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Regions of the Varicella-Zoster Virus Open Reading Frame 63 Latency-Associated Protein Important for Replication In Vitro Are Also Critical for Efficient Establishment of Latency

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Varicella-zoster virus (VZV) open reading frame 63 (ORF63) is one of the most abundant transcripts expressed during VZV latency in humans, and ORF63 protein has been detected in human ganglia by several laboratories. Deletion of over 90% of the ORF63 gene showed that the protein is required for efficient establishment of latency in rodents. We have constructed viruses with a series of mutations in ORF63. While prior experiments showed that transfection of cells with a plasmid expressing ORF63 but lacking the putative nuclear localization signal of the protein resulted in increased expression of the protein in the cytoplasm, we found that ORF63 protein remained in the nucleus in cells infected with a VZV ORF63 nuclear localization signal deletion mutant. This mutant was not impaired for growth in cell culture or for latency in rodents. Replacement of five serine or threonine phosphorylation sites in ORF63 with alanines resulted in a virus that was impaired for replication in vitro and for latency. A series of ORF63 carboxy-terminal mutants showed that the last 70 amino acids do not affect replication in vitro or latency in rodents; however, the last 108 amino acids are important for replication and latency. Thus, regions of ORF63 that are important for replication in vitro are also required for efficient establishment of latency.

Primary infection with varicella-zoster virus (VZV) causes chicken pox, and the virus establishes a latent infection in the cranial nerve and dorsal root ganglia. VZV can subsequently reactivate from these ganglia to cause herpes zoster or shingles. At least six viral genes have been demonstrated to be expressed during latency in humans and in rodents. These are the products of VZV open reading frames (ORFs) 4, 21, 29, 62, 63, and 66 (6, 7, 12, 20).

VZV ORF63 transcripts have been detected during latency in several studies (8, 12, 13). In one study, ORF63 transcripts were the most abundantly expressed viral mRNAs expressed during latency (7). VZV ORF63 transcripts have also been detected in experimentally infected rodents (14, 22, 23). VZV ORF63 protein has also been demonstrated during VZV latency in both human (12, 17, 19) and rodent (10, 14) ganglia.

VZV ORF63 protein is located predominantly in the nucleus during lytic replication in vitro and in cells transfected with a plasmid expressing the protein (10, 26). During latency, however, the protein is present in the cytoplasm of neurons (17, 19). When reactivation occurs, the protein is present in both the nucleus and the cytoplasm (17).

ORF63 is present in two copies in the VZV genome, and each is predicted to encode a 278-amino-acid protein (9). Stevenson et al. (26) showed that the carboxy-terminal 69 amino acids are required for nuclear localization of the protein, while Bontems et al. (2) showed that amino acids 260 to 263 are important for nuclear localization in cells transfected with ORF63. Baiker et al. (1) used plasmids expressing ORF63 mutants to show that amino acids 226 to 229 are also important for translocation of the protein to the nucleus. None of these studies showed whether these sites are important for localization to the nucleus in the context of virus infection.

VZV ORF63 protein is phosphorylated by casein kinase I (2), casein kinase II (2, 26), and ORF47 (15) in vitro. However, no difference was noted in the phosphorylation of ORF63 in cells infected with an ORF47 knockout virus (11). Stevenson and colleagues (26) showed that in transfected cells amino acids 142 to 210 are the predominant sites of phosphorylation. Bontems et al. (2) constructed plasmids with 5 or 10 phosphorylation site substitution mutations (corresponding to amino acids 150 to 224) in ORF63 and showed that these amino acids are important for phosphorylation by casein kinase I or II. In addition, they showed that these phosphorylation sites are important for nuclear localization of the protein in Vero cells. Baiker et al. (1) constructed additional ORF63 mutants and showed that serine residues 165, 173, and 185 are phosphorylated by cellular kinases.

ORF63 protein interacts with the immediate-early ORF62 protein and RNA polymerase 2 (18, 25). A portion of the ORF63 protein colocalizes with the ORF62 protein in the nucleus at both early and late times after infection. The binding domain for ORF62 protein is located in the amino portion of ORF63 protein between amino acids 55 and 67 (1).

We have previously shown that deletion of over 90% of the ORF63 gene (and its repeated gene, ORF70) results in infectious virus that is impaired for replication in vitro and for latency in rodents (5). Here we report the phenotypes of VZV ORF63 carboxy-terminal mutants, internal deletion mutants, and phosphorylation site point mutants.

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A previous study attempted to construct 22 ORF63 mutants and found that only three point mutations (Thr171, Ser181, and Ser185) in ORF63 resulted in viruses that were viable in cell culture. These viruses were impaired for replication in human skin cells but not in T cells (1). In contrast, we found that each of our VZV mutants, including some that were not viable in the previous study, were infectious in cell culture. The carboxy-terminal 70 amino acids, which include the two previously described nuclear localization signals, were dispensable for replication in vitro and for latency in rodents. In contrast, viruses with point mutations of five amino acids which are sites for phosphorylation by cellular kinases or unable to express the carboxy-terminal 109 amino acids of ORF63 were impaired for replication in vitro and for latency.

MATERIALS AND METHODS

Cosmids and transfections. Cosmids VZV NotIA, NotIB, MstIIA, and MstIIB are derived from the Oka vaccine strain of VZV and encompass the entire viral genome (4). Cosmid MstIIA-63DA has identical deletions in both copies of ORF63 that result in loss of codons 24 to 268, with the remaining codons out of frame as previously described (5). Plasmid 63-BclI, which contains ORF63 (VZV nucleotides 106592 to 112215) within a VZV BclI fragment, was also previously described (5). To construct carboxy-terminal mutants of ORF63, plasmid 63-BclI was digested with HpaI and AatII (which cut at VZV nucleotides 110649 and 111366, respectively), and the insert containing ORF63 was cloned into the corresponding sites of plasmid LITMUS 28i (New England Biolabs, Beverly, Mass.). The resulting plasmid was cut with SfoI, AccI, or KpnI, which cut at VZV nucleotides 111005, 111088, and 111208, respectively. The ends of the DNA cut with AccI were blunted with the Klenow fragment of DNA polymerase I, and the ends of the DNA cut with KpnI were blunted with T4 DNA polymerase. A double-stranded oligonucleotide, TAGCTAGGCGCGCCTAGCTA, containing stop codons in all three ORFs and an AscI site, was ligated to each of the linearized fragments. The resulting plasmids were then cut with AatII and HpaI, and the mutated ORF63 sequence was inserted in place of the wild-type ORF63 sequence of plasmid 63-BclI to create plasmids p63KpnI, p63AccI, and p63SfoI.

To produce a plasmid with a deletion in the nuclear localization signal at residues 260 to 263, plasmid 63-BclI was cut with AatII (which cuts at VZV nucleotide 111366) and the linearized DNA was treated with Bal 31 nuclease for 15 to 60 min. After Bal 31 treatment, the DNA was extracted with phenol and precipitated, and the ends were blunted with T4 DNA polymerase and self-ligated. The sequence of the deletion was determined, and two clones were selected, p63-30-4 and p63-15-3. These plasmids have deletions that result in the loss of VZV nucleotides 111335 to 111379 and 111347 to 111364, respectively.

Plasmid pcDNA63-5M contains point mutations that result in serine- or threonine-to-alanine changes at amino acids 150, 165, 171, 181, and 186 as described previously (2). Plasmid pcDNA63-10M has the same mutations as pcDNA63-5M and five additional mutations at amino acids 173, 185, 201, 224, and 244 that result in serine- or threonine-to-alanine changes. The two plasmids were cut with AatII and HpaI, and the mutated ORF63 sequence was inserted in place of the wild-type ORF63 sequence of plasmid 63-BclI to create plasmids p63-5M and p63-10M.

VZV with stop codons in the KpnI site in both copies of ORF63 was constructed by transfecting melanoma cells with 0.5 μg of virion DNA from the ORF63 deletion mutant (5) and 1.5 μg of the VZV BclI DNA fragment from p63KpnI. Cells were passaged each week until cytopathic effects (CPEs) were observed; cell-free virus was prepared, and melanoma cells were infected with dilutions of cell-free virus. The virus was subsequently plaque purified until only the KpnI stop codon mutant DNA and no residual ORF63 deletion mutant DNA could be detected by PCR. An additional round of plaque purification was performed, PCR was again performed to verify that no residual ORF63 deletion mutant DNA could be detected, and the resulting virus was termed ROka63-KpnI. While this strategy was successful for constructing the KpnI stop codon mutant, it proved difficult to plaque purify other mutants from the ORF63 deletion mutant background, likely because most of the other mutants were impaired for replication, similar to the ORF63 deletion mutant.

Each of the mutations, except for the KpnI stop codon mutation, was cloned into cosmid MstIIA by the following procedure. The plasmid containing the ORF63 gene mutant was cut with HpaI and NaeI, and the mutated ORF63 gene was inserted in place of the wild-type ORF63 gene in plasmids ES and AH (5). The mutated plasmid ES was cut with EcoRI and SpeI, and the ORF63 mutated fragment was inserted in place of the wild-type EcoRI-SpeI fragment into plasmid pBSSK+SfiI (5) to produce a plasmid with one copy of ORF63 mutated. The mutated plasmid AH was cut with AvrII and HindIII, and the ORF63 mutated fragment was inserted in place of the wild-type AvrII-HindIII fragment of the ORF63-mutated plasmid pBSSK+SfiI to create a plasmid in which both copies of ORF63 are mutated. Next, the latter plasmid was cut with SfiI and inserted in place of the wild-type SfiI fragment of cosmid MstIIA. The resulting cosmids, were termed MstIIA-63AccI, MstIIA-63SfoI, MstIIA-63AatII30-4, MstIIA-63AatII15-3, MstIIA-63-5M, and MstII-A63-10M.

Recombinant viruses with mutations in ORF63 were made by transfecting melanoma cells with the MstIIA cosmids with mutations in ORF63 along with cosmids MstIIB, NotIA, and NotIB and plasmid pCMV62 by the calcium phosphate transfection procedure (4). Transfected cells were treated with trypsin and passaged weekly until CPE was observed.

Southern blots, immunoblots, immunoprecipitations, immunofluorescence, and growth studies. Virion DNA was prepared from each of the mutants, cut with restriction enzymes, and run on 0.8% agarose gels. After transfer to nylon membranes, the blots were probed with [^{32}P]dCTP-labeled probes corresponding to ORF63 or to the entire VZV genome. Immunoblotting was performed with lysates of virus-infected cells run on polyacrylamide gels and transferred to nylon membranes. The blots were incubated with rabbit antibody to ORF63 (a kind gift from Paul Kinchington) or mouse monoclonal antibody to glycoprotein E (gE) (Chemicon, Temecula, Calif.) followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody and developed by using enhanced chemiluminescence (Pierce Chemical Co., Rockford, Ill.).

Immunoprecipitations were performed using lysates of [^{35}S]methionine-labeled VZV-infected cells. Lysates were precipitated with rabbit antibody to ORF63 or mouse monoclonal antibody to gE, followed by protein A-Sepharose. Immune complexes were run on polyacrylamide gels, and autoradiography was performed. For immunofluorescence studies, VZV-infected cells were grown on glass coverslips, fixed in methanol-acetone (1:1), air dried, incubated with rabbit antibody to ORF63 and then with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody and DAPI (4′,6′-diamidino-2-phenylindole; 1 μg/ml), and examined by fluorescence microscopy.

Analysis of growth in cell culture was performed by infecting melanoma cells with VZV mutants at 37°C. At various times after infection, the cells were treated with trypsin and serial dilutions of infected cells were plated onto six-well dishes. The cells were stained with crystal violet, and plaques were counted.

Analysis of latent infection. Virus inocula were prepared in melanoma cells that were harvested at 3+ CPE (75% of cells with CPE). Female cotton rats (4 to 6 weeks old) were inoculated intramuscularly with 50 μl of cell-associated VZV containing 1.75×10^5 PFU of virus. Animals received six injections on each side of the thoracic and lumbar spine. Five to six weeks after infection, animals were sacrificed, dorsal root ganglia from each side of the spine were removed and pooled, and DNA was isolated as described previously (23). DNA (500 ng) of dorsal root ganglia from VZV-infected animals was amplified by PCR using VZV ORF21 primers, the products were run on agarose gels, and Southern blotting was performed using a radiolabeled probe (23). Serial dilutions of VZV cosmid DNA were added to 500 ng of DNA from uninfected animals, and PCR was performed followed by Southern blotting. The quantity of latently infected DNA was determined by using a phosphorimager.

Statistics. Statistical results were generated using StatXact from Cytel Software Corporation (Cambridge, Mass.). *P* values were computed by using exact permutation distributions.

RESULTS

VZV with the ORF63 nuclear localization signal deleted is not impaired for growth in cell culture. To produce a virus with the nuclear localization site of ORF63 and its duplicate gene, ORF70, deleted, cosmid clones MstIIA-63AatII30-4 and MstIIA-63AatII15-3 were constructed, with amino acids 252 to 267 and 257 to 262 of ORF63 and ORF70 deleted, respectively (Fig. 1). Transfection of melanoma cells with cosmids NotIA, NotIB, MstIIB, and either MstIIA, MstIIA-63AatII15-3, or MstIIA-63AatII30-4 resulted in CPE at 5, 6, or 6 days after transfection, respectively. The resulting viruses were termed ROka, ROka63D-AatII15-3, and ROka63-AatII30-4.

To verify that the deletion mutant viruses had the expected

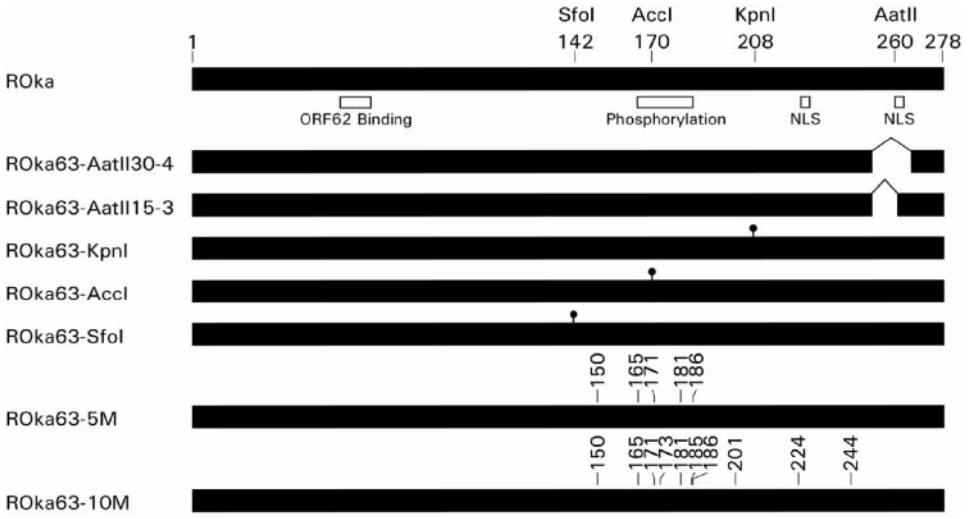

FIG. 1. Map of VZV ORF63 mutants. ROka contains the full-length ORF63 gene, which encodes a 278-amino-acid protein that has an ORF62 binding site, a domain that is highly phosphorylated, and two nuclear localization signals (NLS). ROka63-AatII30-4 and ROka63-AatII15-3 have amino acids 252 to 267 and 257 to 262 deleted, respectively, and lack the carboxy-terminal NLS. ROka63-KpnI, ROka63-AccI, and ROka-SfoI have stop codons (lollipops) that result in failure to translate amino acids 209 to 278, 171 to 278, and 143 to 278, respectively. ROka63-5M and ROka63-10M have alanine in place of serine or threonine at amino acids 150, 165, 171, 181, and 186 and amino acids 150, 165, 171, 173, 181, 185, 186, 201, 224, and 244, respectively.

genome structure, Southern blotting was performed using virion DNA. Digestion of VZV ROka DNA with BclI and AatII generated the expected bands of 4.71 and 0.85 kb, while digestion of ROka63-AatII30-4 or ROka63-AatII15-3 resulted in a 5.6-kb fragment due to the deletion of the AatII site in the nuclear localization signal (Fig. 2A).

Immunoblotting was performed to confirm that ORF63 was expressed in cells infected with the ORF63 deletion mutants. Lysates from cells infected with VZV ROka contained a 45-kDa ORF63 protein, while lysates from cells infected with ROka63-AatII30-4 or ROka63-AatII15-3 showed a slightly smaller ORF63 protein (Fig. 3A). Lysates from each of the ORF63 deletion mutants expressed VZV gE (Fig. 3B).

Infection of cells with ROka63-AatII30-4 showed that the mutant was not impaired for growth in cell culture when compared with the parental virus. The peak titers of VZV ROka and ROka63-AatII30-4 were similar during a 5-day growth analysis (Fig. 4A).

VZV with mutations in several phosphorylation sites are viable in cell culture but impaired for replication. Prior studies attempting to produce VZV with more than one phosphorylation site altered failed to yield infectious virus (1). Two cosmid clones, MstIIA-63-5M and MstIIA-63-10M, were constructed which have mutations in phosphorylation sites. Transfection of melanoma cells with cosmids NotIA, NotIB, MstIIB, and either MstIIA, MstIIA63-5M, or MstIIA63-10M resulted in CPE 7, 15, or 15 days after transfection, respectively. The resulting ORF63 mutant viruses were termed ROka63-5M and ROka63-10M. ROka63-5M has the following mutations: Ser150Ala, Ser165Ala, Thr171Ala, Ser181Ala, and Ser 186Ala. ROka63-10M has the five mutations present in ROka63-5M as well as Ser173Ala, Ser185Ala, Thr201Ala, Ser224Ala, and Thr244Ala (Fig. 1). PCR of virion DNA followed by sequencing confirmed that each of the nucleotide

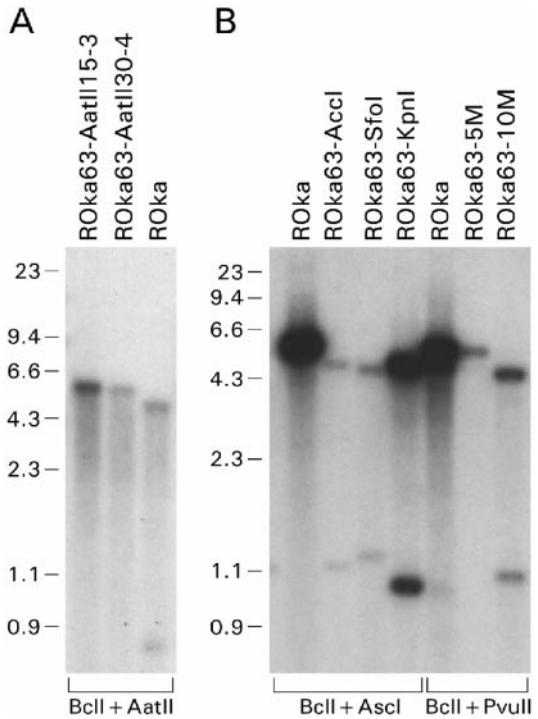

FIG. 2. Southern blots of virion DNA from cells infected with ORF63 deletion mutants. (A) ROka, ROka63-AatII15-3, and ROka63-AatII30-4 DNAs were digested with BclI and AatII and hybridized to an ORF63 probe. (B) ROka, ROka63-AccI, ROka63-SfoI, and ROka63-KpnI DNA were digested with BclI and AscI, and ROka, ROka63-5M, and ROka63-10M were digested with BclI and PvuII, followed by hybridization to an ORF63 probe. Numbers correspond to sizes of DNAs in kilobase pairs.

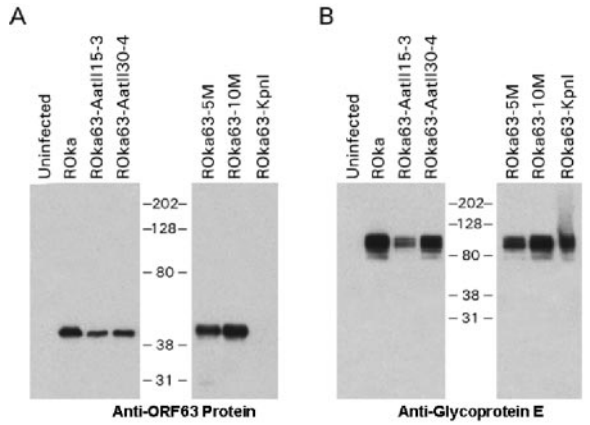

FIG. 3. Immunoblot of ORF63 protein expression in cells infected with ORF63 mutants. (A) A 45-kDa protein reacts with antibody to ORF63 protein in cells infected with ROka, ROka63-5M, and ROka63-10M, a slightly smaller band is detected in cells infected with ROka63-AatII15-3 and ROka63-AatII30-4, and no band is apparent in uninfected cells or cells infected with ROka63-KpnI. (B) Bands of 90- to 100-kDa that react with antibody to VZV gE are present in cells infected with VZV ROka and each of the ORF63 mutants. Numbers correspond to the sizes of proteins in kilodaltons.

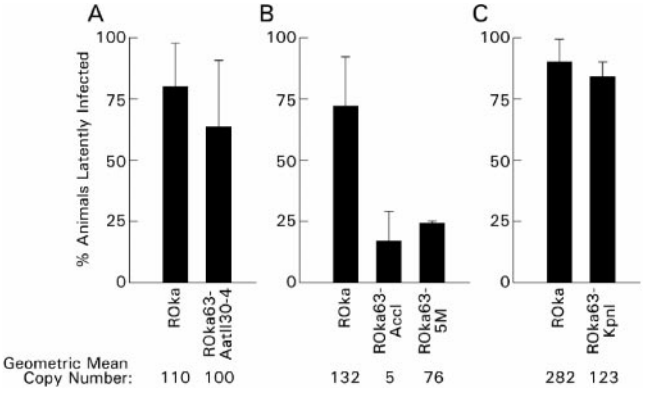

FIG. 5. Percentages of latently infected animals inoculated with different VZV ORF63 mutants. Animals were infected with ROka or ROka63-AatII30-4 (A), ROka, ROka63-AccI, or ROka63-5M (B), and ROka or ROka63-KpnI (C). The percentage of latently infected animals infected with ROka versus ROka63-AatII30-4 ($P = 0.47$) or ROka versus ROka63-KpnI ($P = 0.65$) was not significantly different; however, the percentage of latently infected animals infected with ROka versus ROka63-AccI ($P = 0.0004$) or ROka versus ROka63-5M ($P = 0.012$) was significantly different. Error bars represent the standard errors for two separate experiments. The geometric mean number of VZV genome copies per 500 ng of DNA in PCR-positive ganglia is shown at the bottom.

substitutions engineered into the cosmids was also present in virion DNA.

Southern blotting with virion DNA cut with BamHI showed similar patterns of bands in cells infected with ROka, ROka63-5M, and ROka63-10M, as expected (J. I. Cohen et al., unpublished data). Digestion of ROka63-10M with BclI and PvuII yielded bands of 4.5 and 1.1 kb, in contrast to ROka and ROka63-5M, which showed only a band of 5.6 kb (Fig. 2B). The difference in bands is due to the nucleotide changes (TCG to GCT) responsible for the amino change at 173 (Ser to Ala) in ROka63-10M, which is not present in ROka63-5M. Immunoblots showed that ORF63 protein was expressed at similar levels in cells infected with ROka, ROka63-5M, and ROka63-10M (Fig. 3A). As a control, lysates from cells also expressed VZV gE (Fig. 3B).

Infection of cells with the VZV ORF63 phosphorylation mutants showed that both mutants were impaired for growth in cell culture compared with the parental virus. The peak titers of VZV ROka63-5M and ROka63-10M were about 1 log lower than that of ROka during a 5-day growth analysis (Fig. 4B). The reduction in peak titers was similar to that observed for the ORF63 deletion mutant described previously (5).

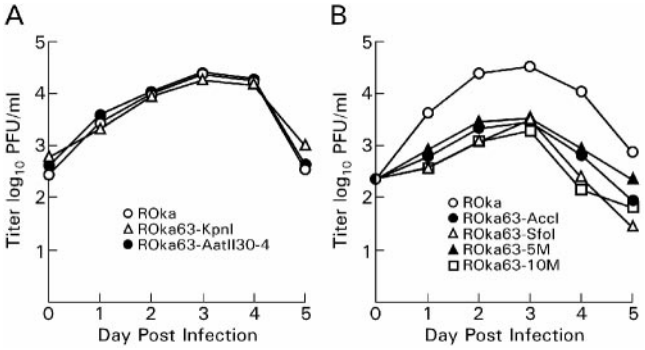

FIG. 4. Growth of ORF63 mutants in melanoma cells. Cells were infected with VZV ROka, ROka63-AatII30-4, or ROka63-KpnI (A) and ROka, ROka63-AccI, ROka63-SfoI, ROka63-5M, or ROka63-10M (B). Each day after infection the cells were treated with trypsin and the virus titer was determined.

VZV with mutations in phosphorylation sites, but not in the nuclear localization site, is impaired for latency. Cotton rats were inoculated intramuscularly along the side of the spine with parental or ORF63 mutant viruses. Five weeks later the animals were sacrificed, thoracic and lumbar ganglia were pooled, and DNA was isolated. PCR followed by Southern blotting was performed to determine whether latent VZV was present.

In two experiments, VZV DNA was present in ganglia from 11 of 17 cotton rats infected with ROka63-AatII30-4, compared with 14 of 18 animals infected with ROka (Fig. 5A). The geometric mean copy number was 100 VZV genomes for cotton rats infected with ROka63-AatII30-4 with PCR-positive ganglia and 110 VZV genomes for animals infected with ROka. Thus, deletion of the ORF63 nuclear localization signal did not affect VZV latency.

In contrast, animals infected with one of the phosphorylation site mutants showed reduced VZV latency. In the two experiments, VZV DNA was present in ganglia from 4 of 17 cotton rats infected with ROka63-5M, compared with 12 of 17 animals infected with ROka (Fig. 5B). The geometric mean copy number of VZV DNA in PCR-positive ganglia was 76 VZV genomes for cotton rats infected with ROka63-5M and 132 VZV genomes for animals infected with ROka.

The carboxy-terminal 70 amino acids of ORF63 are dispensable for replication. To further determine which amino acids are important for replication in vitro and for latency, a series of carboxy-terminal mutants of ORF63 were constructed. Cosmids MstIIA-63SfoI and MstIIA-AccI have an oligonucleotide

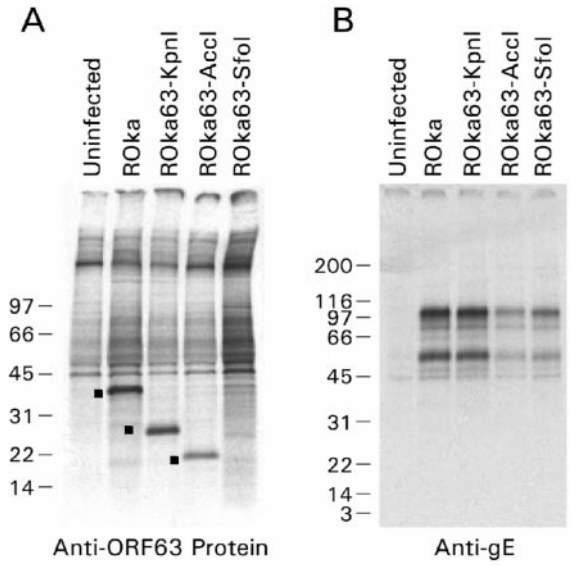

FIG. 6. Immunoprecipitation of ORF63 protein from cells infected with VZV ORF63 mutants. (A) ORF63 protein (squares) is detected in cells infected with VZV ROka (42 kDa), ROka63-KpnI (27 kDa), and ROka63-AccI (22 kDa) but not in cells infected with ROka63-SfoI. (B) gE (60 to 100 kDa) is detected in VZV ROka and each of the mutants. Numbers correspond to the sizes of proteins in kilodaltons.

with stop codons inserted after codons 142 and 170 of ORF63, respectively. Transfection of melanoma cells with cosmids NotIA, NotIB, MstIIB, and either MstIIA, MstIIA-63SfoI, or MstIIA-63AccI yielded CPE at 7, 15, or 15 days after transfection, respectively. The two ORF63 mutant viruses were termed ROka63-SfoI and ROka63-AccI. A third stop codon mutant was made by cotransfecting cells with virion DNA from ROka63D (with both copies of ORF63 deleted) and a plasmid with ORF63 containing an oligonucleotide with stop codons at the KpnI site (after codon 208 of ORF63) and flanking DNA. The resulting virus was plaque purified until only the KpnI mutant could be detected. One additional cycle of plaque purification was performed, and the resulting virus was termed ROka63-KpnI.

Southern blotting was performed to verify that each of the carboxy-terminal mutants had the expected genome configuration. Digestion of ROka, ROka63-AccI, ROka63-SfoI, and ROka-KpnI with BclI and AscI yielded bands of 5.6, 4.5 and 1.1, 4.4 and 1.2, and 4.6 and 1.0 kb, respectively, due to the AscI site inserted into the oligonucleotide containing the stop codons (Fig. 2B). Immunoblotting with antibody to ORF63 protein did not show bands for ROka63-KpnI (or the other stop codon mutants), although gE could be detected with anti-gE antibody (Fig. 3; Cohen et al., unpublished). However, immunoprecipitation with ORF63 protein antibody showed bands of 42, 27, and 22 kDa for lysates from ROka-, ROka63-KpnI-, and ROka63-AccI-infected cells, respectively (Fig. 6A). No bands were detected in lysates from ROka63-SfoI after immunoprecipitation with ORF63 protein antibody. gE was detected in each of the infected cell lysates from the ORF63 stop codon mutants (Fig. 6B). Immunofluorescent staining with antibody to ORF63 protein showed that each of the ORF63 stop codon mutants was expressed in cells infected with the mutant viruses, although the intensity of staining for ORF63 protein expressed by ROka63-SfoI was much less intense than that of the other mutants (Fig. 7A and B).

Analysis of the growth of carboxy-terminal ORF63 VZV mutants in cell culture showed that ROka63-KpnI grew to peak titers similar to those of ROka, while ROka63-AccI and ROka-SfoI grew to lower titers (Fig. 4B). The peak titers for ROka63-AccI and ROka-SfoI were about 10-fold less than that of ROka and similar to those observed with the mutant virus in which nearly all of ORF63 had been deleted (5).

ORF63 truncated for the carboxy-terminal 70 amino acids shows different intracellular patterns depending on the context of the protein. Transfection of cells with plasmids expressing ORF63, or infection of cells with VZV, results in localization of the protein predominantly in the nucleus of cells. In contrast, transfection of cells with an ORF63 truncation mutant with both nuclear localization signals deleted resulted in localization of the protein predominantly in the cytoplasm (1). Infection of cells with ROka or ROka63-KpnI (which does not express the carboxy portion of the protein, which contains the two nuclear localization signals) followed by staining with anti-ORF63 protein antibody showed that the protein localized predominantly in the nucleus (Fig. 7A). Each of the other carboxy-terminal mutants was also expressed in the nucleus, as was the ORF63 protein from ROka63-AatII30-4 (Fig. 7A and B; Cohen et al., unpublished).

While previous experiments showed that transfection of cells with a plasmid expressing the ORF63-10M construct resulted in localization of the mutant ORF63 protein predominantly in the cytoplasm of Vero cells (2), infection of melanoma cells with VZV expressing the ORF63-10M mutant resulted in localization of the protein in the nucleus.

VZV infection of melanoma cells results in formation of polykaryons with rings of nuclei around a central Golgi complex (3). Each of the VZV ORF63 mutants showed the typical pattern of polykaryons (Fig. 7A and B).

The carboxy-terminal 70 amino acids of ORF63 are dispensable for replication and for latency. One month after cotton rats were inoculated with ROka63-KpnI and ROka, ganglia were obtained and latent viral DNA was quantified. The results of two experiments showed that VZV DNA was present in 14 of 18 cotton rats infected with ROka63-KpnI, compared with 16 of 18 animals infected with ROka (Fig. 5C). The geometric mean copy number for PCR-positive ganglia from cotton rats infected with ROka63-KpnI was 123 genomes, and for animals infected with ROka the mean copy number was 282 genomes. VZV DNA was present in 3 of 18 cotton rats infected with ROka63-AccI, compared with 12 of 17 animals infected with ROka (Fig. 5B). The geometric mean copy numbers for PCR-positive ganglia from cotton rats infected with ROka63-AccI was 5 genomes, and for animals infected with ROka the mean copy number was 132 genomes.

DISCUSSION

We have shown that the carboxy-terminal 70 amino acids of the ORF63 protein are dispensable for growth in cell culture and for establishment of VZV latency in cotton rats. In contrast, the carboxy-terminal 108 amino acids or multiple phosphorylation sites prior to the last 70 amino acids of ORF63 are

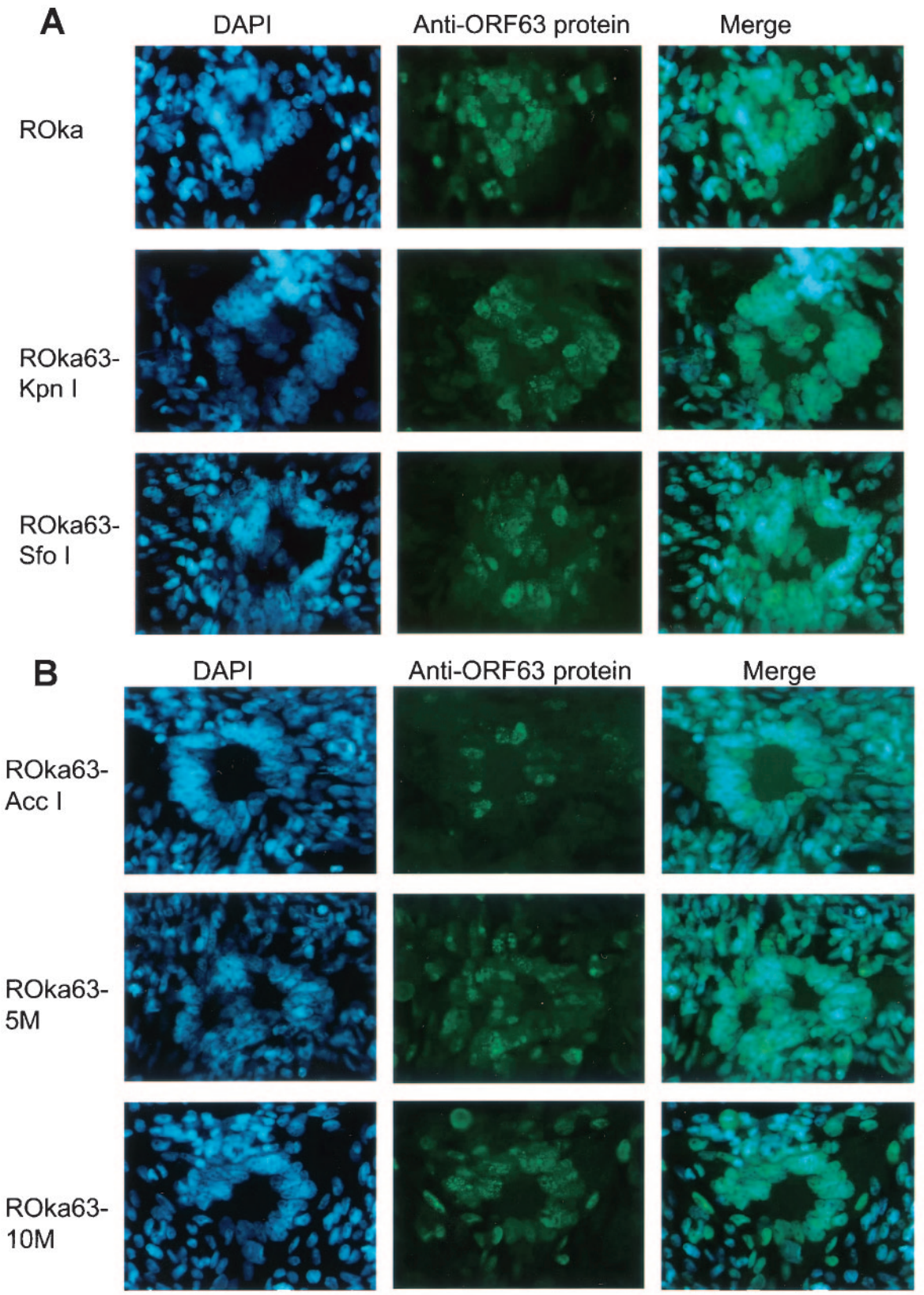

FIG. 7. Immunofluorescence microscopy detects VZV ORF63 mutants in the nucleus of infected cells. Cells were infected with VZV ROka, ROka63-KpnI, or ROka63-SfoI (A) or ROka63-AccI, ROka63-5M, or ROka63-10M (B) and stained with anti-ORF63 protein antibody and DAPI.

important both for growth of VZV in cell culture and for efficient establishment of latent infection.

Prior experiments by Baiker et al. (1) gave very different results for ORF63 mutants than the current study. While we found that deletion of amino acids 252 to 267 of the ORF63 protein (ROka63-AatII30-4) had no effect on growth in cell culture, the prior report stated that deletion of amino acids 260 to 263 of the protein prevented replication of VZV in cell culture. In addition, we showed that amino acids 209 to 278 (ROka63-KpnI) are not important for growth of the virus in cell culture, while the prior report indicated that simultaneous deletion of two regions within the carboxy terminus (amino acids 226 to 229 and 260 to 263) prevented virus replication. Finally, the prior report stated that Ser-to-Ala mutations at amino acids 165, 173, and 186 of the ORF63 protein prevented virus replication. In contrast, we found that VZV with all of these point mutations in ORF63 (ROka63-10M) was impaired for replication but still produced infectious virus. There are several possible explanations for these differences. First, although both our group and Baiker et al. (1) used cosmids derived from the Oka vaccine strain of VZV, the sets of cosmids were not the same and there could be sequence differences in the viral DNA. Second, differences in cosmid transfection conditions may prevent detection of viruses that are impaired in replication. Third, we constructed VZV in which both copies of the ORF63 mutants were located at their native sites in the virus. In contrast, Baiker et al. (1) expressed one copy of each mutant at a single ectopic site in the VZV genome, in a virus in which both native copies of ORF63 were deleted. Thus, expressing an ORF63 mutant at a different location in the VZV genome may affect the viability and growth of the virus.

Cells infected with VZV unable to express the carboxy-terminal 70 amino acids of ORF63 did not have altered nuclear localization of the ORF63 protein compared with those infected with the parental virus. In contrast, two other groups (1, 2) found that transfection of cells with plasmids expressing ORF63 with deletions in this region, which includes two nuclear localization signals, resulted in reduced ORF63 protein in the nucleus. These results suggest that other viral proteins help ORF63 protein to localize to the nucleus. Similar results have been seen with herpes simplex virus proteins. For example, transfection of cells with an ICP4 mutant alone results in diffuse cytoplasmic localization; however, cotransfection of the ICP4 mutant with ICP0 results in movement of a portion of the cytoplasmic ICP4 mutant into the nucleus (21). Since ORF63 protein has been shown to interact with ORF62 protein, and the binding domain in the ORF63 protein was intact in each of the ORF63 mutants, ORF62 may bind to the ORF63 mutants, localizing both proteins to the nucleus. Alternatively, the interaction of ORF63 protein with RNA polymerase 2 (18) may sequester the ORF63 mutants to the nucleus.

Immunofluorescence studies showed that each of the carboxy-terminal mutants produced polykaryons similar to that produced with parental virus (Fig. 7A and B). Interestingly, both ROka63-5M and ROka63-10M also had normal polykaryons. In contrast, abnormal polykaryons were reported in a prior paper for cells infected with ORF63 Thr171, Ser181, or Ser185 point mutants (1). The first two of these point mutations are present in ROka63-5M, and all three of these mutations are in ROka63-10M.

While ORF63 protein is usually localized to the nucleus during lytic infection, the protein localizes to the cytoplasm of neurons during latency (17, 19). It has been suggested that sequestration of ORF63 protein to the cytoplasm in neurons may be important to maintain latency. We found that ORF63 mutants with the nuclear localization site at amino acids 260 to 263 deleted (ROka63-AatII30-4) or with both nuclear localization sites at amino acids 226 to 229 and 260 to 263 deleted (ROka63-KpnI) were not impaired for latency. Furthermore, while all of the ORF63 mutants localized to the nucleus of infected cells, viruses expressing some of these mutants were impaired for latency, while others were not impaired. Therefore, localization of ORF63 protein in infected cells in culture does not appear to correlate with which mutants affect latency in the cotton rat model.

VZV unable to express the carboxy-terminal 70 amino acids of ORF63 (ROka63-KpnI) was not impaired for replication in cell culture or for latency in cotton rats. This region of the protein contains three amino acids that are predicted to be phosphorylated by serine-threonine kinases. These include Thr244, which is a potential target for casein kinase I, and Ser224, which is a putative protein kinase C and/or p34-cdc2 phosphorylation site (2). Thus, these putative phosphorylation sites in ORF63 are not required for replication or latency of VZV.

In contrast, VZV unable to express amino acids 171 to 278 of ORF63 (ROka63-AccI) was impaired both for replication and for latency. These 108 amino acids are located in a region of the protein that is rich in serine and threonine resides that are phosphorylated (1, 2, 26). These include potential targets for casein kinase I (Ser173, Ser185, and Thr201) and casein kinase II (Thr171, Ser181, and Ser186). These results suggest that phosphorylation in this region may be important both for replication and for latency. Three of these serine or threonine residues are changed to alanines (Thr171Ala, Ser181Ala, and Ser186Ala) in VZV ROka63-5M, which like ROka63-AccI, is also impaired for replication and latency. All of the mutated serine or threonine residues in ROka63-5M are putative targets for casein kinase II (2). Taken together, these results imply that phosphorylation in the area of amino acids 171 to 186 of ORF63 is critical for both replication and efficient establishment of VZV latency.

Few studies have examined the role of phosphorylation of herpesvirus proteins in latency. Xia et al. (27) found that deletion of a serine-rich, phosphorylated region of ICP4 from herpes simplex virus type 1 resulted in impairment of replication but not in reduced latency in mice. VZV with a stop codon in ORF66, which encodes a viral protein kinase, was not impaired for latency (24). In addition, VZV unable to express ORF47, which encodes a protein kinase that phosphorylates ORF63 (15), was not impaired for replication or latency in the cotton rat model (24). Thus, while the VZV ORF47 protein can phosphorylate the ORF63 protein, phosphorylation by the viral protein kinase is not required for replication or for latency. Instead, since ROka63-5M (which has mutations in five putative phosphorylation sites) is impaired for replication and latency, these results imply that phosphorylation of ORF63 by cellular proteins might be important for these activities.

The mutations in ORF63 may have affected its transregulatory properties, thereby affecting replication or latency. Bontems et al. (2) showed that certain mutations of ORF63 are important for its transregulatory activities. A plasmid expressing the ORF63-10M mutation lost the ability to transrepress the VZV DNA polymerase promoter compared to wild-type ORF63 when transfected into Vero or ND7 cells. A plasmid expressing the ORF63-KpnI mutation lost its transrepressing activity compared with wild-type ORF63 when transfected into ND7 but not Vero cells. However, a plasmid expressing ORF63 with a deletion in the nuclear localization signal (amino acids 260 to 263) retained its transrepressing activity in both cell types. We found that VZV in which the wild-type ORF63 gene was replaced with the ORF63-KpnI or ORF63 nuclear localization signal mutant gene was unimpaired for growth or latency, while the ORF63-10M mutant was impaired for growth in cell culture and would be predicted to be impaired for latency (since the ORF63-5M mutant was impaired for latency). Thus, the transrepressing activity of the ORF63 mutants in transfected cells does not always correlate with the activity of the protein during replication and latency.

ORF63 mutants that were impaired for replication were also impaired for efficient establishment of latency. In the cotton rat model, VZV may replicate at the site of inoculation and/or in the ganglia. The impaired ability of some of the ORF63 mutants to replicate may have resulted in either less virus reaching the ganglia or less virus replication in the ganglia and therefore impaired establishment of latency. Prior experiments showed that 3 days after infection, high levels of VZV DNA are detected in ganglia of animals infected with a VZV ORF63 deletion mutant (which is impaired for replication) or with the parental virus (5). However, by 6 days after infection the level of viral DNA in ganglia is lower in animals infected with the ORF63 deletion mutant compared to those infected with the control virus. While some of the ORF63 mutants in the present study were impaired for replication in cell culture, high levels of viral DNA were likely present in cotton rat ganglia early after infection. Thus, impairment of replication at the site of inoculation for the ORF63 mutants is unlikely to be the cause for their impaired establishment of latency.

The mutations in ORF63 that impaired latency may have affected the ability of ORF63 protein to interact with other proteins. While the region of ORF63 protein important for binding to ORF62 protein is at the amino portion of the molecule and was unaffected by the mutations in ORF63 described here, ORF63 also interacts with RNA polymerase 2 (18) and the ORF47 protein kinase (15). The region of ORF63 protein that interacts with these two proteins has not been determined. Thus, the interaction of ORF63 protein with RNA polymerase 2, ORF47, or another cellular or viral protein might be critical for its role in latency. ORF63 protein is located in the tegument of virions (16), and the mutations in ROka63-5M or ROka63-AccI may have interfered with the ability of the protein to localize to the tegument. This might result in changes in virion stability or assembly or reduced delivery of the ORF63 protein to cells at the time of infection, thereby impairing replication.

In summary, we found that ORF63 mutations that impaired virus replication in cell culture also impaired the ability of the virus to establish latency. Future experiments will analyze additional ORF63 mutants to determine if mutants can be obtained that are unimpaired for replication but have reduced latency in animal models. Such viruses might be improved candidate vaccines, in that they might induce immunity similar to that induced by the Oka vaccine strain but would be less likely to reactivate and cause herpes zoster.

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