Entry of Alphaherpesviruses Mediated by Poliovirus Receptor-Related Protein 1 and Poliovirus Receptor

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A human member of the immunoglobulin superfamily was shown to mediate entry of several alphaherpesviruses, including herpes simplex viruses (HSV) 1 and 2, porcine pseudorabies virus (PRV), and bovine herpesvirus 1 (BHV-1). This membrane glycoprotein is poliovirus receptor-related protein 1 (Prr1), designated here as HveC. Incubation of HSV-1 with a secreted form of HveC inhibited subsequent infection of a variety of cell lines, suggesting that HveC interacts directly with the virus. Poliovirus receptor (Pvr) itself mediated entry of PRV and BHV-1 but not of the HSV strains tested. HveC was expressed in human cells of epithelial and neuronal origin; it is the prime candidate for the coreceptor that allows both HSV-1 and HSV-2 to infect epithelial cells on mucosal surfaces and spread to cells of the nervous system.

Alphaherpesviruses, including HSV-1, HSV-2, PRV, and BHV-1, infect a variety of cell types in culture, resulting in efficient virus production in a short replicative cycle. Infection in the natural host is characterized by lesions in the epidermis, usually on mucosal surfaces, with spread of virus to the nervous system and establishment of latent infections in neurons. Binding of alphaherpesviruses to cells occurs primarily through an interaction of virion glycoprotein C (gC) with cell surface heparan sulfate, whereas fusion between the virion envelope and cell membrane requires the glycoproteins gB, gD, gH, and gL (*1*).

Several lines of evidence suggest that alphaherpesvirus gD interacts with a cell surface receptor in addition to heparan sulfate to mediate viral entry and that, in certain cell types, HSV-1, PRV, and BHV-1 can use a common gD receptor for entry (*1, 2*). Recently, a gD receptor for entry of HSV-1 and HSV-2 was identified as an additional member of the tumor necrosis factor receptor family, called herpesvirus entry mediator (*3, 4*), and is designated here as herpesvirus entry mediator A (HveA). HveA is the principal receptor for entry of HSV into human lymphoid cells but not into other cell types (*3*). Also, HveA fails to mediate the entry of PRV (*3*). A second mediator of HSV entry identified recently (*5*) was shown to be poliovirus receptor-related protein 2 (*6*). No function and no poliovirus receptor activity have been reported for this protein, and it is therefore designated herpesvirus entry mediator B (HveB). HveB mediates the entry of HSV-2 strains, PRV, and certain viable mutants of HSV-1 but fails to mediate the entry of wild-type HSV-1 strains or BHV-1 (*5*).

These results demonstrate that multiple alphaherpesvirus coreceptors exist, differing in their specificities for individual viruses in the subfamily. Neither HveA nor HveB fits the specifications for a coreceptor that can mediate entry of both HSV-1 and HSV-2 into epithelial cells at the initial site of infection and into neuronal cells for the establishment of latent infection. Also, neither HveA nor HveB serves as a coreceptor for all these viruses—HSV-1, PRV, and BHV-1—and therefore could not be a common coreceptor for these human and animal alphaherpesviruses. Because HveB is closely related to the poliovirus receptor (Pvr) (*7*) and to poliovirus receptor-related protein 1 (Prr1) (*8*), we explored the possibility that one or both of those proteins might mediate the entry of HSV-1 and -2 as well as PRV and BHV-1.

Chinese hamster ovary (CHO) cells express heparan sulfate chains, to which alphaherpesviruses can bind. However, CHO cells are resistant to the entry of HSV-1, PRV, and BHV-1 because of the absence of coreceptors required for virion cell fusion (*3, 5, 9*). CHO cells were transfected with plasmids expressing Pvr or Prr1 (*10*) and then inoculated with virus (*11*) to determine whether expression of the cell proteins could provide the necessary coreceptors for viral entry. Prr1 mediated the entry of several HSV-1 strains and three HSV-1 mutants (ANG, Rid1, Rid2) with amino acid substitutions in gD that preclude the use of HveA for entry (*3*) (Fig. 1). Prr1, designated here as HveC, also enhanced infection by HSV-2 strains (Fig. 1), although the enhancement was not as great because control CHO cells are partially susceptible to HSV-2 infection (*9*). HveC expression rendered CHO cells susceptible to PRV and BHV-1 as well as HSV, entry being a function of virus dose (Fig. 2). Three independently isolated cell lines (CHO–HveC-1, -2, and -3) stably expressing HveC were also capable of being infected by PRV, BHV-1, and HSV-1 (*12*). Pvr mediated the entry of PRV and BHV-1 but not of the HSV-1 strains (Fig. 2). The fact that HveC and Pvr, designated Pvr-HveD, can mediate entry of PRV and BHV-1 does not strictly imply that human cells could support the replication of those viruses; it does suggest, however, that the animal homologs of HveC and Pvr-HveD could mediate entry of those viruses into cells of the

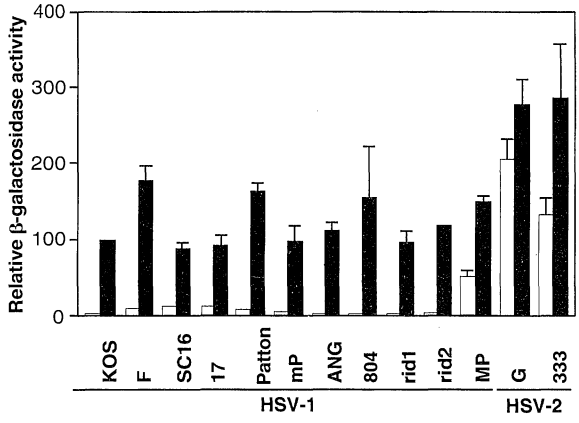

Fig. 1. Enhanced entry of HSV-1 and -2 strains into CHO cells expressing HveC. Subconfluent CHO-IEβ8 cells were transfected with a plasmid expressing HveC (pBG38, solid bars) or a control plasmid (pcDNA3, open bars) and 24 hours later replated in 96-well plates (about 2×10^4 to 4×10^4 cells per well). After 24 hours, the cells were incubated with virus at a range of concentrations and β-Gal activity was quantitated as a measure of viral entry, as described (*3, 11*). The results depicted were for 50,000 pfu per well, in the linear range of plots of virus dose against β-Gal activity. Each experiment was performed with a subset of the viruses that always included HSV-1(KOS). Within each experiment, all values were made relative to the value obtained for the HSV-1(KOS)/HveC infection. Each virus was inoculated in triplicate; the mean values plus SDs for at least two separate experiments are shown.

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natural hosts.

The nucleotide sequence of our isolate of HveC cDNA (GenBank accession number AF060231), as well as I.M.A.G.E. Consortium Clone 287663 (a fragment of HveC cDNA isolated from brain tissue) (*13*) differed from the originally published sequence (*8*) by the absence of single bases at positions 582, 597, and 617. The differences affected the amino acid sequence over a short range but maintained the overall open reading frame (*14*). Diagnostic restriction enzyme analysis of our clone, as well as the corresponding region amplified by polymerase chain reaction (PCR) from a HeLa cell cDNA library, verified our sequence (*12*). The *hveC* gene is located on human chromosome 11 (*8*), which is of special interest because a human gene capable of conferring to Chinese hamster lung cells a susceptibility to HSV-1 infection was previously mapped to human chromosome 11 (*15*). The genes for HveB and Pvr-HveD are located on chromosome 19 (*6*, *16*), whereas that for HveA is on chromosome 1 (*17*).

To identify human cell types in which entry of HSV-1 might be mediated by HveC, we performed reverse transcription–PCR (RT-PCR) analysis with primers specific for HveC cDNA on total RNA isolated from human cell lines and primary cells (*18*). HveC mRNA expression was detected in NT2 cells (teratocarcinoma), SH-SY5Y and IMR-5 cells (neuroblastomas), HL-60 cells (promyelocytic leukemia), primary human diploid fibroblasts, primary human foreskin keratinocytes (Fig. 3), and HeLa cells (*12*) but not in HEL299 cells (embryonic lung fibroblasts) or phytohemagglutinin-activated T cell blasts (Fig. 3) (*18*). As expected, expression of HveC mRNA was also detected in CHO cells that were stably expressing HveC cDNA but not in control CHO cells or in CHO cells stably expressing HveA or HveB (Fig. 3). RT-PCR performed with primers specific for HveA cDNA yielded the expected product in keratinocytes and T lymphoblasts but not in NT2, SH-SY5Y, or IMR-5 cells (*12*). NT2, SH-SY5Y, and IMR-5 cells are susceptible to HSV-1(KOS) infection (*12*) (Fig. 4). HveC is the best candidate for the entry protein used by HSV-1(KOS) in these cells because expression of HveC, but not of HveA, was detected and neither HveB nor Pvr-HveD mediates HSV-1(KOS) entry. Although all four of the herpesvirus entry proteins are expressed in many human tissues and organs, expression of HveA is detected principally in lymphoid organs (*17*, *19*), whereas HveB, HveC, and Pvr-HveD can be expressed in cells of the nervous system (*5*, *7*) or in cells cultured from the nervous system (Fig. 3).

A secreted form of HveA, HveA(200t), binds to virus by an interaction with HSV-1 gD and blocks infection by HSV-1 (*4*). To ascertain whether HveC interacts with virion proteins to mediate entry, we incubated HSV-1 with HveA(200t) or with a secreted form of HveC, HveC(346t) (*20*), prior to infection. Incubation of HSV-1 with either of these truncated proteins inhibited infection of NT2 cells, IMR-5 cells, CHO cell lines stably expressing HveA (*21*) or HveC (*22*) (Fig. 4), and SH-SY5Y cells (*23*) in a dose-dependent manner. Either protein competed with membrane-bound forms of HveA or HveC for interaction with virus, thereby inhibiting infection. As expected, HveA and HveC were expressed on the surface of CHO–HveA-12 and CHO–HveC-1 cells, respectively, according to flow cytometric analysis (*3*, *12*). Recent results demonstrate that HveC(346t) binds purified gD from HSV-1 and HSV-2 in vitro (*22*). Therefore, HveC(346t) may bind the gD present on the HSV-1 virion to inhibit infection, and HveC may be a receptor for virion gD.

Whereas HveA, HveB, and Pvr-HveD are active for entry of subsets of the alphaherpesviruses tested here, HveC mediated entry of all these viruses. Although the gD family members of the alphaherpesviruses share only 10 to 15% amino acid sequence identity, there is general conservation of the positions of six cysteine residues and probably conservation of a domain recog-

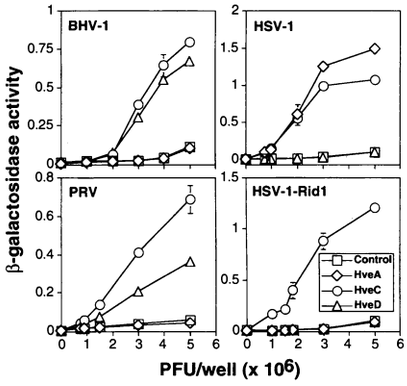

Fig. 2. Enhanced entry of HSV-1, PRV, and BHV-1 into HveC- and Pvr-HveD–expressing CHO cells. Subconfluent CHO-IEβ8 cells (*3*, *11*) were transfected with plasmids expressing HveA (pBec10) (*3*), HveC (pBG38), Pvr-HveD (pBG42.16), or control DNA (pcDNA3). After 24 hours, the transfected cells were replated in 96-well plates (about 2×10^4 to 4×10^4 cells per well) and exposed the next day to HSV-1(KOS), HSV-1(KOS)Rid1, PRV(Kaplan), or BHV-1(Cooper) isolates expressing β-Gal (*11*). Six hours after inoculation, cells were lysed and β-Gal activity was determined as a measure of virus entry (*11*). The infections were done in triplicate and were repeated four times. The mean values plus SDs for a representative experiment are shown.

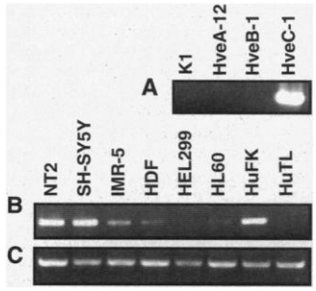

Fig. 3. Expression of HveC mRNA. Total RNA was isolated from established cell lines and primary cells, the cDNA was obtained, and PCR was performed as described (*18*). (**A**) HveC mRNA expression in CHO lines stably expressing HveA (HveA-12), HveB (HveB-1), or HveC (HveC-1) and also the parental CHO cell line (K1) (*18*). (**B**) HveC mRNA expression in human cell lines as described in the text (*18*) and in primary cell cultures: HDF, human diploid fibroblasts; HuFK, human foreskin keratinocytes; HuTL, phytohemagglutinin-activated human T cell blasts (*18*). (**C**) β-Actin mRNA expression detected in the RNA samples from the corresponding lanes in (**B**) (*18*). The β-actin control results are also presented in a figure demonstrating HveB expression in these same cell lines (*5*).

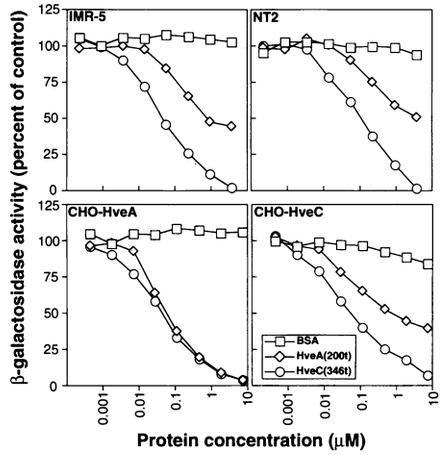

Fig. 4. Blocking HSV-1 infection by secreted HveA and HveC. HSV-1(KOS)tk12 was preincubated with BSA, HveA(200t), or HveC(346t) for 1 hour at 37°C. The cell lines (about 4×10^4 cells per well) were then exposed to the mixtures of virus and protein in 96-well plates for 1 hour at 4°C before transfer to 37°C for 6 hours. CHO–IEβ8/HveA (CHO-HveA) cells and CHO–IEβ8/HveC (CHO-HveC) cells were generated by transfection of CHO–IEβ8 cells (*3*) as described (*21*). Cells were lysed and β-Gal activity was quantitated as a measure of infection by HSV-1(KOS)tk12 (*20*). The values obtained for infections in the presence of added protein were compared with the infection obtained in the absence of added protein to determine percent of control. All values represent the average of at least two experiments performed in triplicate.

nized by HveC. Substitutions at position 27 in HSV-1 gD abrogate entry via HveA (*3*) and enable entry via HveB (*5*) but have no effect on entry via HveC. These findings, coupled with the ability of HveA and HveC to compete for critical sites on virions to block infection, indicate that each entry protein may recognize overlapping but distinct structural domains of gD. Animal homologs of HveB, HveC, and Pvr-HveD probably are the principal coreceptors for entry of animal alphaherpesviruses and may be active for HSV strains, as predicted by experiments that identified gD coreceptors recognized by both animal and human alphaherpesviruses (*2*); however, this remains to be determined.

HSV-1 and HSV-2 strains exhibit differences in pathogenesis, some of which may be attributable to preferences for different entry receptors and, therefore, to targeting of different cell types. However, a common feature of all HSV-1 and HSV-2 strains, and of most alphaherpesviruses, is the ability to replicate in mucosal epithelia and to invade adjacent nerve endings, thereby establishing a latent infection in nerve cell bodies. The results presented here implicate HveC as the prime receptor allowing for HSV-1 and HSV-2 infection of mucosal surfaces and spread to the nervous system and suggest it as a prime target for innovative prophylactic or therapeutic interventions. Moreover, HveC homologs may serve a similar role for infections by animal alphaherpesviruses of their natural hosts and thus may account for the cross-interference patterns observed for human and animal alphaherpesviruses.

REFERENCES AND NOTES

1. P. G. Spear, *Semin. Virol.* **4**, 167 (1993); T. C. Mettenleiter, in *Viral Vectors* (Academic Press, New York, 1995), pp. 367–393.
2. G. Campadelli-Fiume, M. Arsenakis, F. Farabegoli, B. Roizman, *J. Virol.* **62**, 159 (1988); E. A. Petrovskis, A. L. Meyer, L. E. Post, *ibid.*, p. 2196; D. C. Johnson and M. W. Ligas, *ibid.*, p. 4605; D. C. Johnson, R. L. Burke, T. Gregory, *ibid.* **64**, 2569 (1990); A. Karger and T. C. Mettenleiter, *Virology* **194**, 654 (1993); C. C. L. Chase, C. Lohoff, G. J. Letchworth III, *ibid.*, p. 365; W. Lee and A. O. Fuller, *J. Virol.* **67**, 5088 (1993).
3. R. I. Montgomery, M. W. Warner, B. J. Lum, P. G. Spear, *Cell* **87**, 427 (1996).
4. J. C. Whitbeck *et al.*, *J. Virol.* **71**, 6083 (1997); A. V. Nicola *et al.*, *ibid.* **72**, 3595 (1998).
5. M. W. Warner *et al.*, *Virology*, in press.
6. F. Eberle *et al.*, *Gene* **159**, 267 (1995).
7. C. L. Mendelsohn, E. Wimmer, V. R. Racaniello, *Cell* **56**, 855 (1989).
8. M. Lopez *et al.*, *Gene* **155**, 261 (1995).
9. M. T. Shieh *et al.*, *J. Cell. Biol.* **116**, 1273 (1992).
10. The 5′ half of the Prr1 cDNA insert in pBG38 consisted of the Hind III to Bst EII fragment of the I.M.A.G.E. Consortium Clone 140737, obtained from a placenta cDNA library (*13*). The 3′ half of the Prr1 cDNA extended from the Bst EII site to the stop codon and was obtained by two rounds of PCR amplification of a placenta cDNA library (Clonetech) with use of primers int1 (5′-TCCTTCACCGATGGCACTATCC) and 108 (5′-ACACGTACCACTCCTTCTTG) for the first round and primers int1 and 104 (5′-GCTCTAGAGCGGCTACACGTACCACTCCTT) for the second round. The initial thermocycling conditions were 94°C for 1 min, 70°C for 1 min, and 72°C for 1.5 min. After every three cycles, the annealing temperature was decreased 3°C until 55°C was reached, after which cycling was continued to 35 total cycles. Two percent of the first PCR volume was used as template with the second primer set under the cycling conditions. The Prr1 cDNA was ligated into pcDNA3 (Invitrogen) via Hind III and Xba I sites. The Pvr cDNA (α form) was PCR amplified from a HeLa cDNA library (Invitrogen) as described (*16*), with primers pvr01 (5′-TCTGGAGCTTGAAGAAGTGGG) and pvr07 (5′-CACCTTGTGCCCTCTGTCTG) for the first round and pvr01 plus pvr08 (5′-CCTCTCAGTCCCGACGCTGT) for subsequent rounds of amplification. The Pvr cDNA was inserted into pcDNA3 via the Eco RV site to yield pBG42.16.
11. Infectivity assays were performed in 96-well plates as described (*3*). Subconfluent CHO-IEβ8 cells were transfected by using Lipofectamine (GibcoBRL). CHO-IEβ8 cells contain an immediate-early promoter of HSV-1 upstream of the *Escherichia coli lacZ* gene and express β-galactosidase (β-Gal) upon HSV entry, as a result of transactivation by VP16 (*3*). The viruses used for the infectivity assays in Fig. 2 were HSV-1(KOS)tk12 (*5*), HSV-1(KOS)Rid1-tk12 (*5*), PRV(Kaplan)gH$^-$ (*24*), and BHV-1(Cooper)v4a (*25*). These viruses express β-Gal from a *lacZ* cassette inserted within their genomes. PRV(Kaplan)-gH$^-$ virus was propagated on SW78 cells to obtain infectious virus. The description and references for the virus strains used in Fig. 1 were as described (*3*) except for HSV-1(mP) (*26*) and HSV-2(G) (*27*).
12. R. J. Geraghty and P. G. Spear, data not shown.
13. G. G. Lennon, C. Auffray, M. Polymeropoulos, M. B. Soares, *Genomics* **33**, 151 (1996).
14. Nucleotide sequence analysis of pBG38 was performed by the University of Chicago Cancer Research Center DNA Sequencing Facility. The sequence differences were absences of cytosines at base pairs (bp) 582, 597, and 617 [numbered according to sequence published in (*8*)]. These changes yield a deletion of one amino acid residue and the resulting sequence Glu-Ala-Glu-Tyr-Gln-Glu-Ile-Arg-Asn-Pro-Asn-Gly-Thr-Val (amino acids 192 to 205) instead of Glu-Ala-Arg-Val-Pro-Gly-Asp-Ser-Gly-Thr-Pro-Met-Ala-Pro-Val (amino acids 192 to 206).
15. U. Francke and B. Francke, *Somat. Cell Genet.* **7**, 171 (1981).
16. S. Koike *et al.*, *Embo J.* **9**, 3217 (1990).
17. B. S. Kwon *et al.*, *J. Biol. Chem.* **272**, 14272 (1997).
18. SH-SY5Y cells were obtained from D. Pleasure and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. IMR-5 cells were also obtained from D. Pleasure and grown in RPMI medium with 10% fetal bovine serum. CHO K1 and HEL299 were obtained from American Type Culture Collection (ATCC) and grown as described (*5*). NT-2 and HL60 cells were obtained from ATCC and grown in DMEM and 10% fetal bovine serum. The primary human diploid fibroblasts were obtained from M. K. Rundell and the primary human foreskin keratinocytes were obtained from L. Laimins (*28*). The activated T cell blasts were prepared, and CHO–HveA-12 cells were isolated as described (*3*). CHO–HveB-1 cells have been described (*5*). Total RNA was isolated from 1 × 10^6 to 5 × 10^6 cells by using the RNeasy kit (Qiagen). The 3′ RACE kit (GibcoBRL) was used for RT. PCR amplification of HveC sequences was performed with int1 (*10*) and 105 (5′-TCAACACCAGCAGGATGCTC) primers and one round of thermocycling conditions (*10*). The primers int1 and 105 spanned three predicted exons; therefore, amplification of contaminating genomic DNA would result in a band of greater size than the HveC-cDNA–specific (738 bp) band detected. The β-actin control primers and thermocycling conditions have been described (*29*). The samples were subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.
19. H. Hsu *et al.*, *J. Biol. Chem.* **272**, 13471 (1997); S. A. Marsters *et al.*, *ibid.*, p. 14029.
20. HveC(346t) was truncated after amino acid 346 to yield a protein consisting of the predicted ectodomain of HveC, mellitin signal sequence at the NH_2-terminus to facilitate secretion from insect cells, and a histidine tag at the COOH-terminus to facilitate purification from culture medium. Methods for construction of recombinant baculovirus that expressed HveC(346t) and purification of the secreted protein were similar to those described for HveA(200t) (*4, 22*). Blocking experiments were carried out in 96-well plates (about 4 × 10^4 cells per well). Serial dilutions of HveA(200t), HveC(346t), or bovine serum albumin (BSA) were mixed with virus solution [4 × 10^5 plaque-forming units (pfu) of HSV-1(KOS)tk12 per milliliter (*5*), 30 mM Hepes, DMEM, and 10% fetal calf serum]. The mixtures were incubated at 37°C for 1 hour and then chilled on ice. The cells were chilled at 4°C for 10 min before infection. Protein and virus mixture (100 μl) was added to each well and incubated at 4°C for 1 hour, after which the plates were transferred to 37°C for 6 hours. Cells were lysed by adding to each well 100 μl of culture medium containing 1% NP-40. Fifty microliters of cell lysate was transferred to another 96-well dish, 50 μl of chlorophenol red-P-D-galactopyranoside was added, and the kinetics of β-Gal activity was measured at 570 nm.
21. T. Terry-Allison *et al.*, *J. Virol.*, in press.
22. C. Krummenacher *et al.*, *ibid.*, in press.
23. C. Krummenacher, G. H. Cohen, R. J. Eisenberg, data not shown.
24. N. Babic *et al.*, *J. Gen. Virol.* **77**, 2277 (1996).
25. J. M. Miller, C. A. Whetstone, L. J. Bello, W. C. Lawrence, J. C. Whitbeck, *Am. J. Vet. Res.* **56**, 870 (1995).
26. M. D. Hoggan and B. Roizman, *Am. J. Hyg.* **70**, 208 (1958).
27. J. M. Ejercito, E. Kieff, B. Roizman, *J. Gen. Virol.* **2**, 357 (1968).
28. J. T. Thomas and L. A. Laimins, *J. Virol.* **72**, 1131 (1998).
29. J. C. Willey *et al.*, *Am. J. Respir. Cell Mol. Biol.* **14**, 262 (1986).

30. We thank T. Mettenleiter for PRV(Kaplan)gH$^-$, L. Bello for BHV-1v4a, C. Waltenbaugh for use of his spectrometer, N. Susmarski for work with cell lines and virus stocks, W. Hou for technical assistance, and R. Xu for CHO-IEβ8/HveC cells. We also thank R. Montgomery, M. Warner, and P. Speck for helpful discussions, and R. Longnecker and L. Laimins for suggestions on the manuscript. Supported by a grant from the National Institute of Allergy and Infectious Diseases (RO1 AI 36293 to P.G.S.) and by grants from the National Institute of Neurological Disorders and Stroke (NS-36731, NS-30606, and AI-18289 to R.J.E. and G.H.C.). R.J.G. is supported by National Research Service Award F32 AI09471.

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