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

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ABSTRACT The herpesvirus entry mediator C (HveC), previously known as poliovirus receptor-related protein 1 (PRR1), and the herpesvirus Ig-like receptor (HIgR) are the bona fide receptors employed by herpes simplex virus-1 and -2 (HSV-1 and -2) for entry into the human cell lines most frequently used in HSV studies. They share an identical ectodomain made of one V and two C2 domains and differ in transmembrane and cytoplasmic regions. Expression of their mRNA in the human nervous system suggests possible usage of these receptors in humans in the path of neuron infection by HSV. Glycoprotein D (gD) is the virion component that mediates HSV-1 entry into cells by interaction with cellular receptors. We report on the identification of the V domain of HhGR/PRR1 as a major functional region in HSV-1 entry by several approaches. First, the epitope recognized by mAb R1.302 to HhGR/PRR1, capable of inhibiting infection, was mapped to the V domain. Second, a soluble form of HIgR/PRR1 consisting of the single V domain competed with cell-bound full-length receptor and blocked virion infectivity. Third, the V domain was sufficient to mediate HSV entry, as an engineered form of PRR1 in which the two C2 domains were deleted and the V domain was retained and fused to its transmembrane and cytoplasmic regions was still able to confer susceptibility, although at reduced efficiency relative to full-length receptor. Consistently, transfer of the V domain of HhGR/PRR1 to a functionally inactive structural homologue generated a chimeric receptor with virus-entry activity. Finally, the single V domain was sufficient for in vitro physical interaction with gD. The in vitro binding was specific as it was competed both by antibodies to the receptor and by a mAb to gD with potent neutralizing activity for HSV-1 infectivity.

The receptors that mediate herpes simplex virus (HSV) entry into cells have remained elusive for a long time for several reasons. Cell lines lacking receptors are very rare, hampering a genetic approach to the search of the receptors. The virus appears to be able to use alternative receptors (1). Cellular proteins that are able to act as mediators of virus entry when transfected in cells that do not express any other suitable receptor have such a narrow distribution that their actual usage is limited to very specialized cell types. This appears to be the case for herpesvirus entry mediator A (HveA), previously designated HVEM (for herpesvirus entry mediator), which appears to be expressed and functional only in T lymphocytes (2). Recently, the bona fide receptors that mediate HSV-1 entry into human cells were identified as a cluster of molecules belonging to the IgG superfamily (3–5). They have a common structure defined by six conserved cysteines in the ectodomain, which form three domains, one V-like and two C2-like. There are three members known to date: the herpesvirus entry mediator C (HveC) (3), previously known as PRR1, for poliovirus receptor-related protein 1 (6), and HIgR, for herpesvirus Ig-like receptor (5), both of which enable entry of all HSV-1 and -2 strains tested, and HveB (or PRR2) (7), which enables entry of a subset of HSV strains, namely HSV-2 and some HSV-1 gD mutants, but not wild-type HSV-1 strains (4). HIgR and PRR1(HveC) share an identical ectodomain, differ in the transmembrane and cytoplasmic regions, and appear to be splice-variant isoforms (5). Evidence that they can be considered as the bona fide receptors that mediate HSV-1 entry into the most frequently used human cell lines rested on the expression of HIgR/PRR1 proteins in cell lines like HEP-2, HeLa, human fibroblasts, etc., as detected by reactivity to mAb R1.302 to PRR1, and on the ability of the same antibody to block HSV-1 infection in these cells (5). The high level of mRNA expression in samples from nervous system suggests possible usage in humans in the path of neuron infection by HSV (5). The finding that two isoforms—HIgR and PRR1(HveC)—sharing the ectodomain can both mediate HSV entry mapped the functional region of the receptors to their ectodomain (5).

At least four virion glycoproteins, gB, gD, and the heterodimer gH/gL, participate in HSV-1 entry into cells (8–11). Work of the past decade has pointed to gD as the virion component that interacts with cellular receptor(s). The initial observation that expression of gD rendered cells resistant to infection led to the proposal that gD sequesters a putative receptor able to bind the glycoprotein (12). The notion subsequently was strengthened by the findings that incubation of gD-expressing cells with antibodies to gD released the block (1, 13), that viral unrestricted mutants able to overcome the gD-mediated block carry mutations in gD (1, 13, 14), that antidiidiotypic antibodies mimicking gD could bind the surface of commonly used cell lines and blocked virus infectivity and cell-to-cell spread of virus (15), that cells susceptible to HSV infection were able to bind gD in a saturable manner (16), and that soluble forms of gD inhibited virion infectivity (16, 17). No such evidence for involvement of cellular receptors exists for the other glycoproteins, gB and gH/gL.

HSV-1 penetration into cells occurs through a pH-independent fusion event (18, 19). The molecular mechanisms underlying this process remain, in part, obscure, and a model is still lacking that shows how the interaction of gD with its cellular receptor triggers the fusion of the virion envelope with

Abbreviations: gD, glycoprotein D; HSV-1, herpes simplex virus-1; HIgR, herpesvirus receptor/herpes virus Ig-like receptor; hPVRα, human poliovirus receptor-α; PRR1, poliovirus receptor-related protein 1; HveC, herpesvirus entry mediator C; β-gal, β-galactosidase.

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the plasma membrane and recruits the other virion glycoproteins. The aim of the present work was to identify regions of HlgR/PRR1 that are functional in HSV-1 entry and interact with HSV-1 gD. We demonstrate that (i) a major functional region of HlgR/PRR1 with HSV-1 entry activity resides in the V domain of the molecules; (ii) when anchored to cell membrane either through its own transmembrane and cytoplasmic domains, or through transplantation to a structurally related, functionally inactive molecule, the V domain was sufficient to mediate HSV-1 entry into cells, although the collapsed receptor displayed a reduced efficiency compared with full-length molecules; and (iii) the single V domain was sufficient for binding to gD in vitro in a specific fashion, providing evidence for a direct physical interaction between the major functional region of HlgR/PRR1 in virus entry and its viral ligand, gD.

MATERIALS AND METHODS

Cells and Viruses. Cells were grown in DMEM supplemented with 5% fetal calf serum, HlgR/c11, PRR1/c1, and V-TM(PRR1)/Q were obtained by lipofectamine transfection of J1.1–2 cells (5) with pCF18 (HlgR), pLX1.12 (PRR1), or control of the 27 promoter inserted between U1.3 and U1.4 genes, was a gift of B. Roizman and will be described elsewhere. Pelleted extracellular virions were used in all experiments. Infectivity of R8102 was assayed as described (2).

Antibodies. mAb R1.302 to PRR1 and mAb 30 to gD were described (1); mAb HD1 (21) to gD was from Goodwin Institute (Plantation, FL).

Construction, Production, and Purification of Soluble Forms of HlgR and PVR Receptors sVCC(HlgR)-Fc, sV(HlgR)-Fc, and sVCC(PVRc)-Fc. The entire extracellular region of HlgR (amino acids 1–334) was amplified by PCR with primers CFLPRR15 (CGGA ATAG ATCG TCGG TCGG ATGG GCCT TG) and CFLPRR13 (CCGA TCGG CTGG ATAT TGAC CTCC AC). The V domain of HlgR was amplified (amino acids 1–144) with CFLPRR15 and CFLR1V (GGTGG CGGC CGCC ATCA CCGT GAGA TTGA GCTG GC). The extracellular region of PVR (amino acids 1–330) was amplified with primers SBPVPR5 (TTGA TCTG CAGA TGGC CCGA GCCA TGGC CGCC) and SBPVPR3 (ATTT CCTT GGGC CGGC TTGG ACTT GCAC GTTG ACAT). The PCR products were cloned in the Cos Fc Link (CFL) (SmithKline Beecham) vector (22) and transfected in Cos 1 with Eugene 6 (Boehringer Mannheim). The proteins were purified on Affigel protein A. The purity of the protein preparations was checked by silver staining of proteins separated on a 15% SDS-PAGE gel.

Construction of V-TM(PRR1) and V(HlgR)-PVRc Transmembrane Receptors. The V domain of HlgR was amplified with PRR1V5 (TAAT AAGC TTAT GGGT CGGA TCGG TTGG GCCT GC) and PRR1V3 (GGTG TTAG GAGA TTCC ATCA CGCT GAGA TTGG TG). The transmembrane and intracytoplasmic region was amplified by using primers PRR1IC3 (CAAT CTCG GATG GAAT TCCC CTAC CC) and PRR1IC5 (ATTA GGGT CCTT GATG GCCT CGTT ATGG ATGG ATGG ATG). Both PCR products were amplified in the second PCR to get the final cDNA with primers PRR1V5 and PRR1IC3 (23), cloned in the BamHI/HindIII sites of pcDNA3. For the chimera receptor V(HlgR)-PVRc, the V domain was amplified with primers PRR1V5 and R1VIRV3 (GGTG TCTG GGGC TTGG CATC CACG GATG GAGA TTGG TG). The two C domains and transmembrane and intracytoplasmic regions of PVRc were amplified with primers R1VIRCC5 (CAAT CTCG GATG GAAT TCCC CTAC CC) and R1VIRCC3 (GGTG GATG CCTT GATG GCCT CGTT ATGG ATGG ATG). The 1,253-bp cDNA fragment was cloned in BamHI/HindIII sites of the pcDNA3.

Sandwich ELISA for the Soluble Forms of HlgR/PRR1, sVCC(HlgR)-Fc, and sV(HlgR)-Fc. sVCC(HlgR)-Fc, sVCC(HlgR)-Fc, and sV(HlgR)-Fc were bound to microwells by means of anti-HlgG-Fc (Sigma) and reacted with biotinylated mAb R1.302, followed by streptavidin-peroxidase and One Step ABTS (Pierce).

Competition by sVCC(HlgR)-Fc and sV(HlgR)-Fc on HlgR/PRR1-Mediated HSV-1 Infectivity. Aliquots of R8102 were reacted with sVCC(HlgR)-Fc, sV(HlgR)-Fc, or CTLA4-Fc for 1 h at 37°C and absorbed to cells for 2 h at 4°C. Virus was removed. Cells were overlaid with medium containing the sVCC(HlgR)-Fc and sV(HlgR)-Fc at the same concentrations used in the inoculum and incubated for 16 h at 37°C. β-Galactosidase (β-gal) was assayed as described (2).

Competition of HSV-1 Infectivity by Soluble gD. HlgR/c11 cells in 96-well plates were preincubated for 2 h at 4°C with gD-1(A290–299) (24). Viral inoculum in 7.5 μl was added for 90 min at 4°C, removed, and cells were overlaid with DMEM containing glycoprotein. Infection was monitored as above.

In Vitro Binding of gD to Soluble Forms of HlgR/PRR1 by ELISA, gD was purified to homogeneity from HSV-1-infected BHK cells by affinity chromatography to mAb 30 (1) immobilized to Affigel. Microwell plates were coated with 16 nM gD and reacted with sVCC(HlgR)-Fc or sV(HlgR)-Fc, followed by anti-human peroxidase (1:6,000) and o-phenylenediamine (Sigma). For competition ELISA, microwells were coated with gD. Ten nanomolar sV(HlgR)-Fc [representing the saturating amount of sV(HlgR)-Fc for the gD-coated microwell (see Fig. 6.4)] were mixed with increasing concentrations of purified IgG of mAbs HD1, R1.302, or mouse IgG, and binding to gD was detected as described above. In Fig. 6B, dilution 1 corresponds to 1 μM purified IgG.

RESULTS

Construction and Purification of Soluble Forms of HlgR/PRR1 and of Human Poliovirus Receptor-α (hPVRα). Two soluble forms of HlgR/PRR1 and one of hPVRc were constructed in sVCC(HlgR)-Fc, the three domains (one V and two C2) that constitute the ectodomain of HlgR and of PRR1 were fused to the Fe domain of human IgG1. In sVCC(HlgR)-Fc, the single V domain of HlgR/PRR1 was fused to IgG1 Fc. In sVCC(PVRc)-Fc, the ectodomain of hPVRc was fused to the IgG1 Fc. Schematic representation is shown in Fig. 1. COS 1 cells were transfected with the plasmid DNAs, and the soluble receptors were purified from the culture medium by affinity chromatography to protein A. The purity of the protein preparations was checked by silver staining of proteins separated on a 15% SDS-PAGE gel and immunoblotting with a peroxidase-labeled antibody directed to the human Fc portion of IgG (data not shown). Under reducing conditions, the apparent Mr of purified sVCC(HlgR)-Fc and sV(HlgR)-Fc was 70,000 and 45,000, respectively, whereas the predicted Mr values were 59,600 and 38,300, possibly reflecting glycosylation of some of the predicted glycosylation sites present in the ectodomain of HlgR/PRR1. The V domain, as defined here and below, comprises the N-terminal 114 residues, after cleavage of the signal peptide, and PRR1 expresses either one or both isoforms (5). As HlgR and PRR1 share the ectodomain, the epitope recognized by mAb R1.302 must reside in the extracellular region of HlgR that is functional in HSV-1 entry and interacts with HlgR or PRR1. In a sandwich
ELISA that measured the binding of mAb R1.302 to sVCC-(HIgR)-Fc or sV(HIgR)-Fc, the antibody was capable of binding both molecules. The binding was highly specific as mAb R1.302 failed to bind soluble forms of hPVRα (Fig. 2) and of PRR2(HveB) (not shown), two structurally related receptors belonging to the same Ig cluster (6, 7, 22), and sCTLA4-Fc (data not shown), a chimeric protein carrying the V domain of the T cell costimulatory protein CTLA4 (25) fused to IgG1 Fc. These results suggest that a major functional region of HlgR/PRR1 involved in HSV-1 entry into cells resides in the V domain.

A Soluble Form of HlgR/PRR1 Consisting of the Single V Domain Inhibits HSV-1 Infectivity. It has been reported that a soluble form of HveC(PRR1) blocks HSV-1 infectivity (3). It has been reported that receptors belonging to the same Ig cluster (6, 7, 22), and sCTLA4-Fc (data not shown), a chimeric protein carrying the V domain of the T cell costimulatory protein CTLA4 (25) fused to IgG1 Fc. These results suggest that a major functional region of HlgR/PRR1 involved in HSV-1 entry into cells resides in the V domain.

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PRR1 consisting of the V domain fused to its transmembrane and into J1.1–2 cells. J1.1–2 cells were transfected with a deleted form of \( \gamma \alpha \) chimeric receptor consisting of the V domain of HIgR fused to the CC-transmembrane–cytoplasmic regions of hPVRα designated as V(HIgR)-PVRα (C and D), and for comparison with HIgR (E). (B and D) Blocking effect of mAb R1.302 on R8102 infectivity. R1.302, which is capable of inhibiting infection and whose reactive epitope maps to the V domain (Fig. 4B). The number of cells acquiring susceptibility increased when cells were infected at higher multiplicity of infection (100 plaque-forming units per cell) (data not shown). The number of cells acquiring susceptibility was much lower in cultures expressing V-TM(PRRI) than in cultures expressing the full-length HIgR or PRR1. This may be due to either a lower extent of expression of V-TM(PRRI) relative to the full-length molecules or a lower efficiency of the truncated molecule in conferring susceptibility to infection. To discriminate between these two possibilities the extent of expression was compared in V-TM(PRRI)- and HIgR-transfected cells by immunofluorescence with mAb R1.302. The number of fluorescent cells was found to be practically the same (data not shown), suggesting that the deleted version of PRR1 lacking the two C domains is less effective than the full-length counterpart in conferring susceptibility to HSV-1 infection. Parenthetically, the reactivity of mAb R1.302 to cells expressing the deleted and the full-length versions of HIgR/PRR1 confirms that mAb R1.302 is directed to an epitope present in the V domain. Altogether, the results demonstrate that the V domain of HIgR/PRR1 was sufficient to mediate HSV-1 entry into cells, although at reduced efficiency relative to the full-length receptor, and that susceptibility conferred by V-TM(PRRI) correlated specifically with the presence of the V domain.

To confirm this and to investigate the reasons for the lower efficiency of V-TM(PRRI), a second construct was generated in which the V domain of HIgR/PRR1 was transferred to CC-transmembrane–cytoplasmic regions of hPVRα. This receptor was chosen as acceptor of the HIgR/PRR1 V domain because it has an overlapping structure to that of HIgR (6) but fails to mediate entry of any HSV-1 and -2 tested (3). Therefore, it represents the available receptor that is functionally inactive but structurally closer to HIgR/PRR1. Fig. 4C shows that V(HIgR)-PVRα transfected into the resistant J1.1–2 cells conferred susceptibility to HSV-1 infection and had an efficiency comparable to that of full-length HIgR (compare Fig. 4 C and E). Infectivity was abolished by exposure of cells expressing V(HIgR)-PVRα to mAb R1.302 (Fig. 4D), demonstrating that the susceptibility acquired by V(HIgR)-PVRα was a result of transfer of the V domain of HIgR/PRR1. The results confirm that the V domain of HIgR was sufficient to confer susceptibility and, in addition, suggest that the CC backbone of this cluster of molecules augments the virus entry activity located in the V domain and/or participates in virus entry activity with other mechanisms, as noted in the Discussion.

Soluble gD Competes with HSV-1 Infectivity in Cells in Which HIgR Is the Only Available Receptor. Soluble forms of gD inhibit HSV-1 infectivity in Vero cells (16). As the receptors employed by HSV in these cells have not yet been identified, the cellular protein binding gD required for the gD inhibitory effect is unknown. In HveA-transformed cells, gD blocked HSV-1 infectivity (26), consistent with the ability of gD to bind HveA (27). Cells expressing HIgR or PRR1 acquire the ability to bind gD (5, 27). In this series of experiments we verified whether soluble gD can compete with HSV-1 infectivity in cells in which HIgR was the only available receptor. HIgR-cl 11 cells were preincubated with increasing amounts of a soluble recombinant form of gD [gD-1(Δ290–299)] (24), or with fetuin, as a control, unrelated glycoprotein, and subsequently infected with R8102. Results in Fig. 5 show that gD inhibited R8102 infectivity in a dose-dependent manner, while fetuin had no significant effect. The experiment demonstrates that in cells where HIgR was the only available receptor, the binding of HIgR with gD was crucial for HSV-1 entry. The Single V Domain Is Sufficient for Physical Interaction with gD. The above data demonstrate that a major region of HIgR/PRR1 functional in HSV-1 entry resides in the V domain and that this domain is sufficient to mediate HSV-1 infectivity, gD binds to a soluble form of HveA(PRR1) containing the entire ectodomain (27). Here we investigated whether the single V domain of HIgR/PRR1 was sufficient for the physical interaction with gD. For this assay, gD was immobilized to microwells and then reacted with the soluble receptor consisting of the single V-domain, sV(HIgR)-Fc, or with the full-length sVCC(HIgR)-Fc as a positive control. The results in Fig. 6A demonstrate that sV(HIgR)-Fc bound gD in a dose-dependent manner, with a curve essentially similar to that obtained with sVCC(HIgR)-Fc. There was an approximately 30% reduction in the level of saturable binding with sV(HIgR)-Fc relative to sVCC(HIgR)-Fc, suggesting a somewhat higher
Mediating HSV-1 Entry. Evidence for this conclusion rests cumulatively on two series of experiments. First, the mAb R1.302 to HlgR/PRR1, capable of inhibiting infection, reacted with an epitope mapped to the V domain of HlgR/PRR1. Second, a soluble form of the HlgR/PRR1 containing the single V domain competed in a dose-dependent manner with full-length, cell-bound receptor and blocked virus infectivity. Thus, HlgR adds a new member to the list of Ig-like viral receptors whose major functional domains reside in the more external regions. The list includes PVR, the poliovirus receptor, ICAM, the rhinovirus receptor, and CD4, the HIV receptor (29–36). That the N-terminal domains carry functional regions is consistent with the view that, even in the folded molecules, these regions are located more externally and therefore are more likely to interact with the appropriate regions of the virions.

The V Domain Anchored to Cell Membrane Is Sufficient to Mediate HSV-1 Entry into Cells. This conclusion rests on two experiments. First, the receptor-deficient J1.1–2 cells resistant to HSV-1 infection were rendered susceptible when transfected with an engineered form of PRR1 in which the V domain and transmembrane and cytoplasmic regions were retained and fused together but the two C2 domains were deleted. Second, the V domain of HlgR/PRR1 was transplanted to CC-transmembrane–cytoplasmic portion of hPVRα, a structural homologue of HlgR and PRR1 not functional in HSV-1 and -2 entry, generating a chimeric receptor that acquired HSV-1 entry activity. As infectivity was abolished by neutralizing mAb R1.302 in both cases, the receptor activity resided in the V domain. We conclude that the V domain is sufficient to act as a receptor as long as it is anchored to the membrane by suitable transmembrane/cytoplasmic domains.

The Single V Domain Is Sufficient for the in Vitro Physical Interaction of HlgR/PRR1 with gD. gD is a key component of the viral machinery essential for virus entry into susceptible cells, as outlined in the Introduction. Furthermore, gD can interact physically with full-length ectodomain of HveC(PPR1) (27). It was of interest to determine whether the V domain, herein shown to encode a major region functional in HSV-1 entry, was sufficient for in vitro binding to gD, or whether other portions of the ectodomain would mediate this binding. Here we show that a soluble form of HlgR/PRR1 consisting of the single V domain interacted physically with gD in an in vitro binding assay. The binding was specific as it was competed by mAbs to each partner with the ability to neutralize HSV infectivity. Thus, mAb R1.302 to HlgR/PRR1 competed with the ability of gD to bind to the receptor. In a similar fashion, the mAb HD1 to gD with potent neutralizing activity on virion infectivity competed with the binding of gD to its receptor. It can be noted that mAb HD1 recognizes antigenic site Ia of gD, according to the classification of Cohen and Eisenberg (28). The results further indicate that the gD region that interacts with the V domain of HlgR/PRR1 contains the antigenic site Ia, in agreement with the finding that mAbs to this site block binding of PPR1(HveC) to virions (27), and that the gD region interacting with HlgR/PRR1 does not exactly overlap, with that recognized by HveA, which recognizes antigenic sites Ib and VII, but not Ia (26).

Current data provide several lines of evidence that the V domain of HlgR/PRR1 contains the major functional region involved in and sufficient for HSV-1 entry into cells and for physical interaction with gD. Notwithstanding this, we noticed a reduction in the efficiency of the V domain relative to the full-length molecule to interact with virions and with gD, particularly evident in the lower ability of V-TM(PPR1) to mediate susceptibility to HSV-1 infection. Several explanations may account for these reductions, which remain to be investigated further. Thus, for example, the CC domains may provide a spacer that enhances accessibility of virions to the V domain, and/or the CC domains may form a scaffolding for a...
conformation of the V domain suitable to its interaction with virions and gD, as proposed for PVR (34). Alternatively, homo- or heterooligomer formation or association with additional cell surface proteins may be altered as a consequence of the removal of the CC domains. Further yet, the CC domains may have secondary sites that bind gD or may be able to bind the additional glycoproteins, gB and gH/gL, that participate in HSV-1 entry process and thus recruit them to the fusion complex. Consistent with current findings, in the case of poliovirus or HIV interaction with engineered forms of PVR or CD4, respectively, the N-terminal domain was sufficient to render cells susceptible to infection although the virus yields were reduced (32–34). In addition, in the case of poliovirus and rhinovirus receptors, the functional domains need to have a certain degree of flexibility, which may be provided by the CC and interdomain portions, as their cognate binding sites are located in a canyon embedded in the capsid.

Resolution of the interaction at the molecular level of HSV-1 with its bona fide receptors has two major implications. On one hand, it is instrumental to define the interaction between gD and HlgR/PRR1 in terms of minimal size of the functional domain, structural requirements, key residues, etc. In the present study the major functional region of HlgR/PRR1 with HSV-1 entry activity able to physically interact with the viral gD was narrowed to a region of about 114 aa residues. Ultimately, these studies could lead to a model that would allow critical tests of the fusion event between HSV-1 envelope and the plasma membranes and the mode of recruitment of the other viral glycoproteins involved in the process. On another hand, analyses of the interaction of gD with the functional regions of its receptor may lead to practical applications. Infections with HSV-1 are highly prevalent among humans. Pathologic manifestations vary from mucocutaneous lesions of the mouth, face, eyes, or genitals, to involvement of central nervous system, resulting sometimes in encephalitis (see ref. 37). The identification that a major functional domain of HlgR/PRR1 is encoded in the V domain coupled with the demonstration that this domain is sufficient for physical binding to gD and to compete with virion infectivity may provide a basis for the future development of a novel class of anti-HSV agents designed specifically to block HSV-1 infection.

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