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Varicella-zoster virus open reading frame 47 (ORF47) protein is critical for virus replication in dendritic cells and for spread to other cells

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Abstract

Varicella-zoster virus infects human dendritic cells (DCs). We found that VZV infection of DCs resulted in down-regulation of Fas expression on the surface of cells. VZV ORF47 was critical for replication of virus in human immature, but not mature DCs. Immature DCs infected with a mutant virus unable to express ORF47 expressed similar levels of a VZV immediate–early protein as cells infected with parental virus; however, cells infected with the ORF47 mutant expressed lower levels of glycoprotein E. Thus, in the absence of ORF47 protein, there is a block in viral replication between immediate–early and late gene expression. VZV unable to express ORF47 was severely impaired for spread of virus from DCs to melanoma cells. Infection of DCs with parental VZV resulted in a different pattern of phosphoproteins compared with the ORF47 mutant virus. Thus, VZV ORF47 is important for replication in immature DCs and for spread to other cells.
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Keywords: Dendritic cells; Varicella-zoster virus; Varicella; Shingles; Zoster; ORF47

Introduction

Varicella-zoster virus (VZV) infects up to 90% of the human population and is the etiologic agent of varicella (chickenpox). Following resolution of primary infection the virus establishes a lifelong, latent infection in the dorsal root and cranial nerve ganglia. Reactivation of virus results in herpes zoster (shingles). The cellular immune response is important for controlling reactivation and for reducing the severity of primary disease. Persons with impaired cellular immunity are at increased risk for severe chickenpox and are more likely to develop shingles (Gershon et al., 1997; Jura et al., 1989). The cellular immune response to VZV includes NK cells, T cells, and dendritic cells (DCs) (Jenkins et al., 1999; Vossen et al., 2005).

DCs are potent antigen-presenting cells and are important for initiating immune responses to virus infections. Immature DCs express MHC class I and MHC class II molecules, CD40 ligand, and costimulatory molecules such as CD80 and CD86 (Mellman and Steinman, 2001). They have excellent antigen-processing, but poor antigen-presenting capacity. When immature DCs are stimulated by CD40 ligand, tumor necrosis factor-α, interferon-γ (IFN-γ), or interleukin 6 (IL-6), they undergo a morphologic change and become mature DCs. Compared with immature DCs, mature DCs express higher levels of surface CD80 and CD86 and express CD83 which increases their ability to stimulate T cells. Unlike immature DCs, mature DCs are excellent at both antigen processing and antigen presentation.

There are three pathways that DCs use to present antigens to CD8 or CD4 T cells. First, infection of DCs with viruses leads to expression of viral proteins that are processed into 8–10 amino acid peptides in the proteasome. These peptides are loaded onto MHC class I molecules and

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transported to the plasma membrane for presentation to CD8 T cells in a process known as the MHC class I endogenous pathway. Second, DCs can take up apoptotic virus-infected cells or viral proteins. These proteins and particles are processed in endosomes, released into the cytosol for further processing, and presented by MHC class I on the cell surface to CD8 T cells in a process known as the MHC class I cross-presentation pathway. Third, DCs can endocytose viral proteins or inactivated virus in vacuoles, process the proteins, load them onto MHC class II molecules, and transport them to the cell surface for presentation to CD4 T cells. This process is known as the MHC class II exogenous pathway.

Many viruses can productively infect DCs including human immunodeficiency virus (Granelli-Piperno et al., 1998), cytomegalovirus (Riegler et al., 2000), herpes simplex virus (HSV) types 1 and 2 (Jones et al., 2003; Mikloska et al., 2001), Epstein–Barr virus (Li et al., 2002), human herpesvirus 6 (Asada et al., 1999), and VZV (Morrow et al., 2003).

VZV productively infects both immature and mature DCs. Abendroth et al. (2001) showed that VZV-infected immature DCs did not undergo apoptosis, and that the infected cells could transfer virus to human fibroblasts or T cells. Morrow et al. (2003) showed that VZV infection of mature DCs impaired their ability to stimulate allogeneic T cells.

Previous studies from our laboratory showed that VZV mutants unable to express certain genes, such as open reading frame 47 (ORF47) or ORF66, are impaired for growth in human T cells, while other mutants show no difference from parental virus (Moffat et al., 1998; Soong et al., 2000). VZV unable to express ORF47 was even more impaired for spread from human T cells to melanoma cells in vitro. In the present study, we infected immature and mature DCs with a panel of VZV mutants. We found that VZV ORF47 is important for replication of virus in immature, but not mature cells. In the absence of ORF47, VZV replication is blocked between immediate–early and late gene expression. While ORF47 is not required for infection of DCs, it is critical for transfer of VZV from DCs to other cells.

Results

Infection of immature or mature DCs results in down-regulation of surface Fas expression

Human immature DCs were generated from peripheral blood monocytes by incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 for 7 days (Fig. 1, left panel). These cells were defined by their cell surface phenotype (CD1a$^+$ and CD83$^-$). The purity of mature DCs was >90% as assessed by flow cytometry. Immature DCs were then cultured for a further 2 days in medium containing LPS and IFN-γ to stimulate maturation. The mature DCs showed dendrites on their surface (Fig. 1, right panel) and displayed a mature DC phenotype (CD1a$^+$ and CD83$^+$).

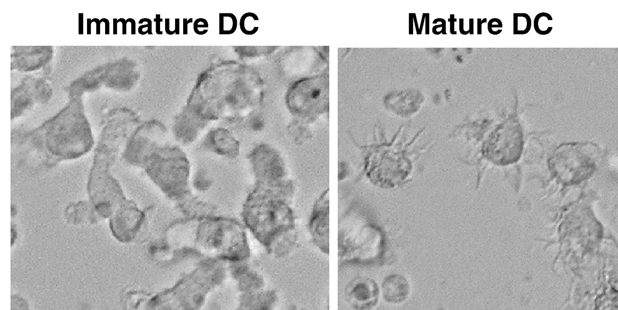

Fig. 1. Morphology of human blood monocyte-derived DCs stimulated by LPS and IFN-γ. Human monocytes were cultured in RPMI containing GM-CSF and IL-4 for 7 days to yield immature DCs. The latter were then incubated with LPS and IFN-γ for 2 more days to yield mature DCs. Dendrites on mature DCs are apparent by microscopy.

Prior studies by Morrow et al. (2003) showed that VZV infection of mature DCs results in reduced surface expression of MHC class I, CD80, CD83, and CD86, but no change in MHC class II, while a study by Abendroth et al. (2001) showed that infection of immature DCs had no effect on MHC class I, class II, or CD86. We incubated VZV-infected immature and mature DCs with antibodies to VZV glycoprotein E (gE) and either Fas (CD95) or MHC class II and determined the percentage of gE-positive cells expressing the various surface markers by flow cytometry. In duplicate experiments we found that levels of Fas were significantly reduced on the surface of both immature (P = 0.007) and mature (P = 0.01) DCs (Fig. 2). In contrast, surface levels of MHC class II were not significantly changed in either immature or mature VZV-infected DCs.

VZV unable to express ORF47 is impaired for replication in immature DCs

Previous studies showed that VZV unable to express ORF47 protein kinase grows to similar titers in human fibroblasts and melanoma cells, but is impaired for growth in human T lymphocytes and skin (Heineman and Cohen, 1995; Moffat et al., 1998; Soong et al., 2000). To determine if ORF 47 is important for VZV infection of DCs, we first infected human immature DCs with recombinant Oka strain VZV (ROka) and VZV unable to express ORF47 protein (ROka47S) in vitro (Fig. 3). Three days after infection, the cells were stained with monoclonal antibodies to VZV gE conjugated with fluorescein isothiocyanate (FITC) and antibody to human CD1a conjugated with phycoerythrin (PE), and then analyzed by flow cytometry. Approximately 24% of immature DCs (CD1a$^+$) infected with VZV ROka expressed VZV gE. In contrast, infection of human immature DCs with VZV unable to express ORF47 (ROka47S) resulted in only about 8% of DCs expressing VZV gE.

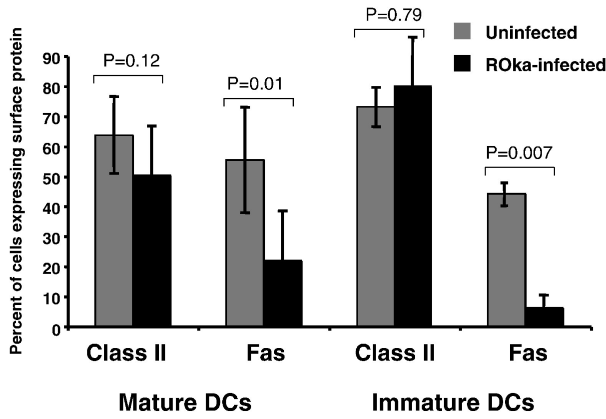

Fig. 2. Surface expression of MHC class II and Fas (CD95) on immature and mature DCs. DCs were inoculated with uninfected human fibroblasts or VZV-infected human fibroblasts for 3 days, and the cells were incubated with monoclonal antibodies to VZV gE and either CD95 or MHC-II. Error bars show the standard error for two independent experiments.

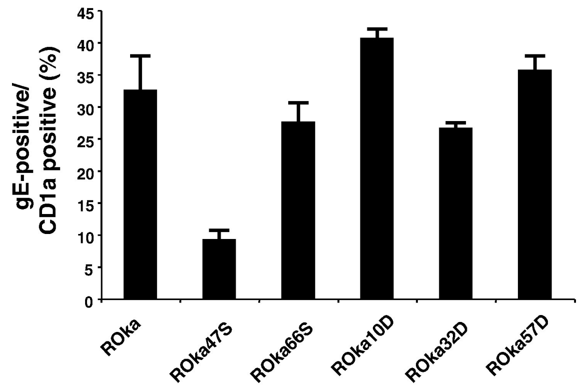

Fig. 4. Percentage of human immature DCs infected with VZV mutants. VZV-infected immature DCs were stained as described in Fig. 2 and analyzed by flow cytometry. Data were obtained from at least three separate experiments and are presented as the mean ± standard error.

In three experiments, 24–39% (mean 32.5 ± 7.7% standard error of mean [SEM] of immature DCs infected with VZV ROka expressed VZV gE (Fig. 4). In contrast, only 8–10% (mean 9.2 ± 1.1% SEM) of immature DCs infected with ROka47S showed VZV gE. This difference was statistically significant ($P < 0.01$).

To determine if other VZV genes are important for virus growth in DCs, we infected immature DCs with four other viral mutants, ROka66S, ROka10D, ROka32D, and ROka57D which are unable to express the ORF66, ORF10, ORF32, and ORF57 proteins, respectively. While ROka66S is partially impaired for growth in T cells (Moffat et al., 1998; Soong et al., 2000), the mutant was not impaired for replication in immature DCs. VZV unable to express ORF10, ORF32, or ORF57 proteins was not impaired for growth in immature DCs (Fig. 4). The mean percentage of DCs expressing VZV gE was 27–40% for the latter four mutants.

VZV ORF47 is not important for replication of VZV in mature DCs

Mature DCs are potent antigen-presenting cells that are important for initiating antiviral immune responses. Morrow et al. (2003) reported that 15–45% of mature DCs expressed VZV antigens 4 days after infection with VZV. To determine which genes are important for VZV infection of mature DCs, we infected mature DCs with VZV ROka, ROka47S, and ROka66S. In three experiments, 15–17% (mean 16 ± 0.6% [SEM]) of mature DCs infected with ROka expressed gE, 12–17% (mean 14 ± 2.3% [SEM]) of mature DCs infected with ROka47S expressed gE, and 12–21% (mean 16.7 ± 6.7% [SEM]) of mature DCs infected with ROka66S expressed VZV gE (Fig. 5). There was no significant

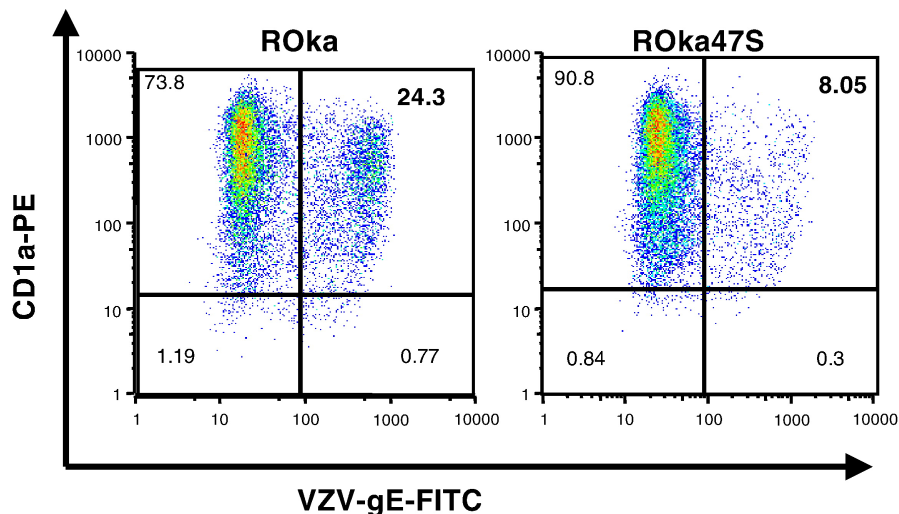

Fig. 3. Flow cytometry analysis of VZV gE on human immature DCs infected with virus. Human DCs were inoculated with VZV ROka and ROka47S infected fibroblasts, stained with PE-conjugated mouse anti-human CD1a monoclonal antibody and FITC-conjugated mouse anti-gE monoclonal antibody, and analyzed by flow cytometry.

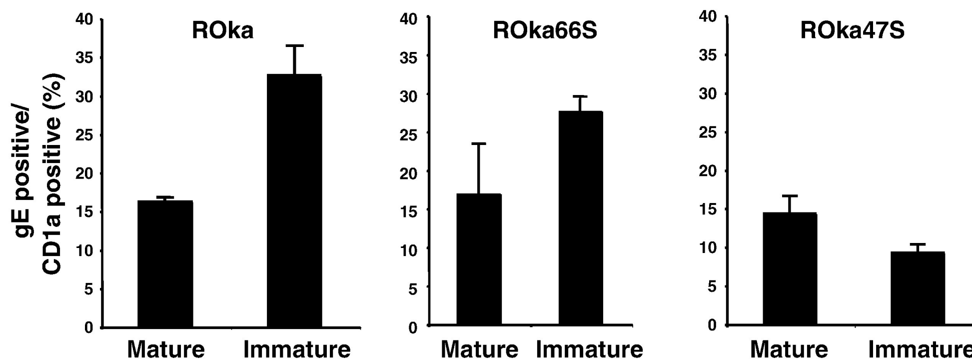

Fig. 5. Percentage of VZV-infected human immature and mature DCs determined by flow cytometry. Immature and mature DCs infected with VZV ROka, ROka47S, or ROka66S were stained as described in Fig. 3 and analyzed by flow cytometry. At least 3 separate experiments were performed and data shown are the mean ± standard error.

difference ($P = 0.23$) in the mean number of cells expressing gE for mature DCs infected with ROka, ROka47S, or ROka66S. Thus, while ORF47 is important for growth of VZV in immature DCs, the viral protein is not critical for replication in mature DCs.

VZV unable to express ORF47 is markedly impaired for transfer of virus from DCs to melanoma cells

To determine if immature DCs infected with VZV produce infectious virus, infected DCs were cultured in vitro for 3 days and added to human melanoma cells in 6-well plates. After incubation at 33 °C for 7 days, the cells were fixed, stained with crystal violet, and the number of plaques was counted. As a control, medium washed from VZV-infected human fibroblasts that had been used to infect immature DCs was used to infect melanoma cells. Inoculation of 1000 ROka-infected immature DCs, of which 32.5 ± 7.7% were gE positive, onto melanoma cells resulted in 18 ± 0.5 plaques; inoculation of 1000 ROka47S-infected DCs, of which 9.2 ± 1.1% were gE positive, onto melanoma cells resulted in no plaques (Table 1). Further dilutions of virus-infected immature DCs showed a >99% reduction in the number of plaques in melanoma cells incubated with ROka47S-infected DCs compared with ROka-infected DCs. Medium obtained from VZV-infected human fibroblasts did not induce plaques in melanoma cells. Thus, VZV unable to express ORF47 is markedly impaired for spread of virus from DCs to melanoma cells.

Table 1
Number of plaques observed on melanoma cell monolayers after inoculation with VZV-infected DCs

No. of DCs added	Mean number of plaques on monolayers infected with	
	ROka-infected DCs	ROka47S-infected DCs
10^5	>300	16.5
10^4	198	0
10^3	18	0

Replication of VZV unable to express ORF47 in DCs is blocked between IE (ORF62) and late (gE) gene expression

Immature DCs infected with VZV ROka47S show reduced expression of gE compared to cells infected with ROka. This could be due to a failure of ROka47S to infect DCs, or to a block in virus replication. To determine which stage in the virus replication cycle is blocked in DCs infected with ROka47S, we infected immature DCs with VZV ROka and ROka47S and performed immunofluorescence microscopy with antibodies to a VZV immediate–early protein (ORF62), a late protein (gE), and a DC marker (CD1a). Infection of immature DCs with VZV ROka or ROka47S showed similar numbers of cells (53% or 47%, respectively) expressing ORF62 protein (Fig. 6). While numerous DCs infected with ROka expressed gE (41% of cells), very few cells infected with ROka47S expressed gE (7% of cells). These results indicate that VZV ROka47S can infect immature DCs, but that virus replication is blocked between immediate–early and late gene expression.

Infection of DCs with VZV ROka results in a different pattern of phosphoproteins compared with ROka47S

VZV ORF47 protein kinase phosphorylates several viral and cellular proteins including VZV gE, gI, and ORF32, ORF47, ORF62, and ORF63 proteins as well as casein and histones (Kenyon et al., 2001, 2002; Ng and Grose, 1992; Ng et al., 1994; Reddy et al., 1998; Sato et al., 2003). To determine which proteins are phosphorylated by ORF47 protein in immature DCs, we radiolabeled cells infected with ROka and ROka47S with [^{33}P]orthophosphate and performed two-dimensional SDS–PAGE gels to separate the labeled proteins. Comparison of phosphoproteins in cells infected with ROka and ROka47S showed bands of 31 kDa and 35 kDa that were present in ROka-infected DCs, but not in ROka47S-infected cells (Fig. 7).

Bands of 31 kDa and 35 kDa that were visible by Coomassie blue staining were excised and mass spectrometry followed by matrix-assisted laser desorption/ionization

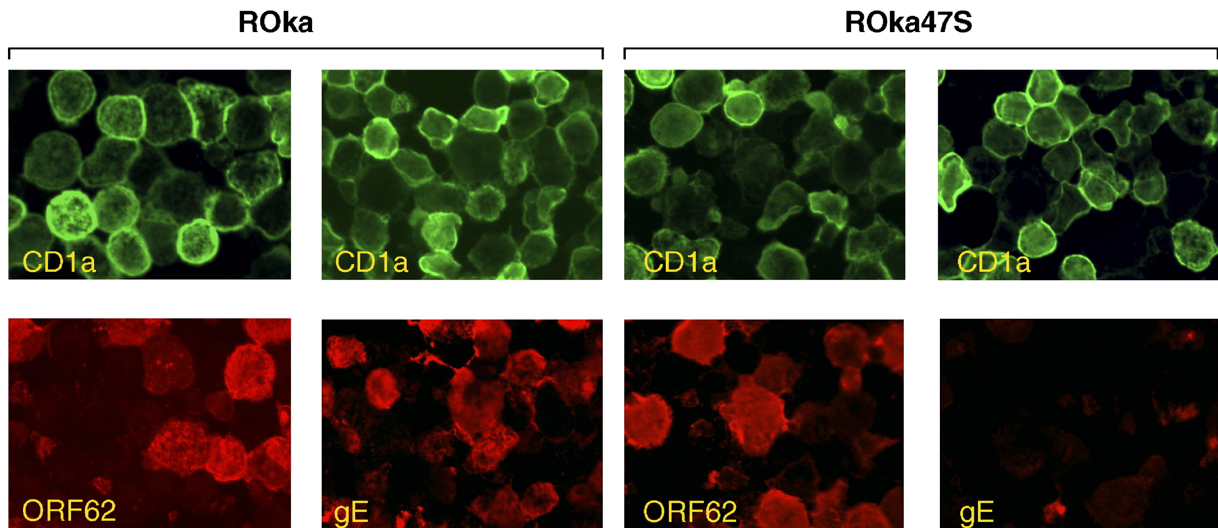

Fig. 6. Immunofluorescent staining of VZV-infected immature DCs with antibodies to DC and VZV proteins. Human immature DCs infected with VZV ROka and ROka47S were stained with anti-CD1a-FITC (green) and anti-VZV IE62 protein or anti-gE monoclonal antibody followed by Fluor-594 (Red)-labeled anti-IgG subtype-specific antibodies.

(MALDI) was performed. The 31-kDa band was identified as the H band of L-lactate dehydrogenase, and the 35-kDa band contained isocitrate dehydrogenase (NAD) subunit alpha. Unfortunately, since antibodies are not available for these human proteins, we were unable to confirm that the proteins are phosphorylated in VZV-infected DCs. Nevertheless, the molecular weights of these phosphoproteins (31 kDa and 35 kDa) are different than those of the previously described viral and cellular proteins phosphorylated by ORF47 protein. Thus, the phosphoproteins detected in DCs infected with ROka, but not ROka47S, are either viral or cellular proteins that have not been previously reported to be phosphorylated by ORF47.

Discussion

We have found that VZV unable to express ORF47 is impaired for growth in immature, but not mature DCs. ORF47 is especially important for transfer of virus from DCs to melanoma cells. The ORF47 protein kinase is important for phosphorylating at least two proteins in immature DCs. These experiments demonstrate the important role of the ORF47 protein in productive infection of immature DCs.

We found that infection of immature or mature DCs with VZV resulted in down-regulation of Fas on the surface of the cells but had no effect on expression of MHC class II. The down-regulation of Fas in immature and mature DCs may help to protect these cells from apoptosis. Other DNA viruses including myxoma virus (Guerin et al., 2002) and adenovirus (Shisler et al., 1997) encode proteins that down-regulate expression of Fas on the surface of cells.

VZV ORF47 was important for growth of the virus in immature DCs, but not mature DCs. While VZV lacking ORF47 could infect immature DCs, as evidenced by

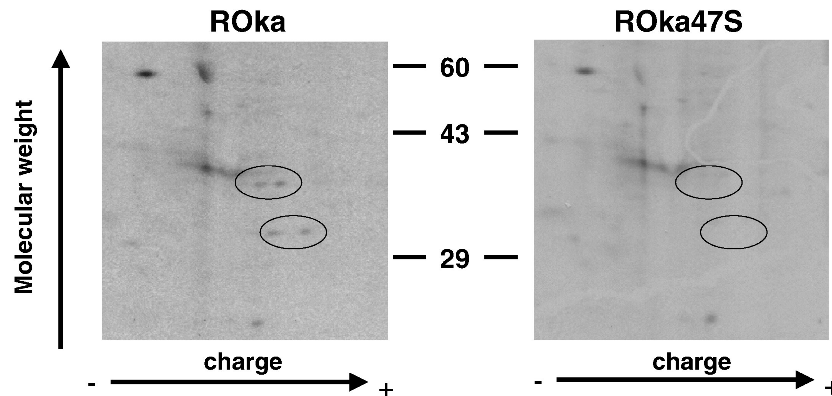

Fig. 7. Two-dimensional gels of phosphorylated proteins. Human DCs were inoculated with VZV ROka and ROka47S-infected human fibroblasts and [^{33}P]orthophosphate-labeled proteins were separated by two dimensional SDS–PAGE gels. Molecular masses of proteins are indicated between the gels. Circles indicate the position of spots that are present in cells infected with VZV ROka, but not ROka47S. The spot on the right at 35 kDa and on the left at 31 kDa were visible with Coomassie blue staining and were excised for digestion by trypsin and subsequent mass spectrometric analysis.

expression of the immediate–early ORF62 protein, there was a block in infection and very few infected cells expressed the late viral protein, gE. These findings are similar to those we previously reported in human T cells (Soong et al., 2000), in which the ORF47 mutant was able to infect umbilical cord blood T cells, but was blocked between immediate–early and late gene expression. In addition, ORF47 is required for growth of VZV in human fetal T cells in the SCID-hu mouse (Moffat et al., 1998). ORF47 is not needed for growth of VZV in melanoma cells or fibroblasts (Heineman and Cohen, 1995; Moffat et al., 1998). Since ORF47 encodes a serine–threonine protein kinase (Ng and Grose, 1992), similar cellular proteins, which may be substrates for ORF47 protein, might be required for the growth of VZV in immature DCs and T cells, but not in mature DCs, melanoma cells, or fibroblasts.

VZV ORF66 was not important for the growth of the virus in immature or mature DCs. ORF66, like OR47, encodes a serine–threonine protein kinase. ORF66 is important for growth of VZV in human T cells both in vitro (Soong et al., 2000), and in the SCID-hu mouse (Moffat et al., 1998). In contrast, ORF66 is not necessary for growth of VZV in human fetal skin, melanoma cells, or fibroblasts (Heineman et al., 1996; Moffat et al., 1998). Therefore, human T cells differ from immature DCs in the importance of the VZV ORF66 protein kinase for VZV replication.

We found that ORF47 is even more important for transfer of VZV from immature DCs to melanoma cells. VZV ROka47S was impaired by at least 198-fold for transfer of virus from DCs to melanoma cells when compared with its parental virus VZV ROka. Previously, we showed that ROka47S-infected human T cells were impaired by approximately 1000-fold for transfer of virus to melanoma cells (Soong et al., 2000). Taken together, these findings suggest that ORF47 may have a similar role in human T cells and in immature DCs.

VZV ORF47 protein kinase is required for phosphorylation of at least two proteins in immature DCs. Phosphoproteins of 31 kDa and 35 kDa in cells infected with ROka, but not ROka47S, were identified by mass spectrometry followed by MALDI as L-lactate dehydrogenase H chain and isocitrate dehydrogenase subunit alpha. A prior study showed that ORF47 protein was important for phosphorylating proteins of 180, 115, 44, 38, and 22 kDa in melanoma cells (Heineman and Cohen, 1995). These studies suggest that ORF47 may phosphorylate different proteins in immature DCs than in melanoma cells. These differences in the phosphoprotein profiles of the two cell types may be important for the marked differences in virus replication in the cells.

DCs play a critical role for antigen presentation and antiviral host defense. A number of herpesviruses productively infect immature DCs (reviewed in Rinaldo and Piazza, 2004). Infection of immature DCs by cytomegalovirus, HSV, or human herpesvirus 6 impairs their maturation and reduces their ability to stimulate T cell proliferation (Beck et al., 2003; Moutaftsi et al., 2002; Salio et al., 1999; Smith et al., 2005). Our observations that ORF47 of VZV is important for virus replication in immature DCs, spread from these DCs to other cells, and for down-regulation of Fas in DCs indicate that this viral protein may be particularly important for VZV to interfere with the immune response as well as for the virus to be transmitted to other cells in the host.

Materials and methods

Cells and viruses

Human diploid fibroblasts (MRC-5), grown in minimum essential medium (Gibco, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine, were used for growth and preparation of virus stocks. Human melanoma (MeWo) cells were used for virus transfer assays. Monocytes were obtained from human peripheral blood and purified by countercurrent elutriation. VZV recombinant Oka vaccine virus (ROka) (Cohen and Seidel, 1993), VZV with stop codons in open reading frame (ORF) 47 (ROka47S) (Heineman and Cohen, 1995) or ORF66 (ROka66S) (Heineman et al., 1996), or VZV with a deletion in ORF10 (ROka10D) (Cohen and Seidel, 1994), ORF32 (ROka32D) (Reddy et al., 1998), or ORF57 (ROka57D) (Cox et al., 1998) have been previously described.

Monocytes were washed with PBS twice and resuspended at 1×10^6 cells/ml in RPMI containing 10% fetal bovine serum, 200 U/ml of IL-4 (Peprotech, Rocky Hill, NJ), 100 ng/ml of GM-CSF (AMGEN, Thousand Oaks, CA), and cultured at 37 °C. The medium was replaced with fresh medium containing cytokines on day 3 and day 5. On day 7, nonadherent cells were collected for infection of VZV. These cells consisted of >90% immature DCs as determined by their $CD1a^+$, $CD83^-$ phenotype using flow cytometry. Mature DCs were generated by transferring the nonadherent immature DCs to 24-well tissue culture plates at 1×10^6 cells/well and culturing them for 2 more days in RPMI containing 10% FCS, 2.5 μg/ml of lipopolysaccharide (LPS) (Sigma, St. Louis, MO), 100 ng/ml of IFN-γ (R&D Systems, Minneapolis, MN), 200 U/ml of IL-4, and 100 ng/ml of GM-CSF at 37 °C. After 2 days the cells consisted of >90% mature DCs as determined by their characteristic dendritic morphology and their cell surface phenotype (i.e., $CD1a^+$ and $CD83^+$) by flow cytometry.

VZV infection of DCs and transfer of virus to melanoma cells

Human fibroblasts in 24-well plates were infected with cells containing 1×10^5 PFU of VZV per well. At 24–48 h after infection (when the cells showed ~80% CPE), 2×10^6 immature or mature DCs were added to each well of VZV-

infected human fibroblasts in RPMI supplemented with 200 U/ml of IL-4 and 100ng/ml of GM-CSF. The 24-well plates were centrifuged for 45 min at 150 × *g* at 4 °C and incubated at 37 °C. After 24 h, the nonadherent DCs were removed and placed into fresh 24-well plates by gently washing the human fibroblasts. The new plate was cultured for 3 more days in fresh medium containing cytokines and VZV-infected DCs were harvested.

For virus transfer assays, VZV-infected DCs were collected 4 days after infection and 10^3–10^5 infected cells were added to 6-well plates containing confluent melanoma cells. After 7 days, melanoma cells were fixed, stained with crystal violet, and the number of plaques was counted. To determine whether cell-free VZV was produced, culture medium from virus-infected DCs was collected 4 days after infection, inoculated into 6-well plates of confluent melanoma cells, and plaques were counted after 7 days.

Staining of cells for flow cytometry and immunofluorescence

VZV-infected DCs were double stained with FITC-conjugated mouse anti-gE monoclonal antibody (Chemicon International, Temecula, CA) and PE-conjugated mouse anti-human CD1a, CD83, CD95 (Fas), or MHC class II monoclonal antibodies (Caltag Laboratories, Burlingame, CA) 4 days after infection. A total of 10^6 cells were stained with the antibodies and analyzed by flow cytometry using Cell Quest software (Becton Dickinson).

For immunofluorescence assays, approximately 10^4 cells were centrifuged onto glass slides for 5 min at 150 × *g*, air dried for 30 min, fixed in methanol–acetone (1:1, vol/vol) at −20 °C for 10 min, and air dried. The cells were stained with FITC-conjugated mouse anti-human CD1a monoclonal antibody (isotype IgG2a) (Dako Corporation, Carpinteria, CA) and anti-VZV immediate–early 62 protein antibody (IgG1) (Chemicon International) or anti-VZV late gE protein antibody (IgG2b), followed by Alexa Fluor 594-labeled goat anti-mouse isotype-specific (IgG1 or IgG2b) antibodies (Molecular Probes, Eugene, OR). Cells were incubated with antibodies for 1 h at 37 °C, washed in PBS, and visualized by fluorescence microscopy.

Cell labeling with radiolabeled orthophosphate and 2-dimensional gel electrophoresis

VZV-infected and mock-infected DCs were collected 4 days after infection and labeled with [^{33}P]orthophosphate for 4 h. After labeling, the cells were lysed in RIPA buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM PMSF). Cell lysates were collected, protein concentrations were determined, and 30 μg of protein was subjected to 2-dimensional gel electrophoresis. The first dimension (isoelectric focusing) was on a 2% polyacrylamide–SDS tube gel (pH 3.5–10 ampholytes), and the second dimension was on a 10% polyacrylamide–SDS slab gel. Gels were stained with Coomassie blue, dried, and autoradiography was performed. [^{33}P]-labeled protein spots present in VZV ROka, but absent in ROka47S infected cell lysates were cut from the gel and mass spectrometry followed by MS-MALDI was performed. Proteins were identified by MS-Fit protein matching.

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