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Induction of conformational changes at the N-terminus of herpes simplex virus glycoprotein D upon binding to HVEM and nectin-1

Eric Lazear [1,b], J. Charles Whitbeck [b], Yi Zuo [b], Andrea Carfí [c], Gary H. Cohen [b], Roselyn J. Eisenberg [a], Claude Krummenacher [a,*]

[a] Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA
[b] Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA
[c] Novartis Vaccines & Diagnostics, Cambridge, MA, USA

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ABSTRACT

Herpes simplex virus entry is initiated by glycoprotein D (gD) binding to a cellular receptor, such as HVEM or nectin-1. gD is activated by receptor-induced displacement of the C-terminus from the core of the glycoprotein. Binding of HVEM requires the formation of an N-terminal hairpin loop of gD; once formed this loop masks the nectin-1 binding site on the core of gD. We found that HVEM and nectin-1 exhibit non-reciprocal competition for binding to gD. The N-terminus of gD does not spontaneously form a stable hairpin in the absence of receptor and HVEM does not appear to rely on a pre-existing hairpin for binding to gD(3C–38C) mutants. However, HVEM function is affected by mutations that impair optimal hairpin formation. Furthermore, nectin-1 induces a new conformation of the N-terminus of gD. We conclude that the conformation of the N-terminus of gD is actively modified by the direct action of both receptors.

Introduction

Entry of herpes simplex virus (HSV) into cells requires the coordinated effort of four essential viral glycoproteins to promote fusion of the viral envelope with a cellular membrane (Connolly et al., 2011; Heldwein and Krummenacher, 2008). Binding of glycoprotein D (gD) to a cell surface receptor is necessary to initiate the membrane fusion process, most likely by triggering gH/gL to activate gB, the fusion protein (Atanasiu et al., 2010a, 2010b). Additionally, gD binding causes down-regulation of receptors from the cell surface and leads to virus endocytosis (Stiles and Krummenacher, 2010; Stiles et al., 2008).

HSV-1 gD can bind several unrelated receptors including nectin-1 (HveC, CD111), HVEM (herpesvirus entry mediator, HveA, TNFRSF14, CD270) and 3-O-sulfated heparan sulfate (Geraghty et al., 1998; Krummenacher et al., 1998; Montgomery et al., 1996; Shukla et al., 1999; Whitbeck et al., 1997). Nectin-1 is a cell-adhesion molecule used by many alphaherpesviruses for entry (Fan et al., 2012; Geraghty et al., 2001) and acts as the main receptor for HSV on neurons and keratinocytes (Huber et al., 2001; Richart et al., 2003; Simpson et al., 2005). The nectin-1 ectodomain consists of three immunoglobulin (Ig) folds with the most distal one having a V-type fold (Di Giovine et al., 2011; Lopez et al., 1995; Narita et al., 2011). The gD binding site is located in the V-domain and overlaps a functional binding site for the natural ligands of nectin-1 (Cocchi et al., 1998; Di Giovine et al., 2011; Fabre et al., 2002; Krummenacher et al., 2002, 1999; Zhang et al., 2011). HVEM is an immune regulatory molecule that interacts with activators and inhibitors of lymphocyte activation (Cheung et al., 2009; Murphy et al., 2006; Pasero et al., 2012). HVEM may have a limited role in viral spread, notably in the eye (Akhtar et al., 2008; Karaba et al., 2012; Tiwari et al., 2005), but may play an important role in modulating the host immune response by interfering with natural HVEM ligands (Kopp et al., 2009; Kopp et al., 2012; Stiles et al., 2010). It is a member of the TNF receptor family with four cysteine-rich domains (CRD) in its extracellular portion (Carfi et al., 2001). The gD binding sites is located on the first two CRDs and overlaps the binding site for the natural ligands BTLA and CD160 (Carfi et al., 2001; Compaan et al., 2005; Connolly et al., 2002; Stiles et al., 2010; Whitbeck et al., 2001).

Conformational changes in HSV glycoproteins play a central role in the process leading from receptor binding to membrane

* Correspondence to: Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, 3800 Spruce Street, Philadelphia PA 19104, USA. Tel.: +1 215 573 8567; fax: +1 215 898 7887.
E-mail address: ckrummen@vet.upenn.edu (C. Krummenacher).
[1] Present addresses: Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO, USA.

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fusion (Heldwein and Krummenacher, 2008). These conformational changes can also be triggered with soluble forms of the receptors and lead to inactivation of virions or blocking of entry (Geraghty et al., 1998). Alternatively soluble receptors were shown to promote entry into receptor-deficient cells (Kwon et al., 2006; Whitbeck et al., 2006).

The structure of the gD ectodomain has been divided into the following regions (amino acid numbering of the mature form of HSV-1 gD from Lys1 to Tyr306). The central core of the gD ectodomain comprises an Ig-like V-fold (aa 55–185) and flanking residues 34–54 and 186–255 (Fig. 1A) (Carfi et al., 2001). This is followed by a functional flexible hinge (aa 256–267) and then by the C-terminus of the native gD ectodomain (aa 268–306) which wraps around the central core and runs antiparallel to residues 23–33 of the N-terminus (Krummenacher et al., 2005). In the structure of gD alone, the first 21 residues are not resolved and presumed extended, but in the structure of gD bound to HVEM they adopt a hairpin structure (Fig. 1B and C). HVEM directly contacts residues 7–15 and 24–30 on each antiparallel strand of this hairpin (Fig. 1C and E) (Carfi et al., 2001; Connolly et al., 2003). Because the first 16 residues of this hairpin occupy the same space as the C-terminus in the receptor-free gD structure, it follows that HVEM binding requires the displacement of the C-terminus from the gD core surface (Krummenacher et al., 2005). Nectin-1 binding also requires displacement of the gD C-terminus from its native position (Fig. 1D) (Di Giovine et al., 2011). In fact, a pocket on the surface of the gD core near the N-terminus is a key binding site for both the nectin-1 Phe129 residue and also for the side chain of gD Trp294 that anchors the C-terminus of native gD (Di Giovine et al., 2011; Krummenacher et al., 2005). Mutations that fix the gD C-terminus to the core are unable to bind either receptor while mutations of the C-terminus that destabilize its interaction with the core increase the affinity for receptors but also result in functionally impaired gD (Krummenacher et al., 2005; Lazear et al., 2008). The C-terminus anchoring motif located around Trp294 is conserved in gD from several alpha-herpesviruses, suggesting a common mechanism of activation upon receptor binding (Krummenacher et al., 2013). A simple model would be that receptor binding is required for positioning and/or altering a functional domain to contact and activate the fusion machinery (Atanasiu et al., 2010a; Lazear et al., 2012). Thus far, determining a region of gD which contacts any other viral glycoprotein during

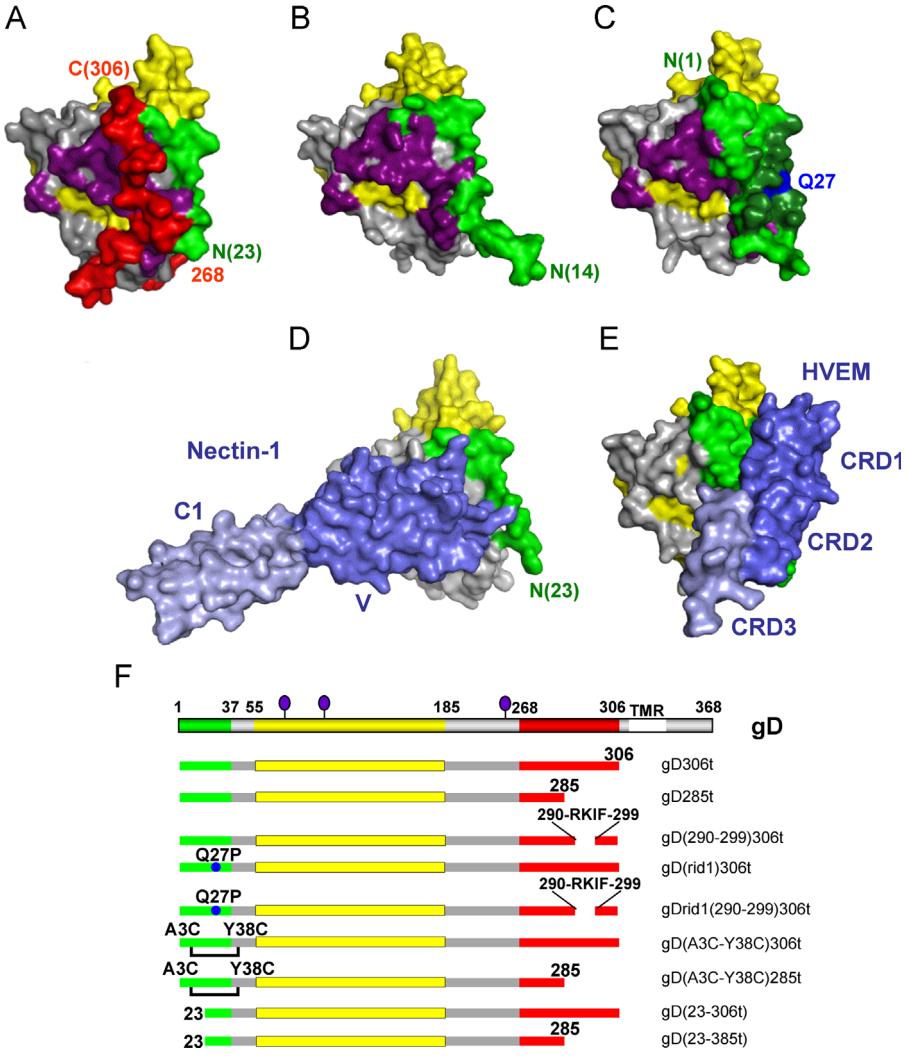

Fig. 1. Conformations of HSV gD and constructs. (A)–(E) Surface representations of structure of gD alone and bound to its receptors. (A) Full ectodomain of gD from a disulfide-bonded dimer of gD(306t) (PDB: 2C36). (B) gD(285t) (PDB: 1L2G). (C) and (E) gD(285t) bound to HVEM(200t) (PDB: 1JMA). (D) gD(285t) bound to nectin-1(346t) (PDB: 3SKU). Colors for gD: green: N-terminus (aa 1–37); red: C-terminus (aa 268–306); yellow: Ig domain (aa 55–185); gray: extensions in Ig core (aa 38–54 and 185–259). The binding site for nectin-1 is shown in purple (panels A-C) and the HVEM binding site is in dark green (panel C). gD residue Q27 is dark blue in panel C. Receptors are shown in blue nectin-1 (V and C1 domains) or HVEM (Cysteine-Rich Domains 1 to 3). All gD residues are not necessarily solved in all structures and only the resolved residues are shown. (F) Stick representations of full-length HSV-1 gD and mutants expressed in baculovirus. Colors are as described above. Amino acid numbering starts at lysine 1 of the mature glycoprotein. Black lines represent engineered disulfide bonds and lollipops indicate *N*-glycosylation sites.

HSV entry has been hampered by the transient or low affinity nature of a functional multi-glycoprotein complex (Eisenberg et al., 2012).

In this study we investigate the influence of the flexible gD N-terminus on the interaction of gD with HVEM and nectin-1. Two mechanisms can be considered to describe the binding of HVEM. First, the extended N-terminus of gD is in a conformational equilibrium and is able to spontaneously form a stable hairpin in the absence of HVEM. In this case, binding of HVEM stabilizes the existing hairpin, thus shifting the equilibrium towards the hairpin conformation. Second, the folding of the N-terminus is directly induced and maintained by HVEM. Here we used a set of gD mutants (Fig. 1F) to investigate these mechanisms. Two observations are in favor of the second possibility. First, the presence of the N-terminus, which can fold over the nectin-1 binding site, does not influence the kinetics of nectin-1 binding. Second, a form of gD with a disulfide bond engineered to lock the N-terminal hairpin does not bind HVEM better than wild type gD. We confirmed the need for HVEM to induce formation of the gD hairpin by taking advantage of the rid1 (Q27P) mutation, which prevents gD from using HVEM (Warner et al., 1998). We show that gD rid1 partially recovers its ability to bind HVEM when the C-terminus of gD is destabilized/opened to facilitate hairpin formation. Altogether these data support the model of HVEM inducing conformational changes in gD rather than simply stabilizing pre-existing gD conformations.

Results

Non-reciprocal competition between HVEM and nectin-1 for binding to HSV gD

The nectin-1 binding site is partially masked by the N-terminal hairpin when HVEM is bound (Fig. 1B vs. C) while, formation of the N-terminal HVEM-binding hairpin on the gD core should be prevented by nectin-1 binding (Fig. 1D). Because of this unusual disposition, we tested how the receptors compete with each other for binding to gD by competition ELISA (Fig. 2). A constant amount of gD ectodomain (gD306t) was incubated with increasing amounts of soluble nectin-1(346t) or HVEM(200t) and then added to HVEM or nectin-1 immobilized on the plate. A decrease in gD detection reflects competition between soluble and immobilized receptors. Nectin-2, which does not bind wild type gD (Connolly et al., 2001; Warner et al., 1998), served as a negative control for competition and, as expected, did not affect the binding of either receptor (Fig. 2, triangles). As anticipated, soluble HVEM competed with immobilized HVEM in a dose-dependent manner (Fig. 2A, squares). Soluble nectin-1 was more efficient than soluble HVEM at blocking gD binding to immobilized HVEM (Fig. 2A, circles). In accordance with the structural data, this result indicates that nectin-1 binding efficiently prevents the formation of the N-terminal HVEM-binding hairpin.

In a reverse setting, soluble nectin-1 prevented gD binding to immobilized nectin-1 as expected (Fig. 2B, circles), but soluble HVEM interfered poorly with nectin-1 binding (Fig. 2B, squares). Formation of a trimolecular complex does not explain this lack of competition as we did not detect nectin-1 binding to a preformed complex of gD-HVEM nor did we detect binding of HVEM to a preformed complex of gD-nectin-1 (not shown). Furthermore, this non-reciprocal competition is not due to a difference in affinity (Krummenacher et al., 1999; Willis et al., 1998b). A similar pattern was observed when HVEM and nectin-1 were competing for binding to gD285t, which lacks the C-terminus and has a higher affinity for both receptors. This indicates that the gD C-terminus is not responsible for the apparent non-reciprocal competition between receptors (data not shown). HVEM may be an inefficient competitor because it must induce a conformational change at the N-terminus of gD and form the hairpin which may be inherently unstable, rather than binding to a more rigid site, like nectin-1.

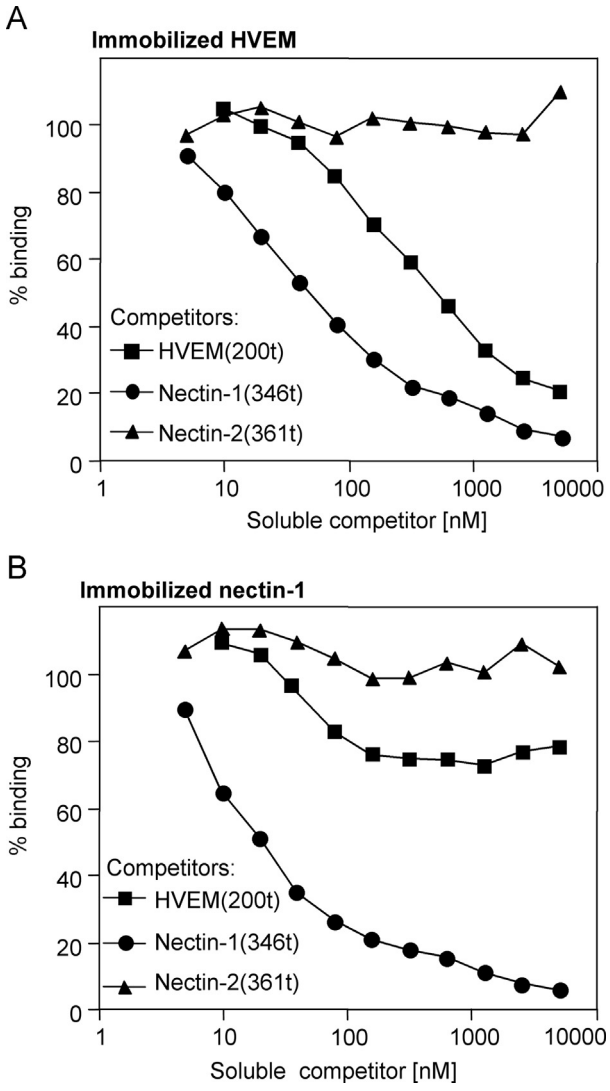

Fig. 2. HVEM and nectin-1 compete for binding to HSV gD in competition ELISA. (A). Plates were coated with HVEM(200t) and incubated with a constant concentration of gD(306t) (0.5 μM) together with increasing concentrations of the indicated competitor. (B). Plates were coated with nectin-1(346t) and incubated with a constant concentration of gD(306t) (1 μM) and increasing concentrations of soluble competitor. gD bound to the immobilized receptor was detected with polyclonal anti-gD IgG R7. Nectin-2(361t) was used as negative control since it does not bind gD(306t). A representative experiment is shown and 100% represents the binding of gD in the absence of competitor.

The flexible N-terminus of gD does not interfere with nectin-1 binding

Although the gD N-terminus (aa 1–32) is not necessary for nectin-1 usage (Yoon et al., 2003), it may influence the formation of the gD-nectin-1 complex. It is possible that the gD N-terminus exists in an equilibrium between an extended or hairpin conformation even when HVEM is not bound. The flexible strand of the hairpin (aa 1–22) folds over the nectin-1 binding site; therefore, if this hairpin forms stably in the absence of HVEM, the interaction with nectin-1 would be partially hindered (Fig. 1B and C). We used forms of gD which lack the first 22 residues to test whether this absence enhances the affinity for nectin. The absence of N-terminal

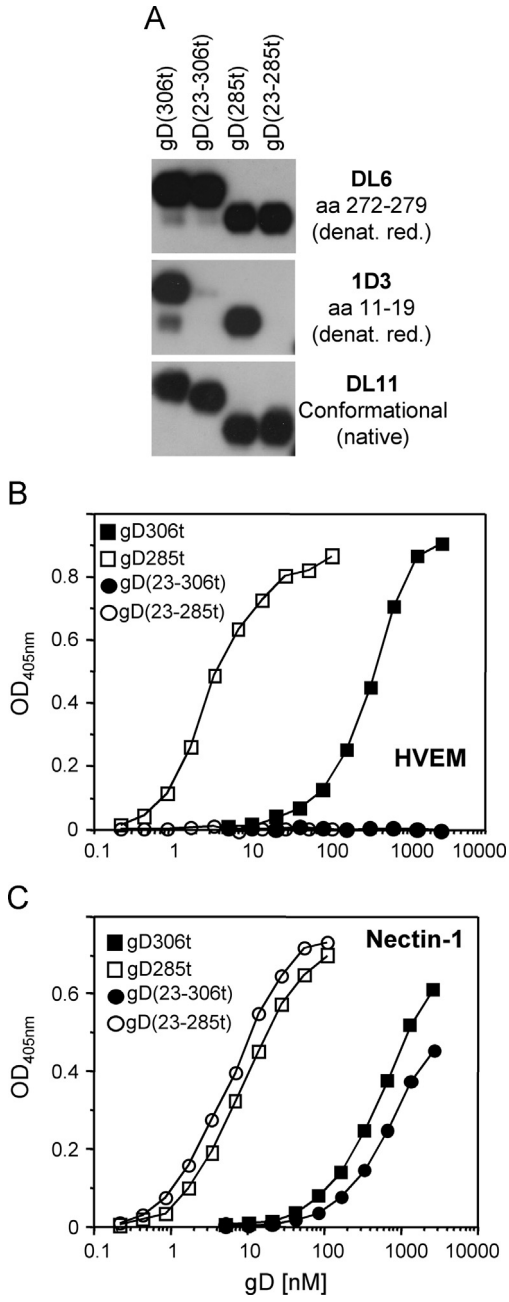

Fig. 3. Characterization of gD lacking residues 1–22. (A). Antigenic characterization by western blot. The indicated proteins were analyzed by PAGE in native or denaturing/reducing conditions. Blots were probed with MAbs DL6, ID3, and DL11. (B). Binding to immobilized HVEM(200t) by ELISA. (C). Binding to immobilized nectin-1(346t) by ELISA. Bound gD was detected with polyclonal antiserum R7.

residues was confirmed by the loss of epitopes for MAb 1D3 (linear, aa 11–19) compared to the control MAb DL6 (linear, aa 272–279) (Fig. 3A) (Chiang et al., 1994). The conformational epitope of the highly neutralizing MAb DL11, which largely overlaps the nectin-1 binding site, is conserved (Connolly et al., 2005; Whitbeck et al., 1999). As expected, deletion of amino acids 1–22 abolished binding to HVEM (Fig. 3B) (Connolly et al., 2003; Yoon et al., 2003). In contrast, binding of gD(23–306t) to nectin-1 was similar to that of the full gD ectodomain gD(306t) (Fig. 3C). Both C-terminal truncations gD(285t) and gD(23–285t) bound nectin-1 with higher affinity than gD(306t) but again the absence of the N-terminus did not allow gD(23–285t) to bind more efficiently than gD(285t) (Fig. 3C). Kinetics analysis by surface plasmon resonance (SPR) also showed that the affinity for nectin-1 was similar between gD(306t) and gD(23–306t), or between gD(285t) and gD(23–285t) (Table 1). The rate of complex formation (k_{on}), which would most likely be affected if the nectin-1 binding site was masked by the gD N-terminus, was also not grossly altered by the N-terminal deletion. Thus, removal of the N-terminal 22 residues did not improve binding of gD to nectin-1. These binding data can be interpreted as (1) the nectin-1 binding site is not obstructed by a stable hairpin, or (2) an unstable hairpin could be easily displaced/opened by nectin-1 without robustly affecting the binding kinetics. The first interpretation appears more consistent with the crystal structure of unbound gD(285t), which shows a disordered N-terminus (Carfi et al., 2001).

Pre-forming the gD N-terminal hairpin does not increase HVEM binding

Since a stable N-terminal hairpin does not form spontaneously, HVEM either induces its formation during binding or, possibly, stabilizes this pre-existing, albeit unfavorable, conformation. In the latter case one would expect that a gD mutant with a pre-formed hairpin would bind HVEM more efficiently than wild type gD. Such a mutant was generated by Connolly et al. by replacing residues Ala3 and Tyr38 with cysteines (Connolly et al., 2005). The disulfide bond created when these two cysteines are juxtaposed, even briefly, during gD synthesis locks the N-terminus in its hairpin position. Using transfected cells, Connolly et al. showed that full-length gD(3C–38C) binds HVEM like wild type gD but fails to bind nectin-1, and that preformation of the loop does not alleviate the need for a receptor for cell–cell fusion (Connolly et al., 2005). To define the biochemical characteristics and kinetics of HVEM binding to gD(3C–38C), we made this double mutation in the context of the soluble gD ectodomain gD(3C–38C)306t and in the shorter form gD(3C–38C)285t and purified these proteins.

The N-terminal linear epitope of MAb 1D3 was present in all constructs but detection by MAb DL11 was abolished in the two 3C–38C mutants (Fig. 4A). The DL11 epitope overlaps the nectin-1 binding site and includes residue Y38 (Connolly et al., 2005; Lazear

Table 1
SPR measurements of purified gD mutants binding to receptors.

	gD306t	gD(23–306t)	gD(3C–38C) 306t	gD(285t)	gD(23–285t)	gD(3C–38C) 285t
Nectin-1 k_{on} (10^3 M)	3.31[a]	4.1	No binding	93.8	143	No binding
Nectin-1 k_{off} (10^{-2} M)	0.575	0.868	No binding	0.669	0.939	No binding
Nectin-1 K_D (10^{-6} M)	1.74	2.11	No binding	0.0713	0.0657	No binding
HVEM k_{on} (10^3 M)	3.01	No binding	4.7	140	No binding	93.7
HVEM k_{off} (10^{-2} M)	1.91	No binding	1.13	1.37	No binding	5.28
HVEM K_D (10^{-6} M)	6.33	No binding	2.39	0.098	No binding	0.56

[a] Absolute values may vary between purified protein batches but represent an accurate comparison between gD mutants and wild type (Willis et al., 1998b).

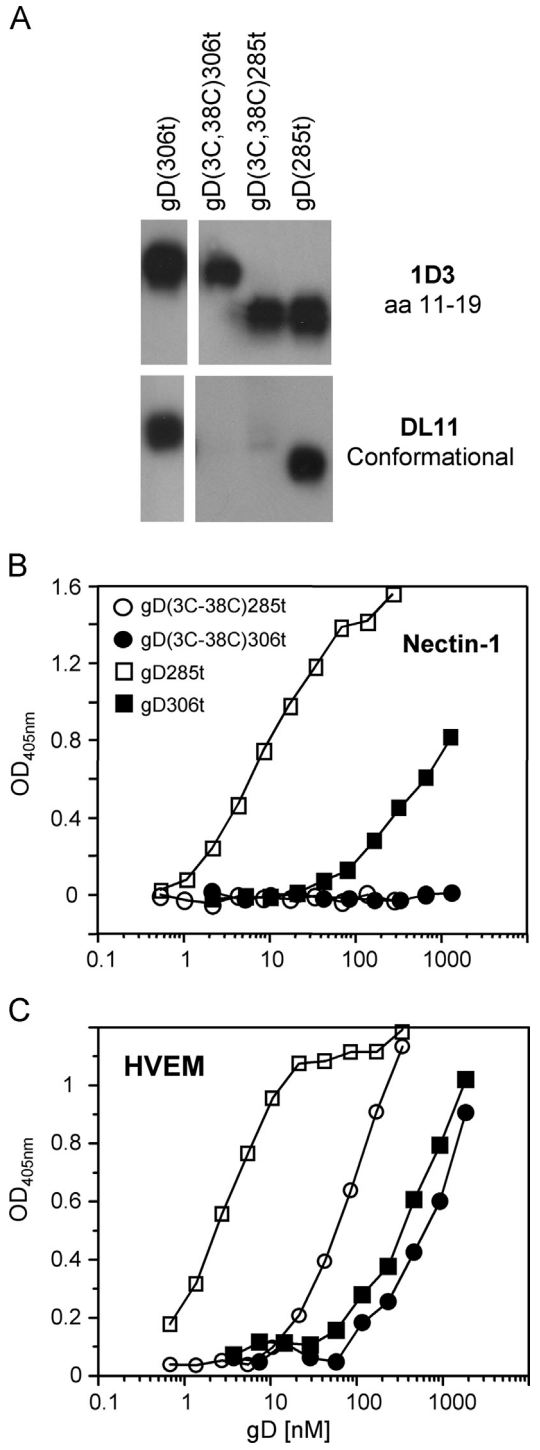

Fig. 4. Characterization of gD(3C–38C) with locked N-terminal hairpin. (A). Antigenic characterization by western blot. The indicated proteins were analyzed by PAGE in native and non-reducing conditions. Blots were probed with MAbs 1D3 and DL11. (B). Binding to Immobilized nectin-1(346t) by ELISA. (C). Binding to immobilized HVEM(200t) by ELISA. Bound gD was detected with polyclonal antiserum R7.

et al., 2008; Whitbeck et al., 1999). Neither gD(3C–38C)306t nor gD(3C–38C)285t bound to nectin-1 (Fig. 4B). In contrast, HVEM bound to gD306t and gD(3C–38C)306t with similar kinetics (Fig. 4C, Table 1). In particular, the k_{on} values were similar for both gD truncated at residues 306, indicating that the locked hairpin did not increase the rate of complex formation of HVEM with the mutant.

Deletion or destabilization of the C-terminus of wt gD favors N-terminal hairpin formation and results in a ~50-fold increase in the rate of complex formation with HVEM (Table 1) (Rux et al., 1998). Thus, we compared HVEM binding to gD(3C–38C)285t and gD(3C–38C)306t. Deletion of the C-terminus also increased the rate of HVEM binding to the 3C–38C mutant (Fig. 4C, compare gD (3C–38C)285t and gD(3C–38C)306t) but not to the extent seen in the wild type gD285t (Fig. 4C). The lower affinity of gD(3C–38C) 285t compared gD285t may be caused by an increased dissociation rate of the gD(3C–38C)285t-HVEM complex (k_{off}) (Table 1). It is possible that a local structural change around the newly engineered disulfide bond renders this hairpin suboptimal for the stability of the complex. Even if its effect is mostly noted in the absence of C-terminus, this is an important caveat to consider in the absence of structure for any of the gD(3C–38C) mutant. However, the hairpin-locking 3C–38C mutant, which can use HVEM to fuse and enter cells (Connolly et al., 2005; Uchida et al., 2009), does not show an increased affinity for HVEM.

Deletion of the gD C-terminus allows binding of gDrid1 to HVEM but does not increase its binding to nectin-1

HSV gD resistance-to-interference mutations rid1 (Q27P) and rid2 (Q27R) abolish binding to HVEM and increase the affinity of gD for nectin-1 (Dean et al., 1994; Krummenacher et al., 1998; Montgomery et al., 1996). The inability of gD rid1 to use HVEM may have two causes. First, the mutation may directly affect an interaction with HVEM, although only the backbone of this residue contacts HVEM (Carfi et al., 2001; Connolly et al., 2003). Second, the mutation may prevent proper formation of the hairpin. In this case, HVEM binding might be rescued by facilitating hairpin formation through removal of the competing C-terminus. Alternatively, if Q27P affects the contact with HVEM, binding might not be rescued even when the C-terminus is not in the way of hairpin formation. Thus, we tried to rescue HVEM binding to the rid1 mutant by destabilizing the gD C-terminus. The Q27P (rid1) mutation was engineered in gD(290–299)306t, which contains a substitution of residues 290–299 by a short linker, and the protein was purified (Chiang et al., 1994) (Fig. 5A). As previously observed, gD(290–299)306t has an increased affinity for both HVEM and nectin-1 (Fig. 5B and C) (Krummenacher et al., 1998; Willis et al., 1998b). However, gDrid1(290–299)306t bound HVEM less well than gD(290–299)306t. Interestingly, gDrid1(290–299)306t and gD306t bound to HVEM equally well. This indicates that a destabilizing substitution at the C-terminus can partially compensate for the rid1 defect at the N-terminus. This suggests that the rid1 mutation may affect hairpin formation (Fig. 5D) so that it prevents HVEM binding when the C-terminus is intact. Because the 290–299 substitution renders virion gD non-functional (despite a high affinity for HVEM and nectin-1), the effect of the rid-1 mutation in the context of this substitution could not be assessed during infection. These in vitro data suggest that the inability of HSV rid1 to use HVEM for entry appears to be caused by ineffective displacement of the C-terminus due to suboptimal formation of the N-terminal hairpin.

The increased affinity of gDrid1 for nectin-1 has been attributed to loosening of the interaction between the gD N- and C-termini thereby facilitating access to the nectin-1 binding site (Krummenacher et al., 2005). This is supported by the structure of unbound gD which shows Gln27 participating in the anchoring pocket for Trp294 from the C-terminus, (Fig. 5E). Indeed, gDrid1 (290–299t) does not bind to nectin-1 better than gD(290–299t) 306t (Fig. 5C). This indicates that when the C terminus is destabilized by the 290–299 deletion, the Q27P mutation has no effect on nectin-1 binding. Although the 290–299 deletion has a stronger effect than the Q27P mutation alone, both mutations

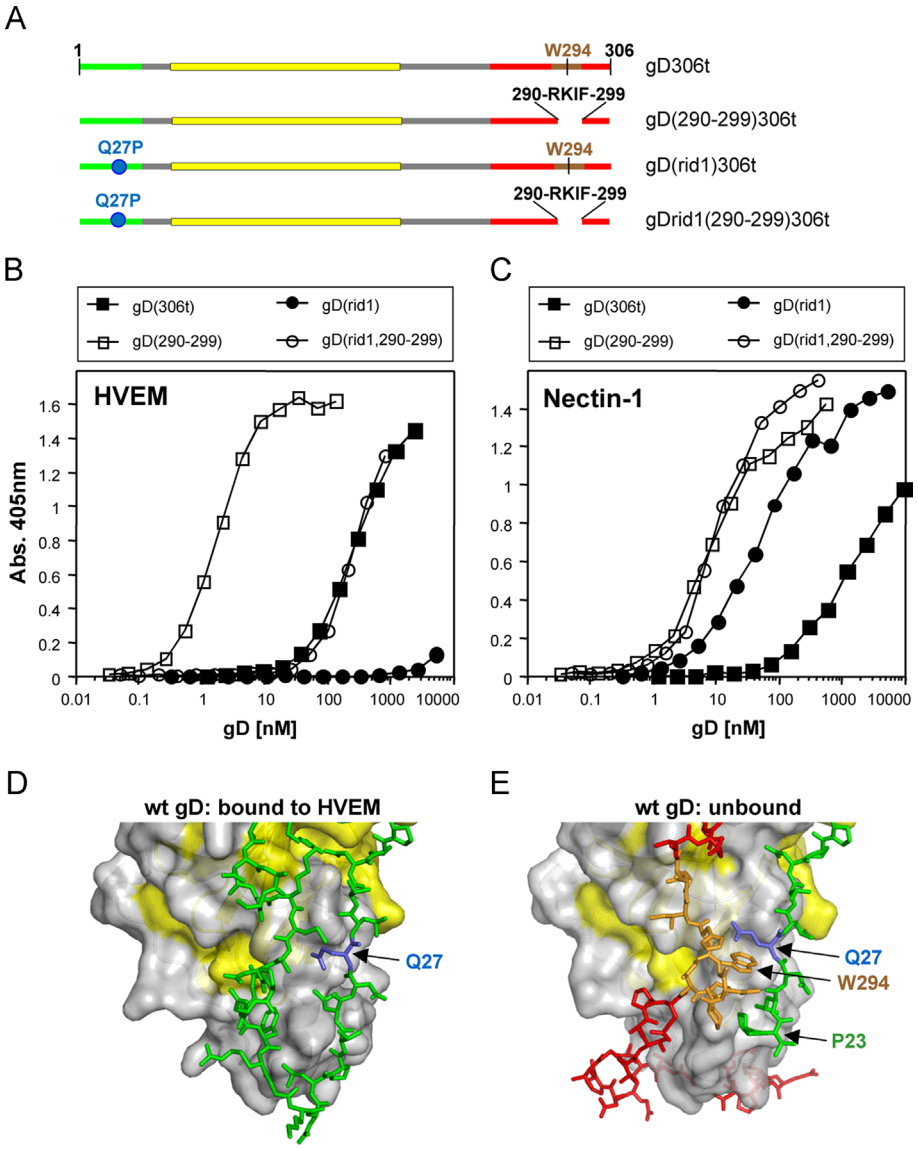

Fig. 5. Binding of gD from HSV strains KOS and rid1 to immobilized receptors by ELISA. (A) Stick diagram of gD mutants colored as in Fig. 1. (B) Binding of gD ectodomains to immobilized HVEM(200t) by ELISA. Black symbols represent gD ectodomain from strain KOS (i.e. gD306t), and rid1 (i.e. gD(rid1)306t). Open symbols represent the corresponding C-terminal mutants gD(290–299)306t and gDrid(290–299)306t. Bound gD was detected with polyclonal antiserum R7. (C) Binding of gD ectodomains to immobilized nectin-1(346t) by ELISA. Symbols are as in B. (D) Structural detail of gD bound to HVEM (for clarity HVEM is not represented). The gD core is shown as a surface and the N-terminal hairpin as green sticks. Gln27 is shown in blue. (E). Structural detail of gD in the absence of receptor. The gD core is shown as surface. The C-terminus is represented in red sticks with residues 290–299 colored gold and the N-terminus is shown as green sticks with Gln27 colored blue. The first residue is Pro23 since flexible residues 1–22 were not resolved.

increase nectin-1 binding by destabilizing the C-terminus and facilitating access to the binding site.

New epitope generated at the gD N-terminus following nectin-1 binding

The conformation of the N-terminus of gD bound to nectin-1 is unknown because residues 1–22 were not resolved in the structure of the complex (Di Giovine et al., 2011; Zhang et al., 2011). Because of steric hindrance by nectin-1, the N-terminus cannot form a stable hairpin as in the structure of gD bound to HVEM. We identified a MAb, MC1, which recognizes residues 7–21 of gD. MC1 does not neutralize virus but competes for gD binding with MAb 1D3 (aa 11–19) which does neutralize entry into HVEM expressing cells (data not shown and (Nicola et al., 1998; Whitbeck et al., 1999)). We used MC1 and 1D3 to ask whether nectin-1 binding affects the conformation of the extended N-terminus. First, MAbs were used to immunoprecipitate gD alone or in complex with nectin-1 (Fig. 6A). The positive control MAb DL6 was able to co-immunoprecipitate nectin-1 with gD while the nectin-1-blocking MAb DL11 failed to co-immunoprecipitate nectin-1 (Nicola et al., 1998). MAb ID3 efficiently immunoprecipitated gD alone or in complex with nectin-1. In contrast, MC1 pulled down gD alone poorly but it efficiently immunoprecipitated the gD-nectin-1 complex. This suggests that the MC1 epitope is poorly available on gD until nectin-1 binds. MC1, like 1D3, failed to co-immunoprecipitate HVEM with gD (data not shown), suggesting that MC1 and HVEM compete with each other for binding to gD. This observation is consistent with the overlap between the MC1 epitope and the HVEM contact site on gD. The increased binding of MC1 to gD bound to nectin-1 was confirmed by SPR (data not shown). To confirm that the nectin-1-induced conformational change is not restricted to soluble proteins, we probed virions with MC1. Binding of MC1 to HSV-1 KOS virions was tested by virus-ELISA. A 60% increase in MC1 binding to HSV gD was observed in the presence of soluble nectin-1(346t) (Fig. 6B). No

change was observed for ID3, DL6 or DL11. Thus MC1 distinguishes two conformations of the N-terminus that were not distinguished in the structures of gD alone or bound to nectin-1.

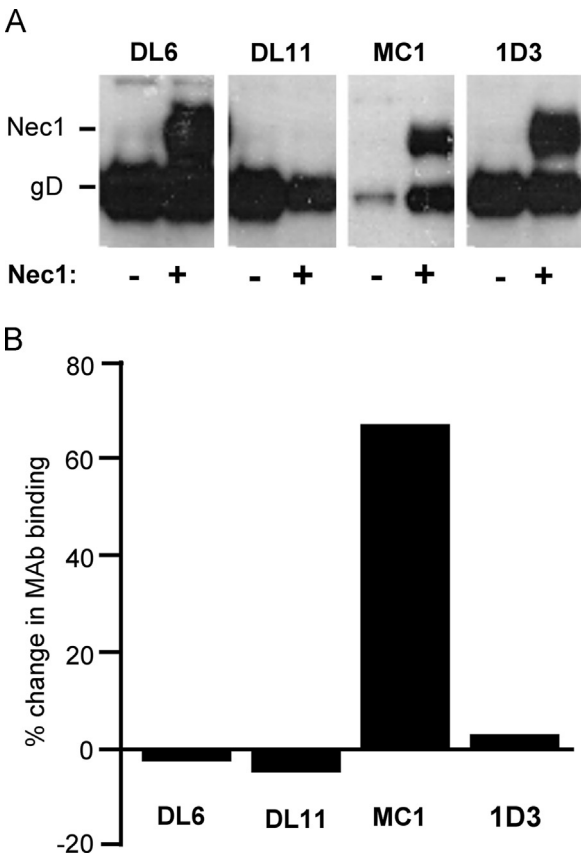

Fig. 6. Increased binding of MAb MC1 to the gD-nectin-1 complex. (A). Immunoprecipitation. Soluble gD285t was pre-incubated alone or with nectin-1(346t), prior to immunoprecipitation with IgG from the indicated mouse MAbs. After SDS-PAGE, the western blot was probed with a mixture of anti-gD R7 and anti-nectin-1 R154 polyclonal rabbit Ig. (B). Virus ELISA. Gradient-purified HSV-1 KOS was immobilized on a plate and incubated with BSA or soluble nectin-1(346t) at 37 °C. Virion gD was then detected with the indicated MAbs. The % change of MAb binding to virion-nectin-1 vs. virion-BSA is indicated.

Discussion

Although HVEM and nectin-1 bind distinct domains of gD to induce a common conformational change, both receptors have a similar affinity for gD, both complexes form with the same kinetics and have comparable stability (Krummenacher et al., 1999; Willis et al., 1998b). Despite these similarities, we observed non-reciprocal competition for binding to gD: i.e. nectin-1 can more readily prevent HVEM binding than the reverse. The contacts sites for HVEM and nectin-1 are not directly overlapping but the folding of the HVEM-binding N-terminus masks the nectin-1 binding site (Fig. 1B and C). A likely explanation for the enhanced ability of nectin-1 to block HVEM is that nectin-1 binds to a rigid surface on the core of gD while HVEM binds a flexible region which must undergo a conformational change during binding. These in vitro data support previous functional observations. First, soluble nectin-1 efficiently blocks virus entry into HVEM expressing cells while soluble HVEM is less efficient at blocking entry into nectin-1 expressing cells (Geraghty et al., 1998). Second, fewer nectin-1 than HVEM molecules are required to allow the same level of entry of HSV-1 KOS into transfected B78H1 cells (Krummenacher et al., 2004). It is therefore possible that structural differences of binding sites may ultimately lead to a preference in receptor usage when both are present on target cells.

Conformation of gD N-terminus during binding to HVEM and nectin-1

In the HVEM-gD complex, the N-terminus forms a hairpin. Comparison of this structure with that of unliganded gD and gD bound to nectin-1 led to two conclusions. First, the N-terminal hairpin masks the nectin-1 binding site and second, its formation requires the displacement of the C-terminus from its native position on the core of gD. Here we used gD mutants to define whether the N-terminus is in a conformational equilibrium and tends to spontaneously form a stable hairpin allowing HVEM binding or whether the folding of the N-terminus is induced and maintained by HVEM. The latter model is supported by two observations suggesting an active role for HVEM in hairpin formation. First, the presence of the flexible N-terminus does not affect the rate of complex formation with nectin-1, indicating that a stable hairpin does not form over the nectin-1 binding site in the absence of HVEM. Second, pre-formation of the hairpin, as in the gD(3C–38C) does not necessarily enhance complex formation with

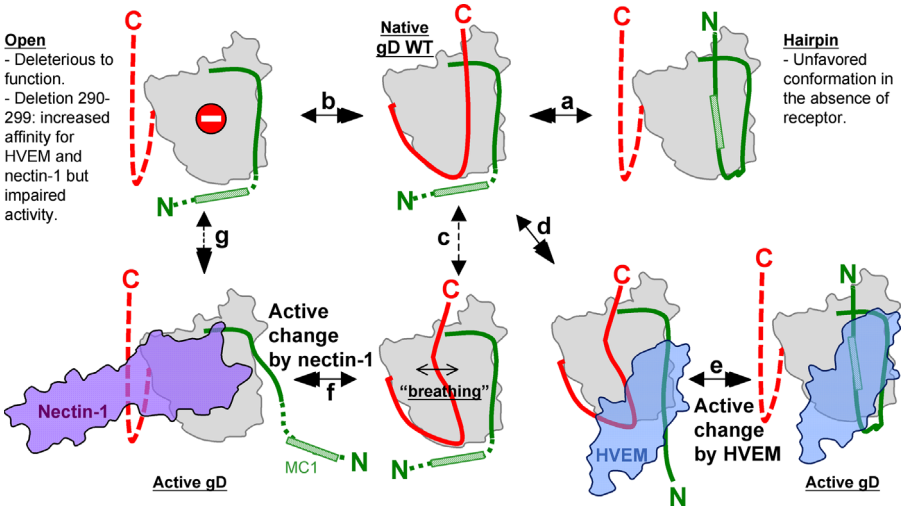

Fig. 7. Model of active induction of conformational changes by receptors. A schematic representation of the receptor-binding face of gD is shown with the core in gray, the C-terminus in red and the N-terminus in green. Dotted lines are not solved in structures and the breathing conformations (bottom center) are hypothetical. The hatched box indicates the MC-1 epitope. Nectin-1 is shown in purple and HVEM in blue. Arrow size symbolizes conformational preference.

HVEM. This suggests that (1) the receptor does not require an initial interaction with a pre-existing N-terminal hairpin and (2) that this hairpin conformation of gD is actively induced by HVEM. A working model is presented in Fig. 7, where arrow sizes are used to indicate favored conformations. Our data suggest that spontaneous folding of the N-terminus is not favored in wild type gD (Fig. 7a). When HVEM binds gD, functional displacement of the C-terminus is achieved indirectly by active folding of the N-terminus (Fig. 7d and e).

Proper formation of the gD N-terminal hairpin is necessary for usage of HVEM during entry. The rid1 mutation (Q27P) prevents the use of HVEM for entry (Montgomery et al., 1996; Warner et al., 1998). The fact that rid1 binding to HVEM can be rescued by destabilization of the C-terminus suggests that the rid1 defect is due to the ineffective formation of the hairpin rather than a perturbation of contacts with HVEM. It is unclear how the Q27P mutation hinders hairpin formation. It may affect the native positioning of the N-terminus on the gD core or weaken interactions between the strands of the hairpin. In either case, the rid1 mutation prevents HVEM from functionally displacing the C-terminus and activating gD. The rid1 mutation itself has a destabilizing effect on the anchoring of the C-terminus (Krummenacher et al., 1998, 2005; Willis et al., 1998b). A more drastic destabilization by a mutation of the C-terminus (i.e. in gDrid1(290–299)) is needed to compensate for the rid1 defect in hairpin formation.

Binding of nectin-1 also affects the conformation of the N-terminus of gD. The first 21 residues were not resolved in the structure of gD alone or gD bound to nectin-1. This is attributed to the intrinsic flexibility of this region. Interestingly, the enhanced detection of gD by MC1 when gD binds to nectin-1 indicates that the gD N-terminus adopts a different conformation when nectin-1 binds. Interestingly, detection of gD by MAb 1D3, which binds to an epitope that overlaps that of MC1, is not affected by nectin-1. This suggests that the rearrangement of the N-terminus is quite specific. Thus MC1 reactivity indicates that a discrete portion of the gD N-terminus is not readily available in its native conformation but becomes exposed, or structurally altered, after nectin-1 binding.

Intermediate conformations and gD activation

It is not yet clear how nectin-1 accesses its binding site in native gD since it is partially masked by the C-terminus. The fact that HVEM and nectin-1 bind soluble gD(306t) with a k_{on} slower than gD(285t) in vitro suggests that each receptor alone is sufficient to displace the C-terminus. An intermediate conformation where the core of gD is partially exposed should be considered in a working model. The main interaction holding the C-terminus in this native position is the insertion of the Trp294 side chain into a pocket on the gD core (Fig. 5). Mutation of this residue or deletion of this region results in a non-functional gD, which has been attributed to an uncontrolled conformational change to an open conformation (Fig. 7b) (Krummenacher et al., 2005; Lazear et al., 2008). Although binding of nectin-1 to the open gD can occur with high affinity, this configuration results in impaired function (Fig. 7g). Thus, any equilibrium between the open and close conformations is likely to strongly favor the close conformation (Fig. 7b). However, the overall interaction between the C-terminus and the core is not robust (Fusco et al., 2005). It is therefore possible that gD is in equilibrium between the close conformation and a partly open conformation that allows for initial receptor interaction (Fig. 7c and e). We postulate that such "breathing" of the C-terminus facilitates the initial interaction with nectin-1 or HVEM without leading to full activation in the absence of receptor, which would be detrimental to the virus (Fig. 7c). The need for an active role of nectin-1 or HVEM in displacing the C-terminus of gD ensures that gD is not prematurely activated. In the context of the viral envelope, where space and movements may be constrained, one cannot exclude the possibility that receptor binding may benefit from other not yet identified cues.

This complex system of activation also suggests that both receptor-binding sites remain protected in native gD until close proximity with the receptor is achieved. The N-terminal HVEM binding hairpin is not stably formed in the absence of receptor and the nectin-1 binding site is partially masked until gD is activated by either receptor. In this way, gD may be at least partially protected from highly neutralizing antibodies that target receptor binding sites (Lee et al., 2013; Whitbeck et al., 1999). This is reminiscent of other viral envelope proteins such as HIV gp120 whose critical chemokine receptor binding site is exposed only after a conformational change is triggered by CD4 binding (reviewed in (Wilen et al., 2012)).

Drastic modifications of gD have been generated to redirect HSV-based viral vectors to exogenous receptors (Campadelli-Fiume et al., 2011; Zhou and Roizman, 2006). Remarkably, viruses carrying a chimeric gD in which the core is replaced by a single-chain immunoglobulin are able to infect target cells that expressed the cognate antigen (Menotti et al., 2008, 2009; Zhou and Roizman, 2006). Although this artificial construct retains the gD N- and C-termini, its mechanism of activation is unclear. Further structural and biochemical data on gD chimeras are needed to determine if the cognate receptor relies on the natural triggering mechanism used during HSV entry.

Overall, this analysis of conformation-based mutants suggests preferred conformations of gD and highlights the active roles of HVEM and nectin-1 in inducing structural changes. The functional consequences of these changes on the fusion machinery is clear but the mode of action of receptor-activated gD will require further structure-function analyses involving all essential entry glycoproteins.

Materials and methods

Proteins

Proteins were purified from baculovirus supernatant as described previously for gD306t, gD285t, nectin-1(346t) and HVEM(200t) (Krummenacher et al., 1998; Rux et al., 1998; Sisk et al., 1994; Whitbeck et al., 1997). The recombinant gDrid1(290–299) was generated by recombination of plasmid pCP277 in Baculogold DNA and selection of recombinant viruses as described previously (Willis et al., 1998a).

The 22-nucleotide deletion at the N terminal of gD was generated by amplifying part of the gD KOS open reading frame in plasmid pRE4 by PCR. The sequence gD(23–306t) was amplified using the forward oligonucleotide (gD23Nterm) TTTTGGATCCGGTCCTGGACCAGCTGACCG and the reverse primer (gD1-3) TTTTCTGCAGTTAATGATGATGATGATGATGGTAAGGCGTCGCGG. The amplified product was cloned into BamH1 and Pst1 restriction sites of the vector pVT-Bac (Tessier et al., 1991) to generate plasmid pCK502 to express gD(23–306) with a C-terminal 6-histidine tag. Sequence for gD(23–285) was amplified using the forward primer gD23Nterm and the reverse primer 285–3B CGGGAATTCACGTGCCTACGGGGTCCTCCAAGA. The product was cloned into BamH1 and EcoR1 restriction sites of pVT-Bac to generate plasmid pCK501. Generation of recombinant baculoviruses was performed as previously described (Willis et al., 1998a). Briefly, plasmids pCK501 and pCK502 were co-transfected with Baculogold DNA (Pharmingen) into Sf9 cells. The recombinant baculoviruses were named Bac-gD(23–285t) and Bac-gD(23–306t).

Expression of gD(3C–38C) mutants in pVT-Bac. Sequence of gD carrying mutations A3C and Y38C was amplified by PCR from plasmid pDL490 (Connolly et al., 2005). The upstream primer 5′ gDA3Cbaculo (GCCGGATCCCAAATATTGCTTGGCGGATG) was used with primer 3′gD1(hisless)306 (TTTTCTGCAGTTAATGGTAAGGCGTC-GCGG) to generate gD(A3C-Y38C)306t or with primer gD285–3B (CGGGAATTCAAGTGCCTACGGGGTCCTCCAAGA) to generate gD(A3C-Y38C)285t. The amplified fragments were digested with BamH1/Pst1 or BamH1/EcoR1 respectively and ligated in vector pVT-Bac digested with the same enzymes. The resulting plasmids pCK521 and pCK530 were co-transfected with Baculogold DNA into Sf9 cells to yield recombinant baculovirus Bac-gD(A3C-Y38C)306t and Bac-gD(A3C-Y38C)285t as previously described (Krummenacher et al., 1998). Recombinant baculoviruses were purified through two rounds of plaque selection on Sf9 cell monolayers. Plaques were tested for gD expression by Western blotting using rabbit polyclonal serum R7 (Isola et al., 1989).

Enzyme-linked immunosorbent assay (ELISA)

Purified HVEM(200t) or nectin-1(346t) diluted to 10 μg/ml in PBS were bound to microtiter plates overnight at 4 °C (Krummenacher et al., 1998). Plates were washed with 0.05% Tween 20 in PBS (PBS-T) and incubated in PBS with 5% milk and 0.05% Tween 20 (PBS-T-milk) for 30 min at room temperature (RT). Plates were washed with PBS-T and incubated with various concentrations of the soluble forms of gD to be tested in PBS-milk for at least 2 h at RT. Plates were washed with PBS-T and incubated at RT in blocking solution containing a 1:1000 dilution of anti-gD rabbit serum R7 (Isola et al., 1989). After being washed with PBS-T the plates were incubated with HRP-conjugated anti-rabbit Ig antibody (1–2 μg/ml) in PBS-milk for 30 min at RT. Plates were then washed with PBS-T and with 20 mM citrate buffer pH 4.5. The HRP substrate (ABTS; Moss, Inc.) in citrate buffer pH 4.5 was added and absorption at 405 nm (A_{405}) was read. Results are presented after subtracting background signal obtained from parallel mock-coated wells.

Competition ELISA

Purified HVEM(200t) or nectin-1(346t) diluted to 10 μg/ml in PBS were bound to microtiter plates overnight at 4 °C (Krummenacher et al., 1998). Blocking is similar to regular ELISA (described above). Purified gD(306t) was diluted in PBS-T-milk to 1 μM and mixed with dilutions of HVEM(200t) or nectin-1(346t). The mixture was added to plates immediately and incubated for 2 h at RT. Detection of gD was performed as indicated above.

Virus ELISA

Gradient-purified HSV was diluted in PBS to 10^8 pfu/ml and 50 μl per well was used to coat the surface of a 96-well ELISA plate. For control wells without virus, 50 μl of PBS was added. After allowing the virus to adsorb for 2 h at RT, the plate was washed in PBS-T and blocked for 30 min at RT using PBS-T-milk. After blocking, plates were washed and individual wells were incubated for 1 h in 50 μl of PBS-T-milk alone or containing 10 μM nectin-1 (346t). Following the incubation with nectin-1(346t), individual MAbs were diluted in PBS-T-milk (1:50 dilution of mouse ascites fluid) and 50 μl per well was added. Duplicate plates containing virus and virus/receptor were incubated for 2 h at 4 °C and 37 °C to distinguish between temperature-dependent and temperature-independent effects of receptor binding. The plates were then washed and incubated for 1 h with peroxidase-conjugated goat anti-mouse antibody (KPL) diluted 1:1000 in PBS-T-milk. The plates were washed, rinsed with 20 mM sodium citrate (pH 4.5) and then incubated in ABTS (Moss) until color development occurred. Absorbance at 405 nm was then determined for each well using a Perkin Elmer Bioassay Reader Data were plotted as the percent change in MAb reactivity with virus in the presence of nectin-1(346t) versus the reactivity in the absence of nectin-1 (346t).

Western blots

Purified forms of gD were separated on 10 or 12% Tris-glycine polyacrylamide gels. For denaturing electrophoresis proteins were boiled in sample buffer containing 1% SDS, 10% glycerol and 20 mM DTT. Non-denaturing electrophoresis was performed according to Cohen et al. (1986). Blots were probed with anti-gD monoclonal antibodies DL11 (Cohen et al., 1986; Muggeridge et al., 1988; Whitbeck et al., 1999), DL6 (Eisenberg et al., 1985; Isola et al., 1989) and 1D3 (Chiang et al., 1994; Cohen et al., 1984) or with polyclonal rabbit serum R7 (Isola et al., 1989).

Surface plasmon resonance (SPR)

Determination of affinities by SPR experiments was carried out on a Biacore X optical biosensor (Biacore AB) at 25 °C following the protocol previously described (Krummenacher et al., 1999; Rux et al., 1998; Willis et al., 1998b) with the following modifications. The running buffer was HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20), pH 7.4. Approximately 1900 response units (RU) of nectin-1(346t) or HVEM(200t) were coupled to flow cell 2 (Fc2) of a CM5 sensor chip via primary amines according to the manufacturer's specifications. Fc1 was activated and blocked without the addition of protein. Soluble gD was serially diluted in HBS-EP. Each gD sample was injected for 2 min to monitor association. Then the sample was replaced by HBS-EP flow, and the dissociation was monitored for 2 min. During the binding and dissociation phases of gD to nectin-1, the flow path was set to include both flow cells, the flow rate was 50 μl/min and the data collection rate was 5 measurements/min. To regenerate the nectin-1 surface, brief pulses of 0.2 M Na_2CO_3 (pH 10.5 for nectin-1 surface or pH 8.0 for HVEM surface) were injected until the response signal returned to baseline. Sensorgrams were corrected for non-specific binding and refractive index changes by subtracting the control sensorgram (Fc1) from the nectin-1 surface sensorgram (Fc2). Data were analyzed with BIAevaluation software, version 3.0. Model curve fitting was done by using a 1:1 Langmuir interaction to measure the rate of association (k_{on}) and the rate of dissociation (k_{off}).

Immunoprecipitation

A set amount of gD(285t) (300 ng) was incubated 1.5 μg nectin-1 (346t), incubated for 1 h at 4 °C. Then 500 ng IgG of MAb DL6, DL11, MC1 or 1D3 were added for 1 h at 4 °C. Finally, Protein A-sepharose was added and incubated for 1 h at 4 °C after which immune complexes were collected, washed with binding buffer and subjected western blot analysis. Blots were probed with combined polyclonal sera R7 (anti-gD) and R154 (anti-nectin-1) (Isola et al., 1989; Krummenacher et al., 1998).

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