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The Varicella-Zoster Virus (VZV) Open Reading Frame 47 (ORF47) Protein Kinase Is Dispensable for Viral Replication and Is Not Required for Phosphorylation of ORF63 Protein, the VZV Homolog of Herpes Simplex Virus ICP22

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To investigate the role of varicella-zoster virus (VZV) open reading frame 47 (ORF47) protein kinase during infection, a VZV mutant was generated in which two contiguous stop codons were introduced into ORF47, thus eliminating expression of the ORF47 kinase. ORF47 kinase was not essential for the growth of VZV in cultured cells, and the growth rate of the VZV mutant lacking ORF47 protein was indistinguishable from that of parental VZV. Nuclear extracts from cells infected with parental VZV contained several phosphorylated proteins which were not detected in extracts from cells infected with the ORF47 mutant. The herpes simplex virus type 1 (HSV-1) UL13 protein (the homolog of VZV ORF47 protein) is responsible for the posttranslational processing associated with phosphorylation of HSV-1 ICP22 (the homolog of VZV ORF63 protein). Immunoprecipitation of ^{32}P-labeled proteins from cells infected with parental virus and those infected with ORF47 mutant virus yielded similar amounts of the VZV phosphoproteins encoded by ORF4, ORF62, ORF63, and ORF68 (VZV gE), and the electrophoretic migration of these proteins was not affected by the lack of ORF47 kinase. Therefore, while the VZV ORF47 protein is capable of phosphorylating several cellular or viral proteins, it is not required for phosphorylation of the ORF63 protein in virus-infected cells.

Varicella-zoster virus (VZV) causes chicken pox following primary infection and shingles upon reactivation from latency. On the basis of its biological and genomic properties, VZV is classified as a member of the alphaherpesvirus subfamily which includes herpes simplex virus types 1 and 2 (HSV-1 and -2). Two conserved genes within this herpesvirus subfamily are predicted to encode protein kinases on the basis of sequence similarity to eukaryotic serine/threonine kinases (1, 8, 9, 16). In VZV these genes are encoded by open reading frame 66 (ORF66) in the unique short region of the genome and by ORF47 in the unique long region of the genome.

The VZV ORF47 gene product possesses kinase activity (11, 12, 18), including the ability to phosphorylate itself, a property common to most eukaryotic protein kinases. Coimmunoprecipitation of the ORF47 protein with other VZV phosphoproteins indicates that ORF47 protein also phosphorylates the VZV ORF62 product but not proteins encoded by ORF4, ORF61, or ORF63. In contrast, HSV-1 UL13 kinase (the homolog of VZV ORF47) induces directly or indirectly the phosphorylation of HSV-1 ICP22 (the homolog of VZV ORF63), whereas the absence of UL13 expression does not affect the phosphorylation of ICP4, the HSV homolog of VZV ORF62 protein (14).

While protein phosphorylation contributes to numerous eukaryotic cellular functions, including gene transcription and translation, the role of the herpesvirus kinases during virus infection remains uncertain. The UL13 kinase of HSV-1 is dispensable for virus replication in cultured cells; however, plaque size and growth rates in some cultured cell lines are reduced in mutants with deletions of UL13 (3, 15). The HSV-1 UL13 kinase influences the accumulation of various HSV-1 mRNAs during infection of cultured cells through the actions of its substrate, ICP22 (14). The level of mRNA encoding the HSV-1 transcriptional activator, ICP0, is reduced in the absence of the UL13 kinase, suggesting that UL13 may play a role in regulating the transcription of HSV-1 genes.

To better define the role of VZV ORF47 for phosphorylation of proteins in infected cells, a VZV mutant unable to express the ORF47 kinase was generated. Here we show that ORF47 protein is responsible for phosphorylation of several proteins in infected cells but is not required for phosphorylation of ORF62 or ORF63.

Generation of VZV containing stop mutations in ORF47. Infectious recombinant VZV was produced by transfection of MeWo cells, a human melanoma cell line, with four overlapping VZV cosmids (Fig. 1) (2). VZV ORF47 is contained in the *Not*I-B cosmid and is expected to encode a 54-kDa protein comprising 510 amino acids (Fig. 1) (4). To produce a cosmid with stop codons in ORF47, the 7,069-bp *Pac*I fragment (VZV nucleotides 76,604 to 83,673) from the *Not*I-B cosmid was cloned into pNEB193 (New England Biolabs, Beverly, Mass.), yielding pNBPac. pNBPac contains the first 506 nucleotides of ORF47. A 927-bp fragment was amplified from pNBPac by PCR. The 5′ primer contained the *Spe*I (nucleotide 82,769) site from VZV, while the 3′ primer contained an *Asc*I site from pNEB193 along with nucleotides 83,654 to 86,676 of VZV. VZV nucleotides 83,663 and 83,666 were changed from G to T in the 3′ PCR primer, so that codons 166 and 167 of VZV ORF47 would both be changed to stop codons in the PCR product. The mutant PCR product was digested with *Spe*I and

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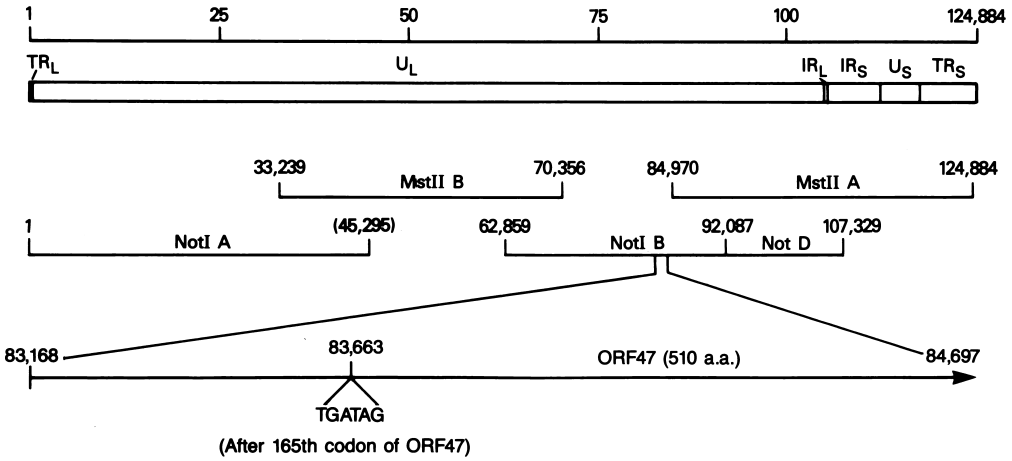

FIG. 1. Construction of recombinant VZV. The prototype VZV genome (Dumas strain [4]) is 124,884 bp in length (top line) and consists of terminal repeats (TR), unique long domains (U_L), internal repeats (IR), and unique short domains (U_S) (second line). The four overlapping cosmids used to generate infectious virus are depicted (third and fourth lines). The cosmids are named according to the restriction fragments from which they were derived. The nucleotide coordinates correspond to the sequence of the prototype strain of VZV. The 3′ end of VZV *Not*I-A terminates within the R3 repeat region, resulting in deletion of the *Not*I site at the 3′ end of the VZV insert (indicated by parentheses). ORF47 protein kinase is encoded within cosmid *Not*I-B. Cosmid *Not*I-B 47S was constructed by replacing ORF47 codons 166 and 167 with stop codons. a.a., amino acids.

*Asc*I and inserted in place of the wild-type *Spe*I-*Asc*I fragment in plasmid pNBPac, resulting in plasmid pO47S. The mutated *Pac*I fragment from pO47S was isolated and cloned into the *Not*I-B cosmid in place of the wild-type *Pac*I fragment. The resulting cosmid, *Not*I-B 47S, contains the ORF47 gene in which codons 166 and 167 have been replaced with stop codons. Two independent clones of cosmid *Not*I-B 47S, A and B, were selected.

The mutated *Not*I-B cosmid (*Not*I-B 47S) and the three parental cosmids with pCMV62 were transfected into MeWo cells by methods previously described (2). Five days after transfection the cells were passaged into 75-cm^2 flasks, and 4 days later cytopathic effects typical of VZV infection were apparent. Two independently derived VZV ORF47 mutants, ROka 47SA and ROka 47SB, were used in parallel for all subsequent experiments.

To verify that the recombinant VZV had the expected mutation, viral DNA from cells infected with the ORF47 mutants was amplified by PCR. Sequence analysis of the PCR products (19) from the ORF47 gene indicated that the mutant viruses had the expected stop codons inserted into the ORF47 gene (7). Southern blot analysis was performed to verify that the viruses produced by cosmid transfections contained no gross genomic changes. Viral DNA from parental recombinant VZV (ROka) and from the two independently derived ORF47 mutants (ROka 47SA and 47SB) was digested with *Bam*HI and *Xho*I and hybridized to the four parental cosmid DNAs that had been labeled by random priming with [^{32}P]dCTP. ROka and ROka 47S DNAs yielded restriction fragments of identical sizes. No unexpected alterations in the genomes of the mutant viruses were detected (7).

VZV ORF47 protein is dispensable for viral replication in cell culture. The inability of VZV ROka 47S to express the ORF47 protein kinase was confirmed by three independent experiments. First, cells infected with either VZV ROka or ROka 47S were metabolically labeled with [^{35}S]cysteine. Lysates of these cells were prepared, and immunoprecipitations were performed with a polyclonal rabbit antiserum directed against the amino terminus of ORF47 (11). Polyacrylamide gel electrophoresis showed a 60-kDa protein, corresponding to the ORF47 protein, expressed in cells infected with VZV ROka but not ROka 47S (Fig. 2A). Second, VZV-infected cells were metabolically labeled with $^{32}P_i$, lysates were prepared, and immunoprecipitations were performed with the anti-ORF47 rabbit serum. A 60-kDa phosphoprotein was present in cells infected with VZV ROka but not ROka 47S (Fig. 2B). Third, nuclear extracts from uninfected, VZV ROka-infected, or ROka 47S-infected cells were prepared and phosphorylated in vitro with [γ-^{32}P]ATP. A 60-kDa phosphoprotein was immunoprecipitated with the ORF47 antiserum from the nuclear extract of VZV ROka-infected cells but not from extracts of

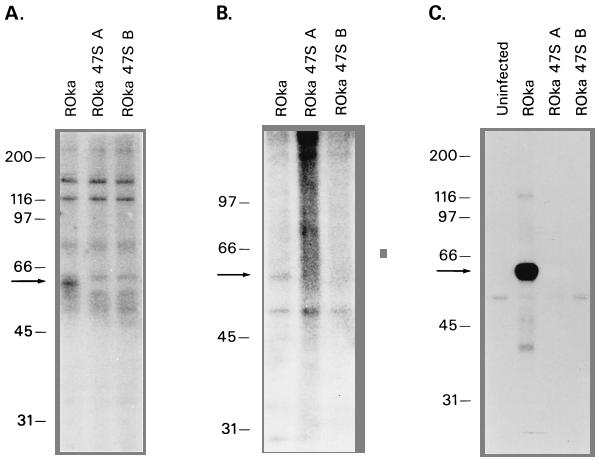

FIG. 2. The VZV ORF47 protein is not expressed during infection with ROka 47S. Cells infected with either ROka or ROka 47S were metabolically labeled for 16 h in medium containing 200 μCi of [^{35}S]cysteine per ml (A) or 300 μCi of $^{32}P_i$ per ml (B). Nuclear extracts were prepared from the labeled cells as previously described (15) and were immunoprecipitated with rabbit polyclonal antibody (diluted 1:200) directed against the VZV ORF47 protein (11). In both panels a 60-kDa protein corresponding to ORF47 is present in ROka- but not ROka 47S-infected cells (arrows). (C) Nuclear extracts (2 mg) prepared from uninfected, ROka-infected, or ROka 47S-infected cells were phosphorylated in vitro with [γ-^{32}P]ATP as previously described (15) and immunoprecipitated with anti-ORF47 protein antibody. The VZV ORF47 protein is present in nuclear extracts from ROka-infected cells but not in nuclear extracts from uninfected or ROka 47S-infected cells. Numbers are sizes of proteins in kilodaltons.

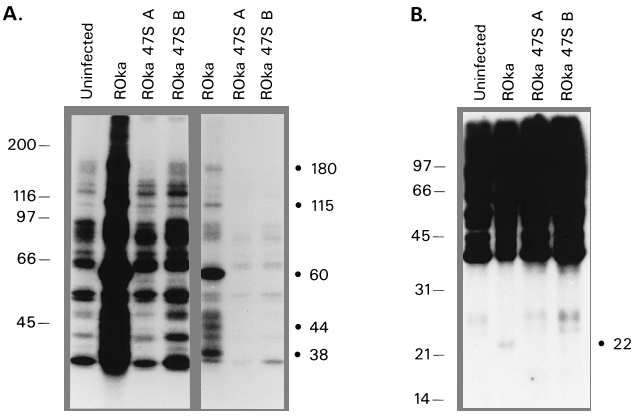

FIG. 3. The VZV ORF47 kinase is responsible for phosphorylation of several proteins in nuclear extracts. Nuclear extracts prepared from uninfected cells or from cells infected with ROka or ROka 47S were phosphorylated in vitro by using [γ-^{32}P]ATP. The proteins were separated by electrophoresis on either 8% (A) or 12% (B) polyacrylamide gels. A lighter exposure of a portion of panel A is included to emphasize the individual protein species. Several phosphoproteins present in the nuclear extracts of ROka-infected cells are not present in the nuclear extracts from uninfected or ROka 47S-infected cells (closed circles). Numbers are sizes of proteins in kilodaltons.

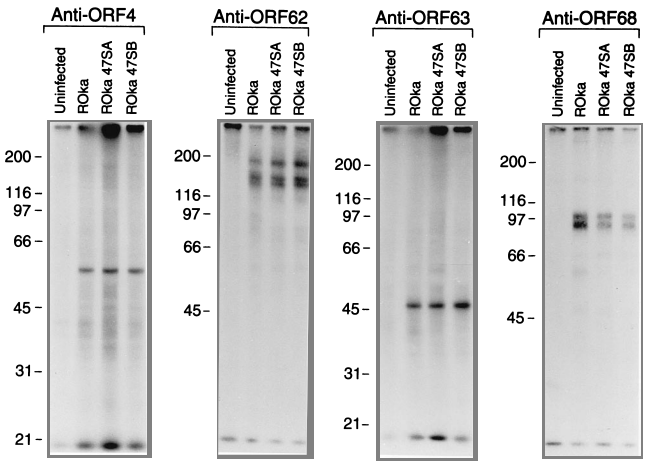

FIG. 4. Phosphorylation of the VZV ORF4, ORF62, ORF63, and ORF68 (gE) proteins is not altered in the absence of ORF47 protein kinase. Uninfected, ROka-infected, and ROka 47S-infected cells were metabolically labeled with $^{32}P_i$. VZV phosphoproteins encoded by ORF4, ORF62, ORF63, and ORF68 were immunoprecipitated from nuclear extracts of the labeled cells. Rabbit antisera specific for ORF4 and ORF63 proteins (10, 12) were used at a dilution of 1:100 or 1:200. Mouse monoclonal antibodies directed against the ORF62 and ORF68 (gE) proteins (Chemicon International, Inc., Temecula, Calif.) were used at a 1:500 or 1:1,000 dilution. Each of the four VZV proteins was phosphorylated to similar extents and migrated at identical rates in the presence and absence of the VZV ORF47 protein kinase.

uninfected or ROka 47S infected cells. These results demonstrate that the VZV ORF47 protein kinase is not expressed in cells infected with the ROka 47S mutants and that the ORF47 protein is dispensable for virus replication in vitro. The molecular mass of the ORF47 protein detected consistently in these studies (60 kDa) was slightly greater than that reported previously (54 kDa) (11, 12, 18). The variation in size may be due to differences in the electrophoresis conditions or in virus strains.

VZV unable to express the ORF47 protein is not impaired for growth in cell culture. The plaque morphologies of cells infected with VZV ROka and ROka 47S were indistinguishable from one another. To further evaluate whether the lack of VZV ORF47 expression affects the growth of the virus in vitro, we measured virus production of ROka and ROka 47S during a 5-day growth analysis by methods previously described (5, 6). MeWo cells were inoculated with virus-infected cells, and each day thereafter the infected monolayers were harvested and the virus titers were determined. The yields of PFU were similar for cells infected with ROka and ROka 47S (7). Therefore, VZV unable to express ORF47 protein is not impaired for growth in cell culture.

VZV ORF47 kinase is responsible for the phosphorylation of several proteins in nuclear extracts. VZV ORF47 protein was phosphorylated in vitro when nuclear extracts from infected cells were incubated with [γ-^{32}P]ATP (Fig. 2C; also see the 60-kDa protein in Fig. 3A). Comparison of other proteins that were phosphorylated in vitro by nuclear extracts from ROka- and ROka 47S-infected cells by both 8% (Fig. 3A) and 12% (Fig. 3B) polyacrylamide gel electrophoresis revealed the presence of several phosphoproteins in ROka-infected cells that were absent from ROka 47S-infected cells. These phosphoproteins of 180, 115, 44, and 38 kDa (Fig. 3A) and 22 kDa (Fig. 3B) may be of viral or cellular origin. Thus, either the ORF47 protein is a broadly active protein kinase, or it induces cellular kinase activity during infection.

Phosphorylation of the VZV ORF4, ORF62, ORF63, and ORF68 (gE) proteins is not altered in the absence of ORF47 protein kinase. To determine the role of the VZV ORF47 protein kinase in the phosphorylation of VZV-encoded proteins, we immunoprecipitated viral phosphoproteins from nuclear extracts of VZV ROka- and ROka 47S-infected cells that had been labeled in vitro with $^{32}P_i$. Each of the phosphoproteins studied, VZV ORF4, ORF62, ORF63, and ORF68 (gE) proteins, was phosphorylated to similar extents in the presence and absence of the ORF47 protein kinase (Fig. 4). Furthermore, the molecular weights of each of the four VZV phosphoproteins studied, including ORF62 protein and gE, which migrate as families of polypeptides on polyacrylamide gels, were identical in extracts of VZV ROka- and ROka 47S-infected cells.

Two VZV proteins, ORF62 and ORF63, were examined by using two-dimensional (2-D) gel electrophoresis (13) to further evaluate whether their phosphorylation was altered by the absence of the ORF47 kinase. Identical bands between pH 5.5 and 6.0 were generated by 2-D gel electrophoresis of ORF62 protein immunoprecipitated from the extracts of ROka- and ROka 47S-infected cells. Similarly, ORF63 protein from extracts of ROka- and ROka 47S-infected cells yielded identical, single broad bands from pH 4.7 to 5.7 on 2-D gel electrophoresis (7). Therefore, we were unable to detect any differences in phosphorylation of VZV proteins resulting from the absence of the ORF47 kinase by 1-D or 2-D gel electrophoresis.

These experiments demonstrate that VZV ORF47 protein, like its HSV homolog, UL13, is not required for growth of the virus in cultured cells. Previous studies showed that the VZV ORF47 protein has protein kinase activity. Immunoprecipitated VZV ORF47 protein phosphorylates both itself and VZV ORF62 protein in vitro (11, 18). In this study, we showed that several proteins contained in nuclear extracts of VZV-infected cells are dependent on the ORF47 protein for phosphorylation. At present, it is not known whether these proteins are of viral or cellular origin. While there was no apparent difference between growth of the VZV ORF47 mutant and that of the parental virus in a human melanoma cell line, the ability of the VZV ORF47 protein to phosphorylate (or induce

the phosphorylation of) a variety of proteins may be important for VZV replication in vivo.

Phosphorylation of VZV proteins encoded by ORF4, ORF62, ORF63, and ORF68 (gE) was compared in cells infected with parental virus and cells infected with VZV unable to express the ORF47 protein. Each of these proteins was phosphorylated to similar extents and had similar molecular weights whether or not the ORF47 kinase was expressed. Similar experiments comparing wild-type HSV-1 and HSV-1 UL13 mutants revealed marked differences in the migration patterns of polypeptides comprising the HSV-1 immediate-early protein, ICP22 (15). In contrast, the VZV homolog of ICP22, ORF63 protein, migrates as a single polypeptide at the same rate whether immunoprecipitated from ROka- or from ROka 47S-infected cells. In addition, more-sensitive experiments using 2-D gel electrophoresis revealed no differences in the phosphorylation of ORF63 protein derived from ROka- and ROka 47S-infected cells. These results suggest that the VZV ORF63 protein is not phosphorylated by the ORF47 protein kinase. Our findings are consistent with previous results obtained with in vitro protein kinase assays (12), in which no phosphorylation of the ORF63 protein by the ORF47 kinase could be demonstrated. Another VZV gene, ORF66, is predicted to encode a protein kinase in VZV-infected cells (18). The ORF66 protein might contribute to the phosphorylation of the ORF63 protein; alternatively, the ORF63 protein may be phosphorylated solely by cellular kinases during VZV infection.

We were also unable to detect a difference in the phosphorylation pattern of VZV ORF62 protein in cells infected with ROka and those infected with ROka 47S by 1-D or 2-D gel electrophoresis. Prior experiments showed that VZV ORF62 protein was phosphorylated in vitro after coimmunoprecipitation with ORF47 protein (12). Taken together these studies indicate that ORF47 protein is capable of phosphorylating ORF62 protein, but other viral or cellular kinases can fully phosphorylate ORF62 protein in the absence of ORF47 kinase. Moreover, the presence of both ORF47 and ORF62 proteins in the virion (18) suggests that phosphorylation of ORF62 protein by ORF47 kinase may occur in the virions themselves, or shortly after the infection of cells (12).

While we were unable to identify a VZV phosphoprotein as a substrate for the ORF47 protein kinase, other viral or cellular proteins may be targets for this protein kinase (17, 20). Phosphorylation of a cellular protein might facilitate the growth of the virus during replication in vivo. Preliminary experiments suggest that HSV-1 that is unable to express the UL13 kinase, the HSV homolog of VZV ORF47, is slightly less lethal in mice than wild-type HSV-1 after intracranial inoculation (3). Future studies of animals infected with VZV ORF47 protein kinase will help to determine the role of the ORF47 protein during infection in vivo.

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