Crystal Structure of Herpes Simplex Virus 2 gD Bound to Nectin-1 Reveals a Conserved Mode of Receptor Recognition

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ABSTRACT

Herpes simplex virus 1 (HSV-1) and HSV-2 are among the most prevalent human pathogens. Both viruses can recognize, via the surface envelope glycoprotein D (gD), human nectin-1 as a functional receptor. Previous studies have successfully elucidated the molecular basis of the binding between HSV-1 gD and nectin-1 by cocrystallography. Despite a high sequence identity between HSV-1 and HSV-2 gDs, the atomic intermolecule details for the HSV-2-gD/nectin-1 interaction remain elusive. Here, we report the crystal structures of both the unbound and the nectin-1-bound HSV-2 gDs. The free-gD structure expectedly comprises an IgV-like core and the surface-exposed terminal extensions as observed in its HSV-1 counterpart but lacks traceable electron densities for a large portion of the terminal elements. These terminal residues were clearly traced in the complex structure as a definitive loop in the N terminus and an α -helix in the C terminus, thereby showing a conserved nectin-1-binding mode as reported for HSV-1 gD. The interface residues in nectin-1 were further mutated and tested for the gD interaction by surface plasmon resonance. The resultant binding patterns were similar for HSV-1 and HSV-2 gDs, further supporting a homologous receptor-binding basis by the two viruses for nectin-1. These data, together with a cell-based fusion assay showing a cross-inhibition of the gD/nectin-1-mediated cell-cell fusion by soluble HSV-1 and HSV-2 gDs, provided solid structural and functional evidence that HSV-1 and HSV-2 recognize nectin-1 via the same binding mode. Finally, we also demonstrated that nectin-1 I80 is an important residue involved in gD interaction.

IMPORTANCE

Despite intensified studies, a detailed picture of the molecular features in the HSV-2-gD/nectin-1 interaction remains unavailable. Previous work focused on HSV-1 gD, which folds into an IgV-like core with large terminal extensions and utilizes the extension elements to engage nectin-1. Here, we report the crystal structures of HSV-2 gD in both the free and the nectin-1-bound forms. The atomic intermolecule details for HSV-2-gD/nectin-1 interaction are clearly presented. The observed binding mode is identical to that reported for its HSV-1 counterpart. This structural observation was further supported by our comparative functional assays showing that nectin-1 mutations similarly affect the ligand-receptor interaction of both virus gDs. Taken together, we provide comprehensive structural and functional data demonstrating a conserved receptor-binding mode between HSV-1 and HSV-2 for nectin-1. Our results also indicate that the tropism difference between the two viruses likely arises from aspects other than the gD/nectin-1 binding features.

Herpes simplex virus (HSV) belongs to the *Alphaherpesvirus* subfamily in the *Herpesviridae* family (1). Two HSV types have been identified thus far, including HSV-1 and HSV-2, both of which can infect humans. The two viruses can infect neurons and thereby cause neurological diseases. They can also establish latency in sensory neurons and result in the infected patient being a lifelong virus carrier (2). Although HSV infections are usually asymptomatic, intermittent reactivation of the latent viruses can lead to severe diseases and sometimes be life-threatening in immunocompromised individuals (1). The asymptomatic nature of HSV infection is also considered to facilitate the virus spread in the population (3). HSV-1 primarily causes oral and ocular lesions and is also related to genital infections, though less frequently (4, 5). HSV-2 causes mainly genital tissue damages (5, 6).

HSV infection starts with the virus entry into susceptible host cells (1, 7). This entry process is a complicated set of multiple events that involve orchestrated interplays between the glycoproteins embedded in the viral envelope and the host surface molecules and receptors (7–9). The current thesis indicates the involve-

ment of at least 5 (of 11) virus envelope glycoproteins in HSV entry, including glycoproteins B, C, D, H, and L (gB, gC, gD, gH, and gL) (7–9). The initial attachment of the virus particles on the cell surface could be mediated by gB or gC, which can recognize sugar moieties like heparan sulfate proteoglycan (HSPG) (10, 11). This is normally followed by a specific interaction between gD and its canonical receptors (12, 13). The final entry step of membrane fusion, then, occurs at either the plasma (14) or the endosome (15) membrane, mediated by an activated core fusion machinery

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composed of gB and gH/gL (16–19). In this cascade of events, the gD-receptor interaction is suggested as a key factor for successful virus entry. In addition to tethering virus particles onto cells expressing specific gD receptors, it is also believed that gD binding to receptor would displace its C-terminal profusogenic domain (PFD), thereby activating the gB/gH/gL fusion executor (20–22). Furthermore, gD is also an important contributing factor to a broad cell-type tropism of HSV by engaging diversified cellular receptors (23). Thus far, a series of host molecules have been reported to be recognized by HSV gD. These include herpesvirus entry mediator (HVEM) (24), nectin-1 (25), nectin-2 (26), and 3-O-sulfonated-heparan sulfate (3-O-S-HS) (27), of which nectin-1 plays an indispensable role in the infection and pathogenesis of both HSV-1 and HSV-2 (28–30).

Nectin-1 belongs to the Ca^{2+} -independent cell adhesion molecule family and functions via homo- or heterodimerization in the formation of adherent junctions (31). The molecule also serves as a functional receptor for both HSV-1 and HSV-2 (12) and is shown to play a more determinant role than other gD receptors (e.g., HVEM) in HSV-2 infection (28, 29). With a truncated gD molecule (spanning residues 1 to 285), which according to previous reports (32–34) shows a much higher affinity for receptors than the longer gD form (spanning residues 1 to 306), the molecular basis of the binding between HSV-1 gD and nectin-1 has been successfully elucidated via costructures (35, 36). It was revealed that the gD molecule contains a V-set immunoglobulin-like (IgV-like) core that is wrapped by large terminal extensions and utilizes the extensive N- and C-terminal extension elements to engage nectin-1 (35, 36). This forms a striking contrast to the binding mode of HSV-1 gD with HVEM, which shows the utilization of an N-terminal hairpin in gD for receptor binding (37). Nevertheless, either way of receptor engagement by HVEM or nectin-1 should displace a “prelocated” C-terminal loop in gD as observed in a previous dimeric structure (38). This characteristic structure is consistent with earlier functional analyses showing that the exposure of the gD PFD is a key step in triggering fusion (20, 21). The current structural investigations, however, are almost exclusively based on the HSV-1 gD protein. Inspired by the high sequence identity (~85%) between HSV-1 and HSV-2 gDs, there has been functional evidence showing that the two viral proteins exhibit similar affinities for nectin-1 (39) and are interchangeable in virus infection (40) and entry inhibition (41). In addition, a recent study reported a complex structure between HSV-2 gD and a human monoclonal antibody, E317, which reveals quite similar structures for HSV-1 and HSV-2 gD proteins (42). These results point to a homologous nectin-1-binding mode of the two viruses. Nevertheless, the molecular basis on the HSV-2 gD recognition by nectin-1 remains elusive, despite an indispensable role of nectin-1 in HSV-2 infection.

In this study, we present the atomic intermolecule details for HSV-2-gD/nectin-1 interaction via the crystal structures of HSV-2 gD in both the free and the receptor-bound forms. We further conduct a thorough mutational analysis characterizing the binding properties of nectin-1 with both HSV-1 and HSV-2 gDs. A conserved receptor binding mode between the two viruses for nectin-1 was solidly confirmed. In addition, we also demonstrated that nectin-1 I80 acts as an important interface residue for gD interaction, in addition to previously identified key amino acids, including N77, M85, and F129 (36, 43, 44).

MATERIALS AND METHODS

Preparation of the proteins. The soluble proteins of HSV-1 gD and nectin-1 were prepared as previously described (35), and HSV-2 gD (the gene of HSV-2 gD was a kind gift from Peng Tao at Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences) was prepared similarly to HSV-1 gD. In brief, the HSV-1 (strain KOS) and HSV-2 (strain 333) gD ectodomain proteins (residues 1 to 285) were expressed in Hi5 cells using recombinant baculoviruses. The GP67 signal sequence was engineered to the N terminus of gD to facilitate protein secretion, and a C-terminal hexa-His tag was used for affinity chromatography purification. The pooled gD proteins from the Ni-nitrilotriacetic acid (Ni-NTA) column were further purified by gel filtration using a Superdex 200 (GE) column.

The nectin-1 ectodomain protein (residues 30 to 335) was obtained by refolding of the *Escherichia coli*-expressed inclusion bodies, which were first added into a buffer consisting of 100 mM Tris (pH 9.0), 400 mM L-arginine, 2 mM EDTA, 4 M urea, 5 mM reduced glutathione, and 1 mM oxidized glutathione and then sequentially dialyzed against water and a buffer containing 10 mM Tris (pH 9.0), and 10 mM NaCl. The refolded protein was then concentrated and loaded onto a Superdex 200 column for further purification.

Comparative mutagenesis study by surface plasmon resonance (SPR). A QuikChange site-directed mutagenesis kit was used to obtain the nectin-1-expressing plasmids with the indicated mutations. The mutant proteins were prepared using the same refolding and purification procedure as described for the wild-type (WT) nectin-1. All the purified proteins were then exchanged to a buffer containing 10 mM HEPES (pH 8.0), 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20, concentrated to approximately 4 mg/ml, and stored at 4°C for further use.

The binding assay was carried out with a BIAcore 3000 system (GE Healthcare). The gD protein (HSV-1 or HSV-2) was immobilized on CM5 chips at ~750 response units using a standard amine coupling method and was then tested for the binding by 1 μM the individual nectin-1 proteins (WT or mutant) at room temperature. The amount of bound protein in response units was recorded for comparison.

Kinetic study. The kinetic analysis was performed by SPR using the BIAcore 3000 system. Gradient concentrations of HSV-1 or HSV-2 gD protein were flowed at 30 $\mu\text{L}/\text{min}$ over nectin-1 (WT or mutant), immobilized at ~750 response units, and tested for binding at 25°C. The running buffer is composed of 10 mM HEPES (pH 8.0), 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20. In each run, 10 mM NaOH was used to regenerate the chip. The binding data were fitted using the 1:1 Langmuir binding model or the steady-state affinity model for dissociation constant (K_d) calculations. The calculated kinetic parameters are summarized in Table 1.

Crystallization and data collection and processing. The crystallization and diffraction data collection for free HSV-2 gD have been described previously (45). To obtain HSV-2 gD/nectin-1 complex crystals, the gD and nectin-1 proteins were mixed at a 3:1 molar ratio and then used to screen the Hampton Research kits via the vapor diffusion sitting drop method. The conditions were then optimized. Diffraction crystals were finally obtained by mixing 1 μL of the protein preparation (3 mg/ml) with 1 μL reservoir solution consisting of 0.2 M sodium chloride, 0.1 M sodium cacodylate (pH 6.0), and 8% (wt/vol) polyethylene glycol (PEG) 8000, followed by incubation at 18°C for about 2 weeks.

The diffraction data of the complex crystal were collected at beamline BL17U of the Shanghai Synchrotron Radiation Facility (SSRF) using the synchrotron radiation at 100 K. The cryoprotectant solution is composed of 15% (vol/vol) glycerol and 85% (vol/vol) reservoir solution. Data were then processed with HKL2000 (46) for indexing, integration, and scaling.

Structure determination. Via molecular replacement using the PHASER (47) program in the CCP4 suite (48), the free HSV-2 gD and the nectin-1-bound complex structures were solved with a free HSV-1 gD structure (PDB code 1L2G, chain A) and an HSV-1-gD/nectin-1 complex structure (PDB code 3U82) as the search models, respectively. The built

TABLE 1 Kinetic statistics of the SPR assay^a

Binding pair	K_{on} ($M^{-1} s^{-1}$)	K_{off} (s^{-1})	K_d (M)	χ^2/RU_{max}^c	Model
HSV-1 gD to nectin-1/WT	5.7×10^5	7.11×10^{-3}	1.25×10^{-8}	0.894/24.2	Langmuir
HSV-1 gD to nectin-1/I80A	— ^b	—	1×10^{-6}	5.44/55.5	Steady-state affinity
HSV-1 gD to nectin-1/tetra-TQEN	1.13×10^6	2.57×10^{-3}	2.27×10^{-9}	2.28/46.3	Langmuir
HSV-2 gD to nectin-1/WT	5.45×10^5	1.04×10^{-2}	1.91×10^{-8}	0.42/24.6	Langmuir
HSV-2 gD to nectin-1/I80A	—	—	1.47×10^{-6}	2.99/46	Steady-state affinity
HSV-2 gD to nectin-1/tetra-TQEN	8.25×10^5	6.44×10^{-3}	7.81×10^{-9}	1.8/47	Langmuir

^a The slow-on/slow-off kinetic data were analyzed by the 1:1 Langmuir binding model. The values of association rate (K_{on}), dissociation rate (K_{off}), and the subsequent dissociation constant (K_d) were calculated and are listed.
^b —, fast-on/fast-off kinetics for which the K_{on} s and K_{off} s cannot be calculated; the steady-state model was used for the K_d determination.
^c The goodness of fit between the experimental data and the calculated model was characterized by the chi-square (χ^2) value, which is less than 1/10 of the maximum response unit (RU_{max}).

structure models were further refined using REFMAC (49) and Phenix (50) and manually adjusted in Coot (51) for several cycles. In each cycle, the stereochemistry of the model was monitored by Procheck (52). The final data processing and structure refinement statistics are summarized in Table 2.

Cell fusion blocking assay. The fusion mediated by HSV gB/gD/gH/gL and the receptors has been validated in various cell types (21, 53). In this study, we set up a cell-based fusion system using CHO-K1 cells as previously reported (53). In brief, the genes of HSV-1 gB, gD, gH, and gL, HSV-2 gD, and human nectin-1 were cloned into the pCDNA4.0-myc-his vector to yield the respective plasmids for protein expression in mammalian cells. The T7 polymerase and T7 luciferase expression plasmids were constructed previously in our lab. The expressing plasmids for HSV-1 gB, gH, gL, HSV-1 or HSV-2 gD, and the T7 luciferase and the plasmids for nectin-1 and the T7 polymerase were separately cotransfected into CHO-K1 cells using Lipo2000 (Invitrogen) according to the manufacturer's instructions. Twelve hours posttransfection, the nectin-1-expressing cells were first incubated for 2 h with gradient concentrations (0.1, 0.5, and 2.5 μ M) of soluble gD proteins of HSV-1 or HSV-2 or hNUDT16 (an irrelevant protein that was prepared as previously described [54]) and then mixed with the cells expressing gB/gD/gH/gL and further incubated for 6 h for cell fusion. The luciferase activity was tested using a luciferase assay system kit bought from Promega.

The virus entry and entry inhibition assays. A genetically modified HSV-1 virus with a luciferase gene has been described previously (55). For the virus entry assay, the WT nectin-1 construct (as used in the cell fusion assay) and the mutant constructs with the indicated mutations (obtained by site-directed mutagenesis) were used to transfect CHO-K1 cells using Lipo2000. The transfection efficiency was characterized by flow cytometry. The cells in a 48-well plate were then incubated with 100 μ l of virus solution (1×10^6 PFU) for 2 h. After washing and a 4-hour incubation in fresh medium, the cells were harvested, lysed, and tested for luciferase activity. The entry inhibition assay follows the same procedure as the one described for the entry assay, except that the virus was incubated with the WT or mutant nectin-1 proteins (final concentration, 100 μ g/ml) for 1 h before it was inoculated into the nectin-1-expressing cells.

Protein structure accession numbers. The coordinates and the related structural factors determined in this study have been deposited into the Protein Data Bank with the PDB codes of 4MYV for the free HSV-2 gD structure and 4MYW for the HSV-2-gD/nectin-1 complex structure.

RESULTS

The structure of free HSV-2 gD. We previously managed to collect a 1.8-Å resolution data set from HSV-2 gD crystals (45). The solved structure, with an R_{work} of 0.189 and an R_{free} of 0.220 (Table 2), includes two gD molecules per asymmetric unit. For each molecule, clear electron densities could be traced for residues V37 to R184 and L195 to R222. These amino acids are arranged into a

TABLE 2 Data collection and refinement statistics

	gD	gD + nectin-1
Data collection		
Space group	P21	P22121
Cell dimensions		
a, b, c (Å)	63.6, 55.4, 65.3	53.3, 170.9, 192.3
α, β, γ (°)	90.0, 96.3, 90.0	90.0, 90.0, 90.0
Wavelength (Å)	0.97916	0.97939
Resolution (Å) ^a	50.0–1.8 (1.86–1.80)	50.0–3.2 (3.31–3.20)
R_{sys} or R_{merge} ^{a,b}	0.085 (0.297)	0.091 (0.492)
$I/\sigma I$ ^a	15.9 (2.71)	15.7 (2.88)
Completeness (%) ^a	96.8 (81.2)	99.7 (99.5)
Redundancy ^a	3.9 (2.8)	4.1 (3.8)
Total no. of reflections	158118	122980
No. of unique reflections	40614	30129
Refinement		
Resolution (Å)	36.3–1.80	38.9–3.19
R_{work}/R_{free} ^c	0.189/0.220	0.196/0.247
No. of atoms		
Protein	2834	8516
Ligand/ion		
Water	407	57
B factors		
Protein	26.0	82.2
Ligand/ion		
Water	29.9	56.0
Root mean square deviations		
Bond lengths (Å)	0.008	0.005
Angles (°)	1.315	0.924
Ramachandran plot ^d		
Most favored region (%)	87.0	83.0
Additionally allowed region (%)	10.7	15.2
Generously allowed region (%)	2.3	1.8
Disallowed region (%)	0	0

^a Values for the outmost resolution shell are given in parentheses.
^b $R_{merge} = \sum_i \sum_{hkl} |I_i - \langle I \rangle| / \sum_i \sum_{hkl} I_i$, where I_i is the observed intensity and $\langle I \rangle$ is the average intensity from multiple measurements.
^c $R_{work} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the structure-factor amplitudes from the data and the model, respectively. R_{free} is the R factor for a subset (5%) of reflections that was selected prior to refinement calculations and was not included in the refinement.
^d Ramachandran plots were generated by using the program PROCHECK.

FIG 1 Structure of the unbound HSV-2 gD. (A) Ribbon representation of the overall structure. The two molecules (chains A and B) present in the asymmetric unit are shown. The IgV-like core is in green, while the N- and C-terminal extensions are highlighted in blue and orange, respectively. The secondary elements are labeled as in HSV-1 gD (37). The loop linking residues R184 and L195 is invisible and is therefore indicated as a dotted curve. The terminal residues are marked. (B, C) Comparison of the free HSV-2 gD structure (green for chain A and blue for chain B) with previously reported gD structures, including those of free HSV-1 gD (red), HSV-1 gD with HVEM (cyan), HSV-1 gD with nectin-1 (orange), and HSV-2 gD with an antibody (magenta). The colored arrows mark, in the individual structures, the positions of the structure termini and the conformations of the loops referred to in the text. Those elements that are invisible in our structure but are clearly traced in the reference structures are circled and labeled. (B) Superimposition of the structures highlighting the external extension region (shown in ribbon). (C) Superimposition of the structures highlighting the IgV-like core.

nine-stranded (A', B, C, C', C'', D, E, F, and G) IgV-like core in the center and several surface components of α -helices ($\alpha 1$, $\alpha 1'$, $\alpha 2$) and loops wrapping the center core on the exterior (Fig. 1A). Overall, this observed structure is very similar to the previously reported gD structures. For example, when superimposed, the free gD structure obtained in this study shows a root mean square deviation (rmsd) of ~ 0.78 Å over 158 equivalent C α atoms to a free HSV-1 gD structure (PDB code 1L2G), an rmsd of ~ 0.85 Å over 160 C α atoms to an HSV-1 gD structure binding with HVEM (PDB code 1JMA), an rmsd of ~ 0.77 Å over 156 C α atoms to an HSV-1 gD structure binding with nectin-1 (PDB code 3U82), and an rmsd of ~ 0.71 Å over 155 C α atoms to the recently reported HSV-2 gD structure in the antibody-binding form (PDB code 3W9E).

Despite a conserved fold as revealed by the low rmsd values, our HSV-2 gD structure exhibits an unexpected feature in the external extension region relative to the aforementioned gD structures. A large portion of the external elements, including those covering residues P23 to R36, A185 to P194, and F223 to A239, lacks traceable electron densities in our structure. The equivalent

amino acids, however, are consistently shown in the reference structures to form a long loop and a short strand (str2) in the N terminus, an intervening G- $\alpha 2$ loop, and a long helix ($\alpha 3$) in the C terminus (Fig. 1B). Further conformational difference was observed in the IgV-like core for the loops connecting strands C'' and D (the C''-D interloop) and strands E and F (the E-F interloop) (Fig. 1C). It is interesting that this E-F interloop also exhibits different conformations for the two molecules in the HSV-2 gD asymmetric unit, with one protomer (chain A) aligning similarly but the other protomer (chain B) orienting distantly from the reference gD structures (Fig. 1C).

The complex structure of HSV-2 gD bound to nectin-1. To elucidate the molecular basis of the nectin-1 recognition by HSV-2 gD, complex crystals were prepared and the structure was successfully solved at 3.2-Å resolution. The constructed model, with an R_{work} of 0.196 and an R_{free} of 0.247 (Table 2), contains two 1:1 binding complexes of the HSV-2 gD to nectin-1 in the asymmetric unit. For each complex, gD residues P23 to P254 and nectin-1 amino acids V33 to E335 are successfully traced in the electron density map. On the whole, the gD molecule shows a typical

FIG 2 Structure of the HSV-2-gD/nectin-1 complex. (A) Ribbon representation of the overall structure. The gD molecule (green for the IgV-like core, blue for the N-terminal extension, and orange for the C-terminal extension) binds to the first IgV-like domain of nectin-1 (colored cyan, orange, and magenta for the first, second, and third Ig-like domains, respectively). The binding interface involves the nectin-1 CC'C'FG rear sheet and the gD terminal extensions, which are circled and labeled. A superimposition comparison of a previously reported complex structure between HSV-1-gD (orange) and nectin-1 (magenta) with the bound structure of HSV-2-gD (green) with nectin-1 (cyan) is highlighted in the black box. (B) Structural comparison of the HSV-2 gD structures in the free (cyan) and the nectin-1 bound (green) forms. The fixed conformations of the terminal elements (highlighted in blue for the N terminus and in orange for the C terminus) in the bound structure are labeled. (C) Detailed view of the binding interface between HSV-2 gD (shown in surface) and nectin-1 (shown in ribbon). The interface residues of gD can be roughly categorized to congregate into surface patches 1 (yellow) and 2 (cyan), which contact mainly the B-C/F-G loop region (orange) and the upper sheet region (green) in nectin-1, respectively. The amino acids involved are shown in sticks and labeled.

structure of an IgV core wrapped by extensive terminal extensions, and nectin-1 folds into an overall rod-like structure with tandemly arranged V-C-C Ig-like domains. As expected, HSV-2 gD binds to the membrane-distal IgV-like domain of nectin-1 (Fig. 2A). In comparison to the free HSV-2 gD structure, many of the untraceable terminal residues are unambiguously observed in the complex structure, presenting as an extended loop in the N terminus and an α -helix (α 3) in the C terminus to engage the receptor (Fig. 2B).

The overall binding mode between HSV-2 gD and nectin-1 is very similar to that observed in a previously reported HSV-1-gD/nectin-1 complex structure (Fig. 2A). The binding relies on the surface components in the terminal extensions of HSV-2 gD engaging the rear (CC'C'FG) sheet of the nectin-1 IgV domain (Fig. 2A). A high complementarity in both the shape and the hydrophobicity character is observed between the two molecules at this interface, bearing extended surface areas of about 938.4 Å² and 946.7 Å² in gD and nectin-1, respectively. We further detailed the interface amino acids with a 4.5-Å resolution cutoff to characterize the receptor/ligand footprints in these two interacting molecules. A total of 24 residues are revealed in HSV-2 gD and are observed to sterically congregate into two main surface patches (patch1 and patch2), as reported for HSV-1 gD (35). The patch1 residues, including P23, L25 to Q27, F223, N227, V231, and Y234,

locate in the distal N-terminal loop and the α 3 helix. While the remaining interface residues of R36 to H39, Q132, R134, P198, and V214 to R222 form the much more extended surface patch2 (Fig. 2C). As expected, essentially the same patches of amino acids are also shown to be responsible for the HSV-1-gD/nectin-1 interaction (Table 3).

As to the receptor, the identified gD footprint in nectin-1 is composed of 26 amino acids, including S59, K61, T63, Q64, T66, Q68, G73 to N77, I80 to N82, M85 to S88, L90, E125, A127 to T131, and N133. Five residues (S59, K61, and F129 to T131) are observed to locate in the B-C and F-G loops, constituting a bottom loop region to contact mainly patch1 of HSV-2 gD. While an upper sheet (the CC'C'FG sheet) region, with the remaining interface residues, interacts in a much more extended manner with HSV-2 gD patch2 (Fig. 2C). This observed amino acid interaction pattern is the same for the two HSV-2-gD/nectin-1 complexes of the asymmetric unit and is also very similar to that observed in the HSV-1-gD/nectin-1 complex structure (35).

Comparative mutagenesis study on nectin-1 binding of HSV-1 and HSV-2 gDs. Based on the complex structures, HSV-1 and HSV-2 gDs contact essentially the same series of rear-sheet residues in nectin-1 for recognition. We therefore further sought functional evidence of a conserved nectin-1-binding mode between the two viral gD proteins. With an alanine-scanning mu-

TABLE 3 Comparison between HSV-1 and HSV-2 gDs for the patch residues locating at the nectin-1 binding interface^a

HSV-2 gD	HSV-1 gD
P23	P23
L25	L25
D26	
Q27	Q27
R36	R36
V37	V37
Y38	Y38
H39	H39
Q132	Q132
R134	
P198	
V214	V214
D215	D215
S216	S216
I217	I217
G218	G218
M219	M219
L220	L220
P221	P221
R222	R222
F223	F223
N227	
V231	T230
Y234	V231
	Y234

^a Based on the HSV-2-gD/nectin-1 complex structure solved in this study and a previously reported structure of HSV-1 gD bound to nectin-1 (PDB code 3U82), the interface residues within a distance of 4.5 Å from the nectin-1 receptor were selected and aligned in a pairwise manner. The two steric patches composed by these amino acids, as referred to in the text, are patch1 (gray background) and patch2 (white background). Clearly shown is that HSV-1 and HSV-2 gDs utilize essentially the same interface residues for nectin-1 recognition.

tagenesis in nectin-1, its interaction with both HSV-1 and HSV-2 gDs were characterized and compared by surface plasmon resonance (SPR). A total of 13 amino acids were selected (based on the complex structure solved in this study and our previously solved HSV-1-gD/nectin-1 structure) and mutated either individually or simultaneously. The mutant proteins were then prepared and purified as described for the wild-type protein and were further tested for the binding to gD (HSV-1 and HSV-2) proteins immobilized at ~750 response units. The binding capacity was thereby characterized as the amount of nectin-1 (WT or mutant), in response units, that is capable of binding to gD when 1 μM individual nectin-1 proteins flow through the chip.

Overall, the resultant binding patterns are very similar for HSV-1 and HSV-2 gDs (Fig. 3A). A series of residues, including K61, T63, Q64, Q76, N82, L90, E125, P130, and N133, do not significantly affect the gD/nectin-1 interaction. Nonetheless, dramatically decreased gD binding is observed for nectin-1 mutants with substitutions for residues N77, I80, M85, and F129, which exhibit about one-half (or less) of the binding response units of the WT nectin-1. This result is also consistent with previous studies reporting the functional indispensability of nectin-1 N77, M85, and F129 in the receptor recognition by HSV-1 gD (36, 43, 44). It is also noteworthy that the tetramutant (simultaneously replacing T63, Q64, E125, and N133 with alanine) nectin-1, which should be disrupted for the receptor homodimerization based on

a previous report (56), shows an increased binding capacity to both gD proteins (Fig. 3A).

We further mapped the nectin-1 residues N77, I80, M85, and F129 onto the binding interface in our complex structure. F129, which locates in the F-G loop, inserts its side chain into a highly hydrophobic pocket formed by F223, V231, and the apolar carbon atoms of Q27 in HSV-2 gD (Fig. 3B). In the upper sheet region, where the remaining three amino acids reside, I80 and M85 are shown to extensively contact HSV-2 gD Y38, Q131, and P221 via strong hydrophobic interactions, while N77 could contribute to the ligand binding by providing H-bond interactions via both the side chain and main-chain nitrogens with gD D215 and M219 (Fig. 3C). For the other interface residues, their role in gD recognition seems to be restricted to a limited number of van der Waals contacts, explaining their individual dispensability.

Cross-inhibition of the gD/nectin-1-mediated cell fusion by soluble HSV-1 and HSV-2 gDs. The conserved nectin-1-binding

FIG 3 Homologous receptor binding between HSV-1 and HSV-2 gDs to nectin-1. (A) A comparative mutagenesis study characterizing the gD/nectin-1 interaction. The gD proteins of HSV-1 (upper panel) and HSV-2 (lower panel) were immobilized on CM5 chips at ~750 response units and were then tested for binding by 1 μM the indicated nectin-1 (WT or mutant) proteins. The amount of bound protein in response units was recorded and presented as a percentage of that of the WT nectin-1 using histograms. Clearly shown is that the interface residue mutations in nectin-1 affect the gD binding similarly for HSV-1 and HSV-2. (B, C) Structural delineation of the role that N77, I80, M85, and F129 play in gD recognition. The binding interface between HSV-2 gD (green) and nectin-1 (cyan) is highlighted for the intermolecular contacts contributed by these four residues. The 2|Fo|–|Fc| electron density map was contoured at 1.0 σ. Dashed lines indicate H bonds. (B) Magnified view of the interface highlighting nectin-1 F129. (C) View of the interface highlighting nectin-1 N77, I80, and M85.

FIG 4 Similar action modes of HSV-1 and HSV-2 gDs in the nectin-1-mediated cell-cell fusion. By comparing the luciferase activities, the cell fusion in the presence of gradient concentrations (0.1, 0.5, and 2.5 μ M) of either HSV-1 or HSV-2 gD or an irrelevant hNUDT16 protein was plotted as the percentage of that without soluble gDs or hNUDT16. The dotted profiles are shown. (Left) HSV-1-gD-mediated cell fusion; (right), HSV-2-gD-mediated cell fusion. All the results are expressed as means $\pm$ standard deviations (SD) from three independent experiments.

mode between HSV-1 and HSV-2 gDs was further confirmed by a cell fusion-based mutual inhibition assay. The gD (HSV-1 or HSV-2) protein, along with the virus fusion executor of gB/gH/gL, and the nectin-1 receptor are separately expressed in CHO-K1 cells. Consistent with previous studies (53), remarkable cell fusion was observed when the nectin-1-expressing cells were mixed with the cells expressing virus gB, gH, and gL of HSV-1 and gD of either HSV-1 or HSV-2. In each case, the fusion is specifically inhibited by the soluble gD protein of its original virus type in a dose-dependent manner. In addition, fusion inhibition patterns similar to those observed in the self-inhibiting tests were recorded when the heterogeneous gD proteins were added to the system (Fig. 4). These observations are consistent with previous reports (41, 57, 58) and demonstrate a very similar mode of action of HSV-1 and HSV-2 gDs in nectin-1-mediated cell-cell fusion.

Nectin-1 I80 is an important gD interaction residue. Based on our comparative mutagenesis study, nectin-1 I80 is a newly identified residue that affects the gD binding, in addition to the previously reported amino acids N77, M85, and F129 (36, 43, 44). We therefore further quantitatively analyzed the contribution of this amino acid to the receptor/ligand engagement. The dissociation constants (K_d s) of nectin-1/I80A bound to HSV-1 and HSV-2 gDs were determined to be 1 μ M and 1.47 μ M, respectively. These values are about 2 orders of magnitude lower than those observed for the WT nectin-1 protein (12.5 nM for HSV-1 gD and 19.1 nM for HSV-2 gD), demonstrating that residue I80 indeed plays an important role in the nectin-1 engagement of gD. We also included the tetra-TQEN mutant protein in the kinetic assay, because these four residues could disrupt nectin-1 homodimerization (56) but did not interfere with the gD/nectin-1 interaction individually (Fig. 3A). The calculated binding affinities, with K_d s of 2.27 nM for HSV-1 gD and 7.81 nM for HSV-2 gD, increased approximately 2- to 5-fold in comparison to WT nectin-1 (Fig. 5 and Table 1).

We further investigated the role of nectin-1 I80 in the virus cell entry with an infection assay using a genetically modified HSV-1 virus containing a luciferase gene (55). CHO-K1 cells were transiently transfected with the nectin-1 (WT or mutant)-expressing plasmids. With similar expression levels (Fig. 6A), mutations of N77A, M85A, and F129A were shown to dramatically impair the

cell entry efficiencies of HSV-1 (Fig. 6B), which is consistent with previous studies (36, 43, 44). An approximate 2.5-fold decrease in the viral invasion was observed for the I80A mutant in comparison to that of the WT protein, demonstrating its functional importance in nectin-1-mediated virus entry. The tetra-TQEN mutant, however, is as capable of mediating virus entry as WT nectin-1 (Fig. 6B). Reciprocally, we further tested in an infection-blocking assay the capability of nectin-1/I80A to inhibit HSV-1 entry into cells by competing with the cell surface nectin-1. Consistent with the result of the infection assay, the nectin-1/I80A mutant exhibits compromised blocking activity against the viral invasion of HSV-1 (Fig. 6C). These results demonstrate that nectin-1 I80 is an important interface residue involved in the gD binding.

DISCUSSION

As with other enveloped viruses, the initiation of virus infection by HSV requires, as a prerequisite, the specific interactions between viral surface glycoproteins and the host cellular receptors (7–9). Although multiple virus envelope proteins other than gD have been shown to be capable of recognizing host molecules (e.g., both gB and gC can engage HSPG [10, 11] and gB also recognizes PILR α [59] and NMHC-IIA [60]), the gD-receptor interaction is considered a key step bridging the attachment and the fusion processes. Of the identified gD receptors, nectin-1 seems to play a more important role in mediating the infection by HSV-2, at least in *in vivo* murine models (28, 29). In comparison to what was seen in the WT or the HVEM knockout mice, an attenuated disease caused by HSV-2 and diminished virus spread were observed in the nectin-1 knockout mice (28). In addition, it was demonstrated that nectin-1, but not HVEM, plays an indispensable role in the HSV-2 infection of the central nervous system by direct cranial inoculation (29). It is therefore an interesting issue to elucidate the molecular basis of the binding between HSV-2 gD and nectin-1. A previous study reported the structure of HSV-2 gD with a neutralizing antibody, E317 (42). The antibody was shown to exert the neutralization activity by disrupting the gD-receptor interactions. The gD/E317 complex structure, therefore, provided the first structural glimpse on the HSV-2-gD/nectin-1-binding basis. In this study, we report a free HSV-2 gD structure and a complex

FIG 5 SPR assay characterizing the binding kinetics of gD (HSV-1 and HSV-2) to the indicated nectin-1 proteins (WT or mutant). The profiles of gD at gradient concentrations flowing through individual nectin-1 proteins immobilized at ~750 response units (RUs) were recorded and are shown. The calculated dissociation constants (K_D s) are labeled.

structure of HSV-2 gD bound with nectin-1. By comparing with the previously reported HSV-1-gD/nectin-1 structures (35, 36), we demonstrated at the atomic level that gDs of the two virus species engage essentially the same patch of nectin-1 residues for receptor recognition. This homologous binding mode was further supported (i) by our comparative mutagenesis study showing that the individual mutation of the interface residues in nectin-1 affects the ligand-receptor interaction similarly for HSV-1 and HSV-2 gDs and (ii) by our fusion inhibition assay showing that soluble gD proteins of the two viruses could mutually inhibit the gD/nectin-1-mediated cell fusion. These comprehensive structural and functional data, together with the previously reported functional evidence (39–41, 57, 58), demonstrate a conserved re-

ceptor-binding mode between HSV-1 and HSV-2 for nectin-1 and imply that the tropism difference between the two viruses likely arises from other aspects than the gD/nectin-1-binding features.

Compared to the HSV-1 gD (free or bound) (35–37) or the bound HSV-2 gD structures (42), the unbound HSV-2 gD structure lacks traceable electron densities for a large portion of the terminal extensions at both the N and C termini. This could be either a result of cleavage—removal of the terminal extensions by accidentally contaminated proteases during crystallization—or an indicative clue of the terminal flexibility. It should be noted that in the thus-far-available complex structures of gD with a receptor or an antibody (35–37, 42), the bound molecules invariably bind

FIG 6 Effects of nectin-1 mutations on HSV-1 entry. (A) Flow-cytometric assay characterizing the expression level of each nectin-1 (WT or mutant) protein. The percentages of the positive transfectants (among 10,000 CHO-K1 cells) are shown. (B) Assay characterizing the cell entry efficiency of HSV-1 via WT or mutant nectin-1. CHO-K1 cells expressing the individual nectin-1 proteins are infected with HSV-1 viruses as specified in Materials and Methods. The histogram shows the virus entry efficiencies of HSV-1 using the indicated nectin-1 mutants in comparison to that using WT nectin-1. (C) Quantification of HSV-1 entry into CHO-K1 cells expressing WT nectin-1 in the presence of soluble nectin-1 proteins of WT and mutant forms. The virus entry with bovine serum albumin (BSA) as an irrelevant-protein control is presented as 100%, and those with the indicated nectin-1 proteins are shown as percentages of that of BSA. All the results are expressed as means $\pm$ SD from three independent experiments. Clearly shown is that nectin-1 mutants (N77A, I80A, M85A, and F129A) with compromised gD-binding capabilities exhibit decreased capacities in inhibiting HSV-1 entry.

to the gD patches that map to these terminal extension elements. In the free HSV-1 gD structure (37), two gD protomers are packed against each other in the crystal via an interface covering the same extension patch. This raises the possibility that a bound partner (a receptor, an antibody, or another gD molecule) stabilizes the otherwise-flexible terminal extension residues of gD into definitive structures. Accordingly, the portion of the terminal extensions were solvent exposed and invisible in the free HSV-2 gD structure rather than being buried with fixed folds as observed in the aforementioned gD structures. Our structure therefore further supports the notion that the moveable character of gD N- and C-terminal segments would suffice for receptor binding, as documented for HSV-1 gD (61). In support of this view, several studies also reported the replacement of the gD N-terminal loop by uPA, interleukin-13 (IL-13), and the HER2 antibody, the chimeric gDs derived from which are functionally active in mediating virus entry into cells expressing their respective cognate receptors (62–64).

We also conducted a thorough mutational analysis for those structurally defined interface residues in nectin-1. In consistency with previous studies (36, 43, 44), individual substitution of N77, M85, and F129 by alanine dramatically impairs the interaction between gD (HSV-1 or HSV-2) and nectin-1. In addition, nectin-1/I80 also plays an important role in the gD/nectin-1 engagement, which was further demonstrated by our kinetic analysis and the virus entry assay. These four amino acids interact extensively with gD and thereby contribute dramatically to the binding via strong H bond and/or hydrophobic interactions. The remaining interface residues, in contrast, do not have a significant role individually. Accordingly, a majority of these amino acids are indeed observed to provide only limited van der Waals contacts in the complex structures and thereby are likely functioning to modulate gD recognition in combination. This is, to our knowledge, the first comprehensive alanine-scanning mutagenesis study on nectin-1 for its interaction properties with gD, which represents a useful supplement to the current structural observations. It is also noteworthy that the I80A mutation leads the kinetic profile of nectin-1 binding to gD to a fast-on/fast-off mode, forming a contrast to the kinetic pattern between WT nectin-1 and gD, which is slow-on/slow-off. We believe that the residue substitution and its resultant change of hydrophobic van der Waals contacts caused the kinetic shift. Similar phenomena of altered K_{on} and/or K_{off} rates resulting from mutations of interface residues have also been reported in other protein-protein interactions (65, 66).

Nectin-1 can form homodimers in solution (35, 56). Based on the solved structures, gD binds to the dimer interface of nectin-1 and would compete against its dimerization (35). In this study, we found that simultaneous mutation of T63, Q64, E125, and N133 to alanine leads to an increased binding affinity between nectin-1 and gD. Although these four residues contact HSV gD, we showed that the individual residues contribute little to the gD/nectin-1 interaction (Fig. 3A). In contrast, they have important roles in nectin-1 homobinding by providing H-bond and salt bridge contacts (56). The increased affinity should therefore be a result of residue substitutions that impaired nectin-1 homodimerization but hardly affected its interaction with gD. This is a functional evidence for a competitive relationship between gD/nectin-1 heterocomplex formation and the nectin-1 dimerization. The result also reminds us of a variant residue usage between gD and the other nectin-1 molecule to engage the same nectin-1 receptor.

This would facilitate the design of a nectin-1 mimic antiviral drug, which can bind to the viral gD protein with high affinity but show low or no specificity for nectin-1. These are interesting issues that are worth pursuing in the future.

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