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Characterization of the Herpesvirus saimiri Orf51 protein

Robert E. Means*

Department of Pathology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

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Abstract

Herpesvirus saimiri (HVS) is a γ_2-herpesvirus sharing genomic colinearity and a high degree of functional homology with HHV-8. To begin exploring the correlates of HVS infectivity and neutralization, we designed and implemented a new reporter assay. Using this assay, we could demonstrate that HVS neutralizing antibodies are present at high levels in naturally infected squirrel monkeys and are strongly induced after pathogenic, experimental infection of common marmosets. Further, we demonstrated that viral entry is influenced by cellular glycosaminoglycans and that, similar to HHV-8, soluble heparin is capable of blocking infectivity. We next cloned and characterized the positional homologue of HHV-8 *K8.1*, HVS *Orf51*. N-glycosidase F treatment indicates that like K8.1, Orf51 is a glycoprotein. Found in the viral particle, it localizes to the endoplasmic reticulum of expressing cells. Like K8.1, Orf51 could bind to agarose-conjugated heparin, implicating this molecule in viral attachment to cells. These studies provide the groundwork for additional experiments into the role that this protein may be playing in viral pathogenicity, persistence, and cell tropism.

Keywords: Herpesvirus saimiri; HHV-8; Kaposi's Sarcoma-associated herpesvirus; *Orf51*; *K8.1*; Glycosaminoglycans; Viral entry; Neutralization

Introduction

Herpesvirus saimiri (HVS) is a γ_2-herpesvirus found in wild squirrel monkeys (*Saimiri sciureus*) where it causes little apparent pathology (Desrosiers and Falk, 1982; Falk et al., 1972). However, infection of various New World primates by HVS causes an acute peripheral T cell lymphoma and lymphocytic leukemia (Wolfe et al., 1971). This virus shares genomic colinearity and high sequence homology with human herpesvirus-8 (HHV-8), a recently described γ_2-herpesvirus that is etiologically associated with Kaposi's sarcoma, Multicentric Castleman's disease and some primary effusion lymphomas (Cesarman et al., 1996; Russo et al., 1996). In vitro, HVS is able to infect a variety of epithelial cell lines and undergoes lytic replication in owl monkey kidney (OMK) cells (Duboise et al., 1996). Further, it is also able to infect several T cell lines and can induce transformation of common marmoset, rabbit, and human T cells (Damania et al., 1999; Duboise et al., 1998a, 1998b; Jung et al., 1991; Whitehouse, 2003). This is in stark contrast to the lack of a good lytic system for the study of HHV-8. The presence of a lytic in vitro system and both persistent and pathogenic animal models has established HVS as a good model for studies of HHV-8.

Infection of cells by members of the herpesviridae family can be broken down into multiple steps, viral attachment to the cell surface followed by fusion and entry. Viral attachment to the cell surface is likely not as simple as a single viral protein binding to a single cellular receptor. Rather, for a number of the herpesviruses, there is an initial attachment to the cell through binding to heparan-sulfate-containing proteoglycans or other glycosaminoglycans (GAGs) (Shukla and Spear, 2001). This is followed by the binding of specific receptors that allow the fusion and entry steps. The HHV-8 K8.1 protein has been shown to interact with cell-surface heparan sulfate and this interaction likely contributes to the broad cell tropism of HHV-8 (Birkmann et al., 2001; Wang et al., 2001). HVS encodes the *Orf51* gene in a similar genomic location; however, little study has been made of this gene or its role in HVS cell entry. In addition to the Orf51 protein, HVS expresses homologues of several other glycoproteins that have been shown to be important in herpesvirus entry, including glycoprotein B (gB), gH, gL, gM, and gN (Ensser et al., 2003). These proteins act to bind the

* Department of Pathology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA. Fax: +1-508-786-1416.
E-mail address: robert_means@hms.harvard.edu.

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herpesviruses to a wide variety of cell-surface receptors and cause fusion either at the cell surface or within endosomes (Spear and Longnecker, 2003). Coexpression of the HHV-8 gB, gH, and gL homologues is sufficient to mediate fusion between the expressing cell and a limited number of epithelial or B cell targets; however, additional viral glycoproteins might be necessary for high-level viral infection. Little is known about the mechanisms of HVS entry, the cell-surface molecules that the virus uses as receptors or even which viral proteins mediate cell binding and fusion.

To begin exploring the mechanisms of HVS entry, we have designed and implemented a new infectivity assay utilizing a virally encoded reporter gene. This assay was used to look at the prevalence of neutralizing antibodies against HVS, the dependence of HVS on GAGs and to begin to explore the role of the Orf51 protein in virus–cell interactions. We have cloned and characterized the *Orf51* gene of the HVS C488 strain. It was found to be expressed as a virally incorporated glycoprotein capable of binding to heparan sulfate. Further, it was found that HVS depends on cell-surface GAGs for its entry into cells, but may possess both Orf51-dependent and -independent entry mechanisms. These results should pave the way for future animal studies to determine the role of Orf51 in vivo and to a better understanding of the mechanisms of the herpesvirus entry process.

Results

Virally encoded SEAP can be used to measure infectivity

Little is known about the requirements for HVS infection of cells. To explore the mechanisms of viral entry, a new infectivity assay was developed utilizing recombinant HVS expressing secreted, engineered alkaline phosphatase (SEAP) under the control of a HCMV promoter. Infection of susceptible cells results in production of SEAP and secretion into the medium where levels can be measured using a sensitive chemiluminescent assay capable of discriminating between engineered and endogenous alkaline phosphatase (Fig. 1A). Two different viruses had been previously constructed, HVS ΔSTP/SEAP and HVS ΔTIP/SEAP (Duboise et al., 1996, 1998b) using a cloning procedure dependent on the detection of SEAP. These viruses had been shown to be competent for establishing a persistent infection of common marmosets, but not for inducing lymphoma (Duboise et al., 1998b). Owl monkey kidney (OMK) cells are capable of supporting high-level HVS lytic replication. To determine whether SEAP production by these viruses could be used to measure the amount of input virus, OMK cells were infected with increasing amounts of virus. Additional cultures were mock-infected in parallel. Measurement of the amount of SEAP activity in the medium at 3 days postinfection

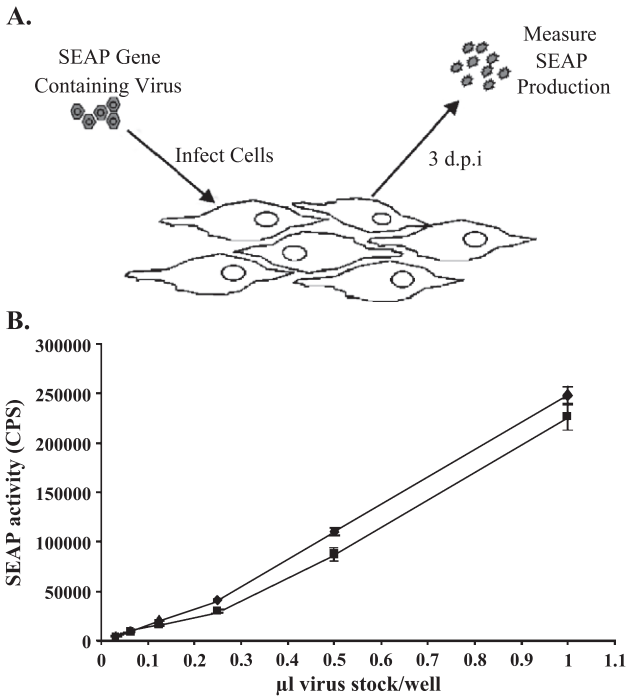

Fig. 1. Overview of the HVS-SEAP infectivity assay. (A) HVS engineered to carry the SEAP gene is used to infect susceptible target cells at different M.O.I. After approximately 3 days, supernatant is collected and the amount of SEAP activity determined by a chemiluminescent assay. (B) Subconfluent OMK cells were infected with increasing amounts of either HVSΔSTP/SEAP (♦) or HVSΔTip/SEAP virus (■). At 3 days postinfection, the amount of SEAP activity in the medium was determined as described in Materials and methods. This graph represents the average of three experiments with the same viral stocks and the standard deviations are indicated. CPS, counts per second; d.p.i., days postinfection.

showed a direct correlation between the amount of input virus and the resulting SEAP activity (Fig. 1B). Measurement of SEAP activity in the mock-infected cultures showed a low background of 500 CPS or less for all of the cultures tested (data not shown). Several stocks of virus were thus tested and although the specific amount of SEAP activity per microliter of virus differed between the stocks, a linear relationship between the amount of input virus and the amount of SEAP activity in the medium was always observed (data not shown).

Naturally infected squirrel monkeys produce neutralizing antibodies against HVS

Blood was taken from six squirrel monkeys (*S. sciureus*, Ss) and tested for the presence of anti-HVS antibodies using ELISA plates coated with purified HVS C488 virus grown in OMK cells. As positive controls, two common marmosets (*Callithrix jacchus*, Cj), another New World primate, experimentally infected with HVS were tested and as a negative control, a Cj that was naïve for HVS was used. Of these animals, four Ss tested highly positive for the presence of anti-HVS antibodies, with detectable titers greater than 1:1000, comparable to the titers seen in the HVS-positive

Table 1
ELISA measurement of anti-HVS C488 titers

Serum source	Serum dilution		
	1:20	1:200	1:1000
Ss 389-98	1.812	1.061	0.168
Ss 430-95	1.712	0.696	0.416
Ss 450-95	1.650	1.055	0.301
Ss 324-97	0.199	0.027	0.007
Ss 89-99	0.082	0.029	0.032
Ss 428-95	2.190	0.923	0.450
Uninfected Cj (−)	0.035	0.021	0.018
Cj 191-95 (+)	2.129	0.809	0.324
Cj 192-95 (+)	>2.500	1.102	0.674

Sera from six Ss and three control Cj were tested against purified HVS C488 coated onto Maxisorb plates as described in Materials and methods. Optical density at 405 nm is reported for each dilution.

Cj (Table 1). One additional animal, Ss 324-97, had only low titers of anti-HVS antibodies, greater than 1:20, but less than 1:200 and one animal, Ss 89-99, had only very weakly detectable antibodies, even at a 1:20 dilution (Table 1).

We next tested whether sera from these animals had detectable neutralizing antibodies against HVS. This was done using a modified version of the infectivity assay. In brief, virus was incubated for 1 h with heat-inactivated serum from each of the animals at a 1:20 final dilution. After incubation, the virus and antibody mixture was overlaid on to OMK cells and the amount of SEAP activity in the medium was measured at 3 days postinfection. One set of wells was left uninfected to serve as background controls. Wells containing virus incubated in the absence of sera or in the presence of sera from a normal, uninfected Cj, were used as negative controls. As a positive control, serum from two Cjs experimentally infected with HVS were used. After measurement, the values for each of the replicates were averaged and the SEAP activity found in the background wells was subtracted from each of the other wells. The values were then divided by the amount of activity found in the well incubated with virus alone to reach a percent infectivity value, with the value of the control wells being 100%. Sera from each of the Ss showing high levels of anti-HVS antibodies also were able to almost completely neutralize viral infectivity (Fig. 2). Ss 324-97, which had low levels of anti-HVS antibodies, was not able to neutralize viral infectivity, while Ss 89-99, with borderline detectable levels of antibodies, reduced HVS infectivity by approximately 30% (Fig. 2). As expected, serum from the uninfected Cj showed no ability to reduce HVS infectivity while sera from the infected animals almost completely neutralized HVS infectivity (Fig. 2). These data demonstrate the utility of this new assay for measuring not only infectivity, but also for testing antiviral compounds.

GAGs influence HVS cell entry

Many of the other herpesviruses require cell-surface GAGs for high-level infection. To test whether HVS had such a requirement, Chinese hamster ovary (CHO) cells with specific defects in GAG production pathways were utilized as target cells for infection by the HVS ΔSTP/SEAP virus. These cells have been previously used to demonstrate the necessity of GAGs for infection of herpes simplex virus type 1, an α-herpesvirus (Shieh et al., 1992; Shukla et al., 1999). The cell lines were created through ethylmethanesulfonate treatment of the parental CHO cell line (Esko, 1992; Esko et al., 1985, 1987, 1988; Lidholt et al., 1992). The PGSA-745 cell line lacks serine-1,3-D-xylosyltransferase, which adds the first sugar in GAG production (Esko et al., 1985). The PGSB-618 cell line lacks galactosyltransferase I and the PGSD-677 cell line lacks both *N*-acetylglucosaminyltransferase and glucuronyltransferase (Esko et al., 1987, 1988; Lidholt et al., 1992). These mutations block the production and cell-surface expression of GAGs including heparan sulfate. Infection of wild-type CHO cells with HVS, while less efficient than OMK cells, resulted in an increase in SEAP activity that directly correlated with the amount of input virus (Fig. 3A). In contrast, infection of the three CHO lines deficient in GAG production showed no increase in

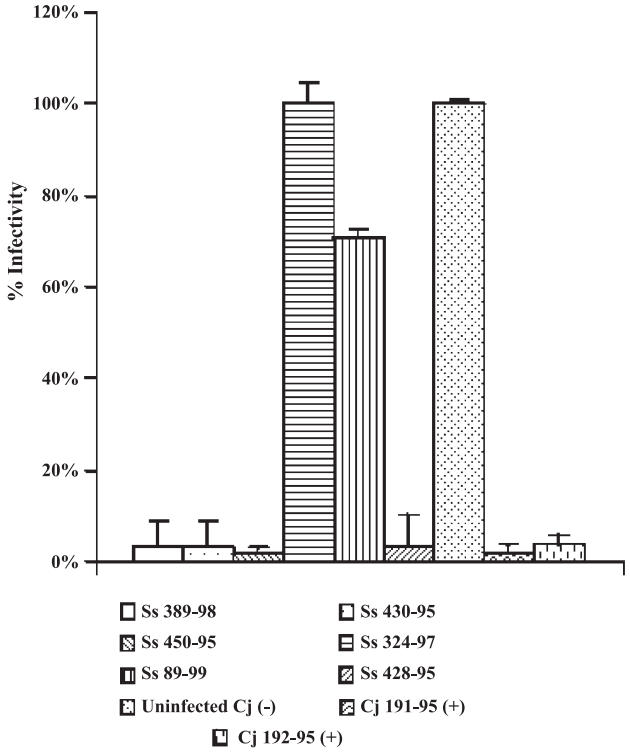

Fig. 2. Neutralization of HVS infectivity. Equal amounts of HVSΔSTP/SEAP were incubated with sera from the indicated animal for 1 h. Treated virus was added to the wells of a 12-well plate containing subconfluent OMK cells. At 3 days postinfection, the amount of SEAP activity in the medium was determined as described in Materials and methods. The percent infectivity was determined by dividing the relative SEAP activity for each well by the amount of SEAP activity resulting from infection of cells with mock-treated virus.

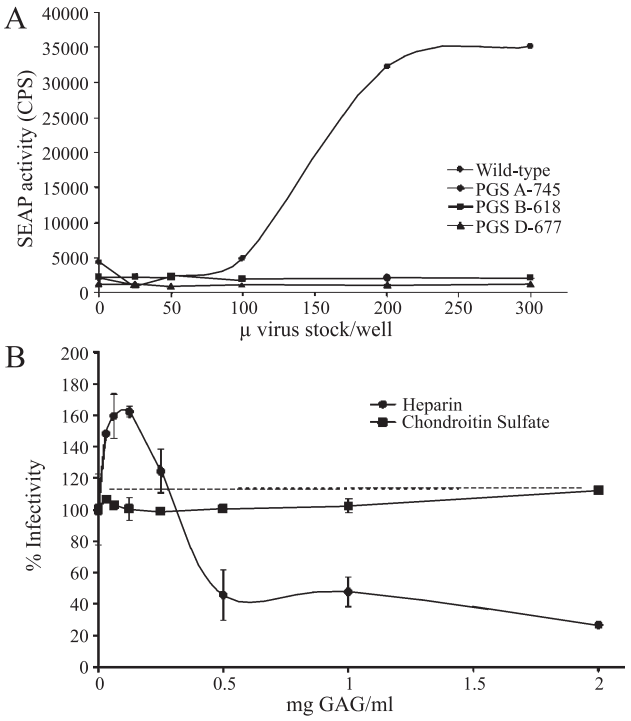

Fig. 3. HVS utilizes GAGs for cell entry. (A) Various concentrations of HVSΔSTP/SEAP were used to infect the indicated wild-type or GAG-production defective CHO cell lines. At 3 days postinfection, the amount of SEAP activity in the medium was determined as described in Materials and methods. Symbols: wild-type cells (●); PGSA-745 cells (♦); PGSB-618 cells (■); PGSD-677 cells (▲). (B) Equal amounts of HVSΔSTP/SEAP were incubated with either heparin or chondroitin sulfate at the indicated concentrations for 1 h. Treated virus was added to the wells of a 12-well plate containing subconfluent OMK cells. Mock-treated virus was added at the same time. Six hours postinfection, either heparin or chondroitin sulfate was added to the cultures containing the mock-treated virus. On the following day, the media was replaced in all of the wells. After two additional days, the amount of SEAP activity in the medium was determined as described in Materials and methods. The percent infectivity was determined by dividing the relative SEAP activity for each well by the amount of SEAP activity resulting from infection of cells with mock-treated virus and treatment with the same concentration of compound. Symbols: chondroitin sulfate (■); heparin (●).

measurable SEAP activity even at the highest viral input (Fig. 3A). These data indicate that HVS, like several other herpesviruses, can utilize GAGs for entry into cells.

HVS entry can be blocked with soluble heparin

To further explore the reliance of HVS on GAGs for entry, we tested whether soluble heparin, structurally similar to heparan sulfate, was capable of blocking virus entry into OMK cells. A constant amount of virus, which had been previously shown to give SEAP activity levels at least 10-fold over background, was incubated with various amounts of heparin for 1 h before infection of OMK cells. As a control for effects of heparin on the OMK cells, an additional set of virus was mock-treated and used to infect OMK cells. Six hours postinfection, heparin at the same concentration as was used for neutralization was added to the mock-treated cultures. As a control for the specificity of heparin, another set of virus was treated with chondroitin sulfate or mock-treated, with chondroitin sulfate being added 6 h after infection. One day postinfection, the medium on all cultures was changed and the cells were incubated for an additional 2 days before SEAP measurement. The amount of SEAP activity in the medium was normalized to the amount of SEAP activity in the medium of cells that were infected with mock-treated virus. At high heparin concentration, there was a dramatic decrease in viral infectivity and this inhibition declined with decreasing amounts of heparin (Fig. 3B). Interestingly, however, low concentrations of heparin enhanced viral infectivity (Fig. 3B), an effect that has been seen with at least one other herpesvirus, human herpesvirus 7 (Secchiero et al., 1997). This neutralizing effect was specific because high levels of chondroitin sulfate had no effect on infectivity. Taken together with the decrease in infection of CHO cells deficient for GAG production, these data indicate that HVS likely utilizes cell-surface heparan sulfate for one step of entry into cells.

The cloning of C488 Orf51

Little is known about the roles that the HVS glycoproteins play in viral entry. HVS is closely related to HHV-8, where it is known that the *K8.1* gene product binds to cell-surface heparan sulfate. We therefore reasoned that the positional homologue of *K8.1*, the *Orf51* gene, might play a role in viral infectivity by binding to cell-surface heparan sulfate. The *Orf51* gene is near the center of the HVS C488 L-DNA region from base 72626 to base 73436. It codes for a protein of 269 amino acids containing nine potential N-linked glycosylation sites and a putative 17 amino acid (a.a.) transmembrane domain (Fig. 4A). Additionally, the first 16 a.a. encode a potential signal sequence. As a source of viral DNA, OMK were infected with HVS strain C488 and incubated until cultures began to show CPE at approximately day 5 postinfection. Total cellular DNA was extracted and used for PCR amplification of the *Orf51* gene using primers that introduced restriction sites into the termini to facilitate cloning into the pEF-myc/his expression vector. After cloning and sequencing, this plasmid was used for PCR amplification of a fragment of the *Orf51* gene encoding the first 240 a.a. up to the transmembrane domain. This PCR product was cloned into the pQE-40 vector and used to generate His_6-tagged Orf51 protein in *E. coli*. Protein was purified using a Ni^{2+} chelate affinity column as described in Materials and methods and used to immunize rabbits for antibody production. Lysates taken from 293T HEK cells transfected with the pEF-Orf51 plasmid showed the presence of a diffuse band running at approximately 52 kDa after Western blotting with the resulting polyclonal anti-Orf51 rabbit sera (Fig. 4B, lane 2), while this was not observed in lysates taken from cells transfected

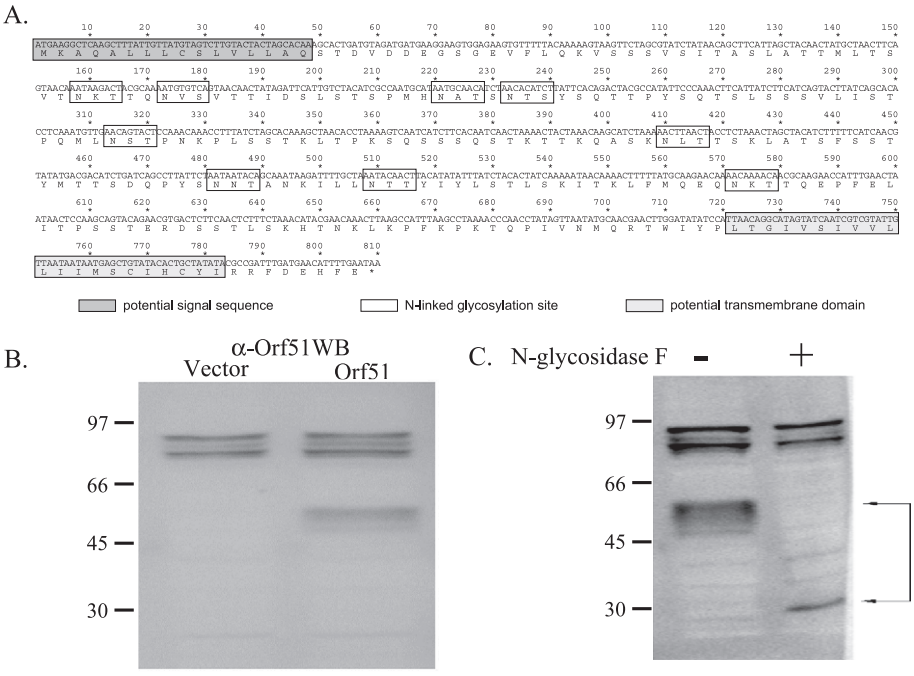

Fig. 4. Characterization of Orf51. (A) Sequence of Orf51 highlighting the locations of the potential signal sequence at the N-terminus, nine potential N-linked-glycosylation sites, and a transmembrane domain near the C-terminus. (B) The pEF-Orf51 or empty pEF vector plasmids were transfected into 293T HEK cells as described in Materials and methods. Approximately 40 h later, cells were harvested, lysed in Lamelli buffer, and resolved on an 8% SDS-PAGE gel. Proteins were then subjected to Western blot detection using a 1:2500 dilution of anti-Orf51 polyclonal sera. (C) The pEF-Orf51 or empty pEF vector plasmids were transfected into 293T HEK cells and harvested as described above. Samples were boiled for 5 min in the presence of SDS and β-mercaptoethanol as described in Materials and methods. An excess of NP-40 buffer either containing 10 U of N-glycosidase F (+ lane) or without enzyme (− lane) was added and the mixture incubated for 3 h at 37 °C. After treatment, 25 μl of the mixture was analyzed by Western blotting using the anti-Orf51 antibody as described. The arrows indicate the shift in molecular weight resulting from treatment.

with the empty pEF vector (Fig. 4B, lane 1). A band of the same molecular weight was observed when the same blot was stripped and reprobed with an anti-His antibody (data not shown). This apparent weight was higher than the 28-kDa molecular weight predicted by the protein sequence. Combined with the diffuse nature of the band and the presence of nine potential N-linked glycosylation sites in the amino acid sequence, we suspected that the altered mobility was due to the posttranslational addition of N-linked carbohydrate moieties.

Orf51 is a glycoprotein

The glycosylation status of Orf51 was examined by treating 293T HEK cell-produced protein with N-glycosidase F, an enzyme capable of removing N-linked glycans. Cells were lysed in SDS buffer and the lysates were boiled in the presence of β-mercaptoethanol. NP-40 and the N-glycosidase F were then added. After incubation with this enzyme, Orf51 ran as a sharp band of ~27 kDa, the predicted molecular weight of the nonglycosylated protein (Fig. 4C). The change in molecular weight, about 25 kDa, suggests that all nine potential N-linked glycosylation sites are likely utilized, assuming a molecular weight of approximately 2.5 kDa per N-linked glycan.

Expression of Orf51 during viral infection

Infection of OMK cells by HVS results in lytic production of virus. Using the anti-Orf51 polyclonal rabbit sera, we examined the production of Orf51 protein at various times postinfection. Cells were infected with a high M.O.I. of HVS C488 and harvested at various times postinfection. Equal amounts of protein were analyzed for Orf51 expression by Western blotting. By 2 days postinfection (d.p.i.), Orf51 protein was readily detectable and the amount of protein increased throughout the 5 days the cultures were monitored (Fig. 5A). Examination of the cultures infected in parallel by immunoflourescent microscopy revealed Orf51 production in a small percentage of cells by 1 d.p.i. with increasing numbers expressing protein at subsequent time points (Fig. 5B). To explore the intercellular localization of Orf51, we transfected 293T HEK cells with either the empty pEF vector or the pEF-Orf51 construct and 2 days post-transfection they were fixed, permeabilized, and stained with the anti-Orf51 antisera. Cells were additionally stained with antibodies against the ER resident protein phosphodiester isomerase (PDI). Examination of these cells by confocal microscopy revealed that the anti-Orf51 antibodies specifically recognize a protein in the pEF-Orf51 transfected cells, which localizes to the nuclear membrane and to a

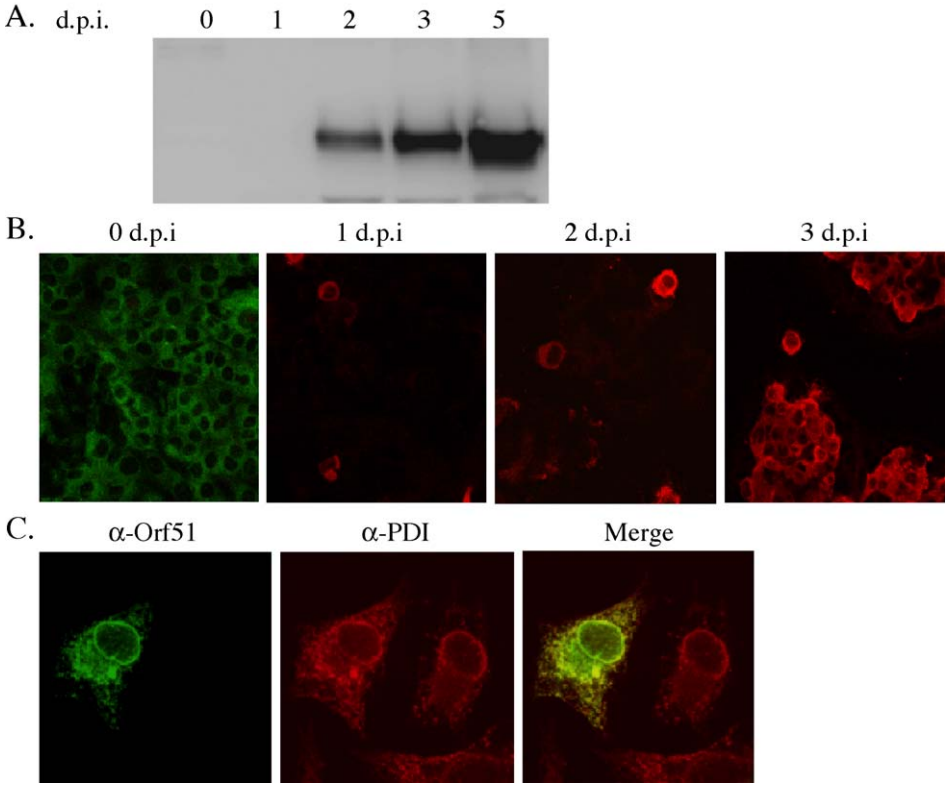

Fig. 5. Expression and localization of HVS Orf51. (A) OMK cells in 10-cm dishes were infected with 10 M.O.I. of wild-type HVS C488. At the indicated days postinfection (d.p.i.), cells were harvested, normalized for total protein content, and analyzed by Western blot for the presence of Orf51 protein. (B) OMK cells, in Lab-Tek II chamber slides, were infected with 10 M.O.I of HVS C488 and incubated for various periods of time as indicated. Cells were then washed, fixed with 4% paraformaldehyde, and permeabilized as described in Materials and methods. Orf51 localization was visualized by indirect immunofluorescence using the anti-Orf51 antibody at a 1:100 dilution and a Texas Red anti-rabbit antibody (Red) at 1:5000. At time 0, cells were stained with a fluorescent green, lipophilic dye to show the monolayer. (C) 293T HEK cells seeded in Lab-Tek II chamber slides were transfected with the pEF-Orf51 or empty pEF vector. Approximately 36 h posttransfection, cells were fixed and permeabilized as described in Materials and methods. Orf51 localization was visualized by indirect immunofluorescence using the anti-Orf51 antibody at a 1:100 dilution and an Alexa 486 anti-rabbit antibody (Green) at 1:5000. Additionally, the localization of PDI was visualized by staining cells with an anti-PDI antibody at a 1:250 dilution followed by an anti-mouse Alexa 594 antibody (Red) at 1:2000.

filamentous structure in the cytoplasm that costains with anti-PDI antibodies (Fig. 5C and data not shown). Parallel cultures of pEF or pEF-Orf51 transfected cells were stained with antibodies against the myc epitope tag that was introduced onto the carboxyl terminus of the Orf51 protein during cloning. Confocal imaging of these cells revealed a pattern of staining that was similar to the one obtained when using the anti-Orf51 antibody (data not shown).

Orf51 is a virion protein

Because we hypothesized that Orf51 was playing a role in viral entry, it follows that it should be present within the viral particle. As a test of this theory, we purified virus and utilized it for ELISA testing of the anti-Orf51 polyclonal rabbit serum. ELISA was performed with normal rabbit serum, serum from a rabbit inoculated with purified, heat-inactivated HVS C488, and the anti-Orf51 polyclonal serum. As expected, no significant reactivity was observed with the normal rabbit serum while the anti-HVS C488 serum showed strong reactivity even at a 1:16,000 dilution (data not shown). The anti-Orf51 serum was strongly positive at a 1:1000 dilution, but had little or no reactivity at a 1:2000 dilution (data not shown). These data suggested that Orf51 was indeed a virion protein.

To further confirm the presence of Orf51 in the viral particle, we next subjected the virion preparations to Western blot analysis with the anti-Orf51 polyclonal sera. Supernatant from HVS C488 infected OMK cells was harvested at 7 d.p.i. when lysis of infected cells was almost complete and spun at low speed to remove cell debris. Cleared supernatant was then subjected to filtration (0.4 μm) and centrifugation at 13,000 × *g* for 1 h to pellet the virions. Pelleted virions were resuspended in buffer and column-purified over Sepharose 4B resin. Eluted virions were pelleted again and resuspended in 0.5% Triton X-100. As a control, HHV-8 was purified in parallel from the supernatant of TPA-stimulated BCBL-1 cells. Equal amounts of HVS and HHV-8 purified virion proteins were boiled in sample buffer containing SDS and β-mecaptoethanol and proteins were separated by SDS-PAGE. The presence of either Orf51 protein or the HHV-8 K8.1 protein in each preparation was determined by Western blotting with the appropriate polyclonal antibody. As expected, the K8.1

protein was easily detected in HHV-8 but not HVS preparations (Fig. 6, right panel). While detection was weaker, we observed a clear band at approximately 52 kDa in the HVS preparation after blotting with the anti-Orf51 antibody (Fig. 6, left panel). We also observed a large, smeared band at a higher molecular weight that may be due to higher order oligomers of Orf51 that were not disassociated by boiling under denaturing conditions.

Orf51 can bind to heparan sulfate

The K8.1 protein of HHV-8 is capable of binding to heparan sulfate molecules (Birkmann et al., 2001; Wang et al., 2001). To determine if HVS Orf51 also had this property and potentially contributed to the ability of HVS to enter into cells, we performed precipitations using heparin bound to agarose beads. As a source of protein, 293T HEK cells were transfected with either pEF-Orf51 or the empty pEF vector. At 36 h posttransfection, cells were harvested and lysed in NP-40-containing buffer. Lysates were subjected to pull down with either agarose bead-conjugated heparin or agarose beads alone. Analysis of the precipitated proteins by Western blot revealed specific pull-down of Orf51 by the heparan-sulfate-conjugated beads, but not unconjugated beads (Fig. 7, lane 2 vs. lane 3). Further, the observed reactivity was specific because no reactivity was seen when heparan-sulfate-conjugated beads were used to immunoprecipitate proteins from pEF-transfected cells (Fig. 7, lane 1). This indicates that Orf51 may be playing a role in viral infection of cells by binding to cell-surface heparan sulfate allowing virus to interact closely with the cell until the fusion receptors are encountered.

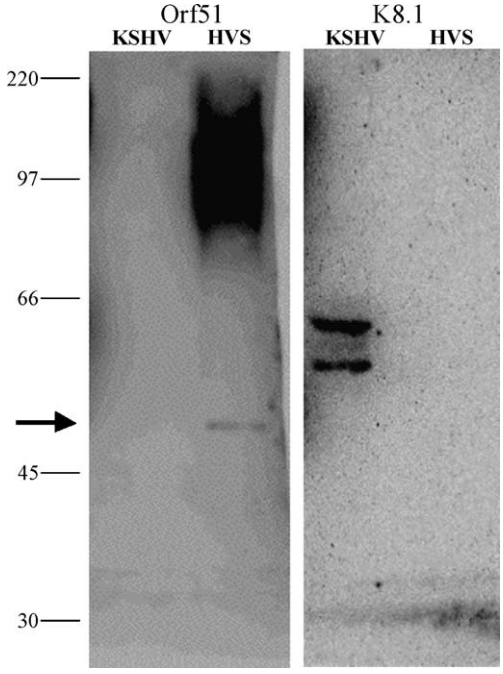

Fig. 6. HVS virion particles contain Orf51 protein. HVS C488 was column purified as described in Materials and methods. In parallel, HHV-8 was purified in a similar manner. Equal amounts of either HVS C488 (lanes 2 and 4) or HHV-8 (lanes 1 and 3) total protein were run out on an 8% SDS-PAGE gel and examined by Western blot for the presence of Orf51 (left panel) or K8.1 (right panel) using the appropriate antibodies. An arrow indicates a band of protein at the appropriate molecular weight of Orf51.

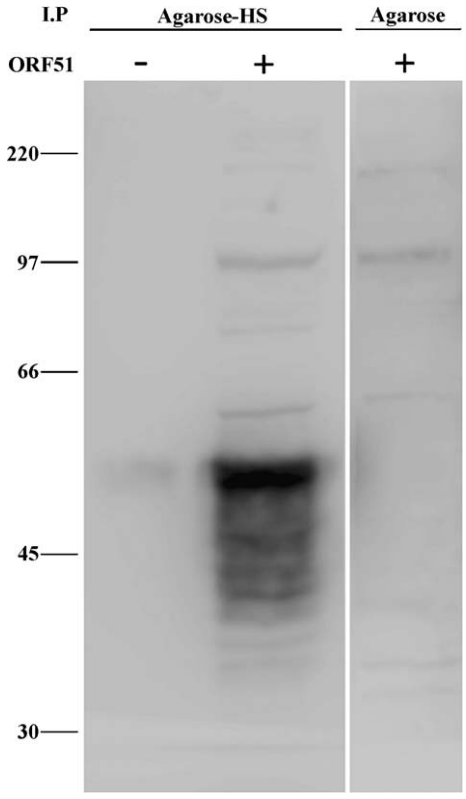

Fig. 7. Orf51 binds heparin-agarose. 293T HEK cells in 10-cm dishes were transfected with either the pEF-Orf51 expression vector or the empty pEF vector. Approximately 36 h posttransfection, cells were harvested and lysed in NP-40 buffer as described in Materials and methods. Each of the lysates was subjected to immunoprecipitation using agarose-conjugated heparan sulfate (Agarose-HS) or unconjugated agarose beads (Agarose). Precipitated proteins were next subjected to Western blotting with the anti-Orf51 polyclonal rabbit serum.

HVS infectivity is not decreased by anti-Orf51 antibodies

Having documented a role for heparan sulfate in HVS entry and an ability of Orf51 to bind the closely related heparin, we next examined the ability of the polyclonal anti-Orf51 serum to neutralize HVS infectivity. As was done with the Ss and Cj sera described above, neutralization assays were performed using a constant amount of virus incubated with various concentrations of anti-Orf51 sera for an hour before infection onto OMK cells. As a negative control, serum from a normal rabbit was used. The neutralizing capacity of two additional sera were tested. Polyclonal antibodies from a rabbit inoculated with purified, inactivated HVS particles and sera taken from a common marmoset experimentally infected with HVS were also tested. As expected, normal rabbit sera showed no

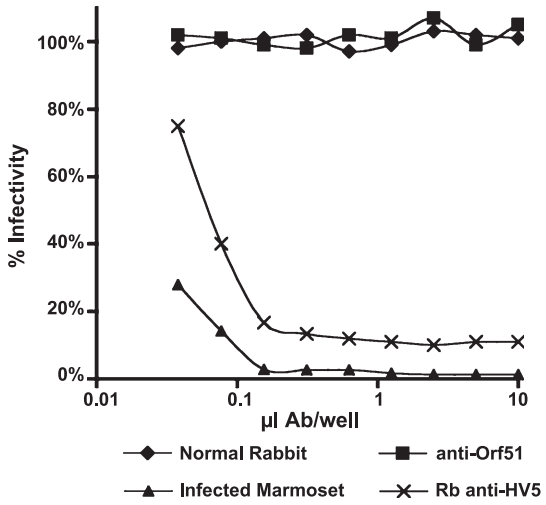

Fig. 8. Neutralization of HVS by various polyclonal sera. Equal amounts of HVSΔSTP/SEAP were incubated for 1 h with nine serial twofold dilutions of the indicated sera starting at 10 µl. Treated virus was added to the wells of a 12-well plate containing subconfluent OMK cells. At 3 days postinfection, the amount of SEAP activity in the medium was determined as described in Materials and methods. The percent infectivity was determined by dividing the relative SEAP activity for each well by the amount of SEAP activity resulting from infection of cells with mock-treated virus.

neutralizing activity, even at the lowest dilution tested (Fig. 8). In stark contrast, both the sera from a rabbit inoculated with HVS particles and the experimentally infected marmoset showed high neutralizing titers with 50% neutralization of virus achieved with 0.06 µl and less than 0.04 µl of serum, respectively (Fig. 8). However, no neutralization of HVS activity was seen with the anti-Orf51 antibody at any tested dilution (Fig. 8). This suggests that Orf51 might not be playing a role in viral entry or that the virus has additional mechanisms for infection that bypass the need for Orf51.

Discussion

In this report, we have demonstrated that HVS interacts with cellular GAGs and that heparan sulfate is likely playing a role in HVS entry. This result is not totally unexpected. A number of other herpesviruses, including members of all three subfamilies, utilize heparan sulfate to differing degrees to aid in the entry process (Compton et al., 1993; Herold et al., 1991; Jacquet et al., 1998; Laquerre et al., 1998; Neyts et al., 1992; Shieh et al., 1992; Shukla and Spear, 2001; Shukla et al., 1999; Spear et al., 1992; WuDunn and Spear, 1989). Herpes simplex virus encodes three different glycoproteins capable of binding to heparan sulfate (Laquerre et al., 1998; Shukla et al., 1999). The closely related γ-herpesvirus, HHV-8, also utilizes heparan sulfate as a target molecule to attach to cells. It does this through at least two viral proteins, K8.1 and its gB homologue (Akula et al., 2001; Birkmann et al., 2001; Wang et al., 2001). In addition, the HHV-8 gB protein also acts to bind one of the viral receptors, α3β1 integrin, through an RGD motif that is not found in the HVS gB (Wang et al., 2003).

The mechanisms by which HVS enters into cells are largely unclear. At a similar genomic location as gp350/220 in EBV, HVS encodes the Orf51 protein. The gB/gH/gL glycoproteins of Epstein–Barr virus (EBV) cooperate with gp350/220 and another glycoprotein, gp42, to mediate cell entry. The gp350/220 protein binds to complement receptor 2 (CR2), also called CD21 (Nemerow et al., 1987; Tanner et al., 1987). This binding to CR2 is followed by gp42 binding to HLA class II molecules, which then allows for virus–cell fusion mediated by the gB/gH/gL glycoproteins (Li et al., 1997). However, epithelial cells are largely CR2 deficient and the gp350/220 and gp42 proteins have been shown to be dispensable with gH playing a greater role in mediating attachment of virus to CR2 deficient cells (Janz et al., 2000; Molesworth et al., 2000). Interestingly, the amount of gp42 present in virus particles is regulated by the viral producer cell, potentially regulating which target cells can be infected (Borza and Hutt-Fletcher, 2002). In this report, we have shown that Orf51 is able to bind to heparan sulfate; however, this does not rule out an ability to bind to a cell-surface protein in a manner similar to gp350/220. Herpesvirus saimiri, like EBV, is able to infect both lymphocytes and epithelial cells, so a producer cell effect is possible. However, until a better system for studying the role of the HVS viral glycoproteins is established, the contribution of the individual glycoproteins will probably remain occult.

Herpesvirus interactions with the glycosaminoglycans has been proposed to allow for initial, relatively nonspecific binding of the virus to target cells. This results in the concentration of virus particles on the surface of these cells and an increased likelihood of interactions between viral proteins receptor proteins and their cognate ligands (reviewed in Shukla and Spear, 2001). However, one of the targets of HVS infection, T lymphocytes, posses only low or undetectable amounts of heparan sulfate proteoglycans (Ibrahim et al., 1999; Manakil et al., 2001). This suggests that the ability of HVS to bind to heparan sulfate might play a larger role in its ability to enter into epithelial cells and that other viral proteins are playing a role in initiating viral interactions with the T lymphocytes. Alternatively, T cells possibly only express heparan-sulfate-modified proteins in response to specific stimuli, such as the up-regulation of proteoglycan syndecan-2 on human macrophages in response to interleukin-1α (Clasper et al., 1999). Binding to heparan sulfate might aid in viral dissemination to T cells in another way. Nonspecific attachment to the surface of nontarget cells such as APC, which then interact with T cells, might serve to facilitate the concentration and transfer of virus to susceptible targets. Each of these possibilities needs to be explored through further experimentation to fully evaluate the im-

portance of heparan sulfate binding to HVS entry and spread in vivo.

The results reported in this paper provide a good basis for the further exploration of the role of GAGs in HVS entry. While we have shown that HVS entry is decreased in cells deficient for GAG production and that soluble heparin is capable of blocking infectivity, this is not formal proof of heparan sulfate proteoglycans acting as receptors for viral entry. Several additional experiments would have to be performed including treatment of target cells with heparanases or sulfation-blocking chemicals like chlorate, followed by an examination of their susceptibility to viral infection. Additional experiments specifically removing heparan sulfate binding proteins from the viral genome to look at effects on infectivity would also address this question and are currently being performed.

Heparan sulfate binding sequences are typically made up of a series of basic residues, arginine, and lysine, separated by nonbasic residues. Several different consensus sites have been described including HSB sequence 1, XBBXBX, HSB sequence 2, XBBBXXBX, and HSB sequence 3, XBXBXXBX (B = basic, X = nonbasic) (Cardin and Weintraub, 1989; Hileman et al., 1998; Sobel et al., 1992). Examination of the Orf51 sequence reveals the presence of at least one stretch of sequence rich in basic residues and containing a consensus heparin binding site starting with residue 214, S**KH**TN**K**L**K**PF**K**P**K**T (X**BB**X<u>X***B*X***B***XX***B***X</u> **B**X, HSB sequence 3 underlined). While this motif is longer than the published HSB sequence 3, it is unclear without further experimentation which residues are critical to heparan sulfate binding. Given that several of the herpesviruses have multiple proteoglycan binding sequences, we are currently exploring whether the HVS gB homologue is capable of binding to heparan sulfate, potentially through a classical HSB domain that has been identified in the ectodomain.

The *Orf51* gene is a positional homologue of the *K8.1* gene of KSHV. Other researchers have shown that KSHV infection can be blocked by α-K8.1 antibodies. The inability to block HVS infection with the anti-Orf51 antibody can be interpreted in several ways. First, there is the possibility that Orf51 is dispensable for the infection of certain target cells, yet required for the infection of others, i.e., endothelial cells vs. T lymphocytes. Another possibility is that although the antibody is capable of recognizing native Orf51 protein, as evidenced by the immunoflorescence data (Figs. 5B,C), critical neutralizing epitopes might not be recognized. The Orf51 antibody was raised against *E. coli*-produced protein, which is nonglycosylated and might not posses the same tertiary structure as mammalian-derived protein. Therefore, the antiserum used in this study may not recognize specific epitopes that could potentially play a role in Orf51 interactions with cell-surface target proteins. Yet, a third possibility is that Orf51 is not playing a role in viral entry; rather, it may be playing a role in viral assembly. To fully determine the role of Orf51 in viral entry, we are currently producing HVS mutants lacking the *Orf51* gene locus.

In addition to presenting the first detailed characterization of a HVS glycoprotein, this report has demonstrated the utility of the SEAP infectivity assay for investigating factors effecting HVS entry into cells. With this assay, we have demonstrated that naturally infected squirrel monkeys, in which virus is apathogenic, make robust neutralizing antibody responses. Equally, in experimentally infected Cj where the virus is invariably fatal, similarly robust responses are made. This eliminates interference with or direction of aberrant antibody production as a mechanism of HVS pathogenicity in Cj. A number of other viruses such as human immunodeficiency virus and its nonhuman primate counterpart simian immunodeficiency virus both show a similar pattern of increased pathogenicity upon zoonotic transmission (Kaur et al., 1998; Mahy and Brown, 2000). The question of what mechanism allows for apathogenic infection of Ss still remains open and is potentially critical in understanding the pathogenic mechanisms of other viruses.

A better understanding of the entry mechanisms of the herpesviruses is critical to devising antiviral vaccines and prophylactic treatments. Defining common mechanisms that multiple herpesviruses utilize, as well as how they differ, could aid in the targeting of newly emerging pathogenic herpesviruses. These results lay the groundwork for a more intense study of the mechanisms by which HVS enters into cells. Additionally, the presented data stresses the common usage of heparan sulfate by the herpesviruses for entry, suggesting possible avenues for new clinical modalities targeting this potential Achilles heel.

Materials and methods

Cell lines, sera, and transfections

The owl monkey kidney (OMK 637) cells have been previously described (Simonds et al., 1975) and were maintained in Modified Eagle's medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (GIBCO). The 293T HEK, Chinese hamster ovary (CHO), PGSA-745 (CRL-2242), PGSB-618 (CRL-2241), and PGSD-677 (CRL-2244) cells were obtained from ATCC and maintained in Ham's F12K medium (GIBCO) supplemented with 10% FCS. BCBL-1 cells were grown in RPMI 1640 supplemented with 10% FCS and were induced for HHV-8 particle production through the addition of 20 ng of phorbol-12-tetradecanoyl-13-acetate (TPA) per milliliter as previously described (Yu et al., 1999). Sera from common marmosets infected with HVS and rabbits inoculated with purified, inactivated HVS particles were the kind gift of Dr. Jae Jung (NEPRC, Southborough, MA). Normal rabbit serum was purchased from GIBCO.

Transfection into 293T HEK cells was performed using Transfectin reagent (Bio-Rad, Hercules, CA). Cells were seeded into 10-cm dishes and grown until ~90% confluent, at which point the medium was discarded and replaced with 10 ml of DMEM + 10% FCS. Two tubes per transfection were prepared. Into tube 1, 15 μg of DNA and 750 μl of DMEM was added. To the second tube, 200 μl of DMEM followed by 30 μl of Transfectin was added. The contents of the two tubes were mixed, incubated for 20 min at room temperature, and the overlaid on the 293T HEK cells. Cells were incubated for 36–52 h in a humidified CO_2 incubator before harvest and analysis.

Whole-virus ELISA

The whole-virion enzyme-linked immunosorbent assay (ELISA) procedure was performed as previously described (Wyand et al., 1996). Briefly, HVS grown in tissue culture was concentrated by ultracentrifugation and by Sepharose chromatography (Pharmacia, Upsala, Sweden). Concentrated, purified virions were then disrupted in Triton X-100 detergent and used to coat flat-bottom 96-well plates (Maxisorb, Nunc, Rochester, NY). Coated wells were incubated with serially diluted serum samples, and bound antibodies were detected with alkaline-phosphatase-conjugated secondary anti- human antibody (SouthernBiotech, Birmingham, AL). The amount of secondary was quantitated using *p*-nitrophenyl phosphate substrate (SouthernBiotech, Birmingham, AL) and measuring the optical density at 404 nm according to the manufacturer's recommendations.

Infectivity and viral neutralization assays

Viral infectivity was measured utilizing the HVS ΔSTP/SEAP and HVS ΔTip/SEAP viruses that have been previously described (Duboise et al., 1996, 1998b). OMK cells were plated into a 12-well dish and when just subconfluent, infected with the indicated amount of virus stock, containing approximately 1×10^7 infectious particles per milliliter, in a total of 1 ml of complete medium. Three days postinfection, cell-free supernatant was harvested and the amount of alkaline phosphatase activity was measured using the Great Escape SEAP reporter assay kit (BD Biosciences, San Jose, CA) according to the manufacturer's recommendations. Neutralization of viral infectivity was measured in a similar fashion. Identical amounts of virus, 0.5 μl containing approximately 5000 infectious particles, were incubated for 1 h at 37 °C in 500 μl of complete medium containing the indicated volume of either potentially neutralizing serum or control serum. Samples were then added to OMK in 12-well dishes containing 500 μl of complete medium. SEAP activity was measured as described above and the percent neutralization was calculated by dividing the amount of SEAP activity in the test well by the amount in the control well containing the same dilution of serum.

Orf51 cloning and antibody production

OMK cells were infected with the C488 strain of HVS. Approximately 7 days postinfection, viral DNA was harvested and subjected to PCR amplification. The following primers were used to amplify Orf51. *Bam*HI-ORF51 5′: 5′-AAGAATTCACCGCCATGAAGACTCAAGTTTTCTT-TATTTGC-3′ *Not*I-Orf51 3′: 5′-GCGGCCGCCACAAAA-TATTCATTAAACTTTCG-3′.

The PCR product was cloned into the pEF vector (Invitrogen, Carlsbad, CA) utilizing the unique *Bam*HI and *Not*I sites introduced during PCR amplification. After complete sequencing to insure that no undesired mutations had been introduced during PCR, this product was used for the amplification of a fragment of the *Orf51* gene, resulting in the elimination of sequences coding for the potential transmembrane domain. This PCR product was placed into the pQE-40 vector (Qiagen, San Diego, CA) for expression as a His_6 tagged-protein. When *E. coli* XL-1 Blue cells (Stratagene, La Jolla, CA) containing plasmid pQE40-Orf51 reached an optical density of approximately 0.6 at 600 nm, 1 mM isopropyl-*b*-D-thiogalactopyranoside was added; cells were harvested 3 h after induction and then solubilized with 6 M guanidine hydrochloride. Due to the presence of the affinity tail, His_6-Orf51 protein was purified to virtual homogeneity in one step by Ni^{2+} chelate affinity chromatography. The purified recombinant His_6-Orf51 protein was used to generate polyclonal antibodies in New Zealand White rabbits. A Ni^{2+} chelate affinity column containing Orf51 protein was used to purify the antigen-specific antibodies. Antibody specific for Orf51 was eluted with high pH solution (0.1 M triethylamine [pH 11.5]).

N-glycosidase F treatment of Orf51

As a source of protein, 293T HEK cells were transfected with the pEF-Orf51 plasmid by the calcium phosphate method. Cell lysates were prepared by the addition of 20 μl of sample buffer containing 10% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol. Samples were boiled for 5 min, and then a 180 μl of a solution containing 1% NP-40 and 1% mercaptoethanol was added. To this mixture, 5 U of N-glycosidase F (Roche, Indianapolis, IN) was added, and samples were incubated at 37 °C for 3 h. At the end of the incubation period, the samples were electrophoresed in a 8% polyacrylamide-SDS gel and subjected to Western blotting as described below.

Immunofluorescence

For immunolocalization of Orf51, A7 cells were seeded into Lab-Tek II chamber slides (Nalge Nunc, Rochester, NY) and transfected with pEF-Orf51 using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's recommendations. Approximately 24 h posttransfection, cells were washed three times with PBS and then fixed

for 30 min with 4% paraformaldehyde (PFA). Cells were permeabilized with 0.1% Triton X-100 and then stained with an anti-His monoclonal antibody (Santa Cruz Biotech) followed by an Texas-Red-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR). Slides were visualized using a Leica TCS SP laser-scanning microscope (Leica Microsystems, Exton, PA). For visualization of Orf51 production during HVS infection, OMK cells were seeded into Lab Tek II chamber slides and infected with HVS C488 at a M.O.I. of 10. At various times postinfection, cells were washed three times with PBS and fixed for 30 min in PFA. As above, cells were permeabilized and stained with the anti-Orf51 antibody followed by staining with a Texas-Red-conjugated anti-rabbit antibody (Molecular Probes).

Heparan sulfate precipitation and Western blotting

Orf51-transfected cells were harvested and lysed with lysis buffer (0.15 M NaCl, 1% Nonidet P-40, 50 mM Tris [pH 7.5]) containing 0.1 mM Na_2VO_3, 1 mM NaF, and protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and bestatin). For precipitation, 30 μl of agarose conjugated-heparan sulfate (Sigma, St. Louis, MO) or unconjugated agarose beads (Sigma) were added to the cell lysate and incubated at 4 °C with gentle rocking for 3 h. The samples were washed 3 times with lysis buffer, resuspended in Lamelli buffer and boiled for 5 min For Western blot detection of proteins, precipitates or whole cell lysates were electrophoresed through a 8% polyacrylamide-SDS gel and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skim milk in phosphate-buffered saline–0.05% Tween 20 for 1 h. The blots were then incubated sequentially with a 1:1000 dilution of anti-His antibody or 1:2500 dilution of anti-Orf51 sera followed by the appropriate secondary reagents. Antibody localization was visualized using a PicoWest chemiluminescence kit (Pierce, Rockford, IL) and a LAS-1000 charge-coupled device camera (Fuji, Inc., Tokyo, Japan).

Viral pellets and immunoprecipitation

To pellet virus, 1 ml of supernatant from either HVS C488-infected OMK cells or TPA-induced, BCBL-1 cells was spun for 3 h at 13,000 × *g* in a refrigerated centrifuge. The supernatants were discarded, and the pellets were resuspended in 30 μl of Lamelli buffer. Each sample was then subjected to two rounds of boiling for 5 min followed by vortexing for 1 min and then subjected to Western blotting with the indicated antibodies.

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