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Insertional Inactivation of the Vaccinia Virus 32-Kilodalton Gene Is Associated with Attenuation in Mice and Reduction of Viral Gene Expression in Polarized Epithelial Cells

JUAN-RAMON RODRIGUEZ, DOLORES RODRIGUEZ, AND MARIANO ESTEBAN*

Departments of Biochemistry and Microbiology and Immunology, State University of New York Health Science Center at Brooklyn, Brooklyn, New York 11203-2098

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The mechanism of poxvirus attachment to cells is poorly understood. We have identified a 32-kDa envelope protein of vaccinia virus which binds to the surface of cultured cells. This binding is specific and selective (J.-S. Maa, J. F. Rodriguez, and M. Esteban, J. Biol. Chem. 265:22174–22180, 1990; C. Lai, S. Gong, and M. Esteban, J. Virol. 65:499–504, 1991). In this investigation, we studied the effect of inactivating the 32-kDa gene (32K gene) on the biology of vaccinia virus. We show that inactivation of the 32K gene decreases by 80% the mortality of mice infected with 32K$^-$ vaccinia virus. This reduction in mortality correlates with diminished viral gene expression in target tissues. In highly polarized epithelial cells, viral gene expression of 32K$^-$ virus was reduced (50 to 60%) at both the apical and basolateral surfaces in comparison with a 32K$^+$ virus. Restriction of virus gene expression in polarized cell surfaces occurs for both intracellular and extracellular forms of infectious 32K$^-$ vaccinia virus. The two infectious forms of vaccinia virus 32K$^+$ infect polarized cells preferentially by the basolateral surface. Our findings provide evidence of the importance of the 32-kDa protein in viral pathogenesis.

Poxviruses have become the focus of extensive studies, not only because they provide a good model system by which to understand the molecular basis of virus-host cell interactions, both with cells in culture and with experimental animals, but also because of their possible use as live recombinant viral vaccines against multiple pathogens (17, 19). The genome of vaccinia virus, Copenhagen strain, has been sequenced and found to potentially encode about 200 different polypeptides (9). Some of these polypeptides perform functions similar to those of their host cell counterparts (16). The viral genes are tightly packed in the genome, and viral transcription proceeds in a cascade fashion (16). Unlike most animal viruses, vaccinia virus in the course of infection gives rise to two fully infectious viral forms produced in the cytoplasm: the intracellular naked virus (INV), which contains a single envelope, and the extracellular enveloped virus (EEV), which acquires two Golgi-derived membranes during morphogenesis and is released from infected cells by fusion of the outermost viral envelope with the cell plasma membrane (5). The majority of the infectious virions produced by most cultured cells in the course of infection are INV, although some cell lines, like rabbit kidney cells (RK-13), can produce up to 30% EEV (20). It is thought that EEV plays a major role in virus dissemination in vivo (21). Although much knowledge has been gained on poxvirus genome organization, transcription, and DNA replication, little is known about the nature of viral proteins that are involved in virus entry (16).

Because virus entry is a major determinant in viral pathogenesis, understanding the structure and function of viral proteins involved in this event will be of great importance in the study of poxvirus infections. Work in our laboratory has identified two envelope proteins of vaccinia virus of 14 and 32 kDa with roles in virus entry. The 14-kDa envelope protein was shown to be involved in virus entry by fusion of the viral envelope with the cell plasma membrane (25). The 32-kDa envelope protein was implicated in virus attachment to cells (15). The gene encoding the 32-kDa protein was sequenced and found to encode a 304-amino-acid protein with a high degree of protein sequence homology to carbonic anhydrases (15, 18). The 32-kDa protein was shown to bind to the surface of cultured cells with specificity, selectivity, and saturability (14). By using ^{125}I-32-kDa protein, it was estimated that there are about 4.5×10^4 binding sites per cell with a high affinity ($K_d = 1.8 \times 10^{-9}$ M), suggesting the interaction of the 32-kDa protein with a specific receptor (14). A potential cell surface-binding domain was localized within the last 50 amino acid residues at the C-terminus, which also contains the transmembrane domain (15). By introducing a stop codon within the coding sequence of the 32-kDa protein, it was observed that this protein was not essential for virus growth in cultured cells (18). A priori, there is an apparent discrepancy between the cell surface-binding characteristics exhibited by the 32-kDa protein and its nonessential role for virus replication in cultured cells. This discrepancy could be explained if vaccinia virus exhibits a more restricted mode of entry into tissues than into cultured cells. In fact, we have shown that vaccinia virus entry is quite selective in highly polarized epithelial cells, MDCK-D11, a tissue culture cell line that could be more like what the virus encounters in vivo (23).

We have described an approach that can easily be used to follow virus replication and tropism in vivo. It is based on the highly sensitive reporter gene luciferase expressed by a vaccinia virus recombinant (26). In this investigation, we have used luciferase production as a means to follow, in tissues and in polarized cells, the replication of two recombinants of vaccinia virus, one that contains a 32-kDa gene (32K gene) and the other with the 32K gene inactivated through insertion of the β-galactosidase gene. Our findings provide evidence that the 32-kDa protein is necessary for

* Corresponding author.

efficient virus replication both in infected animals and in highly polarized epithelial cells.

MATERIALS AND METHODS

Plasmid constructions. A vaccinia virus insertion plasmid, pPR15, containing the luciferase gene under the control of the vaccinia virus late promoter p4b was kindly provided by G. Smith (University of Oxford) (27). An *Eco*RI DNA fragment (1.5 kb) taken from the λgt11 recombinant phage E14, containing the full sequence of the vaccinia virus 32K gene, was cloned into the *Eco*RI site of pUC19 to generate pE14 (15). A 2.8-kb DNA fragment containing the *lacZ* gene under the control of the vaccinia virus late promoter p11 was isolated from plasmid pSC11 (3) after digestion with the restriction enzymes *Xba*I and *Pst*I. This DNA fragment was end-repaired by treatment with mung bean exonuclease and inserted into the *Spe*I site of plasmid pE14, previously digested with *Spe*I and end-repaired with mung bean nuclease. A plasmid, pJR33, was isolated containing the transcription unit p11-*lacZ* flanked by DNA sequences from the 32K gene.

Virus and cells. The WR strain of vaccinia virus was propagated and titrated in African green monkey kidney (BSC-40) cells and purified as described previously (12). BSC-40, HeLa, and mouse Ltk(−) cells were grown in Dulbecco's modified Eagle's medium containing 10% newborn calf serum. Madin-Darby canine kidney cells (MDCK-D11) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. MDCK-D11 is a highly polarized epithelial cell clone (10).

Generation of recombinant viruses. To generate VVLUC 32K$^+$ recombinant virus, BSC-40 cells infected with WR vaccinia virus were transfected with the plasmid pPR15, and recombinant viruses were selected in human 143 cells (TK$^-$) in the presence of 25 μg of 5′-bromodeoxyuridine per ml as described previously (27).

To generate VVLUC32K$^-$, BSC-40 cells infected with VVLUC32K$^+$ were transfected with the plasmid pJR33, and recombinant viruses were selected by the appearance of blue plaques after addition of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (3). Recombinant viruses were plaque purified three times, and a representative virus was selected.

Recovery of extracellular virus. Confluent monolayers of HeLa cells were infected with 0.5 PFU of VVLUC32K$^+$ or VVLUC32K$^-$ per cell. After viral adsorption, cells were washed with phosphate-buffered saline (PBS), and medium containing 2% newborn calf serum was added. At 48 h postinfection (p.i.), the medium was collected, and cell debris was removed by centrifugation at 1,500 rpm for 5 min. The virus present in the cell pellet represents intracellular virus. The virus in the supernatant (extracellular virus) was concentrated by centrifugation at 10,000 rpm for 1 h and resuspended in 1 mM disodium monophosphate buffer (pH 9.0), and this virus was collected after centrifugation at 20,000 rpm for 1 h over a 45% sucrose cushion. The extracellular virus preparation was titrated in the presence of the 14-kDa protein-specific monoclonal antibody MAbC3 (24) to neutralize intracellular virus.

Luciferase assay. Cells infected with VVLUC32K$^+$ or VVLUC32K$^-$ were collected and lysed in luciferase extraction buffer (1% Triton X-100, 25 mM glycil-glycine [pH 7.8], 15 mM magnesium sulfate, 4 mM EGTA [ethylene glycol tetraacetic acid], 1 mM dithiothreitol). Cell debris was removed by centrifugation, and luciferase activity was measured in the presence of luciferin and ATP with a Monolight 2010 luminometer (Analytical Luminiscence, San Diego, Calif.) (23). Tissues from infected animals were washed in PBS, weighed, and homogenized in luciferase extraction buffer (5 μl/mg of tissue) containing 1 mM phenylmethylsulfonyl fluoride and leupeptin (10 mg/ml). The homogenized tissues were sonicated, cell debris was removed by centrifugation, and the supernatants were assayed for luciferase activity (26).

Immunosuppressed mice. Female BALB/c mice, 4 to 6 weeks old (Charles River Breeder Facility), were immunosuppressed by intraperitoneal (i.p.) inoculation (0.2 ml) with cyclophosphamide (Sigma) in PBS at 300 mg/kg of body weight, and 5 days later mice were treated with a second dose of the drug at 150 mg/kg (22). Twenty-four hours later, untreated and drug-treated mice were inoculated i.p. with various doses of VVLUC32K$^+$ or VVLUC32K$^-$ recombinant viruses.

RESULTS

Generation and characterization of a vaccinia virus recombinant with inactivated 32K gene. An insertional plasmid, pJR33, containing the β-galactosidase gene flanked by DNA sequences from the 32K gene was constructed (Fig. 1A). To generate a vaccinia virus recombinant with an inactivated 32K gene, plasmid pJR33 was introduced, by homologous recombination, into the 32K gene locus of the vaccinia virus recombinant VVLUC32K$^+$, which contains the luciferase gene under control of the late virus promoter p4b in the *tk* region (27). Viral recombinants, VVLUC32K$^-$, were selected as blue plaques. Southern blot hybridization analysis and restriction mapping demonstrated that the β-galactosidase gene was introduced into the 32K locus (data not shown). BSC-40 cells were infected with the recombinant virus VVLUC32K$^-$ or with the parental recombinant virus VVLUC32K$^+$, and cell extracts were analyzed by immunoblot with either rabbit anti-vaccinia virus serum (Fig. 1B, lanes 1 to 4) or rabbit anti-32-kDa protein serum (Fig. 1B, lanes 5 to 8). Specific reactivity against the 32-kDa protein was observed in cells infected with VVLUC32K$^+$ (Fig. 1B, lanes 4 and 8). There was no reactivity against a 32-kDa protein in lysates from cells infected with VVLUC32K$^-$ (Fig. 1, lanes 3 and 7).

Because a fusogenic mutant of vaccinia virus from a different strain, IHD-W, has been shown to lack a 32-kDa protein (11), and the nature of this protein is not yet known, we performed a similar immunoblot analysis with lysates of cells infected with the parental strain IHD-J and with the mutant IHD-W. As shown in Fig. 1B, lanes 1 and 5, IHD-J produces an antibody-reacting 32-kDa protein, while IHD-W (Fig. 1B, lanes 2 and 6) lacks reactivity against the 32-kDa protein. By measuring luciferase production in three different cell lines, HeLa, BSC-40, and Ltk(−), infected with VVLUC32K$^+$ or VVLUC32K$^-$, we established that the extent of virus replication was similar for the two recombinant viruses (Fig. 2).

From the results in Fig. 1 and 2, we conclude that the 32-kDa protein is nonessential for vaccinia virus replication in cultured cells, in agreement with previously reported results (18) that viral gene expression is not altered by the lack of 32-kDa protein in the viral envelope and that the fusogenic vaccinia virus mutant strain IHD-W lacks the 32-kDa protein present in the wild-type strains WR and IHD-J.

32-kDa protein is important for efficient virus replication in

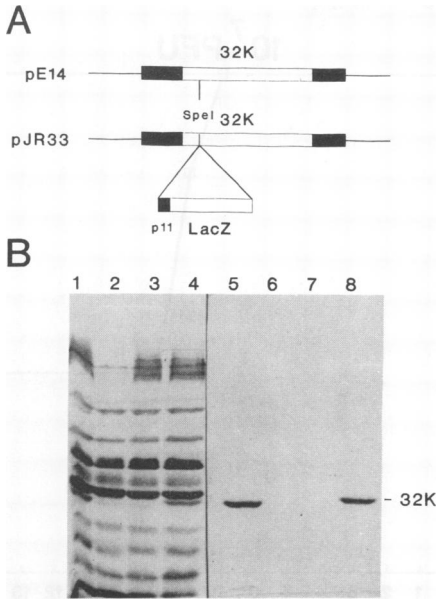

FIG. 1. Characterization of a vaccinia virus recombinant with inactivated 32K gene. (A) Scheme for the construction of the vaccinia virus insertion vector pJR33. A plasmid, pE14, containing the vaccinia virus 32K gene was linearized by the restriction enzyme *SpeI*, at nucleotide position +117 relative to the A at position +1 in the 32K gene initiation codon. The *Escherichia coli lacZ* gene under the control of the vaccinia virus promoter p11 was cloned into this site in the same orientation relative to the 32K gene. The cloning procedures are described under Materials and Methods. (B) BSC-40 cells were infected (5 PFU per cell) with the IHD-J strain of vaccinia virus (lanes 1 and 5), the IHD-W variant (lanes 2 and 6), and the recombinant viruses VVLUC32K$^-$ (lanes 3 and 7) and VVLUC 32K$^+$ (lanes 4 and 8). Cells extracts were prepared at 24 h p.i. and fractionated on 10% polyacrylamide–sodium dodecyl sulfate gels, transferred to nitrocellulose paper, and reacted with rabbit polyclonal antiserum against live vaccinia virus (left side) or with rabbit polyclonal antiserum specific for the vaccinia virus 32-kDa protein (right side). Antibody reactivity was detected by immunoperoxidase staining by procedures previously described (15).

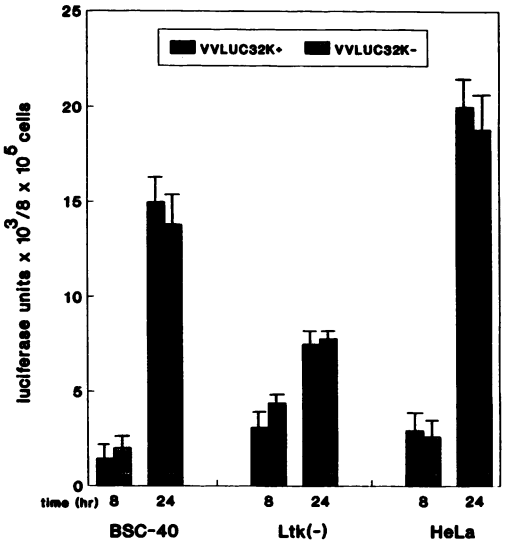

FIG. 2. Luciferase expression in cells of different origin infected with the vaccinia virus recombinants. BSC-40, HeLa, and mouse Ltk(−) cells grown in 24-well trays were infected with the recombinant viruses VVLUC32K$^+$ or VVLUC32K$^-$ at 2.5 PFU per cell. At 8 and 24 h p.i., cells were harvested, and luciferase activity was measured as described under Materials and Methods. Bar denotes standard deviation between duplicate samples.

vivo. To document whether the 32-kDa protein plays a role in vivo, BALB/c mice were inoculated by the i.p. route with 5 × 10^7 PFU of purified VVLUC32K$^+$ or VVLUC32K$^-$ per mouse. The ability of the two different recombinant viruses to replicate in different organs was evaluated by measuring the production of luciferase at 2 days p.i. As shown in Fig. 3, the levels of luciferase were markedly reduced in the spleen, liver, and kidney of mice infected with VVLUC32K$^-$ relative to the luciferase levels found in the same organs from mice infected with VVLUC32K$^+$. These findings also provide evidence that the tropism of VVLUC32K$^-$ is not altered by the inactivation of the 32K gene. Because a reduction in viral gene expression should result in less viral pathogenesis, we next examined whether the virulence of VVLUC32K$^-$ is reduced relative to that of VVLUC32K$^+$. We measured survival in immunosuppressed mice to facilitate the evaluation of the virulence of the vaccinia viruses, as described previously (22). As shown in Fig. 4, all mice inoculated with 10^7 PFU of VVLUC32K$^-$ per mouse survived, while 60% of the animals died after a similar inoculation with VVLUC32K$^+$ (Fig. 4B). When higher doses of virus were used, 10^8 PFU per mouse, all animals in the control group died, while only 20% of the mice inoculated with VVLUC32K$^-$ died (Fig. 4A). The surviving animals were healthy even after 6 months.

From the finding in Fig. 3 and 4, we conclude that the 32-kDa protein of vaccinia virus plays an important role in viral pathogenesis.

Insertional inactivation of the 32K gene results in a virus with reduced gene expression in highly polarized epithelial cells. Since vaccinia virus infects polarized epithelial cells

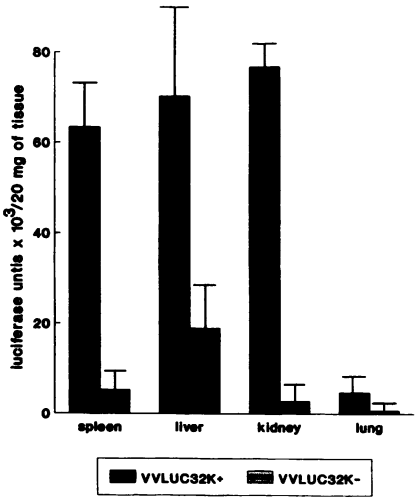

FIG. 3. Reduced luciferase expression in tissues of mice inoculated with VVLUC32K$^-$. BALB/c mice (10 per group) were inoculated i.p. with 5 × 10^7 PFU of purified recombinant virus VVLUC32K$^+$ or VVLUC32K$^-$ per mouse. Two days after virus inoculation, animals were killed, and tissues were removed and tested for luciferase activity. Luciferase activity is represented as luciferase units per 20 mg of tissue. Bar denotes standard deviation of the mean.

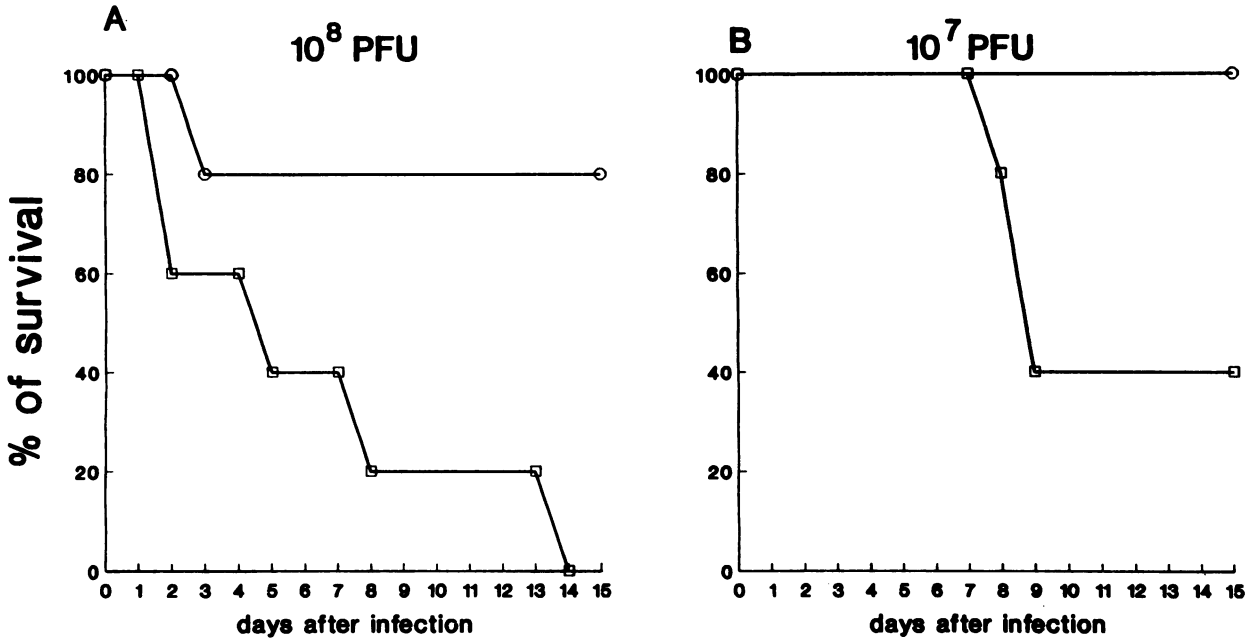

FIG. 4. Reduced virulence of VVLUC32K$^-$ recombinant virus in immunosuppressed mice. Immunosuppressed BALB/c mice (five per group) were inoculated i.p. with 10^8 PFU (A) or 10^7 PFU (B) of purified recombinant virus VVLUC32K$^+$ (□) or VVLUC32K$^-$ (○) per mouse, and survival was followed daily.

preferentially through the basolateral surface and highly polarized epithelial cells may mimic in vivo infections, unlike other cultured cells (23), it was of interest to define whether a virus that lacks the 32-kDa protein behaves like the parental virus in polarized cells. Thus, cells that exhibit high electrical resistance, like MDCK-D11 cells (10), were infected at 10 and 50 PFU per cell by the apical or basolateral surface with VVLUC32K$^+$ or VVLUC32K$^-$, and the luciferase levels were measured at 18 h p.i. The results obtained in three independent experiments were plotted relative to the 100% value for cells infected with VVLUC 32K$^+$ by the basolateral surface. As shown in Fig. 5, a reduction in luciferase levels (~60%) was observed in cells infected with VVLUC32K$^-$ by the basolateral surface at both multiplicities of virus infection. This reduction was also observed at earlier times, 8 h p.i. (not shown). As noted previously (23), infection by the apical surface results in about 20 to 30% of the luciferase expression obtained after infection by the basolateral surface. When the luciferase levels were compared in cells infected with VVLUC32K$^+$ and VVLUC32K$^-$ by the apical surface, a reduction in luciferase production in cells infected with the virus that lacks the 32-kDa protein relative to the parental virus was consistently found.

The experiments in Fig. 5 established that the 32-kDa protein of vaccinia virus is necessary for efficient virus gene expression in highly polarized epithelial cells.

32-kDa envelope protein is necessary for efficient gene expression of extracellular vaccinia virus in polarized epithelial cells. Because there are two forms of infectious vaccinia virus, INV and EEV (see the introduction) and EEV has been implicated as the major infectious form for virus dissemination in vivo (21), it was important to establish whether loss of the 32-kDa protein had any effect on the ability of EEV to infect highly polarized epithelial cells. MDCK-D11 cells were infected by the apical or basolateral surface with the EEV form of both recombinant viruses, VVLUC32K$^+$ and VVLUC32K$^-$, and luciferase production was measured during infection. EEV adsorption was carried out in the presence of MAbC3 to neutralize any contaminating INV present in the inoculum (24). Representative findings are shown in Fig. 6. The most significant observation is that EEV infects polarized epithelial cells preferentially by the basolateral surface and thus behaves like INV. We also found that EEV lacking the 32-kDa protein has reduced luciferase expression by the apical and basolateral surface relative to the 32K$^+$ virus. The lack of 32-kDa protein on the virus particle had no effect on virus yields, since production of EEV was similar in HeLa and BSC-40 cells infected with VVLUC32K$^+$ or with VVLUC32K$^-$ (Table 1). As noted previously (23), the virus titers in MDCK-D11 cells are very low, and direct comparison of virus yields could not be made between polarized cells infected with the 32K$^+$ and 32K$^-$ viruses.

From the results in Fig. 6, we conclude that 32-kDa protein is important for efficient virus gene expression of EEV in highly polarized epithelial cells and that in these cells EEV behaves like INV.

DISCUSSION

Virus attachment and adsorption to cells are major determinants in virus tropism and viral pathogenesis. While a number of receptors have been identified for different animal viruses (31), the nature of the receptor(s) for vaccinia virus has remained elusive. It has been proposed that vaccinia virus enters cells by first interacting with the epidermal growth factor receptor (7), but this interaction has been disputed because the virus can infect cells that lack the epidermal growth factor receptor (30). Several viral envelope proteins of 32 and 29 kDa and a cleavage fragment of the 54-kDa surface tubules have been shown to be important in

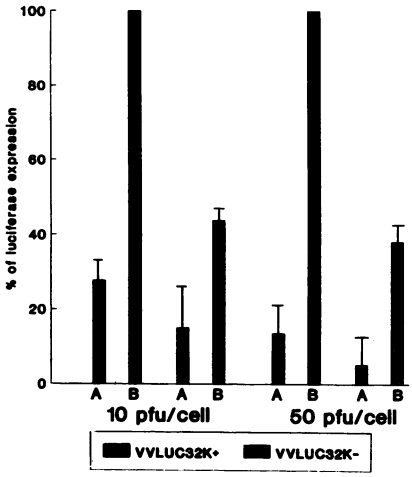

FIG. 5. Reduced luciferase expression in highly polarized epithelial cells infected with VVLUC32K$^-$. MDCK-D11 cells grown to confluency on gelatin-coated Nuclepore filters (12-mm diameter, 3-μm pores) attached to plastic cylinders in 24-well plates were infected with different multiplicities (10 and 50 PFU per cell) of VVLUC32K$^+$ or VVLUC32K$^-$ through the apical (A) or basal (B) surface. Results from duplicate samples (corresponding to 2×10^4 cells) from three independent experiments are represented as a percentage of the value for cells infected through the basal surface with VVLUC32K$^+$, set at 100%.

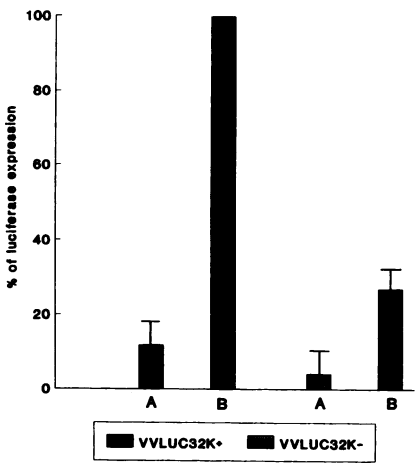

FIG. 6. Reduced luciferase expression in highly polarized epithelial cells infected with the EEV form of VVLUC32K$^-$. MDCK-D11 cells grown as described in the legend to Fig. 5 were infected through the apical (A) or basal (B) surface with the EEV form of VVLUC32K$^+$ or VVLUC32K$^-$ at 10 PFU per cell. The results of duplicate samples (corresponding to 2×10^4 cells) from two independent experiments are represented as described in the legend to Fig. 5.

the binding of the virus to the cell (29). Through binding studies with soluble envelope proteins of vaccinia virus, it was observed that a 32-kDa protein bound strongly to the surface of cultured cells (15). A correlation between the degree of binding of the 32-kDa protein and the ability of the virus to replicate in different cultured cells was also observed, indicating that the 32-kDa protein could play a role in virus attachment to cells (15). Binding studies of ^{125}I-labeled 32-kDa protein to cultured cells of various origins revealed selectivity, specificity, and saturability (14). A potential cell surface-binding domain was localized within the last 50 amino acid residues at the C terminus (15). The requirement of the 32-kDa protein for virus attachment has been disputed because it has been shown that vaccinia virus can replicate efficiently in cultured cells in the absence of the 32-kDa protein (18). However, it was not known whether, under restricted in vivo conditions for virus infection, the 32-kDa protein could play a more selective role in viral infectivity.

In this investigation, we demonstrate that the vaccinia virus 32-kDa envelope protein is indeed necessary for efficient virus infection in vivo. The loss of the 32-kDa protein resulted in about 80% reduction in mortality in virus-infected immunosuppressed mice (Fig. 4). Moreover, through the use of viral recombinants expressing the reporter gene luciferase, we have found that there is a similar reduction (about 80%) in luciferase production in all tissues from animals inoculated with a 32K$^-$ virus versus a 32K$^+$ virus (Fig. 3). The reduction in luciferase production in animal tissues could be due to limited penetration into cells of 32K$^-$ virus, to a deficiency of the mutant virus in any step leading to late virus gene expression, or to both. Our studies measuring virus replication in HeLa and BSC-40 cells have shown that insertional inactivation of the 32K gene has no effect on the extent of luciferase expression (Fig. 2) or in the yields of intracellular or extracellular virus (Table 1). In MDCK-D11 cells, a cell line known to produce very low vaccinia virus yields (23), the pattern of late viral polypeptides was also not affected by the inactivation of the 32K gene (data not shown). The above observations suggest that 32-kDa protein is likely to play a role at an early step in virus infection. Since the luciferase assay measures only gene expression, the experiments with the reporter gene do not

TABLE 1. Yields of intracellular and extracellular forms of 32K$^+$ and 32K$^-$ vaccinia viruses in cultured cells[a]

Virus	Yield (PFU/ml)					
	HeLa			BSC-40		
	24 h p.i.	48 h p.i.	72 h p.i.	24 h p.i.	48 h p.i.	72 h p.i.
32K$^+$						
INV	1.5×10^7	1.15×10^8	1.4×10^8	2.6×10^7	8.5×10^7	5×10^7
EEV	9×10^4	4.5×10^5	1.5×10^5	5.5×10^3	2.2×10^4	1×10^4
32K$^-$						
INV	3.3×10^7	1.5×10^8	1.0×10^8	8×10^7	8.5×10^7	3.5×10^7
EEV	1.5×10^5	9.5×10^5	1.5×10^5	3.5×10^3	1.6×10^4	3.5×10^4

[a] HeLa and BSC-40 cells grown in 6 × 6 wells were infected (0.5 PFU per cell) with VVLUC32K$^+$ or VVLUC32K$^-$ virus. At various times p.i., cells were collected by centrifugation, and intracellular (INV) and extracellular (EEV) virus was prepared as described under Materials and Methods. These two virus populations were titrated in BSC-40 cells with an agar overlay, and results are expressed in plaque-forming units per milliliter.

distinguish between virus binding, membrane fusion, and postfusion events. Repeated attempts to measure directly the binding of radiolabeled (^{3}H) virus particles to MDCK-D11 cells in Nuclepore filters failed to provide clear differences between the 32K$^+$ and 32K$^-$ viruses, possibly because of virus aggregation. Although the basis for attenuation of 32K$^-$ virus is unknown, it is likely that the defect of the mutant virus is in penetration, based on the cell surface-binding characteristics of 32-kDa protein (14, 15) and on the ability of 32K$^-$ virus to replicate efficiently in HeLa and BSC-40 cells.

While in previous studies we reported that in a highly polarized epithelial cells, the INV form of vaccinia virus preferentially infects through the basolateral surface (23), here we found that this selective mode of infection is also observed for the EEV form of vaccinia virus (Fig. 6). These results suggest that cellular determinants important for viral infection are localized at the basolateral surface of MDCK-D11 cells for both the INV and EEV forms of vaccinia virus. However, when MDCK-D11 cells were infected by the apical or basolateral surface with INV and EEV forms of vaccinia virus lacking the 32-kDa protein, a consistent reduction (50 to 60%) in luciferase expression was observed (Fig. 5 and 6). The similar findings obtained with both infectious forms of VVLUC32K$^-$ suggest a common role in viral infection of the 32-kDa protein for INV and EEV. These results could be explained if INV and EEV use the same receptor on the basolateral surface that is recognized by a common viral polypeptide. The 32-kDa protein, if exposed on the envelope of EEV, could be the common ligand for virus attachment. Alternatively, the loss of the 32-kDa protein in the envelope of vaccinia virus could alter the original interaction between envelope proteins, resulting in a virus particle with decreased ability to enter cells by either of the two cell surfaces. Since we have not been able to completely inhibit the virulence and infectivity of VVLUC32K$^-$ either in vivo or in MDCK-D11 cells, this suggests that vaccinia virus uses more than one receptor-viral protein interaction.

Previous studies have found that mice inoculated with IHD-W have a reduction in mortality of about 25%, compared with 70% mortality for animals inoculated with the parental strain IHD-J (21). As shown in this study and in others, IHD-W does not contain the 32-kDa envelope protein (Fig. 1) (11). Since IHD-W might contain other mutations (11), it is not possible to determine with this virus the role of the 32-kDa protein in virulence. However, our findings showing decreased mortality of mice inoculated with vaccinia virus containing only an inactivated 32K gene demonstrate the importance of this envelope protein in virulence.

The results that a vaccinia virus protein is nonessential for growth of the virus in cells in culture but is necessary for virus infection in vivo is not unique for the 32-kDa protein. In fact, deletion of vaccinia virus genes encoding thymidine kinase (2), a growth factor (1), hemagglutinin (8, 28), a 13.8-kDa secreted protein (13), and ribonucleotide reductase (4) have all been shown to decrease virulence in animals but are not essential for virus replication in cultured cells.

In conclusion, our findings provide definitive evidence that the 32-kDa envelope protein of vaccinia virus plays an important role in viral pathogenesis. Clearly, the lack of the 32-kDa protein in the virus particle had an influence on the outcome of vaccinia virus infection in vivo and on the extent of viral gene expression both in target tissues and in highly polarized epithelial cells.

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