probed for ORF11 was then stripped and re-probed for IE62, α-tubulin and RCC1 (regulator of chromosome condensation 1). IE62 was predominantly in the nuclear fraction, the nuclear protein RCC1 was only detected in the nuclear fraction and α-tubulin remained in the cytoplasmic fraction (Fig. 2A). These data indicate that effective separation was achieved during fractionation.

The intracellular localization of ORF11 protein in infected cells was further examined by confocal microscopy. Infected HELF cells were fixed at 10 or 24 h after infection and evaluated for ORF11 distribution relative to the localization of VZV IE62 and gE. Uninfected HELF cells were tested with Ab11 and IE62 antibody showed no staining (Fig. 2B, panel I); in infected cells tested with the Ab11 premunine serum together with IE62 antibody, IE62 was detected but no ORF11 signal was observed (Fig. 2B, panel II). When infected cells fixed at 10 h after VZV infection were co-stained with ORF11 and IE62 antibodies, ORF11 was detected in the nucleus and cytoplasm in cells that expressed IE62 (Fig. 2B, panel III). Infected cells fixed at 24 h post infection had extensive syncytium formation; when these cells were co-stained with Ab11 and a gE antibody, gE was present in a diffuse cytoplasmic distribution, while ORF11 expression was observed in both the nuclei and cytoplasm of gE positive cells; ORF11 was not detectable in cells that had no gE expression (Fig. 2C). These observations were consistent with the Western blot analysis (Fig. 2A), which showed that ORF11 protein was present in both nuclear and cytoplasmic fractions of VZV-infected cells. In addition, when cells were transfected with pCMV11 and fixed after 48 h, ORF11 protein had a diffuse distribution in the nucleus and cytoplasm (Fig. 2D), which was similar to that observed in VZV-infected cells.

ORF11 protein is a component of the VZ virion tegument

To achieve highly sensitive and specific immunogold-labeling in combination with ultrastructural resolution, POKA-infected cells were analyzed by cryo-immuno EM. Using Ab11 antibody together with Protein A conjugated to 15 nm gold particles, we detected dense and specific ORF11 labeling within the tegument of VZ virions, indicating that ORF11 protein was incorporated into VZV particles as a component of the tegument (Fig. 3A). The control experiment in which cryosections of the same infected sample were incubated with Ab11 premunine serum, showed no gold-labeling, indicating the specificity of the Ab11 antibody in immune-EM assays (Fig. 3B).

The effect of ORF11 on VZV gene expression

To determine whether ORF11, as a VZV tegument protein, might contribute to the regulation of VZV gene expression, the effect of ORF11 on promoters of VZV genes was evaluated in transient transfection assays using promoter constructs and the expression of IE or late VZV proteins was assessed in cells infected with a recombinant VZV lacking ORF11.

In transient expression assays, melanoma cells were transfected with three luciferase reporter plasmids that had promoters of VZV gene products including ORF61, ORF10 and gE. When the promoter constructs and pGL3 control vector were individually transfected, telluric reporter plasmids, Renilla luciferase readings with those promoter constructs was low but not at about 300-fold (ORF61), 250-fold (ORF10), and 420-fold (gE) above the promoter-less control, respectively (Fig. 4A). These results were consistent with previous reports that IE62 alone strongly transactivates all three promoters, although with different efficiencies (Berarducci et al., 2007; Che et al., 2007; Ruyechan et al., 2003; Wang, et al., 2009; Yang et al., 2006). In contrast, luciferase expression was similar or about 2-fold higher as compared to the promoter-less control when pCMV11 was co-transfected with the construct in which luciferase reporter gene expression was regulated by the ORF61, ORF10 or the gE promoter. Thus, little or no regulatory effect of ORF11 on these promoters was detected in transient transfection assays.

To further investigate whether ORF11 might contribute to VZV gene expression at the protein level under conditions of viral replication, melanoma cells were infected with POKA or the deletion mutant,
POKAΔ11, at the same inoculum titer. Western blot assays were done to detect IE4, IE62, IE63 and gE over a 4-day time course as shown in a representative analysis in Fig. 4B. At day 1 post-infection, a significant reduction in the levels of IE62 and IE4 was observed in cells infected with POKAΔ11 compared to POKA. By day 2 post-infection, IE4 reached levels similar to those in POKA-infected cells. While IE62 expression had increased substantially by day 2, it continued to remain low compared to POKA-infected cells over the 4-day interval. IE63 production in cells infected with POKAΔ11 was substantially delayed compared to POKA, showing diminished expression for the first 3 days after infection (Fig. 4B). In addition, a very small amount of gE protein was detected in POKA-infected cells at day 1 but not in POKAΔ11-infected cells and levels of gE expression in cells infected with POKAΔ11 remained reduced over the 4-day period in comparison to POKA (Fig. 4B). Thus, ORF11 was required for normal levels of VZV IE4, IE62, IE63 and gE. The intracellular localization of ORF11 during VZV infection. (A) Western blots of the ORF11 detection in nuclear and cytoplasmic fractions. Cellular fractionations were performed from melanoma cells infected with POKAΔ11 at the same inoculum titer. Western blot assays were done to detect IE4, IE62, IE63 and gE over a 4-day time course as shown in a representative analysis in Fig. 4B. At day 1 post-infection, a significant reduction in the levels of IE62 and IE4 was observed in cells infected with POKAΔ11 compared to POKA. By day 2 post-infection, IE4 reached levels similar to those in POKA-infected cells. While IE62 expression had increased substantially by day 2, it continued to remain low compared to POKA-infected cells over the 4-day interval. IE63 production in cells infected with POKAΔ11 was substantially delayed compared to POKA, showing diminished expression for the first 3 days after infection (Fig. 4B). In addition, a very small amount of gE protein was detected in POKA-infected cells at day 1 but not in POKAΔ11-infected cells and levels of gE expression in cells infected with POKAΔ11 remained reduced over the 4-day period in comparison to POKA (Fig. 4B). Thus, ORF11 was required for normal levels of VZV IE4, IE62, IE63 and gE.

**ORF11 binding to RNA in VZV-infected cells**

To assess whether ORF11 protein binds to viral RNA, ORF11 protein was first immunoprecipitated from melanoma cells infected with POKA using Ab11. IE63 mRNA is abundant during VZV infection and therefore was chosen to screen for possible ORF11 protein binding to viral RNAs. Co-immunoprecipitated RNA was reverse transcribed and the 3’ portion of the IE63 RNA was detected by PCR. The single PCR product, which was identified as IE63 by sequencing from the nucleotide 349 to the polyadenylation site, was observed by agarose gel electrophoresis from the infected cell sample immunoprecipitated with Ab11 but was absent when Ab11 was not included (Fig. 5A). Immunoprecipitation was also done with a polyclonal antibody against ORF47, another VZV tegument protein, which can be used in both Western blot and immunoprecipitation experiments (Besser et al., 2003); IE63 mRNA was not detected in the complex immunoprecipitated with or without anti-ORF47 antibody (Fig. 5A). No RT-PCR product was synthesized from the mock-infected cell sample when it was immunoprecipitated with Ab11 or in the absence of the antibody (Fig. 5A). Furthermore, when infected cells were used for RNA immunoprecipitation with the Ab11 pre-immune serum, no IE63 PCR product was observed (data not shown). These results suggested that ORF11 and/or a complex that contains ORF11 can bind to RNA in VZV-infected cells.

**Requirement of the ORF11 N-terminus for its RNA binding activity**

To determine whether ORF11 has the capacity to bind to RNA directly, in the absence of other viral proteins, we used an expression system to make purified ORF11 recombinant protein. The ORF11 gene was initially fused to GST and expressed in E. coli, but full-length ORF11 protein could not be recovered. Therefore we generated constructs that expressed the N-terminal 261 residues and C-terminal 233 residues of ORF11, each including about one third of the ORF11 gene, fused to GST. Samples of IPTG induced and non-induced cell lysates were separated on SD-S-PAGE followed by Coomassie blue stain or NorthWestern blot with an in vitro synthesized IE63 RNA probe that contained +71 to +602 of IE63 mRNA (Fig. 5B). The Coomassie blue stain showed that GST and GST fusion proteins were expressed (Fig. 5B) and the NorthWestern blot indicated that the recombinant GST-tagged ORF11 N-terminal truncation (ORF11N) bound to the IE63 RNA probe, while GST alone and the GST-tagged ORF11 C-terminal truncation (ORF11C) did not (Fig. 5B). Several additional indistinct bands were also detected by NorthWestern blot, but the bands were present in all samples, indicating that they were non-specific. Thus the ORF11 N-terminus but not the C-terminal region has efficient RNA binding activity when expressed in the absence of virus infection.

**Mapping the RNA binding domain of ORF11 protein**

To determine regions of the ORF11 N-terminus that were involved in RNA binding, a series of ORF11 N-terminal truncation mutations fused to GST was generated [Fig. 5C(a)]. Two large ORF11 N-terminal mutants that eliminated residues 1–115 or residues 1–190 [Fig. 5C(a), 2 and 3] exhibited no RNA binding activity [Fig. 5C(b), lanes 2 and 3], whereas the truncation mutation that lacked residues 123–261 and the ORF11 N-terminal 261 residues [Fig. 5C(a), 5 and 7] bound efficiently to the IE63 RNA probe [Fig. 5C(b), lanes 5 and 7]. These results indicated that the ORF11 RNA binding properties were located...
An arginine-rich motif is essential for ORF11 RNA binding activity

Two ORF11 expression constructs, pORF11N/ST1-GST and pORF11N/ST2-GST, were made to investigate whether the arginine residues of the arginine-rich box located at ORF11 N-terminal residues 8–15 were required for the ORF11 RNA binding function [Fig. 5E(a)]. These alanine substitution mutants were expressed in E. coli and purified proteins with the same loading amounts shown by Coomassie blue stain were used for Northwestern blot with the IE63 RNA probe [Fig. 5E(b)]. Substitution of all five arginine residues in the arginine-rich motif inhibited RNA binding, shown by comparison with the GST control; the replacement of the first two arginines with alanine residues decreased RNA binding activity significantly compared to the non-mutated construct, but some residual RNA binding was observed [Fig. 5E(b)]. Thus, the arginine-rich motif located within the first 15 residues of ORF11 N-terminus is necessary for its RNA binding and the first two arginine residues in the motif are required for optimal ORF11 RNA binding function.

Effects of mutating the ORF11 N-terminus and the RNA binding motif in the VZV genome in vitro

Based on these observations using ORF11 N-terminal expression constructs, two VZV recombinants were generated. In POKA11-Nd, the ORF11 RNA binding domain was deleted and in POKA11-Ns, alanines were substituted for arginine residues within the RNA binding motif [Fig. 6A]. The deletion of the ORF11 N-terminus and the presence of the targeted mutations in these VZV recombinants were confirmed by DNA sequencing. Sequencing also showed that the lengths of the ORF11 repeat region, designated R1 in the VZV genome (Davison and Scott, 1986), differed between POKA, POKA11-Nd and POKA11-Ns. Since the R1 region of the cosmid used as a template for generating VZV recombinants was same as that of POKA, the variations of the R1 in POKA11-Nd and POKA11-Ns appear to result from the mutagenesis process. These results confirm an early study showing that VZV repeat regions were difficult to clone and that the size of the repetitions often differed from the template (Davison and Scott, 1986). R1 consists of multiples of 3 bp that encode repeated amino acid sequences. The DNA sequences were converted to the corresponding amino acids and the repeated sequences are grouped as A, B and C, based on their amino acid composition; the A sequence is DAIDDE, the B residues are GEAEE; the C sequence is DAAEE, which in an early study was considered to be a partial copy of A and B, consisting of the first two-amino acids of A linked to the last three-amino acids of B. The ORF11 reiteration in POKA contained 75 amino acids with five A repeats, eight B repeats and one C repeat; the ORF11-Nd reiteration had an additional A repeat and B repeat not present in POKA ORF11 and was 86 amino acids; ORF11-Ns had the shortest reiteration, consisting of 27 amino acids with two A repeats and three B repeats (Fig. 6B).

Western blots were performed to examine whether deleting or mutating the ORF11 RNA binding domain or these variations in the length of the repeat regions affected the synthesis and cellular distribution of the ORF11 protein. Analysis of melanoma cells infected with POKA, POKA11-Nd, and POKA11-Ns showed that ORF11-Ns and ORF11-Nd, like ORF11, were expressed and present in both the nuclear and cytoplasmic fractions of infected cell lysates (Fig. 6C). The apparent reduction in ORF11-Ns and ORF11-Nd levels in the Western blot was probably due to decreased antibody binding affinity because the antibody was generated using an ORF11 N-terminal peptide; alternatively the mutated proteins may be less stable and the differences in the repeat regions could cause the lower binding affinity. The molecular masses of POKA ORF11, ORF11-Ns and ORF11-Nd were about 118, 110 and 104 kDa, respectively, which was consistent with sizes of 794 amino acids for ORF11, 746 amino acids for ORF11-Ns, and 703 amino acids for ORF11-Nd, based on the corresponding DNA sequencing results.

Binding of the VZV ORF11 N-terminus to viral or cellular genes transcripts

To investigate whether the ORF11 RNA binding domain interacted with RNAs of other VZV genes, RNA probes corresponding to VZV IE, early and late genes transcripts, including ORF61 (+17 to +825), IE62 (+23 to +813), ORF29 (−50 to +771) and gE (−29 to +699) were used for Northwestern analysis. The α-actin RNA probe of about 1900 nt was also included. In these experiments, equal amounts of the ORF11 N-terminal 1–261-GST fusion protein or GST, as shown by Coomassie blue stain, were hybridized with in vitro synthesized RNA probes (Fig. 5D). All probes showed binding to the ORF11 N-terminus. Thus, the ORF11 N-terminal domain bound non-selectively to transcripts corresponding to VZV IE, early and late genes and the cellular actin gene in the in vitro RNA binding assays.

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Translated from Chinese
The replication kinetics of POKA11-Nd and POKA11-Ns were next compared to POKA and POKAΔ11. In a 6-day infectious focus assay, the replication of POKAΔ11, POKA11-Nd and POKA11-Ns mutants showed growth kinetics that were similar to POKA (Fig. 7A). In addition, Western blot assays were performed to investigate the expression of IE (IE62) and late (gE) genes in lysates from POKA11-Nd and POKA11-Ns infected cells. Expression did not differ between POKA11-Nd, POKA11-Ns and POKA-infected cells (data not shown). Thus, neither the targeted mutations in the RNA binding domain nor the variations in the ORF11 reiterations altered VZV replication in cultured cells in vitro.

Effects of mutating the ORF11 N-terminus and the RNA binding motif in the VZV genome in skin xenografts in vivo

To investigate the effect of deleting or disrupting the ORF11 RNA binding domain on VZV replication in vivo, skin xenografts were inoculated with fibroblasts infected with POKA, POKA11-Nd, POKA11-Ns and POKAΔ11. A 6-day infectious focus assay was performed to compare the replication of the mutants with that of POKA. The results showed that the replication kinetics of POKA11-Nd and POKA11-Ns were similar to POKA (Fig. 7A). Western blot assays were performed to investigate the expression of IE (IE62) and late (gE) genes in lysates from POKA11-Nd and POKA11-Ns infected cells. Expression did not differ between POKA11-Nd, POKA11-Ns and POKA-infected cells (data not shown). Thus, neither the targeted mutations in the RNA binding domain nor the variations in the ORF11 reiterations altered VZV replication in cultured cells in vitro.

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Infected xenografts were harvested at 10 and 21 days after infection, and virus yields were determined by an infectious center assay. No significant differences were observed between the replication of POKA11-Nd and POKA11-Ns and POKA at either 10 or 21 days post-infection (P < 0.01). As was observed in vitro, the targeted mutations and the accompanying differences in the ORF11 reiterations did not affect VZV growth in skin. In contrast, infectious virus was not recovered from any xenografts inoculated with POKAΔ11 (Fig. 7B).

Discussion

VZV that lacks ORF11 is severely impaired for growth in human skin xenografts in vivo (Che et al., 2008). In these experiments, we demonstrate that ORF11, a predicted tegument protein, is incorporated into the virion tegument and is therefore expected to be released when VZV enter cells. When expressed alone in transfected cells, ORF11 encodes two products with sizes of 118 kDa and 114 kDa. Interestingly, ORF11 is expressed as one 118 kDa product in infected cells. These findings suggest the synthesis of the 114 kDa ORF11 gene product or its stability are diminished during viral infection. ORF11 protein exhibited a diffuse nuclear and cytoplasmic distribution during VZV infection. As infection progressed, the concentration of ORF11 protein increased, but the pattern of ORF11 expression in both the nucleus and cytoplasm remained unchanged. In addition, ORF11 protein exhibited a diffuse nuclear and cytoplasmic distribution during VZV infection. As infection progressed, the concentration of ORF11 protein increased, but the pattern of ORF11 expression in both the nucleus and cytoplasm remained unchanged.
with an HSV-1 recombinant expressing a GFP-tagged UL47 protein (Donnelly and Elliott, 2001b). We used cell-associated virus for VZV infection, while cell-free virus was used in the HSV-1 experiments. However, since this difference in cellular distribution between ORF11 and HSV-UL47 was also evident in the transfected cells, it seems unlikely that the type of inoculum affected the observations in infected cells. Thus, ORF11 intracellular localization and trafficking differ from that of its HSV-1 UL47 homolog in both infected and transfected cells.

Although the replication kinetics of the VZV recombinant with the ORF11 deletion was similar to that of wild-type POKA in tissue culture (Che et al., 2008; Zhang et al., 2010), we found that the synthesis of IE4, IE62, IE63 and gE proteins in POKAα11-infected cells was delayed or reduced compared to POKA-infected cells. Significant reductions of IE62 and IE4 proteins were evident in POKAα11-infected cells at 24 h after inoculation but IE4 levels were similar to those in POKA-infected cells by 48 h and IE62 levels had increased substantially. In contrast, we noted that the pattern of gene expression observed in cells infected with the two RNA binding mutants, POKAα11-Nd and POKAα11-Ns, was indistinguishable from POKA over a 4-day time course. The observations with POKAα11 suggest that the lower levels of expression of essential regulatory proteins that occur in the absence of ORF11 at early times after VZV infection are tolerated and that expression of major IE gene products recovers rapidly, allowing normal replication of the ORF11 null mutant in vitro. Furthermore, we showed that ORF11 protein alone had either little or no effect on the activities of ORF61, ORF10 and gE promoters. The delays in IE4, IE62, IE63 and gE synthesis may reflect a need for synergistic effects of ORF11 with other viral proteins to achieve the usual kinetics and levels of expression of key VZV proteins. It is also possible that ORF11 has a function related to virus entry and uncoating or virion assembly.

Our experiments demonstrate that ORF11 is an RNA binding protein, making ORF11 the third of the UL47 homologues in alphaherpesviruses known to exhibit RNA binding capacity. RNA binding proteins are important in many aspects of cellular biology, especially in the regulation of gene expression where they are involved in every stage and are also often important for viral effects on the protein expression profile of the host cell to produce an environment that supports efficient viral replication (Sandri-Goldin, 2004). Given its capacity to bind RNAs, the ORF11 protein could also be involved in the post-transcriptional control of RNA metabolism, as suggested for other cellular or viral-encoded RNA binding proteins. Moreover, HSV-1 UL47 has been reported to bind at least two viral transcripts that are packaged into virions and these packaged mRNAs can be expressed in newly infected cells (Sciortino et al., 2001, 2002). Whether VZV virions contain viral mRNAs has not been investigated, but if so, ORF11 could be involved in their virion incorporation and delivery into newly infected cells, promoting the efficient initiation of infection.

We demonstrated that ORF11 N-terminus is required for its RNA binding function but the C-terminus is not, and that an arginine-rich motif within the ORF11 RNA binding domain was essential for the RNA binding function. We did not test whether the central region of the ORF11 protein was involved in RNA binding but we note that the RNA binding domains of HSV-1 and BHV-1 UL47 are located in the N-terminus. The arginine-rich motif of ORF11 is partially conserved in HSV-1 UL47 protein but it is not required for RNA interactions (Verhagen et al., 2006a), suggesting that ORF11 and HSV-1 UL47 achieve RNA binding by a related but not identical process. Nevertheless, the fact that an arginine-rich motif in the ORF11 protein mediates direct binding to RNA is consistent with the overall role of such motifs in UL47 and a number of other viral RNA binding proteins, such as HIV-Rev, human cytomegalovirus (hCMV) UL69 and Epstein-Barr virus (EBV) EB2 (Donnelly et al., 2007; Hirai et al., 2003; Toth et al., 2006; Truant and Cullen, 1999; Weiss and Narayana, 1998; Zapp et al., 1991).

The evaluation of VZV recombinants that had a deletion or substitutions altering the ORF11 RNA binding domain as mapped by ORF11 mutagenesis in plasmid constructs showed no impairment in growth as compared to POKA in cultured cells; this finding was predicted since ORF11 protein is dispensable for VZV replication in vitro (Che et al., 2008). When the growth of POKAα11-N and POKAα11-Ns was assessed in human skin xenografts in SCID mice in vivo, deleting or disrupting the ORF11 RNA binding domain also had no effect on replication when compared to POKA, indicating that ORF11 RNA binding capacity is either not required or can be fully compensated for during VZV infection of differentiated human skin cells in vivo.

We have noted that the sizes of the ORF11 reiterations differed between the POKAα11-Nd, POKAα11-Ns and POKA recombinants. These differences can be explained by the fact that these regions are difficult to clone and the size of the reiterations often differs from the template (Davison and Scott, 1986). The VZV genome contains five tandem direct repeats of short G+C-rich sequences, three of which are located within open reading frames, including ORF11, ORF14 and ORF22, and appear to encode repeated amino acid residues. Similar polypeptide-encoding reiterations are found in other herpesviruses (McGeoch et al., 1985, 1988; Rixon and McGeoch, 1984). However, whether differences in the repeated amino acid sequences alter the function of the protein is not known. In our experiments, the ORF11 mutants did not differ from POKA in their growth characteristics, suggesting that the variable sizes of the repeat regions in ORF11 did not affect VZV replication in vitro and in human skin in vivo.

In summary, this study showed that VZV ORF11 encodes a multifunctional protein. ORF11 is a structural component of the tegument. ORF11 contribute to the regulation of essential VZV genes in cultured cells by an as yet undefined mechanism and has the capacity to bind viral and cellular gene transcripts. The evidence that disrupting the RNA binding function of ORF11 did not alter VZV skin infection together with the finding that ORF11 protein is required for normal levels of IE and gE protein expression in vitro, suggests that the impaired VZV growth in skin in the absence of ORF11 may be explained by these effects on the kinetics of viral protein synthesis or a possible function in virus entry and uncoating or assembly rather than the lack of functions related to its RNA binding capacity.

Materials and methods

Generation of antiserum to ORF11

To generate an ORF11-specific antiserum, ORF11 N-terminal was amplified by PCR with primers P1 and P2 (Table 1) using PfuUltraTM-HF DNA polymerase (Stratagene, La Jolla, CA) and the VZV cosmid pvFsp73 as a template (Nizumida et al., 2003). The PCR product was inserted into pGEX-2 T vector (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) to yield the expression construct pORF11N–GST. GST-tagged protein was purified using a Glutathione Sepharose 4B MicroSpin column (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ). Rabbit premimmune serum was obtained and anti-ORF11 serum (Ab11) was collected at day 77 after five immunizations with the GST-ORF11 protein (Josman LLC™, Inc., CA).

Western blot

POKA-infected melanoma cells were harvested after 48 h infection and whole cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer as described previously (Che et al., 2006). Cell lysate was separated by SDS-polyacrylamide gel electrophoresis (PAGE), electro-transferred onto immobilon-polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) and reacted overnight at 4 °C with Ab11 (1:20,000 dilution). Immunoblot of the same protein was also analyzed by using anti-ORF4 polyclonal antibody (kindly provided by Dr. Kinchington, University of Pittsburgh, PA) as a loading control for viral protein.
Table 1
Primer sequences and their VZV genome localization.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Genomic localization</th>
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<tbody>
<tr>
<td>P1</td>
<td>TATGCCACCGGTCCATATACCGG</td>
<td>13589–13612</td>
</tr>
<tr>
<td>P2</td>
<td>GAGGACTCTACCAACCATATTG</td>
<td>14348–14370</td>
</tr>
<tr>
<td>P3</td>
<td>CTACCAACGCTGTGCGCTGCGTATACCGA</td>
<td>13588–13609</td>
</tr>
<tr>
<td>P4</td>
<td>CTACCGTGCACAGGCGATATCGTCTAATACCACAGAC</td>
<td>13610–13623</td>
</tr>
<tr>
<td>P5</td>
<td>GGTACGCAATATCAGATGGCAC</td>
<td>13633–13654</td>
</tr>
<tr>
<td>P6</td>
<td>GGATTGAGCAGCGTTATAAATGACCCGACCTGCAATAC</td>
<td>13587–13609</td>
</tr>
<tr>
<td>P7</td>
<td>TGCTACCCAAGGCGGAG</td>
<td>13936–13954</td>
</tr>
<tr>
<td>P8</td>
<td>AAGCCC</td>
<td>13936–13954</td>
</tr>
<tr>
<td>P9</td>
<td>GCTGCACAATCCGCTGCACAGGCGATATCGTCTAATACCACAGAC</td>
<td>13610–13623</td>
</tr>
<tr>
<td>P10</td>
<td>GATATCGTCTAATACCACAGAC</td>
<td>13633–13654</td>
</tr>
<tr>
<td>P11</td>
<td>CCTGACCAATATCAGATGGCAC</td>
<td>13622–13654</td>
</tr>
<tr>
<td>P12</td>
<td>TGCTACCCAAGGCGGAG</td>
<td>13630–13641</td>
</tr>
<tr>
<td>P13</td>
<td>GCAGTTACATCCGCTGCACAGGCGGAG</td>
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</tr>
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<td>P14</td>
<td>GCTGATATTATATACCGGACCTGCAATAC</td>
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<td>P15</td>
<td>TCGACGGATCACATCCGCTGCACAGGCGGAG</td>
<td>13592–13620</td>
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<td>P16</td>
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<td>P26</td>
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<tr>
<td>OligoDT+</td>
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<td>122-GST, pORF11/116</td>
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<td>adaptor</td>
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<td>122, 16</td>
</tr>
<tr>
<td>IE63 RT</td>
<td>ATGCCAATCTGAGGCGCACTTTTCATTTGC</td>
<td>11035–11052</td>
</tr>
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</table>

*Portions of sequences shown in bold indicate restriction enzymes sites. The underlined portion contains the introduced or mutated VZV sequence.

The nuclear and cytoplasmic extracts from cells were prepared by using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) and followed the manufacturer’s instructions. For the comparison of VZV IE and late protein expression, 1 × 10^6 melanoma cells were seeded per well in 6-well plates 1 day before infection. Cells were infected with log_{10} 40 plaque forming units (PFU) of POKA or POKA11 per well. Equivalent amounts of cell lysates extracted from infected cells over a time course of 1-, 2-, 3- and 4-day post-infection were resolved on SDS-PAGE followed by Western blot analysis with rabbit polyclonal antibodies against IE4, IE63 (kindly provided by Dr. Knitchking) and IE62 (kindly provided by Dr. Ruyechan, University of Buffalo, NY), respectively, and with mouse monoclonal antibody against gE (MAb6812, Millipore Biosciences, Temecula, CA).

Plasmid construction

The full-length ORF11 sequence was generated by PCR using primers P3 and P4, with the psvp73 cosmids as a template. The PCR product was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) to obtain an ORF11 expression plasmid, pCMV11. The IE62 expression plasmid pCMV62 and luciferase reporter plasmids pGL3-IE62, pGL3-ORF10 and pGL3-gE were reported previously (Berarducci et al., 2007; Che et al., 2007; Wang et al., 2009).

Plasmids pORF11/1-261-GST, pORF11/16-261-GST, pORF11/116-261-GST and pORF11/191-261-GST, expressing residues 1–261, 16–261, 116–261, 191–261, respectively, as GST fusion proteins, were generated by PCR with primers P1 and P2; P7 and P8; P2 and P9; and P2; the cosmid psvp73 was used as the template (Table 1). Plasmids pORF11/1-122-GST, pORF11/16-122, and pORF11/1–22, expressing ORF11 amino acid residues 1–122, 16–122, and 1–22, respectively, were constructed using cosmids psvp73 as a template; PCR was done with three pairs of primers, including P1 and P10; P7 and P10; and P1 and P11. The plasmid pORF11N/ST1-GST, which expresses the ORF11 N-terminal 1–122 aa with the arginine-rich motif RRQSRQRR mutated to AAQAIAAQ as a GST fusion protein (the underlined residues show the mutated amino acids) was generated through four PCRs. The first PCR was done with the plasmid pORF11/1–261-GST as a template and primers P2 and P10, the second PCR was synthesized by using the first PCR product as a template and primers P13 and P10, the third PCR was done using the second PCR product as a template and primers P14 and P10, and the last PCR was obtained by using the third PCR product as a template and primers P15 and P10. Plasmid pORF11ST2-GST, expressing the ORF11 N-terminus with mutation of the first two arginines in the arginine-rich motif to alanines, was generated with pORF11N/ST1-GST plasmid as a template and primers P16 and P10. Plasmid pORF11C-GST, in which ORF11 C-terminal tagged with GST, was constructed by PCR with the pair of primers P5 and P6 and the cosmids psvp73 as a template. The resulting PCR fragments were inserted into Small-cut pGEX-2T vector.

Reporter gene assays, cryo-immuno electron microscopy and confocal microscopy

Reporter gene assays and cryo-immuno EM samples were prepared as described previously (Che et al., 2007, 2008). For confocal microscopy, human embryonic lung fibroblasts (HEL) were fixed with 4% formaldehyde at 10 and 24 h after VZV infection. After blocking with 1% fish gelatin for 1 h at RT, cells were incubated with a murine monoclonal anti-IE62 antibody (kindly provided by Dr. Ruyechan) and Ab11, or murine monoclonal anti-gE for 1 h at RT. Cells were washed and incubated for 1 h at RT with fluorescein isothiocyanate-labeled anti-rabbit and Texas Red-labeled anti-mouse secondary antibodies (Jackson ImmunoResearch, Inc.). Cell nuclei were counterstained with Hoechst 23338 (Invitrogen, Carlsbad, CA). Analysis was performed with a Leica TCS-SP confocal laser scanning microscopy (Heidelberg, Germany).

DNA immunoprecipitation and detection using RT-PCR

Cells were lysed using RNase-free lysis buffer [50 mM Tris (pH 7.6), 150 mM NaCl, 2.5 mM MgCl2, 0.1% NP-40, and protease inhibitor] and incubated at 4 °C for 10 min. Cells were then sonicated for 30 s and centrifuged at 4 °C for 10 min at 2851 RCF. The supernatant was collected in a fresh tube and RNasin (Invitrogen, Carlsbad, CA) was added at 1 U/μl. Immunoprecipitation was performed with 400 μl of cell extracts and 5 μl of Ab11 or anti-ORF47 antibody. After the final wash, the beads were resuspended in 300 μl of lysis buffer and extracted once with phenol and once with phenol-chloroform (pH 4.6). The extracted nucleic acid was ethanol precipitated and dissolved in 100 μl elution buffer (1 mM EDTA, Applied Biosystems/Ambion, Austin, TX). After treatment with 10 units of DNase I (Applied Biosystems/Ambion, Austin, TX) for 1 h at 37 °C, each sample was extracted by phenol-chloroform (pH 4.6), precipitated by ethanol, and resuspended in 40 μl RNase-free water. RT-PCR was done with the samples purified by RNA immunoprecipitation. cDNA was synthesized using an oligo(dt)-containing adaptor primer (Table 1). PCR was then done with an ORF63 gene-specific primer and an adaptor primer. The resulting PCR product was cloned and sequenced.

Synthesis of RNA probes and Northern blot

PCR products corresponding to VZV IE, early and late genes, and to cellular gene α-actin, were cloned under the control of a T7 promoter (Che et al., 2006). 1 μg of plasmid DNA was linearized and incubated for 1 h at 37 °C in a final reaction volume of 20 μl, containing 1 × T7 RNA polymerase reaction buffer, 10 mM ATP, CTP, and GTP, 2 μl of T7 RNA polymerase (Applied Biosystems/Ambion, Austin, TX); and 5 μl of [α-32P]-UTP (3,000 Ci/mmol) (GE Healthcare, Bio-Sciences Corp.,


Donnelly, M., Verhagen, J., Elliott, G., 2007. RNA binding by the herpes simplex virus type 1 nucleocapsid protein UL47 is mediated by an N-terminal arginine-rich domain that also functions as its nuclear localization signal. J. Virol. 81 (5), 2283–2296.


Zhang, Y., McKnight, J.L., 1993. Herpes simplex virus type 1 UL46 and UL47 deletion mutants lack VP11 and VP12 or VP13 and VP14, respectively, and exhibit altered viral thymidine kinase expression. J. Virol. 67 (3), 1482–1492.