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The Varicella-Zoster Virus ORF66 Protein Induces Kinase Activity and Is Dispensable for Viral Replication

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Varicella-zoster virus (VZV) open reading frames (ORFs) 47 and 66 encode proteins that are homologous to a family of eukaryotic serine-threonine kinases. Prior studies showed that the VZV ORF47 protein has kinase activity in vitro and is dispensable for replication in cultured cells. To examine the role of the ORF66 protein during infection, we constructed VZV recombinants that are unable to express either the ORF66 protein (ROka 66S) or both the ORF47 and ORF66 proteins (ROka 47S/66S). VZV unable to express ORF66 grew to titers similar to those of the parental VZV (ROka) in vitro; however, VZV lacking both ORF66 and ORF47 grew to titers lower than those of ROka. Nuclear extracts from ROka 66S- or ROka 47S-infected cells showed a 48-kDa phosphoprotein(s); a phosphoprotein with a similar size was not present in nuclear extracts from ROka 47S/66S-infected cells. To determine the role of the ORF66 protein in the phosphorylation of specific VZV-encoded proteins, we immunoprecipitated known VZV phosphoproteins (ORF4, ORF62, ORF63, and ORF68 proteins) from nuclear extracts of phosphate-labeled cells infected with ROka, ROka 66S, or ROka 47S/66S. Each of the VZV phosphoproteins was phosphorylated to a similar extent in the presence or absence of either the ORF66 protein or both the ORF66 and ORF47 proteins. From these studies we conclude (i) neither ORF66 alone nor ORF66 and ORF47 in combination are essential for VZV growth in cultured cells, (ii) ORF66 either is a protein kinase or induces protein kinase activity during infection, and (iii) the VZV phosphoproteins encoded by ORF4, ORF62, ORF63, and ORF68 do not require either ORF66 alone or ORF66 and ORF47 for phosphorylation in vitro.

Varicella-zoster virus (VZV) is a member of the alphaherpesviruses subfamily along with herpes simplex virus types 1 and 2 (HSV-1 and -2). Like the other alphaherpesviruses, VZV has two open reading frames (ORF47 and ORF66) predicted to encode protein kinases on the basis of homology to known serine/threonine protein kinases (1, 12, 20, 30). ORF47 of VZV encodes a 54- to 60-kDa phosphoprotein (13, 23), and ORF66 encodes a 48-kDa phosphoprotein (31).

Little is known about the function of VZV ORF66. While VZV ORF47 has homologs in the alpha-, beta-, and gammaherpesvirus subfamilies, VZV ORF66 has homologs only in the alphaherpesvirus subfamily. Stevenson and colleagues (31) have shown that in cells infected with vaccinia virus expressing ORF66, the ORF66 protein is located exclusively in the cytoplasm. Immunoprecipitation of ORF66 protein from cells infected with either VZV or vaccinia virus expressing ORF66, followed by incubation in vitro with [γ-^{32}P]ATP, did not result in phosphorylation of the protein (31). Thus, while ORF66 is homologous to the HSV-1 UL3 protein kinase, evidence that the ORF66 protein possesses kinase activity in vitro has not been presented.

Substrates have been identified for the HSV-1 homologs of VZV ORF47 and ORF66. The ORF47 homolog, encoded by HSV-1 UL13, induces the phosphorylation of the HSV-1 regulatory protein ICP22 (27, 28). The ORF66 homolog, encoded by HSV-1 US3, mediates the posttranslational modification of the membrane phosphoprotein encoded by HSV-1 UL34 (29). However, the functional importance of the UL34 protein as a substrate for HSV-1 US3 protein has been questioned since the UL34 phosphorylation site (29) is absent from the UL34 homologs of other alphaherpesviruses, including VZV and equine herpesvirus 1 (18). The US3 protein kinase of HSV-2 has been associated with the phosphorylation of a 14- to 22-kDa protein believed to be the US9 tegument protein (4) and an 80-kDa protein identified as the UL12 alkaline exonuclease (6). Thus, it has been postulated that the US3 protein kinases of HSV-1 and HSV-2 have substrates other than the UL34 protein. Although none have been definitively identified, cellular proteins may also be substrates for the alphaherpesvirus kinases.

The HSV-1 proteins encoded by UL13 and US3 either alone or in combination are dispensable for replication of the virus in cell culture (3, 26, 28). Similarly, both of the corresponding homologs in pseudorabies virus (PRV) are dispensable for its replication in vitro (8). We have constructed VZV mutants that are unable to express either the ORF66 protein or both the ORF66 and ORF47 proteins. Neither ORF66 nor the combination of ORF66 and ORF47 is essential for growth of VZV in cell culture; however, VZV unable to express both proteins grows to lower titers in vitro than does the parental virus. The analysis of cells infected with the ORF66 and ORF47 mutants indicated that the ORF66 protein induces kinase activity in vitro.

Generation of VZV unable to express the ORF66 protein. Recombinant VZV can be produced by cotransfection of four overlapping cosmids which span the entire viral genome (Fig. 1). The VZV ORF66 protein is encoded by nucleotides 113,037 to 114,215 (7) within the *Mst*IIA cosmid. To construct a cosmid that is unable to express the ORF66 protein, the VZV *Mst*IIA cosmid was digested with *Hha*I by the RecA-assisted restriction endonuclease cleavage procedure (10). A 60-base oligonucleotide, ATCCAACAAATTGTGACGTTAT

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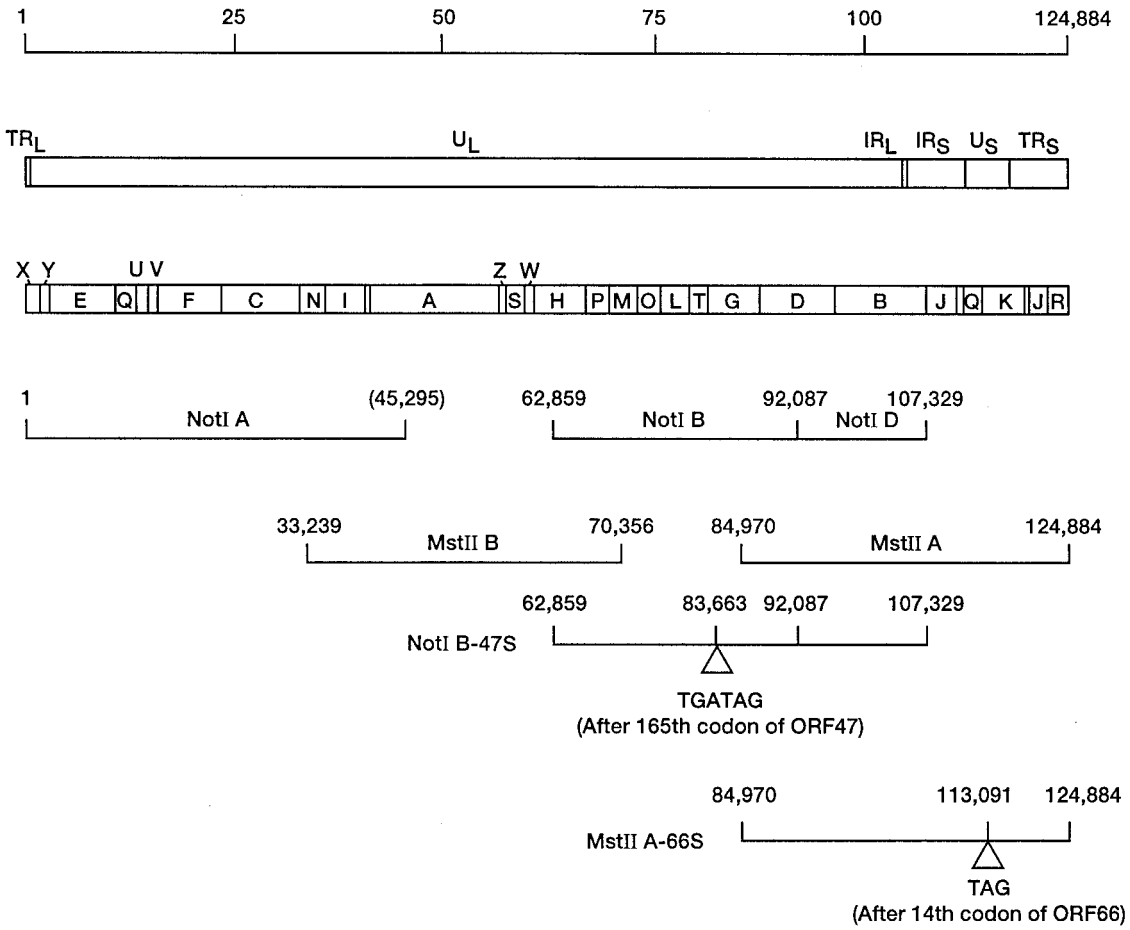

FIG. 1. Construction of recombinant VZV. The prototype VZV genome (Dumas strain) is 124,884 bp in length (top line) and consists of terminal repeat (TR), unique long (U_L), internal repeat (IR), and unique short (U_S) DNA domains (second line) (7). A *Bam*HI restriction endonuclease map is shown (third line). The four overlapping cosmids used to generate infectious virus are depicted (fourth and fifth lines). The nucleotide coordinates correspond to the sequence of the prototype strain of VZV. The 3′ end of VZV *Not*IA 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). Cosmid *Not*IB-47S contains two consecutive in frame stop codons after the 165th codon of ORF47 (sixth line). Cosmid *Mst*IIA-66S contains stop codons in all three ORFs after the 14th codon of ORF66 (seventh line).

ATATCCCAAGGCAAAGGCCGCTCCCGTCATAGCAA ATA, complementary to ORF66 and centered at the *Hha*I site (VZV nucleotide 113,091) was synthesized. Ninety nanograms of the 60-mer was annealed to 1 μg of VZV cosmid *Mst*IIA in the presence of the *Escherichia coli* RecA protein for 15 min at 37°C. The remaining, unprotected *Hha*I sites in the *Mst*IIA cosmid were methylated by using *Hha*I methylase at 37°C for 1 h. After being heated to 65°C for 15 min to inactivate the methylase and dissociate the RecA-oligonucleotide complex, the DNA was cut with *Hha*I and blunted with T4 DNA polymerase. A 20-base oligonucleotide, TAGCTAGGCGCGCC TAGCTA, was inserted into the *Hha*I site. This oligonucleotide contains an *Asc*I site and stop codons in all three open reading frames. The resulting cosmid, VZV *Mst*IIA-66S, contains the ORF66 gene with stop codons after the 14th codon of ORF66. Two independent clones, A and B, were selected and sequenced at the site of the oligonucleotide insertion; in both cases a cytosine residue (VZV nucleotide 113,092) had been unexpectedly deleted during the cloning procedure.

The mutated cosmid (*Mst*IIA-66S) was transfected into MeWo cells with the other three parental cosmids by methods previously described (2). Cytopathic effects typical of VZV infection were apparent 9 days after transfection, indicating the presence of infectious recombinant VZV. Two independent ORF66 mutants, ROka 66SA and ROka 66SB, were used in parallel for all experiments. VZV recombinants unable to express both ORF66 and ORF47 were also generated. *Mst*IIA-66S and *Not*IB-47S, a cosmid that contains stop codons in ORF47 (13), along with the remaining two parental cosmids were cotransfected into MeWo cells. Again, cytopathic effects were noted 9 days after transfection. Two independent clones with stop codons in both ORF66 and ORF47, ROka 47S/66SA and ROka 47S/66SB, were isolated.

Southern blot analysis was performed to verify that the viruses derived from cosmid *Mst*IIA-66S had the expected mutation. ROka 66S or ROka 47S/66S DNA was digested with *Sac*I and *Asc*I, transferred to nitrocellulose, and hybridized to the four parental cosmid DNAs that had been labeled with [^{32}P]dCTP. A 6.1-kb fragment present in ROka was cleaved into two fragments of 4.9 and 1.2 kb because of the presence of the new *Asc*I site in the inserted oligonucleotide (Fig. 2A and B). To verify that ROka 47S/66S had the expected mutations in ORF47, viral DNAs were amplified from infected cells by PCR with primers 5′-CACGCTCAATGAACAGTTATG-3′ and 5′-TGAGGCCGGAATGCGTATGCCTTCGAAAAGCTTTG

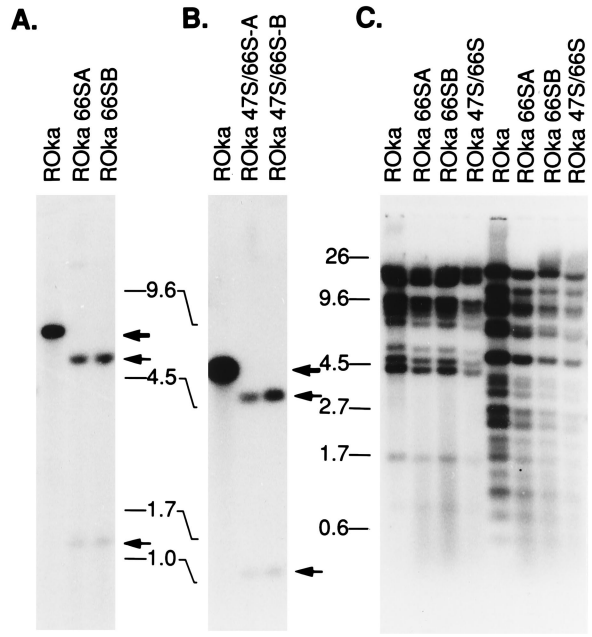

FIG. 2. Southern blot analysis of recombinant VZV DNA. (A and B) Virion DNA isolated from nucleocapsid preparations was digested with *Asc*I and *Sac*I and probed with a radiolabeled *Sac*I fragment (VZV nucleotides 111,912 to 117,989). A 6.1-kb fragment present in ROka DNA was replaced by 4.9- and 1.2-kb fragments in ROka 66S and ROka 47S/66S DNA (arrows). (C) ROka, ROka 66S, and ROka 47S/66S virion DNAs were digested with *Eco*RI (C, lanes 1 to 4) and *Bam*HI (C, lanes 5 to 8) and probed with all four radiolabeled cosmid DNAs. The DNA fragments produced by *Eco*RI or *Bam*HI digestion are identical in size. The numbers refer to the sizes (in kilobase pairs) of DNA.

AGTGCGTTACGCCGTAACGCACTGC-3′ (VZV nucleotides 83,521 to 83,541 and 84,533 to 84,592, respectively). Sequence analysis of the PCR products (33) indicated that the viruses contained the expected stop codons in ORF47. Southern blot analysis of *Eco*RI or *Bam*HI digests of each of the viral DNAs showed identical bands, indicating that no gross genomic changes had occurred (Fig. 2C).

The ORF66 protein is dispensable for viral replication in cell culture in the presence or absence of the ORF47 kinase. VZV ORF66 and ORF47 encode 48- and 60-kDa proteins, respectively (13, 31). The inability of VZV ROka 66S and ROka 47S/66S to express the ORF66 protein was confirmed by immunoprecipitation of [^{35}S]methionine-labeled extracts from infected cells with an antibody specific for ORF66 protein. To produce the antibody to the ORF66 protein, the carboxyl portion of ORF66 was amplified from cosmid VZV MstIIA by PCR with oligonucleotides CCGGGAATTCCAGGTGATGT TTGTGTGGGAGAC and CGGGAATTCTTAATCTCCAA CTTCCATTGGATT (VZV nucleotides 113,685 to 113,708 and 114,195 to 114,218, respectively). The PCR product was cut with *Eco*RI, and the VZV DNA was cloned into the *Eco*RI site of plasmid pGEX-2T (Pharmacia Biotech, Piscataway, N.J.). The resulting plasmid, pGEX-66, encodes the glutathione-*S*-transferase protein fused to amino acids 217 to 393 of the VZV ORF66 protein. *E. coli* cells were transformed with pGEX-66, and the fusion protein was purified and used to immunize the rabbits. Immunoprecipitation with the ORF66-specific rabbit serum revealed a 48-kDa protein in cells infected with VZV ROka but not in cells infected with ROka 66S or ROka 47S/66S (Fig. 3A). Immunoprecipitation of another aliquot from each of the [^{35}S]methionine-labeled extracts with antibodies to VZV gE indicated that each of the infected cell

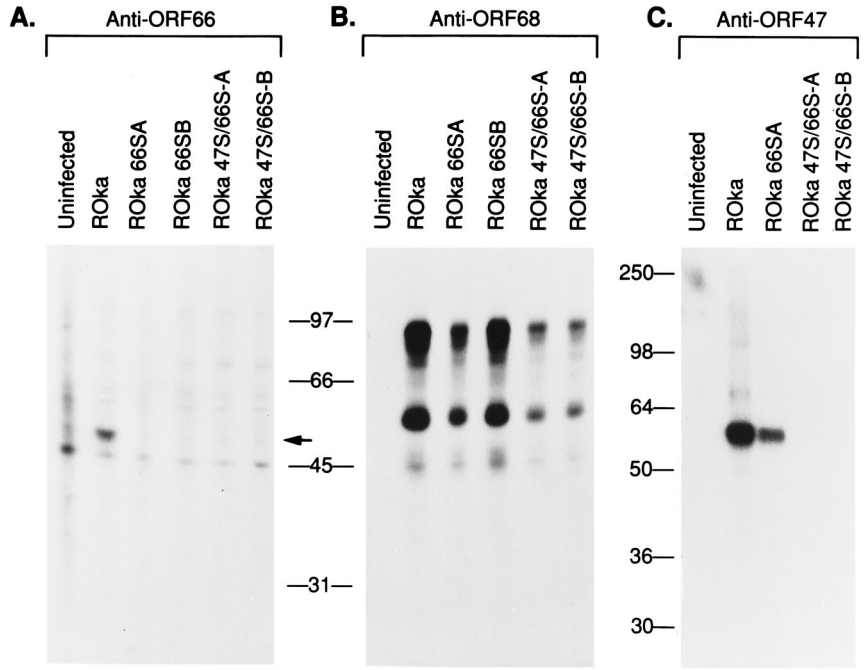

FIG. 3. VZV ORF66 protein is not expressed during infection with ROka 66S; neither ORF66 nor ORF47 protein is expressed during infection with ROka 47S/66S. (A) Uninfected cells or cells infected with ROka, ROka 66S, or ROka 47S/66S were labeled with [^{35}S]methionine. By immunoprecipitation with ORF66-specific antiserum, a 48-kDa protein was detected in extracts from cells infected with ROka (arrow) but not in extracts from uninfected cells or cells infected with ROka 66S or ROka 47S/66S. (B) By immunoprecipitation with antibodies to VZV gE, similar amounts of VZV gE were detected in each of the infected cell extracts. (C) Nuclear extracts prepared from uninfected cells or cells infected with ROka, ROka 66S, or ROka 47S/66S were phosphorylated in vitro and immunoprecipitated with anti-ORF47 protein antibodies. The VZV ORF47 protein is present in ROka- and ROka 66S-infected cells but not in nuclear extracts from uninfected or ROka 47S/66S-infected cells. The numbers are the sizes (in kilodaltons) of the proteins.

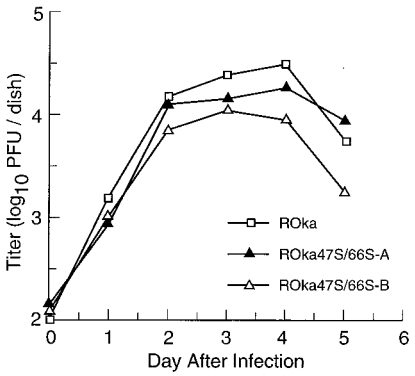

FIG. 4. VZV unable to express both the ORF47 and ORF66 proteins is impaired for growth in cell culture. Melanoma cells were inoculated with ROka- or ROka 47S/66S-infected cells. Aliquots were harvested daily for 5 days following infection, and the titers in melanoma cells were determined. Day 0 value is the titer of virus in the VZV-infected cell inoculum. Each experiment was performed in duplicate, and the $\log_{10}$ of the mean number of plaques per dish at each time point is indicated.

extracts contained VZV gE (Fig. 3B), confirming that the lack of detectable ORF66 protein was not due to uninfected cells.

The inability of VZV ROka 47S/66S to express ORF47 protein was verified by immunoprecipitation of in vitro-phosphorylated nuclear extracts (13) from infected cells with an anti-ORF47 antibody (a kind gift of C. Grose [23]). A 60-kDa phosphoprotein was present in nuclear extracts prepared from VZV ROka or ROka 66S but not from ROka 47S/66S-infected cells (Fig. 3C).

VZV unable to express both the ORF66 protein and the ORF47 kinase is impaired for growth in cell culture. The plaque morphologies of cells infected with VZV ROka and ROka 66S were indistinguishable from each other. However, cells infected with ROka 47S/66S yielded smaller plaques than did cells infected with parental VZV. The size of plaques produced by ROka 47S/66S was 0.48 ± 0.22 mm (mean ± standard deviation), while the size of plaques produced by ROka was 0.82 ± 0.25 mm ($P < 0.01$; Student's t test). In contrast, the plaque size for cells infected with ROka was similar to that infected with ROka 66S (0.74 ± 0.27 mm). To further evaluate the growth characteristics of VZV ROka 66S and ROka 47S/66S in vitro, we measured virus production in cells infected with these mutants and the parental virus during 5 days of growth. The yield of plaque forming units was similar for cells infected with ROka and ROka 66S (14); however, the yield of plaque forming units was less during the first 3 days for ROka 47S/66S-infected cells, consistent with the difference in plaque size (Fig. 4). Previous studies showed that VZV unable to express ORF47 grew to titers similar to those of parental VZV (13). Therefore, while VZV unable to express ORF66 or ORF47 proteins was not impaired for growth in cell culture, VZV lacking both ORF66 and ORF47 proteins grew to lower titers in vitro.

The ORF66 protein has or induces kinase activity in vitro. Protein kinase assays were performed with nuclear extracts from cells infected with ROka, ROka 66S, ROka 47S, or ROka 47S/66S to compare the relative contributions of the VZV ORF66 and ORF47 proteins to the total kinase activity of infected cells (Fig. 5). Nuclear extracts prepared from ROka- or ROka 66S-infected cells and incubated with [γ-^{32}P]ATP showed a 60-kDa phosphoprotein which has previously been identified as the ORF47 kinase (13). As expected, this protein was not present in nuclei of cells infected with ROka 47S or ROka 47S/66S. No obvious differences were noted between proteins phosphorylated by nuclear extracts from ROka 66S-infected cells and from ROka-infected cells; however, nuclear extracts from ROka 47S/66S-infected cells failed to phosphorylate a 48-kDa species (Fig. 5, arrow) which was phosphorylated by extracts of ROka 66S. A phosphoprotein with a similar size was present in ROka 47S-infected cells. Therefore, the ORF66 protein either has kinase activity or induces the activity of another protein kinase in nuclei of VZV-infected cells. In the absence of the ORF66 protein, the ORF47 kinase apparently phosphorylates the same protein or a protein with a similar size. Thus, the two VZV proteins may share a common substrate.

Previously, we showed that a phosphorylated form of ORF47 protein could be immunoprecipitated from nuclear extracts of VZV-infected cells labeled with $^{32}P_i$ (13). In the present study, we were unable to immunoprecipitate an ORF66 phosphoprotein from similar radiolabeled extracts. Stevenson and colleagues (31) were able to detect an ORF66 phosphoprotein from whole-cell extracts of $^{32}P_i$-labeled cells. Our data suggest either that the phosphorylated form of the ORF66 protein is present in much lower abundance in infected cell nuclei than is the ORF47 kinase or that the ORF66 protein is phosphorylated less efficiently in the strain of VZV that we used.

Phosphorylation of the VZV ORF4, ORF62, ORF63, and ORF68 (gE) proteins is not altered in the absence of ORF66 protein. To determine the role of the VZV ORF66 protein in the phosphorylation of VZV-encoded proteins, we immunoprecipitated viral phosphoproteins from nuclear extracts of VZV ROka-, ROka 66S-, or ROka 47S/66S-infected cells that had been metabolically labeled in vitro with $^{32}P_i$. Each of the proteins studied, VZV ORF4, ORF62, ORF63, and ORF68 (gE) proteins, was phosphorylated to a similar extent in the presence or absence of either the ORF66 protein or both the ORF66 and ORF47 proteins (Fig. 6). In addition, the apparent molecular weights of each of the four VZV phosphoproteins

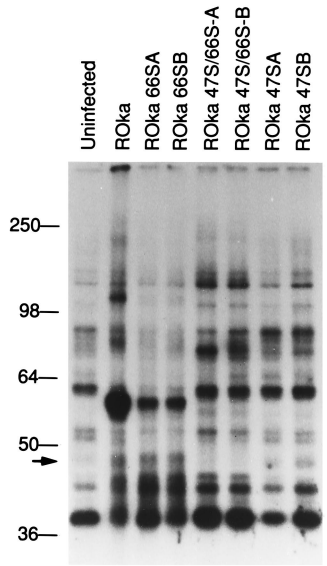

FIG. 5. VZV ORF66 protein has or induces kinase activity in vitro. Nuclear extracts prepared from uninfected cells or from cells infected with ROka, ROka 66S, ROka 47S/66S, or ROka 47S were phosphorylated in vitro with [γ-^{32}P]ATP. The proteins were separated by polyacrylamide gel electrophoresis. A 48-kDa phosphoprotein(s) is present in nuclear extracts from ROka-, ROka 66S-, and ROka 47S-infected cells but not in nuclear extracts from ROka 47S/66S-infected cells (arrow). The numbers are the sizes (in kilodaltons) of the proteins.

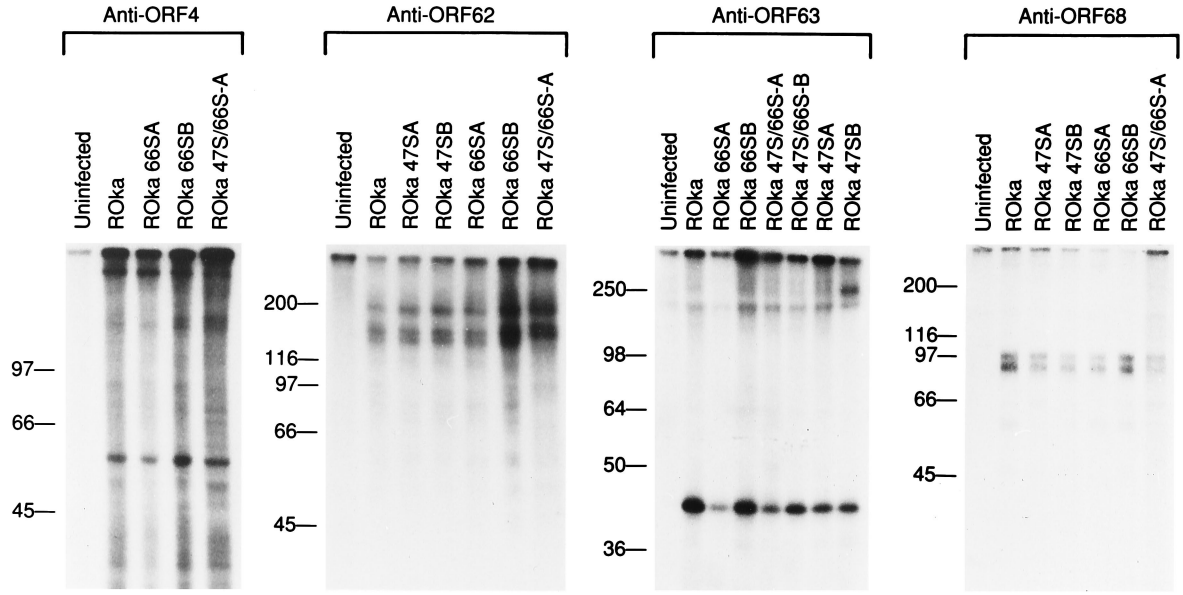

FIG. 6. Phosphorylation of the VZV ORF4, ORF62, ORF63, and ORF68 (gE) proteins is not altered in the absence of ORF66 or ORF66 and ORF47 proteins. Uninfected cells and cells infected with ROka, ROka 66S, ROka 47S/66S, and ROka 47S were metabolically labeled with $^{32}P_i$. VZV phosphoproteins encoded by ORF4, ORF62, ORF63, and ORF68 were immunoprecipitated from nuclear extracts of the labeled cells. Rabbit antisera specific for ORF4 (22) and ORF63 (24) proteins were used at a dilution of 1:100 or 1:200, respectively. Mouse monoclonal antibodies directed against the ORF62 and ORF68 (gE) proteins (Chemicon International, Inc., Temecula, Calif.) were used at a 1:500 and 1:1,000 dilution, respectively. Each of the four VZV proteins was phosphorylated to a similar extent and migrated at an identical rate in the presence or absence of the VZV ORF66 or ORF47 proteins. The numbers are the sizes (in kilodaltons) of the proteins.

studied, including the ORF62 protein and gE which migrate as families of polypeptides on polyacrylamide gels, were identical in extracts of ROka-, ROka 66S-, and ROka 47S/66S-infected cells.

We have shown that the VZV ORF66 and ORF47 proteins together are dispensable for replication of VZV in cell culture. VZV unable to express either ORF66 or ORF47 (13) grew to titers similar to those of parental virus in cell culture; however, VZV unable to express both proteins grew to titers lower than those of parental virus in vitro. HSV-1 with a deletion in the US3 gene (the homolog of ORF66) grows to titers similar to those of the wild-type virus in vitro (19, 26). While HSV-2 unable to express US3 grows to titers similar to those of the wild-type virus in most cell lines, the US3 mutant fails to grow in peritoneal macrophages (17, 25). PRV with a stop codon in the US3 protein kinase gene grows to slightly lower titers than does the wild-type virus in some cell lines but shows a marked impairment in growth in other cells in vitro (9, 15, 16). Explant cultures infected with the PRV US3 mutant showed impairment in egress of virions from the nucleus, with accumulation of virions in the perinuclear space; however, this effect was not seen with the same mutant in other cells (32). Thus, the VZV ORF66 mutant might be impaired for growth in certain cell types or in explant cultures.

While we have shown that VZV ORF66 protein induces kinase activity, our studies do not formally show that the ORF66 protein is itself a protein kinase. The observations that the ORF66 protein contains motifs that are conserved with eukaryotic protein kinases (1, 12, 20, 30) and that the homologs of ORF66 in HSV-1, HSV-2, and PRV are protein kinases (5, 11, 26, 34) provide support that VZV ORF66 may have direct kinase activity.

VZV ORF66 protein kinase is not necessary for the phosphorylation of VZV ORF4, ORF62, ORF63, and ORF68 (gE) proteins. Ng and colleagues have shown that VZV ORF47 encodes a protein kinase that can phosphorylate VZV ORF62 protein in vitro (24). However, the ORF47 protein, like ORF66 protein, is not required for phosphorylation of ORF62 protein during infection of cells in vitro (13). Furthermore, each of the four VZV phosphoproteins tested was phosphorylated to a similar extent in cells infected with virus that was unable to express both VZV ORF66 and ORF47 proteins. Thus, it is likely that the four VZV phosphoproteins described above can be phosphorylated by cellular kinases in vitro. While substrates for ORF66 and ORF47 proteins have not yet been identified, we found that these proteins are responsible for the phosphorylation of a 48-kDa protein(s) in VZV-infected cells, and thus the ORF66 and ORF47 proteins may share a common substrate.

When HSV-1, HSV-2, or PRV US3 mutants were tested in animals, the HSV-1 and HSV-2 mutants showed 1,000- to 10,000-fold-reduced virulence in mice (21, 25) and the PRV mutant was attenuated in pigs (16). After challenge with virulent PRV, pigs that received the PRV UL3 mutant were completely protected from infection and excreted no virus after challenge with the wild-type virus (15). The ability of ORF66 to induce the phosphorylation of a cellular or viral protein may be important for VZV replication during natural infection. The impaired growth of VZV unable to express both ORF47 and ORF66 proteins implies that these two proteins together have an important role in VZV replication. Future studies of animals infected with the VZV ORF66 mutant will help to determine the role of this protein during infection in vivo.

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