JOURNAL OF VIROLOGY, July 2002, p. 7255–7262 Vol. 76, No. 14
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.14.7255–7262.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

Amino Acid Substitutions in the V Domain of Nectin-1 (HveC) That Impair Entry Activity for Herpes Simplex Virus Types 1 and 2 but Not for Pseudorabies Virus or Bovine Herpesvirus 1

Wanda M. Martinez and Patricia G. Spear*

Department of Microbiology-Immunology, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611

Received 28 December 2001/Accepted 12 April 2002

The entry of herpes simplex virus (HSV) into cells requires the interaction of viral glycoprotein D (gD) with a cellular gD receptor to trigger the fusion of viral and cellular membranes. Nectin-1, a member of the immunoglobulin superfamily, can serve as a gD receptor for HSV types 1 and 2 (HSV-1 and HSV-2, respectively) as well as for the animal herpesviruses porcine pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV-1). The HSV-1 gD binding domain of nectin-1 is hypothesized to overlap amino acids 64 to 104 of the N-terminal variable domain-like immunoglobulin domain. Moreover, the HSV-1 and PRV gDs compete for binding to nectin-1. Here we report that two amino acids within this region, at positions 77 and 85, are critical for HSV-1 and HSV-2 entry but not for the entry of PRV or BHV-1. Replacement of either amino acid 77 or amino acid 85 reduced HSV-1 and HSV-2 gD binding but had a lesser effect on HSV entry activity, suggesting that weak interactions between gD and nectin-1 are sufficient to trigger the mechanism of HSV entry. Substitution of both amino acid 77 and amino acid 85 in nectin-1 significantly impaired entry activity for HSV-1 and HSV-2 and eliminated binding to soluble forms of HSV-1 and HSV-2 gDs but did not impair the entry of PRV and BHV-1. Thus, amino acids 77 and 85 of nectin-1 form part of the interface with HSV gD or influence the conformation of that interface. Moreover, the binding sites for HSV and PRV or BHV-1 gDs on nectin-1 may overlap but are not identical.

Various members of the alphaherpesvirus subfamily, including herpes simplex virus (HSV) type 1 (HSV-1) and HSV type 2 (HSV-2), enter mammalian cells through similar multistep mechanisms. After the initial attachment of virus to cell surface heparan sulfate via viral glycoprotein C (gC) and/or glycoprotein B (gB), viral glycoprotein D (gD) interacts with any one of several different cellular receptors. This interaction triggers the fusion of viral and cellular membranes, a poorly understood process that requires additional viral glycoproteins (glycoproteins H [gH] and L and gB) and possibly other cellular molecules (reviewed in references 39 and 40).

Nectin-1, nectin-2, and CD155, related members of the immunoglobulin superfamily, are gD receptors that mediate the entry into cells of one or more alphaherpesviruses (5, 40). Nectin-1, the receptor with the broadest entry activity, mediates the entry of all commonly tested strains of HSV-1 and HSV-2 as well as of the animal alphaherpesviruses porcine pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV-1) (10, 14). Nectin-2 mediates the entry of certain HSV-1 variants containing substitutions in amino acid 25 or 27 of gD (Rid variants) and of PRV and has weak entry activity for HSV-2 (22, 44). CD155, the poliovirus receptor, has entry activity for PRV and BHV-1 but not for HSV-1 or HSV-2 (14). Mammalian homologs of nectin-1 (identified in mice, pigs, cows, and monkeys) and nectin-2 (in mice) share a high degree of amino acid identity with their human counterparts and exhibit similar herpesvirus entry activities (25, 28, 38). However, the human and mouse forms of nectin-2 differ in entry specificity, as mouse nectin-2 mediates the entry of PRV only and not of the HSV-1 Rid variants (HSV-1/Rid) or of HSV-2.

Nectin-1 and nectin-2 are expressed in a variety of cell lines and tissues relevant to HSV pathogenesis, including cells of epithelial and neuronal origins (5, 10, 14, 40, 44). Both molecules engage in transhomophilic or heterophilic interactions with other nectin family members, localize to cell junctions, and mediate cell-cell adhesion (21, 35, 37, 42). In cells with complex junction structures, such as epithelial cells, nectin-1α and nectin-2 localize to cadherin-based adherens junctions via interactions of carboxy-terminal sequences with L-afadin, an F-actin binding protein (29, 41, 42).

The nectins share a similar ectodomain structure consisting of an N-terminal variable domain-like (V) immunoglobulin domain and two constant domain-like (C) immunoglobulin domains. Differentially spliced transcripts of the nectin-1 and nectin-2 genes can be translated to yield multiple isoforms that differ in the sequences of the transmembrane and cytoplasmic regions (5, 40). All membrane-bound isoforms expressed from the same gene exhibit indistinguishable entry activities, indicating that the critical domains for HSV entry reside in the ectodomain. Several lines of evidence have indicated that the V domains of nectin-1 and nectin-2 contain the gD binding site. A soluble form of the V domain of nectin-1 can block HSV entry into nectin-1-expressing cells and bind to soluble HSV-1 gD (9, 20). Similar results were obtained with a soluble form of the V domain of nectin-2 and HSV-1 variant U21 gD (22). Recombinant nectin-1 with the two C domains deleted can mediate HSV entry, although inefficiently (9). Also, a chimeric receptor containing the V domain of nectin-1 fused to

* Corresponding author. Mailing address: Northwestern University, The Feinberg School of Medicine, Department of Microbiology-Immunology, Mailcode S213, 320 E. Superior St., Chicago, IL 60611. Phone: (312) 503-8230. Fax: (312) 503-1339. E-mail: p-spear@northwestern.edu.

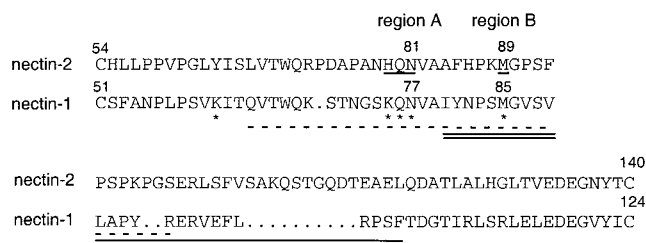

FIG. 1. Alignment of nectin-1 and nectin-2 amino acid sequences in the N-terminal V domain. The amino acid sequence contained between the two conserved cysteines of the V domain of nectin-1 is aligned below the corresponding sequence of nectin-2. Numbers shown above the amino acids correspond to their positions from the initial methionine. Regions in nectin-2 previously shown to be critical for HSV entry activity and referred to as regions A and B are indicated by single underlining (23). The asterisks indicate the amino acids of nectin-1 mutated in this study. The linear epitopes for anti-nectin-1 MAbs CK6 and CK8 were mapped to a region indicated by double underlining (18), and the region of nectin-1 identified as sufficient to confer HSV entry activity to CD155 is indicated by a broken line (8). The sequences shown are those in GenBank entries for nectin-1 (accession number AF060231) and nectin-2 (accession number AF058448).

part of CD4 exhibits full alphaherpesvirus entry activity (12). In addition, anti-nectin-1 monoclonal antibodies (MAbs) that block HSV-1 entry into cells map to the V domain (9, 18).

Studies of sequences within the V domain of nectin-1 have narrowed the HSV-1 gD-interacting region to amino acids 64 to 104. HSV-1 entry and gD binding to nectin-1 were inhibited by two anti-nectin-1 MAbs whose linear epitopes mapped to amino acids 80 to 104 (18). In addition, a chimeric receptor containing nectin-1 amino acids 64 to 94 in place of the corresponding amino acids of CD155 gained the ability to mediate HSV-1 entry and bind to soluble gD (8).

Recently, specific amino acids within the V domain of nectin-2 that are critical for HSV entry activity were identified (23). Chimeric molecules were constructed between the human and mouse forms of nectin-2 to identify sequences of human nectin-2 that could confer HSV entry activity on the chimera. Our results showed that two small regions (regions A and B; Fig. 1) in the V domain of human nectin-2 were independently able to transfer HSV entry activity to mouse nectin-2. The critical amino acids within these regions are at positions 79 to 81 and 89. These results suggested that the gD binding domain of nectin-2 may overlap regions A and B.

The goal of this study was to determine whether amino acids 75 to 77 and 85 of nectin-1, the amino acids in positions homologous to regions A and B of nectin-2 (Fig. 1), are critical for HSV-1 entry and gD binding as well as for entry and gD binding by other alphaherpesviruses. Nectin-1 mutants were constructed in which these amino acids were replaced with alanines or other amino acids in different combinations. The mutants were tested for anti-nectin-1 MAb binding, soluble gD binding, and HSV entry activity. We found that nectin-1 mutants containing substitutions in both amino acid 77 and amino acid 85 lost the ability to mediate the entry of HSV-1 and HSV-2 and failed to bind to soluble forms of HSV-1 and HSV-2 gDs. However, these mutants were not significantly impaired for the entry of PRV and BHV-1, indicating that HSV and the animal alphaherpesviruses differ in their interactions with nectin-1. Interestingly, mutants containing single substitutions in either amino acid 77 or amino acid 85 had a significantly reduced ability to bind to soluble forms of HSV-1 and HSV-2 gDs, but their entry activities were not drastically reduced compared to that of wild-type nectin-1; these results suggested that a reduced affinity for gD does not necessarily eliminate HSV entry activity.

MATERIALS AND METHODS

Cells and viruses. Chinese hamster ovary (CHO-K1) cells were provided by J. Esko (University of California, San Diego) and grown as previously described (30). PEAK cells, used to produce the gD:Fc proteins, were purchased from Edge Biosystems and grown according to the manufacturer's instructions. The β-galactosidase (β-Gal) reporter viruses used were HSV-1(KOS)tk12 and HSV-1(KOS)Rid1/tk12 (44); gH-negative PRV(Kaplan) (2), provided by T. Mettenleiter (Federal Research Center for Virus Diseases of Animals, Insel Reims, Germany); BHV-1(Cooper)TK-bgal+v4a (27), provided by L. Bello (University of Pennsylvania); and HSV-2(333)gJ−, described below. PRV was propagated and titers were determined on gH-expressing VeroSW78 cells (2), and BHV-1 was propagated and titers were determined on MDBK cells. All other viruses were propagated and titers were determined on Vero cells.

The reporter virus HSV-2(333)gJ− was engineered to contain a cytomegalovirus-*lacZ* cassette in place of part of the glycoprotein J gene. Plasmid pCR5 contains HSV-2(333) DNA sequences from nucleotides 3801 to 4311 and 4481 to 5127 inserted upstream and downstream, respectively, of the cytomegalovirus-*lacZ* cassette of pCR3 (derived from pSP73 [Promega]). This plasmid was cotransfected with HSV-2(333) genomic DNA into Vero cells, and recombinant viruses expressing β-Gal were plaque purified as previously described (32). The appropriate localization of the insert was verified by PCR amplification across the upstream and downstream junctions. This reporter virus, contrary to wild-type HSV-2, has a reduced ability to infect CHO-K1 cells but can still infect CHO cells expressing the appropriate human gD receptors. Revertants with the deletion or insertion repaired remained unable to infect CHO-K1 cells; the mutation responsible for the decrease in CHO-K1 infectivity has not yet been identified (C. Rowe, W. M. Martinez, and P. G. Spear, unpublished observations).

Plasmids. Plasmid pBG38 containing the nectin-1α gene in pcDNA3 has been described elsewhere (14). The nectin-1 coding sequences were excised from pBG38 by digestion with *Hin*dIII and *Xma*I and cloned into pUC19 to generate pWM69. The plasmids used to generate the soluble gD:Fc fusion proteins have been described elsewhere: pBG64 [HSV-1(KOS) gD:Fc] (13), pAF1 [HSV-1(KOS)Rid1 gD:Fc], pBG74 [BHV-1 gD:Fc], and pBG75 [HSV-2(333) gD:Fc] (12).

To generate nectin-1 mutants, site-directed mutagenesis was performed with a PCR-based system according to the manufacturer's instructions (QuikChange site-directed mutagenesis kit; Stratagene). The template for the PCRs was pWM69. All mutants were sequenced across the appropriate region to ensure the presence of the intended nucleotide substitutions only and then were recloned into the *Hin*dIII and *Xma*I sites of pBG38. The plasmids coding for the nectin-1 mutants were as follows: pWM105 (K75A), pWM107 (Q76A); pWM103 (N77A), pWM104 (QN76-77AA), pWM108 (KQN75-77AAA), pWM106 (M85A), pWM109 (M85F), pWM114 (QN76-77AA and M85F [QN76-77AA;M85F]), pWM115 (KQN75-77AAA;M85F), pWM116 (K61A;QN76-77AA), pWM117 (K61A;KQN75-77AA), pWM129 (N77A;M85F), and pWM130 (N77A;M85A).

Soluble gD:Fc fusion proteins. Soluble gD:Fc fusion proteins were produced as described previously (12). Briefly, PEAK cells were transfected with the gD:Fc-expressing plasmids by the calcium phosphate method according to the manufacturer's instructions (Edge Biosystems). Cells were incubated in Dulbecco modified Eagle medium with no serum, and supernatants were collected 24 and 48 h later. The concentrations of gD:Fc hybrids in the supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) with rabbit immunoglobulin G as the standard.

CELISA for detection of binding of MAbs and gD:Fc hybrids to cells. A cell ELISA (CELISA) was performed as described previously (13). Briefly, subconfluent CHO-K1 cells in six-well dishes were transfected with the appropriate plasmids by using Lipofectamine (Gibco BRL) and replated on 96-well dishes 24 h later. After 18 h, cells were exposed to blocking solution (phosphate-buffered saline plus 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 3% bovine serum albumin) for 30 min and then exposed to ascites containing a MAb or to culture supernatants containing soluble gD:Fc. The antibodies used included CK6 and CK8

TABLE 1. Summary of nectin-1 mutations and phenotypes[a]

Nectin-1 mutant	Critical amino acid(s) mutated	Binding of gD:Fc derived from:			Entry activity for		
		HSV-1	HSV-1/Rid1	HSV-2	HSV-1	HSV-1/Rid1	HSV-2
K75A	75 or 76 only	+	+	+	+	+	+
Q76A		+	+	+	+	+	+
N77A	77 or 85	−	↓	−	↓	↓	↓
M85A		−	↓	−	↓	↓	↓
M85F		−	↓	−	↓	↓	↓
QN76-77AA		−	↓	−	↓	↓	↓
KQN75-77AAA		−	↓	−	↓	↓	↓
K61A;KQN75-77AAA		−	↓	−	↓	↓	↓
N77A;M85A	77 and 85	−	−	−	−	−	−
N77A;M85F		−	−	−	−	−	−
QN76-77AA;M85F		−	−	−	−	−	−
KQN75-77AAA;M85F		−	−	−	−	−	−

[a] +, wild type; ↓, reduced; −, undetectable. All the mutants exhibited wild-type entry activity for PRV and BHV-1. BHV-1 gD:Fc bound to all mutants at levels comparable to those observed with wild-type nectin-1.

(18), provided by G. Cohen and R. Eisenberg (University of Pennsylvania), and R1.302 (10), provided by M. Lopez (INSERM, Marseille, France), all diluted 1:500. Subsequently, cells were fixed and sequentially exposed to biotin-conjugated secondary antibody (Sigma), AMDEX streptavidin-conjugated horseradish peroxidase (Amersham), and substrate solution containing 3,3′,5,5′-tetramethylbenzidine (BioFX). At various times after substrate addition, plates were read at 370 nm. Alternatively, the reactions were stopped by the addition of stopping solution (BioFX), and plates were read at 410 nm.

Viral entry assays. Viral entry assays were performed as previously described (30). Briefly, CHO-K1 cells were transfected as described above and replated on 96-well plates 24 h later. Cells were then exposed to serial dilutions of β-Gal-expressing virus diluted in phosphate-buffered saline plus 0.1% glucose and 1% heat-inactivated serum. After 6 h, cells were washed, incubated with a solution containing the β-Gal substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and analyzed as described previously (30).

RESULTS

Construction of nectin-1 mutants and cell surface expression. Figure 1 presents an alignment of sequences between the two conserved cysteines of the V domains of nectin-1 and nectin-2 and indicates the positions of amino acids in nectin-2 previously shown to be critical for entry activity (23). Four nectin-1 mutants were constructed by replacing the amino acids in homologous positions, amino acids 75, 76, 77, and 85, with alanine. Methionine 85 was also replaced with phenylalanine, based on the finding that nectin-2 mutant M89F had lost HSV entry activity (23). Also, two mutants were engineered to contain multiple alanine substitutions in amino acids 75 to 77, and six nectin-1 mutants were constructed in which substitutions in amino acids 75 to 77 were combined with substitution M85F or M85A or substitution K61A in an adjacent region (Fig. 1). A list of the nectin-1 mutants examined in these studies is shown in Table 1.

To determine whether the nectin-1 mutants retained proper conformation and were expressed on the cell surface, CHO-K1 cells were transfected with plasmids expressing wild-type nectin-1 or the nectin-1 mutants or with a control plasmid and were tested by a CELISA for the binding of anti-nectin-1 MAbs (Fig. 2). CK6, CK8, and R1.302 are anti-nectin-1 MAbs that compete with gD for binding to nectin-1 and can inhibit HSV entry into nectin-1-expressing cells (9, 10, 18). The epitopes recognized by CK6 and CK8 have been mapped to amino acids 80 to 104 by using synthetic peptides (Fig. 1). R1.302 recognizes a conformational epitope. As shown in Fig. 2, all the nectin-1 mutants retained the ability to bind to R1.302 at levels comparable to those observed for wild-type nectin-1, indicating that the overall conformation and cell surface expression of the mutant receptors were not drastically altered. For mutant M85F, the levels of CK6 and CK8 binding were reproducibly somewhat reduced, whereas for mutants N77A, M85A and N77A;M85A, the levels were reproducibly enhanced. These results are perhaps not surprising given that the CK6 and CK8 epitopes have been mapped to a region overlapping Met85 and adjacent to Asn77. It is likely that these amino acid substitutions affect the accessibility or actual conformation of the CK6 and CK8 epitopes.

Entry activities of nectin-1 mutants. CHO-K1 cells are resistant to the entry of most alphaherpesviruses unless engineered to express a gD receptor. To determine the entry activities of the nectin-1 molecules, CHO-K1 cells were transfected with plasmids expressing wild-type nectin-1 or the nectin-1 mutants or with a control plasmid. The transfected cells were exposed to serial dilutions of reporter alphaherpesviruses (HSV-1, HSV-1/Rid1, HSV-2, PRV, and BHV-1) which contain a *lacZ* cassette in the viral genome and express β-Gal upon entry into cells. β-Gal activity was measured 6 h after initial viral exposure and used as an indication of viral entry.

The results obtained with the HSV strains are shown in Fig. 3. Seven of the 11 nectin-1 mutants retained significant levels of entry activity for HSV-1, HSV-2, and HSV-1/Rid1. The four mutants that contained substitutions in both amino acid 77 and amino acid 85 (N77A;M85F, N77A;M85A, QN76-77AA;M85F, and KQN75-77;M85F) were severely impaired in HSV entry activity, more so for HSV-1 and HSV-2 than for HSV-1/Rid1. The rest of the mutants showed entry activities for the three viruses that were either somewhat reduced (particularly QN76-77AA and KQN75-77AAA) or comparable to that observed with wild-type nectin-1. Consistent differences among the mutants with single amino acid substitutions were most evident for the entry of HSV-1, as depicted by the comparison of entry at a single input viral dose within the linear portion of

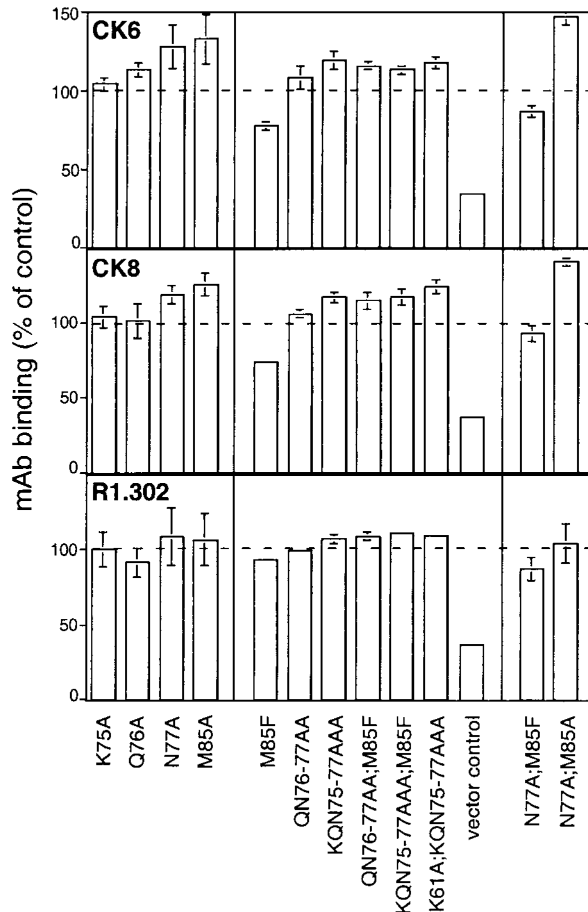

FIG. 2. Cell surface expression of nectin-1 mutants. CHO-K1 cells were transfected with plasmids expressing nectin-1 mutants or wild-type nectin-1 or with a control plasmid. After 48 h, the cells were reacted with anti-nectin-1 MAbs and fixed. Binding was detected with a biotinylated secondary antibody followed by streptavidin-horseradish peroxidase. The results are expressed as optical densities at 370 nm (OD_{370}) normalized to the binding obtained with wild-type nectin-1 (set to 100% and represented in the graph by a broken line). Values shown are the means and standard deviations of triplicate determinations. Vertical lines dividing the data represent separate experiments. The OD_{370} for binding of the antibodies to wild-type nectin-1 ranged from 1.25 to 2.5. CK6 and CK8 recognize linear epitopes, and R1.302 recognizes a conformational epitope, all in the V domain.

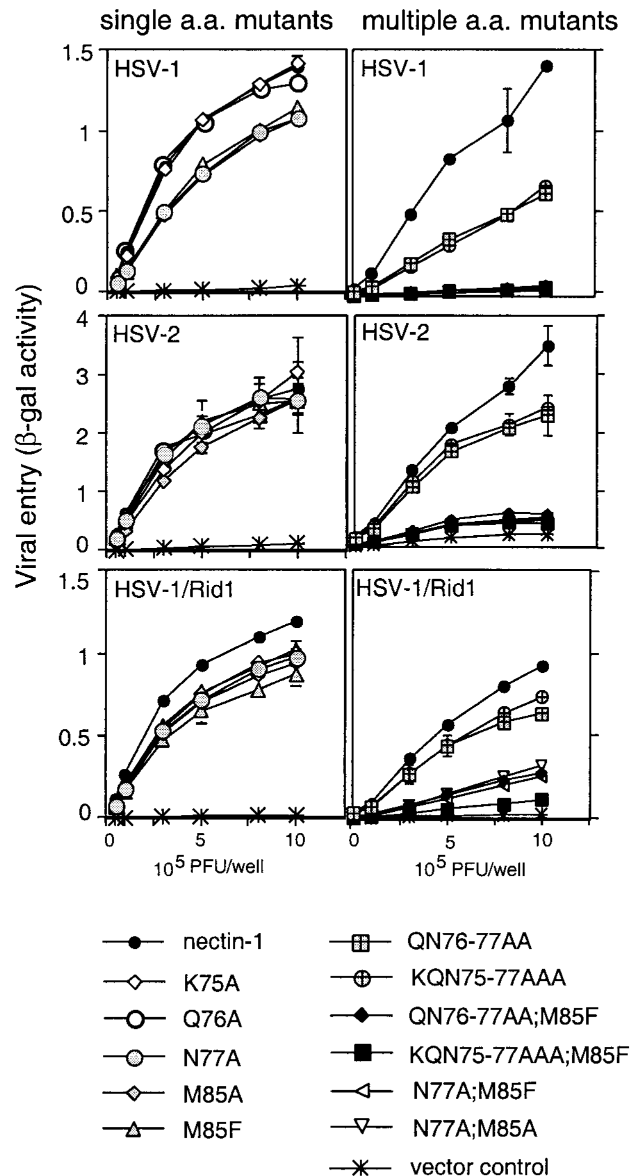

FIG. 3. HSV entry activities of wild-type nectin-1 and nectin-1 mutants. CHO-K1 cells transfected with plasmids expressing wild-type nectin-1 or nectin-1 mutants or with a control plasmid were replated on 96-well plates (2×10^4 to 4×10^4 cells per well) and exposed to serial dilutions of β-Gal reporter viruses (HSV-1, HSV-1/Rid1, and HSV-2) for 6 h. Then, the β-Gal substrate ONPG was added, and the reaction products were quantitated with a spectrophotometer. Values shown (optical density at 410 nm) are the means and standard deviations for triplicate samples. Similar results were obtained in two other experiments. a.a., amino acid.

the dose-response curve (Fig. 4). Single substitutions in amino acids 77 and 85 but not in amino acid 75 or 76 reduced HSV-1 entry activity compared to the entry activity obtained with wild-type nectin-1. Further reduction was detected with mutants containing multiple substitutions in amino acids 75 to 77, suggesting that the addition of the Q76A mutation enhanced the entry defect seen with the N77A mutant. Combining the substitution in amino acid 77 with one in amino acid 85 but not with one in amino acid 61 (outside of regions A and B) eliminated HSV-1 entry activity. The results of entry assays with PRV and BHV-1 are shown in Fig. 5. Surprisingly, none of the nectin-1 mutants was significantly impaired for the entry of PRV or BHV-1 (data are shown for representative mutants in Fig. 5).

Binding of gD to nectin-1 mutants. To assess the effects of the nectin-1 mutations on gD binding, CHO-K1 cells expressing wild-type nectin-1 or the various mutants were exposed to serial dilutions of soluble forms of gD, and binding was quantitated by a CELISA. The soluble proteins used were gD:Fc hybrids containing the gD ectodomains of HSV-1, HSV-1/Rid1, HSV-2, and BHV-1 fused to the Fc portion of rabbit

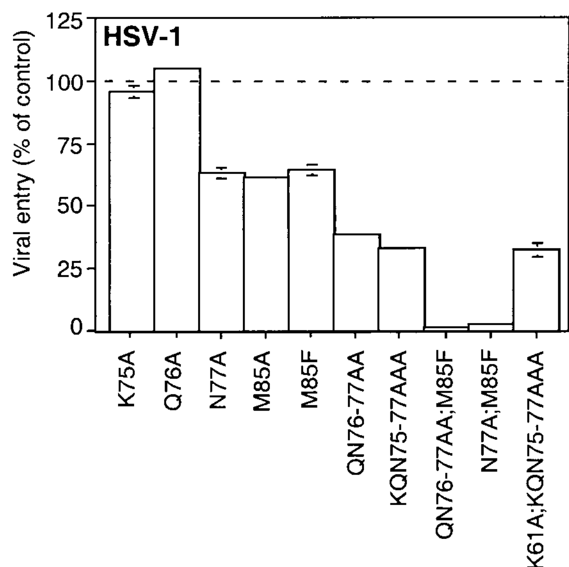

FIG. 4. HSV-1 entry activities of wild-type nectin-1 and nectin-1 mutants at a single input concentration of HSV. Data from Fig. 3 are presented to show the relative entry activities of wild-type and mutant forms of nectin-1 after exposure of the transfected cells to HSV-1 at a single input concentration of 3×10^5 PFU/well. The values obtained with the nectin-1 mutants were normalized to the value obtained with wild-type nectin-1, which was set to 100% and which is represented in the graph by a broken line. Standard deviations of triplicate determinations for each sample are shown.

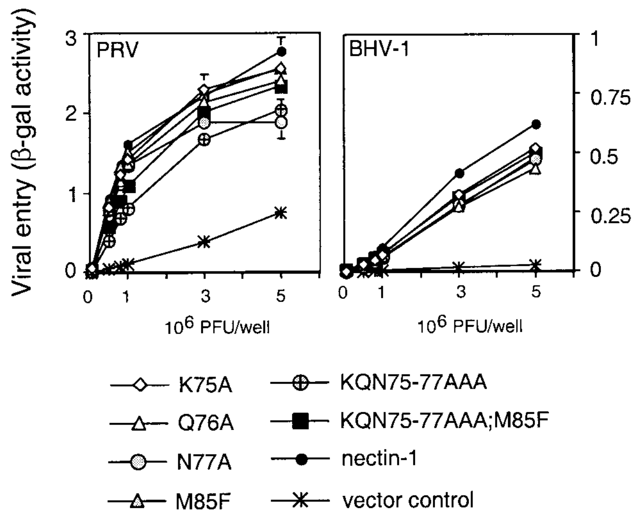

FIG. 5. Entry activities of wild-type nectin-1 and nectin-1 mutants for PRV and BHV-1. CHO-K1 cells transfected with plasmids expressing wild-type nectin-1 or nectin-1 mutants or with a control plasmid were replated on 96-well plates (2×10^4 to 4×10^4 cells per well) and exposed to serial dilutions of β-Gal reporter viruses (PRV and BHV-1) for 6 h. Then, the β-Gal substrate ONPG was added, and the reaction products were quantitated with a spectrophotometer. A representative experiment is shown. Values shown (optical density at 410 nm) are the means and standard deviations for triplicate samples.

immunoglobulin G (12). The results of a representative experiment are shown in Fig. 6.

Cells expressing nectin-1 or Q76A did not differ in their ability to bind to HSV-1 gD:Fc, HSV-1/Rid1 gD:Fc, or HSV-2 gD:Fc. Surprisingly, the rest of the mutants failed to bind to HSV-1 gD:Fc or HSV-2 gD:Fc, even though some of the receptors, such as mutants N77A, M85A, M85F, and QN76-77AA, were able to mediate HSV-1 and HSV-2 entry. This set of mutants bound to HSV-1/Rid1 gD:Fc at intermediate levels compared to the binding seen with wild-type nectin-1. Nectin-1 mutants containing substitutions in both amino acid 77 and amino acid 85 (QN76-77AA;M85F, N77A;M85A, and N77A;M85F) exhibited no significant binding to any of the HSV gD:Fc hybrids. Wild-type nectin-1 and all the nectin-1 mutants bound to BHV-1 gD:Fc at similar levels (data not shown).

The inability to detect the binding of HSV-1 or HSV-2 gD:Fc to any of the mutant receptors exhibiting partial viral entry activity is probably not due entirely to the low affinity of binding of these soluble molecules to nectin-1 or to insufficient sensitivity of the assay. The amount of HSV-2 gD:Fc required to achieve 50% binding to nectin-1-expressing cells was comparable to that for HSV-1/Rid1 gD:Fc (Fig. 6), suggesting similar affinities of interactions. Nevertheless, HSV-1/Rid1 gD:Fc exhibited intermediate levels of binding to the mutant receptors, whereas HSV-2 gD:Fc did not, despite similar effects of the mutations on the entry of the cognate viruses (Fig. 3). We cannot yet explain the all-or-none binding activities of HSV-1 gD:Fc and HSV-2 gD:Fc compared to the intermediate levels of binding observed with HSV-1/Rid1 gD:Fc. The Rid1 mutation (Q27P) must be responsible, perhaps in ways discussed below. The Rid1 mutation has been reported to increase the affinity of HSV-1 gD for nectin-1 (20), consistent with the lower concentrations of HSV-1/Rid1 gD:Fc than of HSV-1 gD:Fc required to achieve 50% binding to cells expressing nectin-1 (Fig. 6). The interactions of the soluble forms of HSV-1 gD:Fc and HSV-2 gD:Fc with the mutant receptors probably do not reflect the interactions of authentic virion-associated gD, whereas the binding of HSV-1/Rid1 gD:Fc to nectin-1 and the mutant receptors appears to correlate better with the viral entry activities of the receptors (23, 26).

DISCUSSION

We have identified two amino acids in separate regions (region A, amino acid 77; region B, amino acid 85) of the V domain of nectin-1 that are critical for gD binding and HSV entry activity. The simultaneous substitution of these amino acids resulted in mutants with dramatic decreases in gD binding and entry activities for HSV-1, HSV-2, and HSV-1/Rid1. However, single substitutions in amino acid 77 or 85 were sufficient to reduce HSV-1 and HSV-2 gD binding, even though they did not eliminate HSV entry activity (Table 1). These results suggest that weak interactions between gD and nectin-1, which probably occurred with the single substitutions, are sufficient for HSV entry activity. Another interesting finding is that substitutions of amino acids 77 and 85 did not impair entry activities for the animal alphaherpesviruses PRV and BHV-1, indicating that these viruses interact with nectin-1 in a manner different from that of HSV and that other regions of nectin-1 are probably more critical for the binding of PRV and BHV-1 gDs. Thus, although the binding sites for HSV-1 and

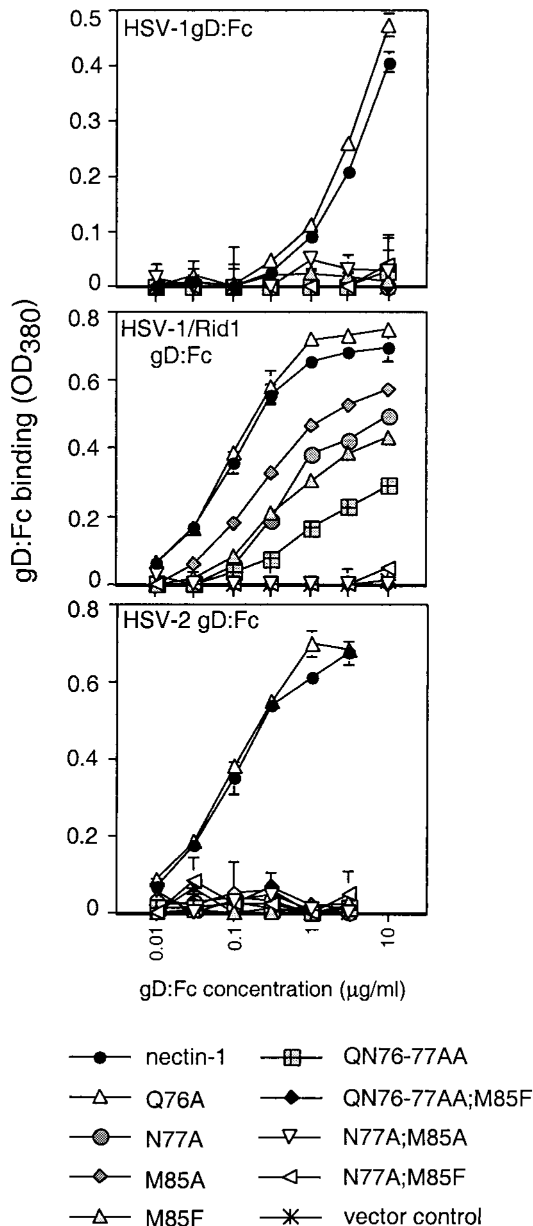

FIG. 6. Binding of soluble gD molecules to wild-type nectin-1 and nectin-1 mutants. CHO-K1 cells transfected with plasmids expressing nectin-1 mutants or wild-type nectin-1 or with a control plasmid were replated on 96-well plates. The cells were incubated with serial dilutions of culture supernatants containing the various gD:Fc molecules, at the concentrations indicated, for 30 min and fixed. Binding was detected with a biotinylated secondary antibody followed by streptavidin-horseradish peroxidase. Values shown (optical density at 380 nm [OD_{380}]) are the means and standard deviations of triplicate determinations.

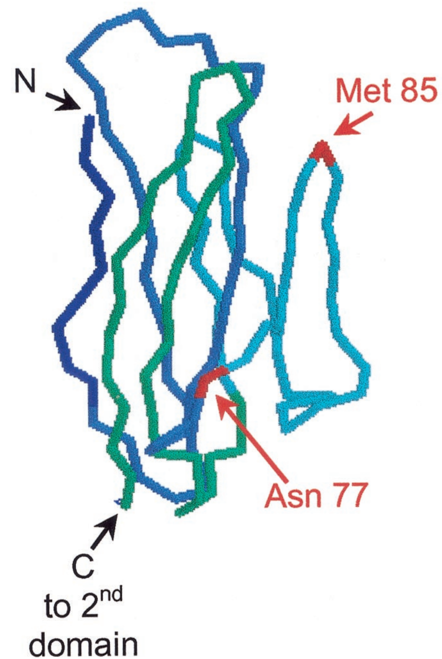

FIG. 7. Locations of the critical mutations on a model of the V domain of nectin-1. The model presented is based on the model of the poliovirus receptor, CD155, deposited in the Brookhaven Protein Database as entry 1DGI (16). The backbone trace of the V domain is colored from dark blue at the N terminus (N) to green at the C terminus (C), which is connected to the second immunoglobulin-like domain. Red coloring denotes the predicted locations of Met85 and Asn77. Amino acid substitutions at both of these positions significantly reduced entry activities for HSV strains but not for PRV or BHV-1.

PRV gDs on nectin-1 appear to overlap, based on competitive binding experiments (11, 28), they cannot be identical.

Amino acids 77 and 85 are predicted to lie in loops flanking the putative C′ β strand of the V domain, based on the homology of nectin-1 to CD155, the poliovirus receptor (3, 16) (Fig. 7). The corresponding C′C″ loop and, specifically, the amino acid localized to the position homologous to that of Met85 of nectin-1 in CD4 and in the poliovirus receptor have been shown to be critical for interactions with human immunodeficiency virus and poliovirus, respectively (1, 4, 31, 33). Thus, homologous regions in members of the immunoglobulin superfamily that serve as virus receptors seem to be important for interactions with viral entry proteins. A previous study of nectin-1 made use of a chimeric approach to identify a larger region encompassing the C′C″ loop as being important for gD interactions and HSV-1 entry (8). Substitution of the corresponding sequences in CD155 with amino acids 64 to 94 of nectin-1 was sufficient to transfer HSV-1 gD binding and entry activity. However, smaller regions or specific amino acids involved in gD interactions were not identified. A smaller substitution (amino acids 77 to 94 of nectin-1 replacing the corresponding sequences of CD155) resulted in greatly reduced HSV-1 gD binding and entry activity, even though it contained amino acids 77 and 85. Most likely the CD155 structure into which amino acids 77 to 94 were inserted does not allow amino acids 77 and 85 and adjacent sequences to assume the appropriate conformation for interactions with HSV gD.

The construction of nectin-1 mutants in this study was based on the results of previous studies with nectin-2 in which the amino acids in homologous positions were found to be crucial for HSV entry activity. However, in contrast to the results obtained with the M85F substitution in nectin-1, the corresponding mutation (M89F) in nectin-2 by itself was sufficient to eliminate HSV entry activity (19). This finding is in keeping

with the hypothesis that the interaction of nectin-1 with gD is of a higher affinity than that of nectin-2 with gD (22). Amino acid substitutions that merely reduce the affinity of gD binding to nectin-1 may eliminate binding to nectin-2. We have been unable to detect gD–nectin-2 binding by a CELISA, and others have detected only weak binding of soluble nectin-2 to gD by an ELISA under conditions that readily reveal the binding of nectin-1 to gD (22, 23). Thus, although homologous regions in nectin-1 and nectin-2 are critical for interactions with HSV gD, the affinities and natures of the interactions differ.

Although the nectin-1 mutations described here had similar effects on the entry of all three HSV strains tested, they had much different effects on the binding of the cognate soluble gD:Fc hybrids to cells expressing the receptors. The range of binding observed with HSV-1/Rid1 gD:Fc correlated best with the entry activities of the wild-type and mutant forms of nectin-1 (Fig. 3 and 6). Why did the HSV-1 and HSV-2 gD:Fc hybrids fail to bind to most of the receptor mutants? One possible explanation is based on the reported effects of the Rid1 mutation. The dissociation constant for the binding of a soluble form of HSV-1/Rid gD (N-terminal 306 amino acids) to the nectin-1 ectodomain was determined by surface plasmon resonance (20) to be about 20-fold lower (0.17×10^{-6} M) than that for the binding of soluble wild-type HSV-1 gD (3.2×10^{-6} M). This increase in affinity conferred by the Rid1 mutation (Q27P) was due entirely to a higher on rate for the interaction. In the same study (20), deletion of 21 amino acids from the C terminus of the 306-amino-acid soluble form of gD was shown to have even larger effects of a decreased dissociation constant (and thus an increased affinity for nectin-1) and an increased rate of association. Thus, regions of gD both N-terminal to and C-terminal to a domain critical for stable binding to nectin-1 (encompassing amino acids 77 and 85) influence negatively the rate of association for wild-type gD. Given that the N terminus of HSV-1 gD can assume multiple conformations (6), it seems possible that stable binding to nectin-1 requires a change in the conformation of the N terminus for wild-type gD but not for the Rid1 form of gD, consistent with the differences in the rates of association. This scenario implies that the wild-type form of nectin-1, but none of the receptor mutants except for Q76A, can induce this change for wild-type forms of HSV-1 gD:Fc and HSV-2 gD:Fc, whereas the receptor mutants must have some activity in this regard for authentic gD in virions, perhaps because the C terminus is tethered. The unexpected results obtained from quantitation of the binding of the various forms of gD:Fc hybrids to the various nectin-1 mutants may reflect artifactual folding of some of these soluble hybrid proteins but also demand further exploration of dynamic changes in gD and receptors that are required for stable gD–nectin-1 binding and the triggering of membrane fusion.

Interactions between HSV virions (or other alphaherpesviruses) and the cell surface are complex, involving multiple ligand-receptor interactions (40). The binding of alphaherpesvirus virions to cells is usually mediated by interactions of gC or gB with cell surface heparan sulfate, interactions that are probably of a higher affinity (45) than the binding of gD to one of its receptors (20, 46). Subsequent interactions of gD with a receptor may stabilize virus binding so that washes with glycosaminoglycan can no longer elute the virus (17, 24), but gD cannot very efficiently mediate the initial virus binding (43). However, once the virus is bound via attachment to heparan sulfate, the binding of gD to one of its receptors would be facilitated and need not require a high affinity. Also, the relatively high abundance of gD in virions probably allows for binding to receptors with increased avidity (15). Our results confirm the observation that relatively weak interactions between gD and receptors are sufficient to trigger subsequent steps in the entry process (25).

The finding that none of the nectin-1 mutants was impaired for the entry of PRV and BHV-1 was unexpected. Soluble PRV gD can compete with HSV-1 gD for binding to nectin-1 (11, 28). Also, antibodies against nectin-1 that block HSV-1 entry also block PRV entry (28). In addition, the coexpression of HSV, PRV, or BHV-1 gD with nectin-1 can interfere with the use of nectin-1 for entry by any of these alphaherpesviruses (7, 13, 34). These studies indicated that the binding sites on nectin-1 for PRV, BHV-1, and HSV-1 gDs are similar or overlap or that the binding of one gD causes a conformational change in the receptor that prevents the binding of another gD. Our studies demonstrate that regions in nectin-1 important for HSV and PRV or BHV-1 entry are distinct even though they may overlap. The results of previous studies with nectin-2 chimeras also support this hypothesis, since substitutions in region B resulted in decreased entry activity for HSV-1/Rid but not for PRV (23). Further studies of nectin-1 are required to identify other structural determinants important for alphaherpesvirus entry activity.

Nectin-1 is a cell adhesion molecule that localizes to cell junctions. Previous studies have reported that soluble HSV-1 gD can inhibit cell adhesion mediated by nectin-1, presumably by competing for regions important for transhomophilic interactions (19, 36). Experiments in our laboratory are ongoing to determine the structural requirements of nectin-1 and nectin-2 for viral entry activity and for cell-cell adhesion in order to determine whether these activities are influenced by similar or different regions of the V domain. The identification of mutations in nectin-1 important for HSV entry activity sheds light on the molecular interactions between HSV and nectin-1 and may also aid studies geared toward the production of antiviral agents that target the entry process.

ACKNOWLEDGMENTS

We thank N. Susmarski, M. L. Parish, and A. Fridberg for technical assistance and C. Rowe, G. Cohen, R. Eisenberg, and M. Lopez for reagents.

This work was supported by grant R37 AI36293 from the National Institute of Allergy and Infectious Diseases. W.M.M. was supported by Public Health Service fellowship F31 GM19765.

REFERENCES

1. **Arthos, J., K. C. Deen, M. A. Chaikin, J. A. Fornwald, G. Sathe, Q. J. Sattentau, P. R. Clapham, R. A. Weiss, J. S. McDougal, C. Pietropaolo, R. Axel, A. Truneh, P. J. Maddon, and S. R. W.** 1989. Identification of the residues in human CD4 critical for the binding of HIV. Cell **57:**469–481.
2. **Babic, N., B. G. Klupp, B. Makoschey, A. Karger, A. Flamand, and T. C. Mettenleiter.** 1996. Glycoprotein gH of pseudorabies virus is essential for penetration and propagation in cell culture and in the nervous system of mice. J. Gen. Virol. **77:**2277–2285.
3. **Belnap, D. M., B. M. McDermott, Jr., D. J. Filman, N. Cheng, B. L. Trus, H. J. Zuccola, V. R. Racaniello, J. M. Hogle, and A. C. Steven.** 2000. Three-dimensional structure of poliovirus receptor bound to poliovirus. Proc. Natl. Acad. Sci. USA **97:**73–78.
4. **Bernhardt, G., J. Harber, A. Zibert, M. deCrombrugghe, and E. Wimmer.** 1994. The poliovirus receptor: identification of domains and amino acid residues critical for virus binding. Virology **203:**344–356.

5. **Campadelli-Fiume, G., F. Cocchi, L. Menotti, and M. Lopez.** 2000. The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells. Rev. Med. Virol. **10:**305–319.
6. **Carfi, A., S. H. Willis, J. C. Whitbeck, C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and D. C. Wiley.** 2001. Herpes simplex virus glycoprotein D bound to the human receptor HveA. Mol. Cell **8:**169–179.
7. **Chase, C. C. L., K. Carter-Allen, C. Lohff, and G. J. Letchworth.** 1990. Bovine cells expressing bovine herpesvirus 1 (BHV-1) glycoprotein IV resist infection by BHV-1, herpes simplex virus, and pseudorabies virus. J. Virol. **64:**4866–4872.
8. **Cocchi, F., M. Lopez, P. Dubreuil, G. Campadelli-Fiume, and L. Menotti.** 2001. Chimeric nectin 1-poliovirus receptor molecules identify a nectin 1 region functional in herpes simplex virus entry. J. Virol. **75:**7987–7994.
9. **Cocchi, F., M. Lopez, L. Menotti, M. Aoubala, P. Dubreuil, and G. Campadelli-Fiume.** 1998. The V domain of herpesvirus Ig-like receptor (HIgR) contains a major functional region in herpes simplex virus-1 entry into cells and interacts physically with the viral glycoprotein D. Proc. Natl. Acad. Sci. USA **95:**15700–15705.
10. **Cocchi, F., L. Menotti, P. Mirandola, M. Lopez, and G. Campadelli-Fiume.** 1998. The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. J. Virol. **72:**9992–10002.
11. **Connolly, S. A., J. C. Whitbeck, A. H. Rux, C. Krummenacher, S. Van Drunen Littel-van den Hurk, G. H. Cohen, and R. J. Eisenberg.** 2001. Glycoprotein D homologs in herpes simplex virus type 1, pseudorabies virus, and bovine herpes virus type 1 bind directly to human HveC (Nectin-1) with different affinities. Virology **280:**7–18.
12. **Geraghty, R. J., A. Fridberg, C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and P. G. Spear.** 2001. Use of chimeric nectin-1(HveC)-related receptors to demonstrate that ability to bind alphaherpesvirus gD is not necessarily sufficient for viral entry. Virology **285:**366–375.
13. **Geraghty, R. J., C. R. Jogger, and P. G. Spear.** 2000. Cellular expression of alphaherpesvirus gD interferes with entry of homologous and heterologous alphaherpesviruses by blocking access to a shared gD receptor. Virology **268:**147–158.
14. **Geraghty, R. J., C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and P. G. Spear.** 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science **280:**1618–1620.
15. **Handler, C. G., R. J. Eisenberg, and G. H. Cohen.** 1996. Oligomeric structure of glycoproteins in herpes simplex virus type 1. J. Virol. **70:**6067–6075.
16. **He, Y., V. D. Bowman, S. Mueller, C. M. Bator, J. Bella, X. Peng, T. S. Baker, E. Wimmer, R. J. Kuhn, and M. G. Rossmann.** 2000. Interaction of the poliovirus receptor with poliovirus. Proc. Natl. Acad. Sci. USA **97:**79–84.
17. **Karger, A., and T. C. Mettenleiter.** 1993. Glycoproteins gIII and gp50 play dominant roles in the biphasic attachment of pseudorabies virus. Virology **194:**654–664.
18. **Krummenacher, C., I. Baribaud, M. Ponce de Leon, J. C. Whitbeck, H. Lou, G. H. Cohen, and R. J. Eisenberg.** 2000. Localization of a binding site for herpes simplex virus glycoprotein D on herpesvirus entry mediator C by using antireceptor monoclonal antibodies. J. Virol. **74:**10863–10872.
19. **Krummenacher, C., I. Baribaud, J. F. Sanzo, G. H. Cohen, and R. J. Eisenberg.** 2002. Effects of herpes simplex virus on structure and function of nectin-1/HveC. J. Virol. **76:**2424–2433.
20. **Krummenacher, C., A. H. Rux, J. C. Whitbeck, M. Ponce de Leon, H. Lou, I. Baribaud, W. Hou, C. Zou, R. J. Geraghty, P. G. Spear, R. J. Eisenberg, and G. H. Cohen.** 1999. The first immunoglobulin-like domain of HveC is sufficient to bind herpes simplex virus glycoprotein D with full affinity, while the third domain is involved in oligomerization of HveC. J. Virol. **73:**8127–8137.
21. **Lopez, M., M. Aoubala, F. Jordier, D. Isnardon, S. Gomez, and P. Dubreuil.** 1998. The human poliovirus receptor related 2 protein is a new hematopoietic/endothelial homophilic adhesion molecule. Blood **92:**4602–4611.
22. **Lopez, M., F. Cocchi, L. Menotti, E. Avitabile, P. Dubreuil, and G. Campadelli-Fiume.** 2000. Nectin2α (PRR2α or HveB) and nectin2δ are low-efficiency mediators for entry of herpes simplex virus mutants carrying the Leu25Pro substitution in glycoprotein D. J. Virol. **74:**1267–1274.
23. **Martinez, W. M., and P. G. Spear.** 2001. Structural features of nectin-2 (HveB) required for herpes simplex virus entry. J. Virol. **75:**11185–11195.
24. **McClain, D. S., and A. O. Fuller.** 1994. Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. Virology **198:**690–702.
25. **Menotti, L., E. Avitabile, P. Dubreuil, M. Lopez, and G. Campadelli-Fiume.** 2001. Comparison of murine and human nectin-1 binding to herpes simplex virus glycprotein D (gD) reveals a weak interaction of murine nectin-1 to gD and a gD-dependent pathway of entry. Virology **282:**256–266.
26. **Menotti, L., M. Lopez, E. Avitabile, A. Stefan, F. Cocchi, J. Adelaide, E. Lecocq, P. Dubreuil, and G. Campadelli-Fiume.** 2000. The murine homolog of human nectin1δ serves as a species nonspecific mediator for entry of human and animal αherpesviruses in a pathway independent of a detectable binding to gD. Proc. Natl. Acad. Sci. USA **97:**4867–4872.
27. **Miller, J. M., C. A. Whetstone, L. J. Bello, W. C. Lawrence, and J. C. Whitbeck.** 1995. Abortion in heifers inoculated with a thymidine kinase-negative recombinant of bovine herpesvirus 1. Am. J. Vet. Res. **56:**870–874.
28. **Milne, R. S. B., S. A. Connolly, C. Krummenacher, R. J. Eisenberg, and G. H. Cohen.** 2001. Porcine HveC, a member of the highly conserved HveC/nectin 1 family, is a functional alphaherpesvirus receptor. Virology **281:**315–328.
29. **Miyahara, M., H. Nakanishi, K. Takahashi, K. Satoh-Horikawa, K. Tachibana, and Y. Takai.** 2000. Interaction of nectin with afadin is necessary for its clustering at cell-cell contact sites but not for its *cis*dimerization or *trans*interaction. J. Biol. Chem. **275:**613–618.
30. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87:**427–436.
31. **Morrison, M. E., Y.-J. He, M. W. Wien, J. M. Hogle, and V. R. Racaniello.** 1994. Homolog-scanning mutagenesis reveals poliovirus receptor residues important for virus binding and replication. J. Virol. **68:**2578–2588.
32. **Pertel, P. E., and P. G. Spear.** 1996. Modified entry and syncytium formation by herpes simplex virus type 1 mutants selected for resistance to heparin inhibition. Virology **226:**22–23.
33. **Peterson, A., and B. Seed.** 1988. Genetic analysis of monoclonal antibody and HIV binding sites on the human lymphocyte antigen CD4. Cell **54:**65–72.
34. **Petrovskis, E. A., A. L. Meyer, and L. E. Post.** 1988. Reduced yield of infectious pseudorabies virus and herpes simplex virus from cell lines producing viral glycoprotein gp50. J. Virol. **62:**2196–2199.
35. **Reymond, N., S. Fabre, E. Lecocq, J. Adelaide, P. Dubreuil, and M. Lopez.** 2001. Nectin4/PRR4, a new afadin-associated member of the nectin family that trans-interacts with nectin1/PRR1 through V domain interaction. J. Biol. Chem. **276:**43205–43215.
36. **Sakisaka, T., T. Taniguchi, H. Nakanishi, K. Takahashi, M. Miyahara, W. Ikeda, S. Yokoyama, Y. F. Peng, K. Yamanishi, and Y. Takai.** 2001. Requirement of interaction of nectin-1α/HveC with afadin for efficient cell-cell spread of herpes simplex virus type 1. J. Virol. **75:**4734–4743.
37. **Satoh-Horikawa, K., H. Nakanishi, K. Takahashi, M. Miyahara, M. Nishimura, K. Tachibana, A. Mizoguchi, and Y. Takai.** 2000. Nectin-3, a new member of immunoglobulin-like cell adhesion molecules that shows homophilic and heterophilic cell-cell adhesion activities. J. Biol. Chem. **275:**10291–10299.
38. **Shukla, D., M. Dal Canto, C. L. Rowe, and P. G. Spear.** 2000. Striking similarity of murine nectin-1α to human nectin-1α (HveC) in sequence and activity as a glycoprotein D receptor for alphaherpesvirus entry. J. Virol. **74:**11773–11781.
39. **Spear, P. G.** 1993. Entry of alphaherpesviruses into cells. Semin. Virol. **4:**167–180.
40. **Spear, P. G., R. J. Eisenberg, and G. H. Cohen.** 2000. Three classes of cell surface receptors for alphaherpesvirus entry. Virology **275:**1–8.
41. **Tachibana, K., H. Nakanishi, K. Mandai, K. Ozaki, W. Ikeda, Y. Yamamoto, A. Nagafuchi, S. Tsukita, and Y. Takai.** 2000. Two cell adhesion molecules, nectin and cadherin, interact through their cytoplasmic domain-associated proteins. J. Cell Biol. **150:**1161–1175.
42. **Takahashi, K., H. Nakanishi, M. Miyahara, K. Mandai, K. Satoh, A. Satoh, H. Nishioka, J. Aoki, A. Nomoto, A. Mizoguchi, and Y. Takai.** 1999. Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with afadin, a PDZ domain-containing protein. J. Cell Biol. **145:**539–549.
43. **Terry-Allison, T., R. I. Montgomery, M. S. Warner, R. J. Geraghty, and P. G. Spear.** 2001. Contributions of gD receptors and glycosaminoglycan sulfation to cell fusion mediated by herpes simplex virus 1. Virus Res. **74:**39–45.
44. **Warner, M. S., R. J. Geraghty, W. M. Martinez, R. I. Montgomery, J. C. Whitbeck, R. Xu, R. J. Eisenberg, G. H. Cohen, and P. G. Spear.** 1998. A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2 and pseudorabies virus. Virology **246:**179–189.
45. **Williams, R. K., and S. E. Straus.** 1997. Specificity and affinity of binding of herpes simplex virus type 2 glycoprotein B to glycosaminoglycans. J. Virol. **71:**1375–1380.
46. **Willis, S. H., A. J. Rux, C. Peng, J. C. Whitbeck, A. V. Nicola, H. Lou, W. Hou, L. Salvador, R. J. Eisenberg, and G. H. Cohen.** 1998. Examination of the kinetics of herpes simplex virus glycoprotein D binding to the herpesvirus entry mediator, using surface plasmon resonance. J. Virol. **72:**5937–5947.