JOURNAL OF VIROLOGY, Aug. 2007, p. 7844–7851 Vol. 81, No. 15
0022-538X/07/$08.00+0 doi:10.1128/JVI.00325-07

Varicella-Zoster Virus IE63, a Major Viral Latency Protein, Is Required To Inhibit the Alpha Interferon-Induced Antiviral Response▽

Aruna P. N. Ambagala and Jeffrey I. Cohen*

Medical Virology Section, Laboratory of Clinical Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received 13 February 2007/Accepted 8 May 2007

Varicella-zoster virus (VZV) open reading frame 63 (ORF63) is the most abundant transcript expressed during latency in human sensory ganglia. VZV with ORF63 deleted is impaired for replication in melanoma cells and fibroblasts and for latency in rodents. We found that replication of the ORF63 deletion mutant is fully complemented in U2OS cells, which have been shown to complement the growth of herpes simplex virus type 1 (HSV-1) ICP0 mutants. Since HSV-1 ICP0 mutants are hypersensitive to alpha interferon (IFN-α), we examined the effect of IFN-α on VZV replication. Replication of the ORF63 mutant in melanoma cells was severely inhibited in the presence of IFN-α, in contrast to other VZV mutants that were similarly impaired for replication or to parental virus. The VZV ORF63 mutant was not hypersensitive to IFN-γ. IFN-α inhibited viral-gene expression in cells infected with the ORF63 mutant at a posttranscriptional level. Since IFN-α stimulates gene products that can phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF-2α) and inhibit translation, we determined whether cells infected with the ORF63 mutant had increased phosphorylation of eIF-2α compared with cells infected with parental virus. While phosphorylated eIF-2α was undetectable in uninfected cells or cells infected with parental virus, it was present in cells infected with the ORF63 mutant. Conversely, expression of IE63 (encoded by ORF63) in the absence of other viral proteins inhibited phosphorylation of eIF-2α. Since IFN-α has been shown to limit VZV replication in human skin xenografts, the ability of VZV IE63 to block the effects of the cytokine may play a critical role in VZV pathogenesis.

Varicella-zoster virus (VZV) is a neurotropic alphaherpesvirus. Primary VZV infection begins with inoculation of the respiratory mucosa, followed by cell-associated viremia and the rash of chickenpox. During primary infection, the virus establishes latency in sensory ganglia and can subsequently reactivate to cause shingles. At least six VZV open reading frames (ORFs), ORF4, -21, -29, -62, -63, and -66, are expressed during latency (11–13, 29, 38). ORF63 is the most prevalent and abundant VZV transcript expressed in latently infected human ganglia (11, 13).

VZV ORF63 encodes a 278-amino-acid protein, which is located in the tegument of virions (32) and is expressed as an immediate-early (IE) protein (14). IE63 is extensively modified in infected cells as a 45-kDa protein (14) that is phosphorylated by the VZV ORF47 kinase (30) and cellular casein kinases (5). IE63 binds to IE62, the major transactivator protein of VZV, and up-regulates expression of the VZV glycoprotein I (gI) promoter (36). IE63 has been reported to act as a transcriptional repressor for a number of VZV and heterologous viral and cellular promoters (5, 17). IE63 promotes the survival of cultured primary human neuronal cells (22). Point mutations in phosphorylation sites prior to the last 70 amino acids of IE63 result in mutant viruses impaired for replication (3, 8) and for establishment of latent infection in rodents (8). During lytic replication in vitro, IE63 is located predominantly in the nucleus; however, during latency, the protein is detected in the cytoplasm of sensory neurons (18, 35, 37).

* Corresponding author. Mailing address: Laboratory of Clinical Infectious Diseases, National Institutes of Health, 10 Center Dr., Building 10, Room 11N234, Bethesda, MD 20892. Phone: (301) 496-5265. Fax: (301) 496-7383. E-mail: jcohen@niaid.nih.gov.

▽ Published ahead of print on 16 May 2007.

The innate immune response is critical for defense against invading viruses. In response to virus infection, a number of signal transduction pathways are activated, including activation of genes that encode type I interferons (IFNs), such as IFN-α and IFN-β. These IFNs inhibit VZV replication in vitro (4, 15). VZV infection of human skin xenografts in SCID mice results in down-regulation of IFN-α in infected cells and increased expression of IFN-α in the surrounding uninfected epidermal cells, which may delay the appearance of skin lesions (33, 34). These findings indicate that IFN-α plays an important role in VZV pathogenesis.

Previously, we reported the generation of a recombinant VZV in which over 90% of both copies of ORF63 were deleted (7). The ORF63 deletion mutant was impaired for replication in human melanoma cells and for establishment of latency in rodents. Here, we report that the ORF63 deletion mutant is hypersensitive to human IFN-α compared to parental virus or other VZV deletion mutants that are impaired for replication in human melanoma cells. IFN-α confers cellular resistance against virus infection by at least two cellular proteins, double-stranded RNA-activated protein kinase (PKR) and 2′-5′oligoadenylate synthetase. Activated PKR phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2α), thereby preventing initiation of translation. We demonstrate that cells infected with the VZV ORF63 deletion mutant have increased levels of phosphorylated eIF-2α (eIF-2α-P) compared to those infected with parental virus, suggesting that expression of the ORF63 protein is necessary to overcome virus-induced phosphorylation of eIF-2α. Finally, we show that transient expression of IE63 diminishes basal levels of eIF-2α-P. These studies indicate that VZV ORF63 has a major role in the ability of VZV to overcome the innate immune response to the virus.

MATERIALS AND METHODS

Cells and viruses. MeWo human melanoma cells were provided by Charles Grose, and U2OS and Saos-2 osteosarcoma cells and Cos cells were obtained from the American Type Culture Collection (Manassas, VA). Recombinant Oka VZV (ROka) was obtained from cosmid clones derived from the attenuated Oka strain of VZV. VZV ROka63D, ROka61D, and ROka67D have ORF63, -61, and -67 deleted (7, 9, 10). Recombinant adenovirus Ad63 expressing VZV IE63 and green fluorescent protein and recombinant adenovirus Adblank expressing green fluorescent protein alone were described previously (23).

Immunoblots and Northern blots. For detection of VZV proteins, cells were lysed in CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} buffer (1% CHAPS [T. J. Baker, Phillipsburg, NJ] in Tris-buffered saline [19.98 mM Tris, 136 mM NaCl, pH 7.4]) supplemented with Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for 30 min on ice. For detection of eIF-2α, cells were lysed in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer, followed by sonication and boiling. To induce eIF-2α phosphorylation, cells were treated with 1 μM thapsigargin (Sigma-Aldrich, St. Louis, MO) for 30 min. Proteins were separated on 4 to 20% Tris-glycine sodium dodecyl sulfate-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. Rabbit polyclonal antibodies to VZV ORF61 protein (42), IE62 (42), IE63 (42), thymidine kinase (TK) (a gift from Christine Talarico), ORF4 protein (39), phosphorylated eIF-2α (Biosource International, Camarillo, CA), and mouse monoclonal antibodies to VZV glycoprotein E (gE) (Chemicon, Temecula, CA), cellular β-actin (Sigma-Aldrich), and eIF-2α (Biosource International) were used as primary antibodies. Horseradish peroxidase-conjugated goat anti-mouse antibodies (Pierce, Rockford, IL) and horseradish peroxidase-conjugated goat anti-rabbit antibodies (Pierce) were used as secondary antibodies.

Total RNA extracted from MeWo cells using the RNeasy mini kit (QIAGEN, Valencia, CA) was electrophoresed on 1% agarose gels containing 6.7% formaldehyde, blotted onto nylon membranes, and hybridized with [^{32}P]dCTP probes. Gene-specific probes were generated by PCR from HeLa cell cDNA (Stratagene, La Jolla, CA) or VZV cosmids using 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′ for human β-actin, 5′-CACC CAATCGCCTCAACTAT-3′ and 5′-TGTCCTGAACGGCATTAACA-3′ for VZV TK, 5′-GCAGTAACTCCTCAACCAAG-3′ and 5′-ATCGTAGAAGTG GTGACGTT-3′ for VZV gE, and 5′-TCTCCAAAACCACCGATGAACG-3′ and 5′-CGACGAGGAAGAGGATGAAGACG-3′ for VZV ORF62. Membranes were washed, exposed to phosphorimager screens, and scanned using a STORM 860 PhosphorImager, and bands were quantified using the Image Quant program (Molecular Dynamics, Sunnyvale, CA).

Plaque reduction and immunofluorescence assays. Monolayers of MeWo and U2OS cells grown in six-well-plates were either not treated or treated for 18 h with 1,000 U per ml of recombinant human IFN-α-2b (Intron A; Shering Corporation, Kenilworth, NJ) or recombinant human IFN-γ-1b (Actimmune; Intermune Inc., Brisbane, CA). In order to determine the effects of IFN on VZV plaquing efficiency, the cells were infected with serial dilutions of ROka, ROka63D, ROka61D, or ROka67D and incubated at 34°C. Seven (for ROka) or 10 (for VZV mutants) days after infection, the cells were stained with crystal violet and the plaques were counted. To determine the effects of IFN-α on VZV replication in U2OS and MeWo cells, the cells were pretreated with IFN-α for 18 h and infected with ROka or ROka63D at a ratio of 1:200 (infected to uninfected cells). Four days after infection, virus titers were determined by plating on MeWo cells.

VZV-infected cells were grown on glass coverslips, fixed in methanol-acetone (1:1 [vol/vol]), air dried, incubated with mouse monoclonal antibodies to VZV gE (Chemicon) or IE62 (Chemicon) followed by fluorescein isothiocyanate-conjugated goat anti-mouse antibody and DAPI (4′, 6′-diamidino-2-phenylindole) (1 μg/ml), and examined by fluorescence microscopy. The surface areas of immunofluorescent foci were measured using Image J software (http://rsb.info.nih.gov/ij/).

Transient-transfection assays. Monolayers of Cos cells grown in six-well plates were transfected with control plasmid (pCI) or plasmid expressing VZV IE63 (pCI-63) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total amount of plasmid DNA used was 3.2 μg for each transfection. Two days after transfection, the cells were harvested, lysed, and subjected to immunoblotting.

RESULTS

VZV with ORF63 deleted grows to titers equivalent to those of the parental virus in U2OS cells. VZV ROka63D, which has ORF63 deleted, is markedly impaired for growth in melanoma cells. U2OS osteosarcoma cells can fully complement the growth of herpes simplex virus type 1 (HSV-1) ICP0 null mutants and HSV-1 VP16 mutants (47, 48) and partially complement the growth of VZV ORF61 deletion mutants (10). To determine whether U2OS cells can complement the growth of ROka63D, MeWo and U2OS cells were infected with ROka or ROka63D, and 7 days postinfection, the cells were fixed and stained with antibody to VZV gE in an immunofluorescent-focus assay (Fig. 1A). Infection of MeWo cells with ROka63D resulted in fluorescent foci smaller (367,000 ± 71,800 units of surface area) than those observed in cells infected with ROka (822,000 ± 171,000 units of surface area) ($P < 0.0001$; Wilcoxon test). However, fluorescent foci in U2OS cells infected with ROka63D (117,000 ± 21,200 units per surface area) were similar in size to those infected with ROka (120,000 ± 14,600 units per surface area) ($P = 0.476$; Wilcoxon test). Infection of Saos-2 osteosarcoma cells with VZV ROka resulted in fluorescent foci (109,000 ± 18,600 units per surface area); however, no foci were observed in these cells infected with ROka63D.

To corroborate these observations, we determined the titers

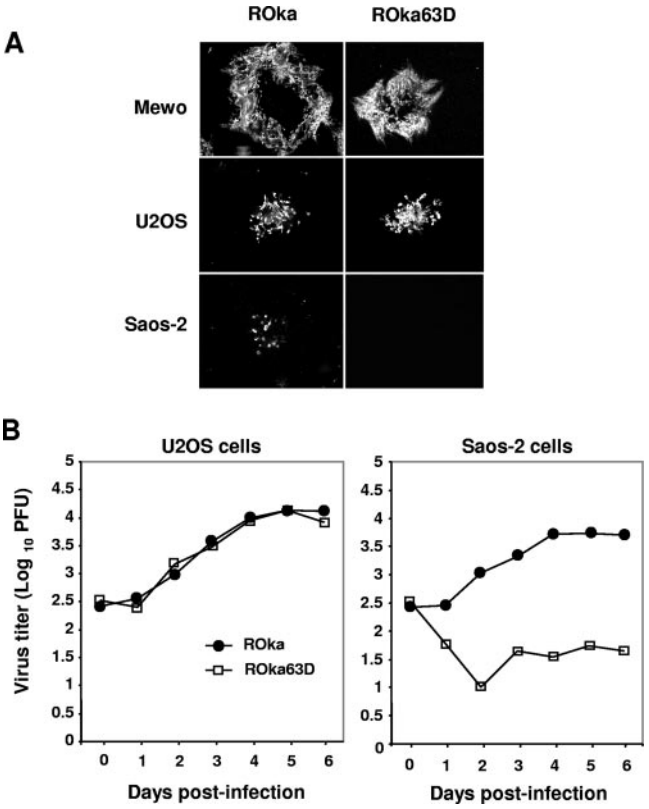

FIG. 1. (A) Immunofluorescent foci of ROka and ROka63D in MeWo, U2OS, and Saos-2 cells. Cells infected with ROka or ROka63D were stained with anti-VZV gE mouse monoclonal antibody, followed by fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G antibody. (B) Growth of ROka and ROka63D in U2OS and Saos-2 cells. U2OS and Saos-2 cells were inoculated with VZV-infected MeWo cells. Cells were harvested on days 1 to 6 after infection, and virus titers were determined by plating on MeWo cells. The virus titer on day zero represents the titer of the input inoculum.

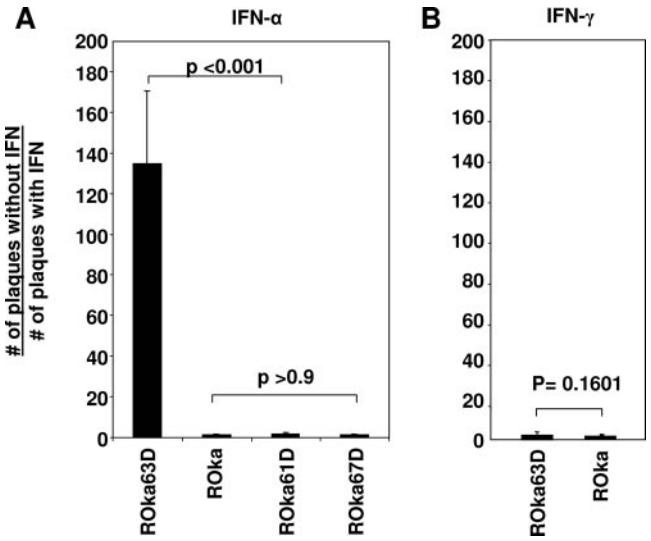

FIG. 2. Effect of IFN-α (A) and IFN-γ (B) on plaque formation by ROka and VZV deletion mutants in MeWo cells. MeWo cells were untreated or pretreated with IFN-α (1,000 IU/ml) or IFN-γ (1,000 IU/ml) for 18 h and infected with ROka, ROka63D, ROka61D, or ROka67D. Plaques were counted 6 days (for ROka) or 10 days (for deletion mutants) after infection. The efficacy of IFN treatment was measured by the ratio of the number of plaques in IFN-untreated wells to the number of plaques in IFN-treated wells. The results from three experiments were analyzed by one-way analysis of variance, and the Welch modified *t* test was used to calculate *P* values. The error bars represent standard deviations.

of ROka and ROka63D in MeWo, U2OS, and Saos-2 cells each day for 6 days. As reported previously, ROka63D titers were lower than those of ROka in MeWo cells (data not shown). In contrast, ROka63D grew to titers similar to those of ROka in U2OS cells (Fig. 1B). ROka63D replication was not complemented by growth in Saos-2 cells, as expected, based on the immunofluorescent-focus assay.

Growth of VZV with ORF63 deleted is severely impaired in the presence of IFN-α. Previous studies by Mossman et al. (40) showed that the growth of HSV-1 ICP0 mutants is fully complemented in U2OS cells and that these HSV-1 mutants are hypersensitive to IFN-α. Since the growth of ROka is fully complemented in U2OS cells, we determined if ROka63D is also exquisitely sensitive to IFN-α in a plaque reduction assay. MeWo cells pretreated with human IFN-α showed a 1.3-fold reduction in the number of plaques produced by ROka compared to cells treated with culture media alone (Fig. 2A). In contrast, pretreatment of MeWo cells with IFN-α reduced the number of plaques produced by ROka63D by 135-fold. To determine if IFN-α hypersensitivity of ROka63D is a direct consequence of the impaired growth of ROka63D, we tested the effect of IFN-α on the growth of two other small-plaque mutants of VZV, ROka61D and ROka67D. Pretreatment of MeWo cells with IFN-α reduced ROka61D and ROka67D plaques only by 1.8- and 1.4-fold, respectively, compared with culture media alone.

To determine whether ROka63D is also hypersensitive to human IFN-γ, we performed plaque reduction assays using MeWo cells pretreated with IFN-γ. IFN-γ inhibited the plaque formation of ROka63D in MeWo cells to the same extent as that of ROka (Fig. 2B). Taken together, these results demonstrate that VZV ROka63D is hypersensitive to IFN-α, but not to IFN-γ.

To investigate whether exogenous IE63 allows ROka63D to overcome the effect of IFN-α, we infected MeWo cells with a replication-defective adenovirus expressing VZV IE63 (Ad63) or control replication-defective adenovirus (Adblank) for 5 h, treated the cells with IFN-α for 18 h, and then infected them with ROka63D. Seven days after infection, plaques were observed in cells treated with IFN-α and infected with Ad63 and ROka63D; however, no plaques were noted in cells treated with IFN-α and infected with Adblank and ROka63D (Fig. 3). Plaques were observed in cells not treated with IFN-α and infected with ROka63D (Fig. 3) and in cells treated with IFN-α

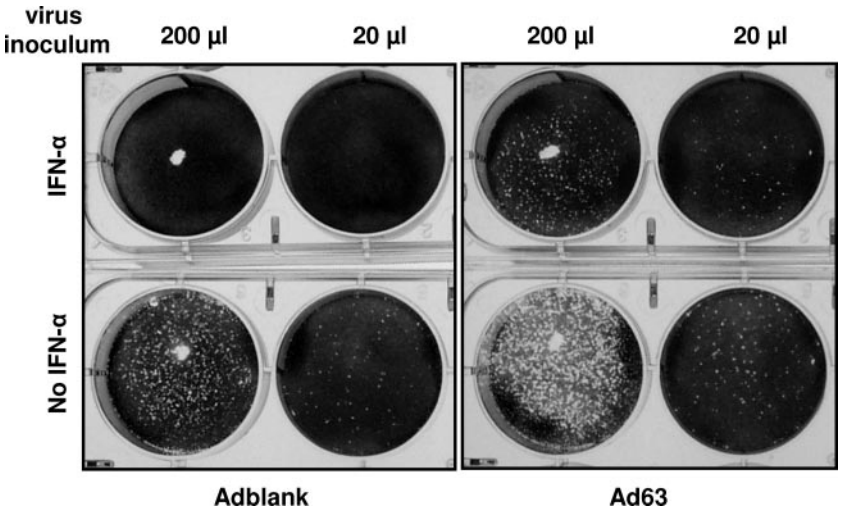

FIG. 3. VZV IE63 expressed in *trans* overcomes the effect of IFN-α in ROka63D-infected cells. MeWo cells were infected with control adenovirus (Adblank) or adenovirus expressing VZV IE63 protein (Ad63) for 5 h and treated with IFN-α (1,000 IU/ml) for 18 h. The cells were then infected with serial dilutions of ROka63D, and 10 days after infection, the monolayers were stained with crystal violet.

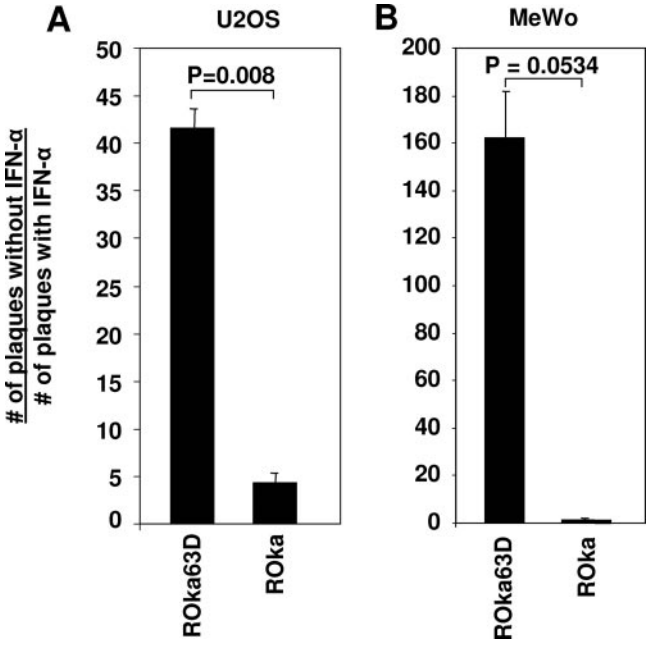

FIG. 4. Effects of IFN-α on replication of ROka and ROka63D in U2OS and MeWo cells. Monolayers of U2OS and MeWo cells were untreated or pretreated with IFN-α (1,000 IU/ml) for 18 h. The cells were then infected with ROka or ROka63D. Four days after infection, the cells were harvested and the titer of each virus was determined on MeWo cells. The efficacy of IFN-α treatment was measured as described in the legend to Fig. 3. The error bars represent standard deviations.

and infected with ROka and Adblank or Ad63 (data not shown).

VZV with ORF63 deleted is also hypersensitive to IFN-α in U2OS cells. While HSV-1 ICP0 null mutants are hypersensitive to IFN-α compared to wild-type virus in Vero cells, they show the same level of sensitivity to IFN-α as wild-type virus in U2OS cells (40). Therefore, we looked at the replication of ROka and ROka63D in U2OS cells pretreated (or not treated) with IFN-α and determined virus titers on MeWo cells. IFN-α reduced the titer of ROka63D in U2OS cells 42-fold and reduced the titer of ROka 4-fold (Fig. 4A). Similarly, IFN-α treatment reduced the titer of ROka63D in MeWo cells 162-fold and reduced the titers of ROka 1.5-fold (Fig. 4B).

IFN-α impairs viral-gene expression in cells infected with ROka63D at a posttranscriptional level. To determine the effect of IFN-α on VZV gene expression, we performed immunofluorescent-focus assays using antibodies to VZV gE or IE62. Treatment of MeWo cells with or without IFN-α, followed by infection with ROka, ROka63D, ROka61D, or ROka67D, showed that expression of gE was severely impaired only in cells treated with IFN-α and infected with ROka63D (Fig. 5A). The level of expression of VZV IE62 was also severely reduced in cells treated with IFN-α and infected with ROka63D compared to cells treated with IFN-α and infected with ROka (Fig. 5B).

To confirm the immunofluorescence data, immunoblots were performed using lysates of MeWo cells pretreated or not treated with IFN-α and infected with ROka, ROka63D, or ROka61D for 2 days (Fig. 6A). In the absence of IFN-α, ROka-infected cells expressed the highest levels of IE62, VZV TK (a putative early protein), and gE (a late protein), while cells infected with ROka63D and ROka61D expressed similar levels of these proteins. In cells treated with IFN-α, levels of the three viral proteins in ROka61D-infected cells were comparable to those in cells treated with IFN-α and infected with ROka. However, cells infected with ROka63D showed markedly reduced levels of each of the three viral proteins compared with ROka or ROka61D. Quantification of the proteins in cells treated with IFN-α showed that the levels of IE62, TK, ORF61 protein, and gE were 7.2-fold, 3.9-fold, 9.4-fold, and 25.2-fold lower in cells infected with ROka63D than in those infected with ROka (Fig. 6B). Antibody to IE63 and ORF61 protein confirmed that cells infected with ROka63D and ROka61D did not express these proteins. Thus, there was a marked reduction in the expression of all three kinetic classes of viral proteins in ROka63D-infected cells pretreated with IFN-α compared with ROka or ROka61D-infected cells pretreated with IFN-α.

To determine whether the effect of IFN-α on ROka63D gene expression occurs at the level of virus gene transcription, Northern blots were performed, and the levels of IE62, TK, gE, and β-actin transcripts were quantified. The quantities of transcripts were similar for all three kinetic classes of viral genes in cells infected with ROka, ROka63D, and ROka61D (Fig. 7). Thus, IFN-α inhibits viral-gene expression in ROka63D-infected cells at a posttranscriptional level.

VZV IE63 is required to inhibit phosphorylation of eIF-2α by the host cell in response to virus infection. IFN-α induces a variety of IFN-stimulated genes, including PKR. Activated PKR phosphorylates eIF-2α, resulting in inhibition of translation and therefore inhibition of virus replication (26, 28, 31). Since many viruses encode proteins that inhibit or reverse phosphorylation of eIF-2α, we measured the level of eIF-2α-P in ROka and ROka63D-infected cells (Fig. 8). Thapsigargin, a potent inducer of eIF-2α-P (45), was used as a positive control. eIF-2α-P was undetectable in MeWo cells infected with ROka or ROka61D or in uninfected cells. eIF-2α-P was detected in ROka63D-infected cells or cells treated with thapsigargin. The levels of total eIF-2α were similar for virus-infected cells and uninfected cells. These results indicate that VZV IE63 is required to overcome phosphorylation of eIF-2α, which is induced by virus infection.

VZV IE63 inhibits phosphorylation of eIF-2α in the absence of other viral proteins. To determine whether VZV IE63 is sufficient to overcome phosphorylation of eIF-2α, we transfected Cos cells with a plasmid encoding VZV IE63 (pCI-63) or empty vector (pCI). Two days later, the amounts of phosphorylated and total eIF2 were assessed in transfected cells by immunoblotting and quantified by densitometry (Fig. 9). Cells transfected with pCI-63 showed an eightfold reduction in the amount of eIF-2α-P compared to cells transfected with pCI. These results suggest that VZV IE63 is sufficient to inhibit eIF-2α phosphorylation.

DISCUSSION

We have shown that growth of VZV with ORF63 deleted can be fully complemented by U2OS cells and that the mutant virus is markedly impaired for growth in the presence of IFN-α. Further-

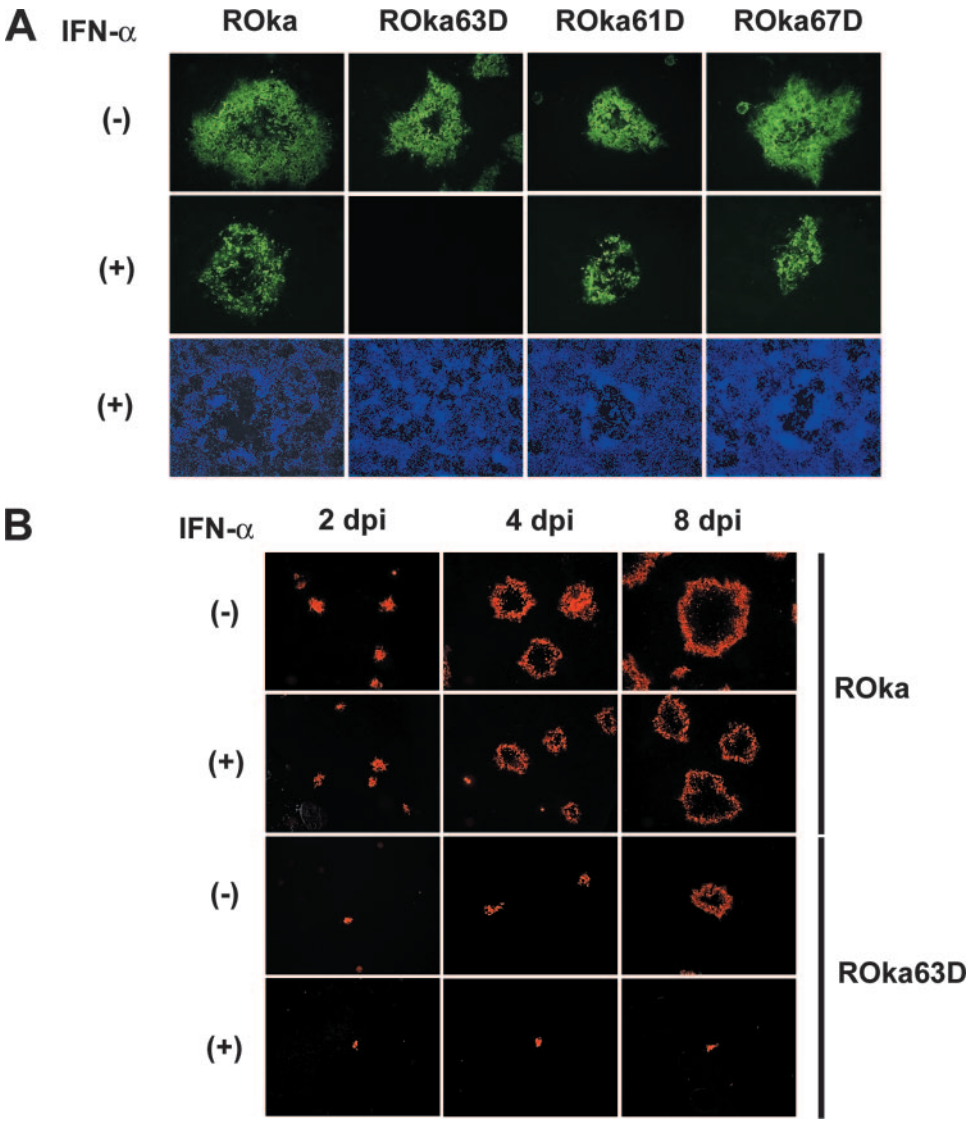

FIG. 5. Immunofluorescence staining with antibodies to VZV gE and IE62 of VZV-infected, IFN-α-untreated, or IFN-α-pretreated MeWo cells. MeWo cells treated with human IFN-α (1,000 IU/ml) or untreated were infected with ROka, ROka63D, ROka61D, or ROka67D. On the indicated days after infection, the cells were fixed and stained with antibodies to VZV gE (A) or VZV IE62 (B) or with DAPI (A, bottom). The data are representative of one of two independent experiments.

more, we found that IE63 inhibits phosphorylation of eIF-2α expressed in the absence or presence of other viral proteins. These results indicate that IE63 plays a critical role in inhibiting the innate immune response to VZV by the host.

While VZV with ORF63 deleted is impaired for growth in melanoma and Saos-2 cells and human diploid fibroblasts (7, 23), the mutant was fully complemented in U2OS cells. U2OS cells complement the growth of a number of virus mutants, including HSV-1 mutants in ICP0 (48) and VP16 (47) and VZV mutants in ORF61 (10) and ORF63. Each of these herpesvirus mutant proteins is important for regulating gene expression. Yao and Shaffer (48) postulated that U2OS cells may encode a cellular factor that activates viral-gene expression and reduces the need for a viral transactivator. More recently, Hancock et al. (19), using somatic cell hybrids formed between U2OS cells and human fibroblasts, suggested that U2OS cells are defective either for chromatin-dependent nuclear repression or for a signal transduction pathway that activates nuclear repression in response to virus infection. Thus, at present it is uncertain how U2OS cells complement the growth of HSV and VZV mutants.

Since HSV-1 ICP0 mutants are fully complemented in U2OS cells and are hypersensitive to IFN-α (20, 40), we postulated that a VZV ORF63 deletion mutant might also be markedly impaired for growth in the presence of IFN-α. The ORF63 deletion mutant was hypersensitive to IFN-α while other mutants, like ROka63D, that are impaired for growth in melanoma cells were not hypersensitive to IFN-α. While both HSV ICP0 and ROka63D are hypersensitive to IFN-α, there are several differences in their responses to the cytokine. First, HSV ICP0 mutants are resistant to IFN-α in U2OS cells, while the VZV ORF63 mutant remained hypersensitive to IFN-α in

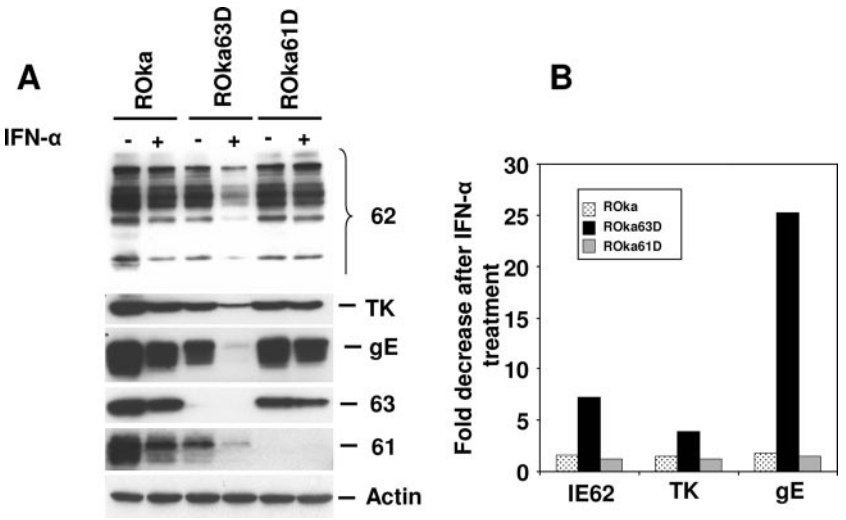

FIG. 6. Immunoblots of VZV IE, putative early, and late proteins in MeWo cells untreated (−) or pretreated (+) with IFN-α. (A) MeWo cells untreated or pretreated with IFN-α (1,000 IU/ml) were infected with ROka, ROka63D, or ROka61D. Cell lysates were prepared 2 days after infection and immunoblotted with antibodies to VZV IE62, TK, gE, IE63, ORF61 protein, or cellular β-actin. (B) The VZV protein bands were quantified using a densitometer and normalized to the corresponding β-actin band. The reduction (*n*-fold) in protein expression due to IFN-α treatment was determined as the ratio of normalized densitometry values from IFN-α-untreated cells to that from IFN-α-treated cells. The data are representative of one of two independent experiments.

U2OS cells. There was however, a slight reduction in hypersensitivity of the ORF63 deletion mutant in IFN-α-treated U2OS cells compared with melanoma cells (plaquing efficiencies were reduced 42-fold versus 162-fold, respectively). Second, HSV ICP0 mutants are hypersensitive to IFN-γ (20), while VZV with ORF63 deleted is little affected by IFN-γ. Harle et al. (20) reported that IFN-γ reduced the plaquing efficiency of an HSV ICP0 mutant by 45-fold but reduced plaque formation by wild-type HSV by only 3-fold. In contrast, IFN-γ reduced the plaquing efficiency of the VZV ORF63 deletion mutant by only 2.5-fold and that of the parental virus by 1.2-fold. Third, the effect of IFN-α on HSV-1 ICP0 mutants occurs at the transcriptional level, while the effect on the VZV ORF63 mutant occurred at a posttranscriptional level. Mossman et al. (40) demonstrated that accumulation of IE and early viral transcripts was severely impaired in IFN-α-treated Vero cells infected with HSV-1 ICP0 mutants and that expression of the corresponding IE and early viral proteins was also reduced in those cells. In contrast, IFN-α had little or no effect on VZV transcripts of different kinetic classes in ORF63 mutant-infected cells. However, there was a marked reduction in viral proteins in IFN-α-treated cells infected with the ORF63 deletion mutant compared with parental VZV, especially in the expression of a late VZV protein. These observations suggest

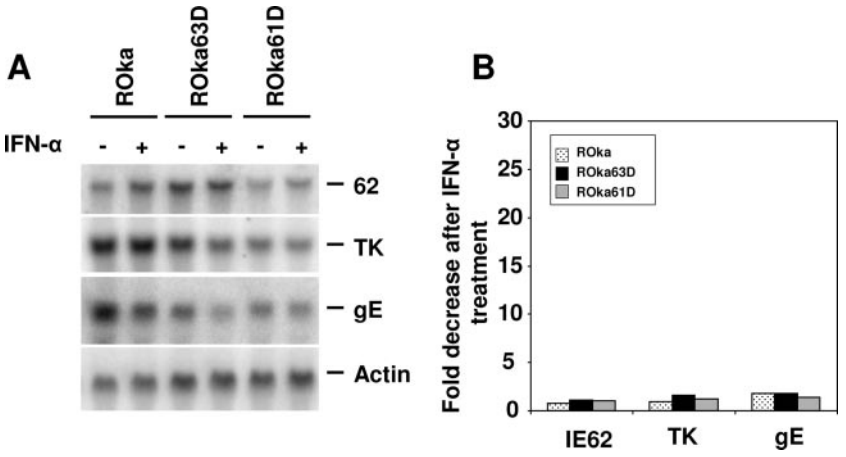

FIG. 7. Northern blots of VZV IE, putative early, and late transcripts in MeWo cells in the presence (+) or absence (−) of IFN-α. (A) MeWo cells untreated or pretreated with human IFN-α (1,000 IU/ml) were infected with ROka, ROka63D, or ROka61D. Total RNA was isolated 2 days after infection, and Northern blots were performed using radiolabeled DNA probes specific for VZV IE62, TK, gE, and cellular β-actin. (B) Bands were quantified using a densitometer, and each band was normalized to the area of the corresponding β-actin band. Reduction (*n*-fold) in mRNA expression due to IFN-α treatment was calculated as the ratio of densitometry values from IFN-α-untreated cells to that from IFN-α-treated cells. The data are representative of one of two independent experiments.

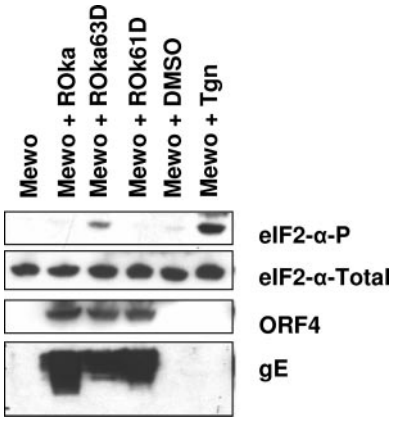

FIG. 8. Immunoblot of eIF-2α phosphorylation in ROka, ROka63D, or ROka61D-infected MeWo cells. Mewo cells were infected with ROka, ROka63D, or ROka61D; cell lysates were prepared 2 days after infection; and immunoblots were performed using antibodies to phosphorylated or total eIF-2α, VZV ORF4, or gE. Lysates from thapsigargin (Tgn)-treated MeWo cells were used as a positive control, and lysates from uninfected or dimethyl sulfoxide (DMSO)-treated cells were negative controls for eIF-2α-P. The data are representative of one of three independent experiments.

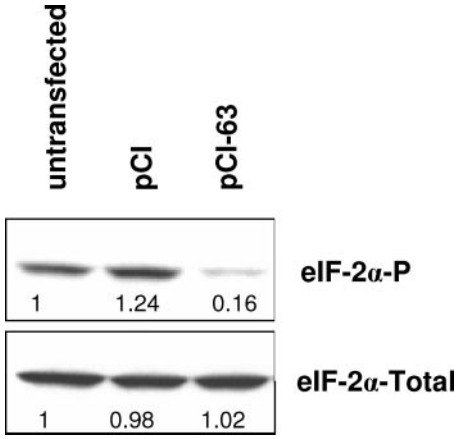

FIG. 9. Immunoblot of phosphorylated and total eIF-2α in Cos cells expressing VZV ORF63. Cos cells were transfected with the indicated plasmids, and 2 days later, cell lysates were prepared and immunoblotted with antibodies to phosphorylated or total eIF-2α. The numbers below the bands indicate signal intensities relative to that of untransfected cells. The data are representative of one of three independent experiments.

that VZV IE63 and ICP0 use different mechanisms to overcome the antiviral effects of IFN-α.

Type I IFNs exert their effects in target cells by inducing expression of a large number of IFN-stimulated genes (27). These genes encode proteins that include PKR, 2′-5′oligoadenylate synthetase, and the Mx proteins that interfere with viral transcription and translation processes. PKR is activated in response to double-stranded RNA molecules generated during virus infection and phosphorylates eIF-2α. In mammals, eIF-2 delivers Met-tRNAi to the ribosome in a GTP-dependent manner to initiate protein synthesis. The inactive eIF-2–GDP complex is released from the ribosome and is rapidly converted to active eIF-2–GTP by eIF-2B (16). Phosphorylation of eIF-2α increases the affinity of eIF2 for eIF2B up to 100-fold, leading to competitive inhibition of eIF-2B. Since eIF-2B is present in limited amounts in the cells, a small increase in eIF-2α phosphorylation leads to a marked decrease in protein synthesis.

Viruses have evolved several mechanisms to interfere with eIF-2α phosphorylation and prevent an inhibition of protein synthesis in infected cells (46). HSV-1 encodes two gene products, γ34.5 and Us11, which prevent accumulation of eIF-2α-P. While γ34.5 mediates dephosphorylation of eIF-2α (21), Us11 binds to and inhibits the activation of PKR and its ability to phosphorylate eIF-2α (6, 44). These two HSV proteins exert their functions at distinct phases of viral replication (41). Cells infected with HSV-1 mutants lacking either of the two proteins show increased levels of eIF-2α-P compared to wild-type virus (41). Since melanoma cells infected with a VZV ORF63 deletion mutant have increased levels of eIF-2α-P compared to those infected with parental VZV, these findings indicate that IE63 is required to overcome virus-induced phosphorylation of eIF-2α. The observation that transient expression of IE63 in cells reduces the level of eIF-2α-P indicates that IE63 alone is sufficient for modulating the phosphorylation of eIF-2α.

At present, it is uncertain how VZV induces phosphorylation of eIF-2α in the absence of IE63. Desloges et al. (15) demonstrated that the levels of phosphorylated and total PKR were not significantly affected in VZV-infected melanoma cells compared with uninfected cells. Thus, PKR may not contribute to phosphorylation of eIF-2α in VZV-infected cells. In addition to PKR, three other kinases, PERK, GCN2, and HRI, phosphorylate eIF-2α (1). Several different viruses, including human cytomegalovirus (24), bovine viral diarrhea virus (25), and hepatitis C virus (43), activate PERK.

The observations that VZV IE63 reduces the level of eIF-2α-P and that VZV with ORF63 deleted is markedly impaired for growth in the presence of IFN-α have important implications for VZV pathogenesis. IE63 is expressed both during lytic infection and during latency. The effect of IE63 on IFN-α may be important during acute infection with VZV. The increase in IFN-α expression in uninfected epidermal cells adjacent to VZV-infected cells in human skin xenografts in mice suggests that IFN-α may be partly responsible for the long incubation period of varicella (33). SCID mice infused with antibody to the IFN-α/β receptor had 10-fold-higher titers of virus in VZV-infected human skin xenografts and larger skin lesions than animals that received no antibody. The importance of IFN-α is also reflected by the observation that treatment of immunocompromised children with IFN-α reduces the severity of varicella (2). IE63 is also expressed during latency in neurons, and the viral protein may protect virus-infected neurons from the effects of IFN-α during latency and/or reactivation. Thus, the ability of VZV IE63 to inhibit IFN-α antiviral activity may be important for blocking the innate immune response to the virus during primary infection and reactivation from latency.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases.

We thank Randall J. Cohrs for Ad63 and Adblank.

REFERENCES

1. **Anderson, P., and N. Kedersha.** 2002. Visibly stressed: the role of eIF2, TIA-1, and stress granules in protein translation. Cell Stress Chaperones **7:**213–221.
2. **Arvin, A. M., J. H. Kushner, S. Feldman, R. L. Baehner, D. Hammond, and T. C. Merigan.** 1982. Human leukocyte interferon for the treatment of varicella in children with cancer. N. Engl. J. Med. **306:**761–765.
3. **Baiker, A., C. Bagowski, H. Ito, M. Sommer, L. Zerboni, K. Fabel, J. Hay, W. Ruyechan, and A. M. Arvin.** 2004. The immediate-early 63 protein of varicella-zoster virus: analysis of functional domains required for replication in vitro and for T-cell and skin tropism in the SCIDhu model in vivo. J. Virol. **78:**1181–1194.
4. **Balachandra, K., D. Thawaranantha, P. I. Ayuthaya, J. Bhumisawasdi, K. Shiraki, and K. Yamanishi.** 1994. Effects of human alpha, beta and gamma interferons on varicella zoster virus in vitro. Southeast Asian J. Trop. Med. Public Health **25:**252–257.
5. **Bontems, S., E. Di Valentin, L. Baudoux, B. Rentier, C. Sadzot-Delvaux, and J. Piette.** 2002. Phosphorylation of varicella-zoster virus IE63 protein by casein kinases influences its cellular localization and gene regulation activity. J. Biol. Chem. **277:**21050–21060.
6. **Cassady, K. A., and M. Gross.** 2002. The herpes simplex virus type 1 U(S)11 protein interacts with protein kinase R in infected cells and requires a 30-amino-acid sequence adjacent to a kinase substrate domain. J. Virol. **76:**2029–2035.
7. **Cohen, J. I., E. Cox, L. Pesnicak, S. Srinivas, and T. Krogmann.** 2004. The varicella-zoster virus open reading frame 63 latency-associated protein is critical for establishment of latency. J. Virol. **78:**11833–11840.
8. **Cohen, J. I., T. Krogmann, S. Bontems, C. Sadzot-Delvaux, and L. Pesnicak.** 2005. 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. J. Virol. **79:**5069–5077.
9. **Cohen, J. I., and H. Nguyen.** 1997. Varicella-zoster virus glycoprotein I is essential for growth of virus in Vero cells. J. Virol. **71:**6913–6920.
10. **Cohen, J. I., and H. Nguyen.** 1998. Varicella-zoster virus ORF61 deletion mutants replicate in cell culture, but a mutant with stop codons in ORF61 reverts to wild-type virus. Virology **246:**306–316.
11. **Cohrs, R. J., and D. H. Gilden.** 2007. Prevalence and abundance of latently transcribed VZV genes in human ganglia. J. Virol. **81:**2950–2956.
12. **Cohrs, R. J., and D. H. Gilden.** 2003. Varicella zoster virus transcription in latently-infected human ganglia. Anticancer Res. **23:**2063–2069.
13. **Cohrs, R. J., J. Randall, J. Smith, D. H. Gilden, C. Dabrowski, H. van Der Keyl, and R. Tal-Singer.** 2000. Analysis of individual human trigeminal ganglia for latent herpes simplex virus type 1 and varicella-zoster virus nucleic acids using real-time PCR. J. Virol. **74:**11464–11471.
14. **Debrus, S., C. Sadzot-Delvaux, A. F. Nikkels, J. Piette, and B. Rentier.** 1995. Varicella-zoster virus gene 63 encodes an immediate-early protein that is abundantly expressed during latency. J. Virol. **69:**3240–3245.
15. **Desloges, N., M. Rahaus, and M. H. Wolff.** 2005. Role of the protein kinase PKR in the inhibition of varicella-zoster virus replication by beta interferon and gamma interferon. J. Gen. Virol. **86:**1–6.
16. **Dever, T. E.** 2002. Gene-specific regulation by general translation factors. Cell **108:**545–556.
17. **Di Valentin, E., S. Bontems, L. Habran, O. Jolois, N. Markine-Goriaynoff, A. Vanderplasschen, C. Sadzot-Delvaux, and J. Piette.** 2005. Varicella-zoster virus IE63 protein represses the basal transcription machinery by disorganizing the pre-initiation complex. Biol. Chem. **386:**255–267.
18. **Grinfeld, E., and P. G. Kennedy.** 2004. Translation of varicella-zoster virus genes during human ganglionic latency. Virus Genes **29:**317–319.
19. **Hancock, M. H., J. A. Corcoran, and J. R. Smiley.** 2006. Herpes simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression. Virology **352:**237–252.
20. **Harle, P., B. Sainz, Jr., D. J. Carr, and W. P. Halford.** 2002. The immediate-early protein, ICP0, is essential for the resistance of herpes simplex virus to interferon-alpha/beta. Virology **293:**295–304.
21. **He, B., M. Gross, and B. Roizman.** 1997. The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1α to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **94:**843–848.
22. **Hood, C., A. L. Cunningham, B. Slobedman, A. M. Arvin, M. H. Sommer, P. R. Kinchington, and A. Abendroth.** 2006. Varicella-zoster virus ORF63 inhibits apoptosis of primary human neurons. J. Virol. **80:**1025–1031.
23. **Hoover, S. E., R. J. Cohrs, Z. G. Rangel, D. H. Gilden, P. Munson, and J. I. Cohen.** 2006. Downregulation of varicella-zoster virus (VZV) immediate-early ORF62 transcription by VZV ORF63 correlates with virus replication in vitro and with latency. J. Virol. **80:**3459–3468.
24. **Isler, J. A., A. H. Skalet, and J. C. Alwine.** 2005. Human cytomegalovirus infection activates and regulates the unfolded protein response. J. Virol. **79:**6890–6899.
25. **Jordan, R., L. Wang, T. M. Graczyk, T. M. Block, and P. R. Romano.** 2002. Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. J. Virol. **76:**9588–9599.
26. **Katze, M. G.** 1995. Regulation of the interferon-induced PKR: can viruses cope? Trends Microbiol. **3:**75–78.
27. **Katze, M. G., Y. He, and M. Gale, Jr.** 2002. Viruses and interferon: a fight for supremacy. Nat. Rev. Immunol. **2:**675–687.
28. **Kaufman, R. J.** 1999. Double-stranded RNA-activated protein kinase mediates virus-induced apoptosis: a new role for an old actor. Proc. Natl. Acad. Sci. USA **96:**11693–11695.
29. **Kennedy, P. G., E. Grinfeld, and J. E. Bell.** 2000. Varicella-zoster virus gene expression in latently infected and explanted human ganglia. J. Virol. **74:**11893–11898.
30. **Kenyon, T. K., J. Lynch, J. Hay, W. Ruyechan, and C. Grose.** 2001. Varicella-zoster virus ORF47 protein serine kinase: characterization of a cloned, biologically active phosphotransferase and two viral substrates, ORF62 and ORF63. J. Virol. **75:**8854–8858.
31. **Kibler, K. V., T. Shors, K. B. Perkins, C. C. Zeman, M. P. Banaszak, J. Biesterfeldt, J. O. Langland, and B. L. Jacobs.** 1997. Double-stranded RNA is a trigger for apoptosis in vaccinia virus-infected cells. J. Virol. **71:**1992–2003.
32. **Kinchington, P. R., D. Bookey, and S. E. Turse.** 1995. The transcriptional regulatory proteins encoded by varicella-zoster virus open reading frames (ORFs) 4 and 63, but not ORF 61, are associated with purified virus particles. J. Virol. **69:**4274–4282.
33. **Ku, C. C., J. Besser, A. Abendroth, C. Grose, and A. M. Arvin.** 2005. Varicella-zoster virus pathogenesis and immunobiology: new concepts emerging from investigations with the SCIDhu mouse model. J. Virol. **79:**2651–2658.
34. **Ku, C. C., L. Zerboni, H. Ito, B. S. Graham, M. Wallace, and A. M. Arvin.** 2004. Varicella-zoster virus transfer to skin by T cells and modulation of viral replication by epidermal cell interferon-alpha. J. Exp. Med. **200:**917–925.
35. **Lungu, O., C. A. Panagiotidis, P. W. Annunziato, A. A. Gershon, and S. J. Silverstein.** 1998. Aberrant intracellular localization of varicella-zoster virus regulatory proteins during latency. Proc. Natl. Acad. Sci. USA **95:**7080–7085.
36. **Lynch, J. M., T. K. Kenyon, C. Grose, J. Hay, and W. T. Ruyechan.** 2002. Physical and functional interaction between the varicella zoster virus IE63 and IE62 proteins. Virology **302:**71–82.
37. **Mahalingam, R., M. Wellish, R. Cohrs, S. Debrus, J. Piette, B. Rentier, and D. H. Gilden.** 1996. Expression of protein encoded by varicella-zoster virus open reading frame 63 in latently infected human ganglionic neurons. Proc. Natl. Acad. Sci. USA **93:**2122–2124.
38. **Meier, J. L., R. P. Holman, K. D. Croen, J. E. Smialek, and S. E. Straus.** 1993. Varicella-zoster virus transcription in human trigeminal ganglia. Virology **193:**193–200.
39. **Moriuchi, H., M. Moriuchi, H. A. Smith, and J. I. Cohen.** 1994. Varicella-zoster virus open reading frame 4 protein is functionally distinct from and does not complement its herpes simplex virus type 1 homolog, ICP27. J. Virol. **68:**1987–1992.
40. **Mossman, K. L., H. A. Saffran, and J. R. Smiley.** 2000. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. J. Virol. **74:**2052–2056.
41. **Mulvey, M., J. Poppers, D. Sternberg, and I. Mohr.** 2003. Regulation of eIF2α phosphorylation by different functions that act during discrete phases in the herpes simplex virus type 1 life cycle. J. Virol. **77:**10917–10928.
42. **Ng, T. I., L. Keenan, P. R. Kinchington, and C. Grose.** 1994. Phosphorylation of varicella-zoster virus open reading frame (ORF) 62 regulatory product by viral ORF 47-associated protein kinase. J. Virol. **68:**1350–1359.
43. **Pavio, N., and M. M. Lai.** 2003. The hepatitis C virus persistence: how to evade the immune system? J. Biosci. **28:**287–304.
44. **Peters, G. A., D. Khoo, I. Mohr, and G. C. Sen.** 2002. Inhibition of PACT-mediated activation of PKR by the herpes simplex virus type 1 Us11 protein. J. Virol. **76:**11054–11064.
45. **Prostko, C. R., M. A. Brostrom, and C. O. Brostrom.** 1993. Reversible phosphorylation of eukaryotic initiation factor 2 alpha in response to endoplasmic reticular signaling. Mol. Cell Biochem. *127-***128:**255–265.
46. **Schneider, R. J., and I. Mohr.** 2003. Translation initiation and viral tricks. Trends Biochem. Sci. **28:**130–136.
47. **Smiley, J. R., and J. Duncan.** 1997. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. J. Virol. **71:**6191–6193.
48. **Yao, F., and P. A. Schaffer.** 1995. An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. J. Virol. **69:**6249–6258.