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ARTICLE

HSV-1 Virions Engineered for Specific Binding to Cell Surface Receptors

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Expression of specific peptide epitopes on the surface of virions has significant potential for studying viral biology and designing vectors for targeted gene therapy. In this study, an HSV-1 amplicon plasmid expressing a modified glycoprotein C (gC), in which the heparan sulfate binding domain was replaced with a His-tag, was used in generating HSV-1 virions. Western blot analysis demonstrated the presence of modified gC in the purified virions. The amplicon vectors were packaged using a gC^-, $lacZ^+$ helper virus to generate a mixture of high-titer helper virus ($lacZ^+$) and amplicon vectors (GFP^+), which expressed modified gC in the virion envelope. His-tagged virions bound to 293 6H cells expressing a cell surface pseudo-His-tag receptor four-fold more efficiently than to parental 293 cells and also proved more effective than wild-type virus in binding to both cell types. Binding resulted in productive infection by the modified virions with expression of reporter genes and cytopathic effect comparable to those of wild-type virions. Thus, not only can HSV-1 tropism be manipulated to recognize a non-herpes simplex binding receptor, but it is also possible to increase the infective capacity of the vectors beyond that of the wild-type virus via specific ligand receptor combinations.

Key Words: virus entry, HSV receptors, targeting, virion envelope, heparan sulfate, gene therapy, HSV-1 glycoproteins

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) vectors have several advantages as gene delivery vehicles, including the relative ease of vector construction, their large transgene capacity, and their broad host range [1,2]. For some applications, their use may be enhanced by modifying the virion so as to target infection to specific cell types. HSV-1 virions consist of four distinct subcompartments: (1) an inner core containing the linear double-stranded DNA viral genome; (2) an icosadeltahedral proteinaceous capsid surrounding the core; (3) an amorphous tegument that contains proteins, such as VP16 and *vhs* (virus host shutoff); and (4) an outer lipid bilayer envelope containing at least 12 glycoproteins. HSV-1 infection involves binding of the virion to the cell surface followed by fusion of the virion envelope with the plasma membrane and subsequent entry of the nucleocapsid into cytoplasm. Virus attachment and penetration involve a cascade of interactions between several envelope glycoproteins and host cell surface molecules [3]. The initial binding of virus to susceptible cells is mediated by the heparan sulfate (HS) binding domains of glycoproteins C (gC) and B (gB) in the virion envelope [3,4]. These domains bind with high affinity to HS glucosaminoglycan side chains found on many cell surfaces.

Interactions of gC and gB with HS have been studied extensively and their respective binding domains have been defined [4–9]. HSV-1 gC-negative mutants are impaired in binding and penetration but remain highly competent for replication [4]. Mutant viruses lacking both gC and gB are further impaired in binding compared to

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gC-negative virus, suggesting that gB also contributes significantly to HS binding [7]. Following gC and gB binding to HS, glycoprotein D (gD) interacts with one of several cellular receptors, five of which have been identified—HveA (herpes virus entry A) [10], HveC (nectin-1 [11]), HveB (nectin-2 [12]), nectin-3 [13], and HS modified by a 3-O-sulfotransferase [14]. Although it was initially thought that gD binding to its receptor had a higher affinity than gC for HS [15,16], recent work by Rux *et al.* [17] has demonstrated that the affinity of gC to HS is 30-fold higher than that of gD for any of its receptors. Following gD–receptor interaction, a pH-independent fusion event, facilitated by gB and the additional glycoproteins H and L, occurs between the virion envelope and the host cell plasma membrane [18–20] to allow the capsid and associated tegument proteins to enter into the cytoplasm.

Several lines of evidence support gC as the major mediator in virus attachment to cells via HS moieties of cell surface proteoglycans [4]. First, heparin, a molecule chemically similar to HS, inhibits the binding of HSV to cells by masking the HS binding domain on the virus envelope [21]. Second, masking HS on the cell surface, also by heparin treatment or by selection of cell lines defective in HS synthesis, significantly reduces virus binding [22–25]. And third, gC and gB proteins isolated from infected cells, as well as virions, bind to immobilized heparin on affinity column [4]. Our studies have focused on modifying gC for retargeting binding as its contribution to binding is 40% greater than that of gB [9] and also because gB is essential for virus penetration.

Targeting HSV-1 vectors requires at least two kinds of modifications to the viral envelope. First, the natural tropism of HSV-1 should be reduced. And second, the vector should express new ligands capable of binding to specific cell surface receptors to decrease the infection of nonspecific tissues while preserving the natural mechanism of virus entry through fusion of the virus envelope with the cell surface membrane. Reducing native HSV-1 tropism can potentially be achieved by deleting the native ubiquitous cell recognition sites found in viral envelope glycoproteins and replacing these sequences with cell-specific ligands to allow selective entry only into cells that bear receptors for those ligands. Mutational analysis across the external N-terminal domain of gC has defined the major HS binding determinant to lie between amino acids 33 and 123 [8]. Therefore, modifications to this region would be predicted to alter the native tropism of HSV-1 virions to HS motifs. Targeting also depends on the identification of appropriate ligand/receptor combinations and a strategy for introducing ligands into the viral envelope while maintaining its infectivity.

The present study was designed to develop a versatile method for targeting HSV-1 infection by redirecting virion attachment to unique cell surface receptors. In an effort to alter the natural tropism of HSV-1 virions, the HS binding domain of gC was replaced with a His-tag sequence. Re-engineered HSV-1 virions expressing the modified gC, as well as virions with wild-type gC, were evaluated for binding to parental human 293 cells (naturally permissive to HSV-1 infection) and a line derived from them (293 6H) expressing a pseudo-His-tag receptor. The results showed that gC-modified virions bound at least four times more efficiently to 293 6H cells compared to 293 cells and compared to wild-type virus.

RESULTS

Modified gC in Virions

To assess the expression and processing of modified gC, we transfected the amplicon plasmid encoding modified gC (pCONGAH; Fig. 1) into Vero cells. Since gC is under the control of its own promoter, viral transactivating

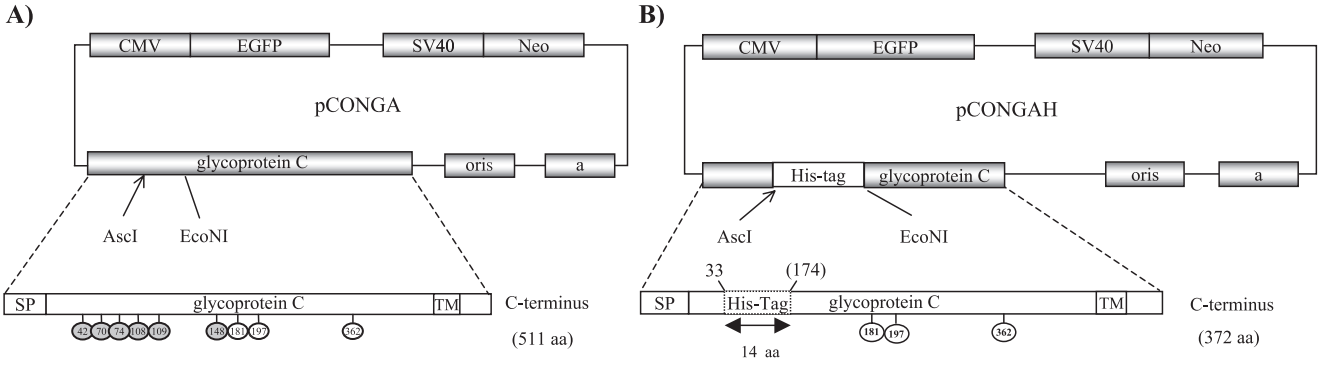

FIG. 1. Structure of HSV-amplicon vectors pCONGA and pCONGAH encoding wild-type gC and gC*mut*His-tag. (A) Amplicon pCONGA bears the full-length HSV-1 gC gene with its own promoter as well as transgene cassettes for GFP under the CMV promoter and *neo*^{R} under the SV40 promoter. (B) In amplicon pCONGAH a His-tag sequence was inserted in-frame within gC in pCONGA, replacing amino acid residues 33 to 174 with His-tag sequences to create the recombinant fusion protein gC*mut*His-tag. Abbreviations: SP, signal peptide; TM, transmembrane domain; circles, N-linked glycosylation sites; aa, position number (those in the HS binding domain are shaded).

proteins are required for expression. We induced the transfected cells to express gC mutant proteins by infection with either gC-deleted (gCΔ2-3) or gC wild-type (hrR3) virus, resulting in the production of both amplicon vector and helper virus virions. We harvested the different populations of virions carrying only gC*mut*His-tag (CONGAH/gCΔ2-3) or both gC*mut*His-tag and gC*wt* glycoproteins (CONGAH/hrR3) and concentrated them, with hrR3 and gCΔ2-3 virus as controls. To assess the extent of glycosylation of gC*mut*His-tag in the envelope, we treated purified virions with N-glycosidase F. We analyzed incorporation of gC proteins into the virions by Western blot using both gC and His-tag antibodies to evaluate the size of immunoreactive gC and presence of the His-tag (Fig. 2). As predicted, the gC*mut*His-tag (Fig. 2A, lanes 6 and 8, and 2B, lanes 5 and 7) showed increased mobility in SDS–PAGE gels (apparent MW 50 kDa), compared to gC*wt* (apparent MW 97 kDa) (Fig. 2A, lane 4), consistent with the deletion of 139 residues, the removal of six potential N-glycosylation sites at positions 42, 70, 74, 108, 109, and 148, and the insertion of 6 His residues. A markedly higher level of gC*mut*His-tag versus gC*wt* protein was observed in the purified virions obtained by transfection with pCONGAH and infection with hrR3 compared to hrR3 alone. Treatment with N-glycosidase F yielded faster migrating immunoreactive bands consistent with glycosylation of gC in the virions. The apparent size of the protein core (44 kDa) of the modified gC was consistent with the calculated molecular weight of gC*mut*His-tag. Increasing enzyme concentration in the reaction did not alter this pattern, suggesting that digestion was complete under these conditions (data not shown). As expected, both gC and His-tag antibodies failed to detect any proteins from the gC-deleted virus, gCΔ2-3 (Figs. 2A and B, lanes 1 and 2). These data suggest that by using this method: (i) virions can incorporate gC*mut*His-tag into the envelope; (ii) the gC*mut*His-tag is incorporated at concentrations greater than gC wild type, even when gC is expressed at the same time by helper virus; and (iii) gC*mut*His-tag is glycosylated at the remaining glycosylation sites.

Infectivity of gC-Modified Virions

Modification of the HSV-1 virion to express modified envelope components may affect binding to cellular receptors and/or penetration into the cell cytoplasm. These two processes can be dissociated from one another either at low temperatures or by using neutralizing antibodies that block binding, but not penetration [34,35]. To assess whether gC*mut*His-tag was exposed on the virus envelope and able to mediate His-tag receptor binding activity, we applied CONGAH virions to parental 293 cells and 293-derived cells stably expressing a pseudo-His-tag receptor (293 6H) at 4°C. Using a monoclonal anti-HA antibody, we confirmed that 293 6H cells express the His-tag receptor (42 kDa) in abundant amounts with no cross-reacting proteins in control 293, Vero, and 2-2 cells (Fig. 3).

We compared the relative binding efficiencies to 293 6H and 293 cells of virions bearing only the His-tagged gC with those of gC wild-type or gC-null virions over different time intervals at 4°C (Fig. 4). We quantitated relative infectivity by subsequent incubation at 37°C and assessment of lacZ expression 24 h later. (*Note*. Given that the ratio of helper virus to amplicon vector was greater than 100:1, only lacZ, and not GFP, was assessed.) Wild-type gC virus binding efficiency at 4°C for 1 h was ~1000-fold less than at 37°C for 1 h on both 293 and 293 6H cells (data

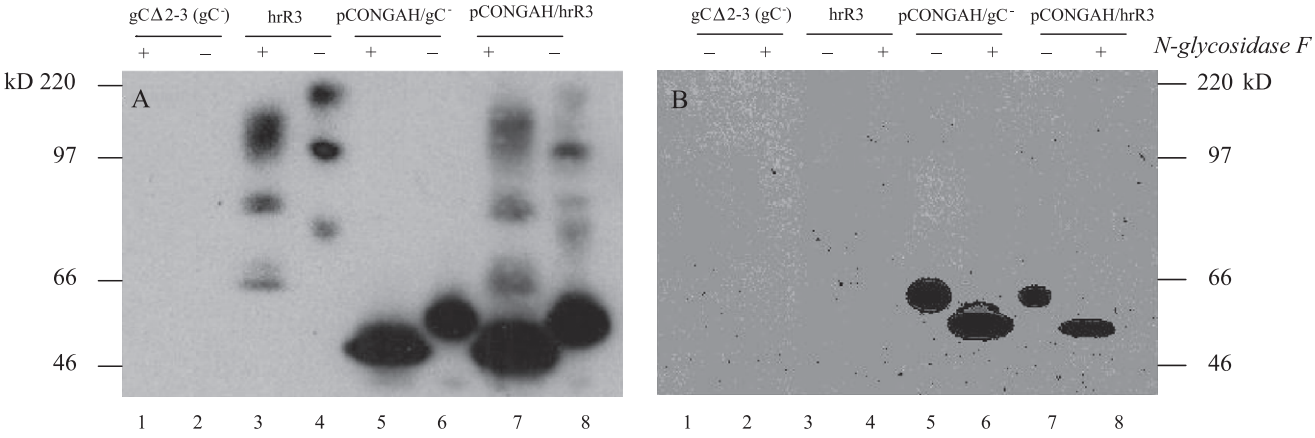

FIG. 2. Characterization of gC*mut*His-tag in virions. 293 cells were transfected with amplicon encoding gC*mut*His-tag. Twenty-four hours posttransfection, the cell monolayers were infected with hrR3 or gC-deleted virus. The resulting virions were purified and solubilized and either mock digested (–) or incubated with N-glycosidase F (+). Samples were then separated via SDS–PAGE, blotted onto nitrocellulose, and probed (A) with a rabbit polyclonal antibody to gC or (B) with a murine monoclonal antibody to the His-tag sequences. hrR3 and gCΔ2-3 virion were used as controls.

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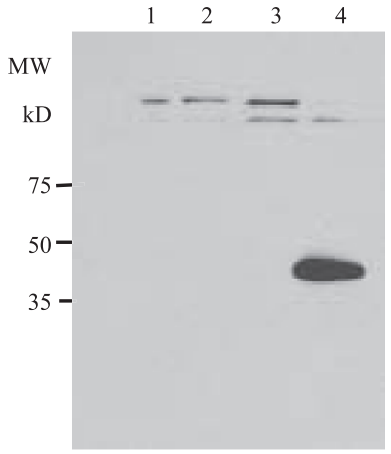

FIG. 3. Western blot characterization of 293 6H cells. Cell lysates of Vero (lane 1), Vero 2-2 (lane 2), 293 (lane 3), and 293 6H cells (lane 4) were subjected to SDS–PAGE gel followed by Western blotting using the monoclonal anti-HA-Ab, which specifically recognized the pseudo-receptor present in 293 6H cells.

not shown). For both wild-type and mutant gC viruses, doubling the incubation time at 4°C from 1 to 2 h increased the number of bound virus moderately (~40%), with cells detaching from the plates at longer time points. After a 2-h incubation on cell monolayers at 4°C, the binding capacity of CONGAH virions for 293 6H cells was about 4.5 times more efficient compared to parental 293 cells or to wild-type gC virions for either 293 or 293 6H cells. As expected, gC⁻ virion binding/infectivity was reduced 50% compared to wild-type gC virions. In a parallel experiment, CONGA (wild-type gC) containing an *Asc*I restriction site prepared with mutant (gCΔ2-3) or wild-type gC (hrR3) virus resulted in comparable binding profiles to wild-type gC (data not shown), indicating normal function of the modified, wild-type gC. In all cases, binding resulted in a productive infection by the modified virions, with expression of the reporter gene and induction of cytopathic effect comparable to virions bearing wild-type gC. Thus, the addition of a His-tag in place of the HS binding domain in gC did not block the ability

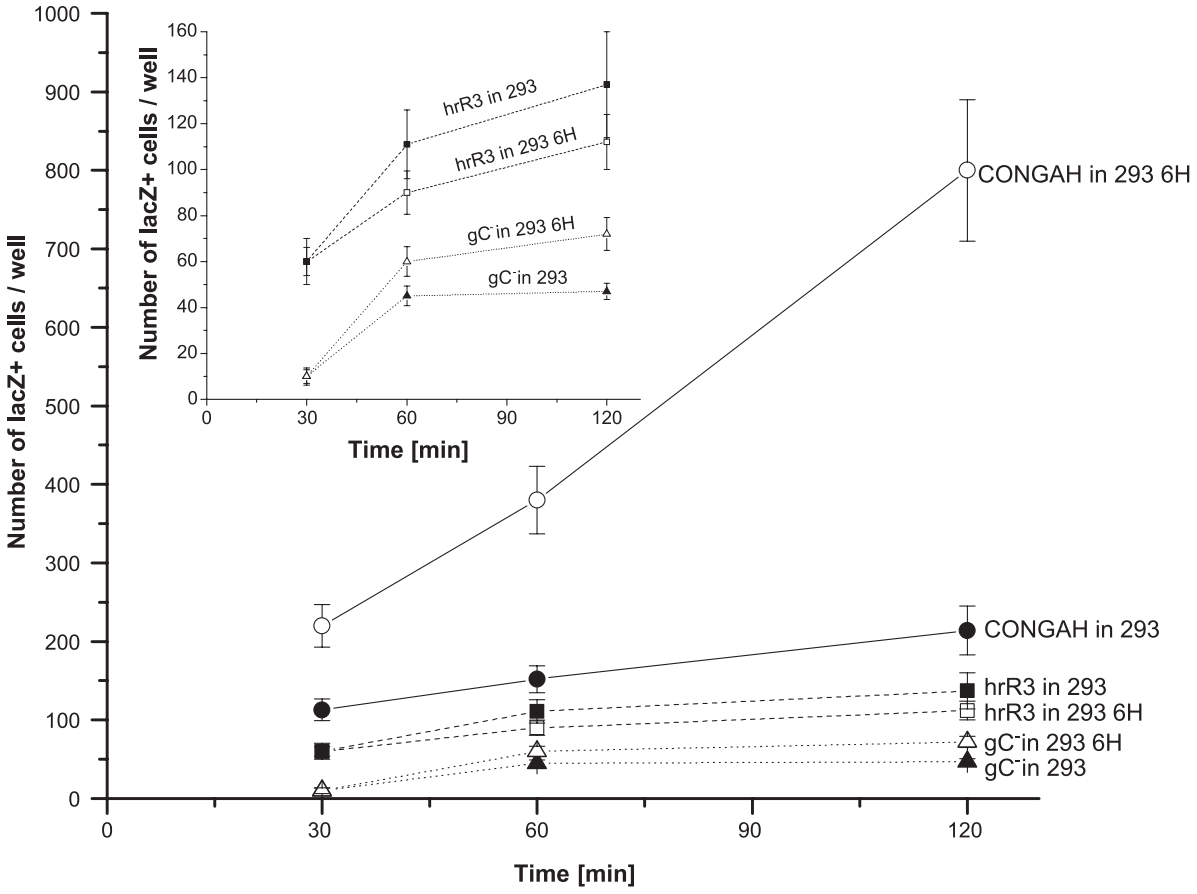

FIG. 4. Binding efficiencies of CONGAH and other virions to 293 and 293 6H cells. The binding efficiencies of CONGAH, hrR3, and gC⁻ viruses (all of them lacZ⁺) were assessed using 293 and 293 6H cells. Cells were incubated at 4°C with viruses (m.o.i. = 0.1). The viruses were allowed to bind to the cell surface for 0 to 120 min, after which the unbound virus was removed by washing. Cells were then incubated at 37°C for 24 h and stained for lacZ. Control experiments using hrR3 and gC⁻ virus are expanded in the inset. Error bars, means ± SEM.

of the virus to infect cells, and entry of HSV virions was both targeted to specific cell surface receptors and enhanced over wild-type virus binding to native 293 cells and to cells bearing those receptors.

Specificity of His-Tagged gC Binding to Pseudo-receptor-Bearing Cells

We carried out two assays to evaluate the specificity of binding of His-tag virions to 293 6H cells. First, we used a competition assay to determine whether a soluble His-tag protein (C-NF1-His-tag) could inhibit gC*mut*His-tag virus binding to 293 6H cells. This experiment indicated that binding of CONGAH virions to 293 6H cells was inhibited up to 80% by this His-tag protein (Fig. 5). This was quantitated as the percentage decrease in number of lacZ$^+$ cells relative to unblocked CONGAH infection (~1000 versus 200 lacZ$^+$ cells/well in the absence or presence, respectively, of C-NF1-His-tag protein). As expected this His-tag protein did not inhibit binding of CONGAH and hrR3 virions to 293 cells. These data suggest that the soluble His-tag fusion protein and the gC*mut*His-tag virions compete for the same pseudo-His-tag receptor binding sites on the cells.

Second, we confirmed exposure of gC*mut*His-tag protein on the surface of virions by using mouse anti-His-tag antibody to block binding of virions expressing this recombinant protein to 293 6H cells and counted the number of cells expressing lacZ$^+$ in the presence or absence of anti-His-tag antibodies (Table 1). The binding of CONGAH virions was inhibited about 50% using the His-tag antibody at 1:100. In contrast, His-tag antibodies did not neutralize hrR3 binding. Treatment with anti-myoglobin antibodies also did not alter virus binding of CONGAH compared to hrR3 (data not shown). This indicates that the His-tag on intact CONGAH virions was accessible to the anti-His-tag mAb and that this interaction blocked binding to the pseudo-His-tag receptors.

TABLE 1: Virus neutralization assay

Virus/amplicon[a]	+His-tag Ab (No. lacZ$^+$ cells)		No Ab (No. lacZ$^+$ cells)
	1:50	1:100	
hrR3	41	44	43
CONGAH	19	20	52

[a] hrR3 virus and CONGAH virus (10^3 transducing units in 10 μl) were incubated for 30 min at room temperature with different dilutions of mouse mAb against the His-tag (1:50 and 1:100). The treated virus preparations were then used to infect 2.5×10^5 293 cells for 1 h at 4°C in a 24-well plate. The cells were washed three times and then incubated at 37°C for 24 h. Cells were stained for lacZ and lacZ$^+$ cells per well counted. Results are from a representative experiment.

DISCUSSION

This study demonstrates a simple, versatile means to target infection of HSV-1 virions to specific cells based on replacing the HS binding domain of the gC glycoprotein with an epitope for a cell surface receptor. An amplicon plasmid was engineered to contain unique restriction sites flanking this domain such that it could be readily replaced with other peptide ligands. As a model system for redirecting viral tropism, a unique His-tag element was incorporated into gC. Virions packaged in cells transfected with such an amplicon and infected with helper virus deleted for gC sequences expressed only modified gC in the envelope. When helper virus encoding intact gC was used, the virions contained an abundance of modified gC over wild-type gC, presumably due to higher levels of expression following transfection versus infection. This modified gC was shown to be present in purified virions and was able to increase binding to cells expressing a pseudo-His-tag receptor by over four-fold compared to

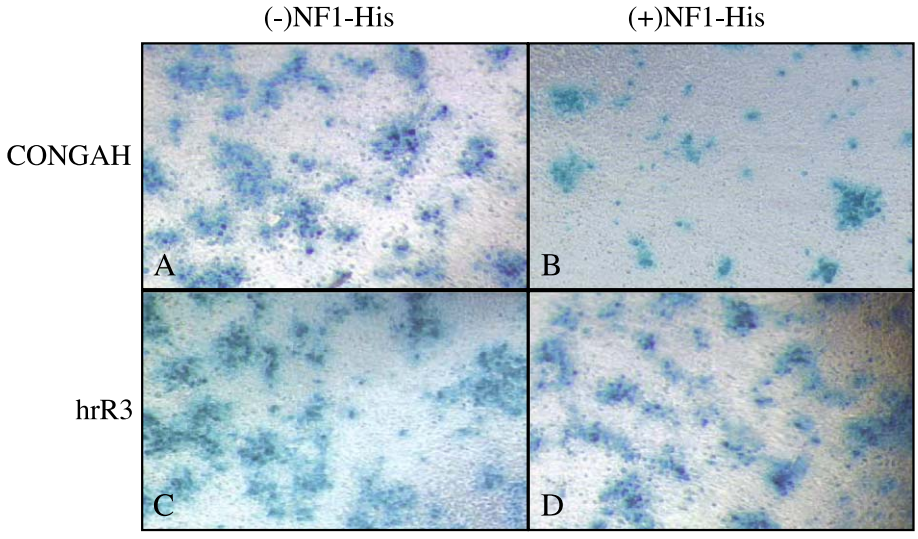

FIG. 5. Competition for binding. 293 6H cells were incubated (at 4°C for 30 min) with (A and B) CONGAH or (C and D) hrR3 virus in (A and C) the absence or (B and D) the presence of His-tag-receptor competitor protein (NF1-His), then washed, and incubated at 37°C. Twenty-four hours later the cells were stained for lacZ. Representative fields are shown.

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cells without this receptor. Further, the infectivity of the modified virions was greater than that of wild-type virions for these cells, indicating the capacity not only to target, but also to increase the infectivity of HSV-1 virions.

Notably in this study the efficiency of HSV-1 infection was greater for the His-tag-engineered virions to cells bearing the His-tag receptor than that of wild-type virions to the parental non-His-tag-expressing cells, indicating that rather than being compromised, infectivity was enhanced. These results suggest that the gC*mut*His-tag in the modified envelope has a higher affinity for the pseudo-His-tag receptor than does wild-type gC for HS. This observation has important implications. The ability to increase infectability may allow investigators to inject fewer viruses in *in vivo* targeting experiments without losing efficiency of infection and thereby increase the safety profile of this vector. Further, based on Western blot analysis, a markedly higher level of gC*mut*His-tag protein in virions was obtained by transfection with pCONGAH followed by infection with hrR3 containing wild-type gC, compared to the amount of wild-type gC obtained by hrR3 infection alone. Thus, it appears that retargeting of HSV-1 virus may be achieved by propagation of any of the commonly used HSV-1 recombinant virus vectors (e.g., [36,37]) in cells transfected with modified gC expression cassettes. Although this method facilitates evaluation of ligands for retargeting of HSV-1 virions, manipulation of HSV-1 glycoproteins by deletion or substitution of peptide sequence may result, in some cases, in altered glycoprotein processing such that they are not incorporated into the virion envelope or interfere with infectivity.

HSV-1 recombinant and amplicon vectors have shown great promise in a number of therapeutic paradigms, e.g., cancer vaccination [38], oncolytic cancer therapy [39], pain amelioration [36], and growth factor delivery [40–42]. Vectors are typically delivered to specific sites in the body by direct injection into the local area of interest. However, in some applications disseminated delivery with cell-specific targeting could be advantageous. For example, a single direct intratumoral injection of vector can provide relatively efficient gene delivery to a single focal region in the tumor, but does not address local invasion, distant migration, or diffuse seeding by tumor cells [39,43]. HSV-1 vectors in current use possess a broad host range and can therefore infect normal cells in addition to tumor cells. Lack of specificity could limit the utility of HSV-1 vectors for modalities such as in cancer gene therapy because the therapy could result in toxicity to normal cells, particularly when the therapeutic gene encodes a toxic protein. The potential for toxicity to normal cells is increased by systemic delivery with sequestration of HSV-1 vectors delivered through the vasculature in the liver, kidney, and lung [44], thus reducing the number of vector particles available for delivery to cancer cells.

The success of the present strategy in retargeting successful infection of HSV-1 may lie in the limited alteration in the envelope and high-level expression of the modified envelope protein. gC is responsible for about 60% of the binding capacity of the virion to HS on the cell surface, while gB contributes the remaining binding activity. However, since gB also has a role in the virion entry process, modification of gB may inadvertently compromise infectability. The binding role of gB and any wild-type gC in virions containing modified gC is presumably minimized by the greater abundance of the modified gC. In fact the efficiency of binding can be increased over that of gC and gB in wild-type virions by choosing ligands with high binding affinity to specific cell surface receptors. In this regard it may be important to choose ligands that do not enhance receptor-mediated endocytosis, as endocytosis of the HSV-1 virion is less efficient than fusion in mediating successful infection of cells, as endocytosis exposes the virion and its contents to degradative enzymes in the endosomal/lysosomal compartments of the cell. In contrast, fusion delivers the tegument/capsid directly into the cytoplasm where it can move along microtubules to the cell nucleus in a protected manner and deposit its DNA directly into the nucleus [45].

In previous studies HSV virions have been altered either to express unique ligands in the envelope in addition to the normal glycoprotein complement or to replace or modify specific envelope glycoproteins with other proteins; however, these vectors typically had a reduced ability to induce a productive infection and were impaired in spreading to the neighboring cells compared to wild-type virus. In one study, a novel cellular glycoprotein encoding CD4, a T-lymphocyte marker, was introduced into the HSV viral envelope [46] in addition to the normal glycoprotein complement, though specific binding to CD4-responsive cells was not tested. In another study, pseudorabies gB introduced into a mutant gB^- HSV-1 virus was able to complement the lethal defect in HSV-1 gB^- virus, but the reverse reaction was not possible [47]. Thus, these experiments suggested the possibility of modifying HSV-1 virus tropism with other viral glycoproteins. Mixed infections of HSV-1 and HSV-2 have been used to pseudotype the two viruses, which then acquire the ability to infect an expanded range of cell types. Although mixtures of HSV-1/HSV-2 recombinant viruses have been isolated and used for infections, they were principally used to physically map glycoprotein genes in combination with type-specific cytotoxic T-cell reactivities [48] and were not studied for targeted infectivity or altered host range. More recently, recombinant HSV-1 mutant virus deleted for the HS binding domain of gC was engineered to express erythropoietin (EPO) (incorporated in the N-terminal region of a truncated form of gC) to target binding to EPO-receptor-bearing cells [9]. This experiment suggested for the first time the possibility for targeting HSV-1 binding to a non-HSV-1 cell surface

receptor. However, a marked reduction in productive infection was observed, due to cellular endocytosis of the virus. Modification of gD in HSV-1 has also been tested [49] by incorporating the vesicular stomatitis glycoprotein G into a gD-deficient HSV vector. The resulting pseudotyped virus was able to infect 293 cells and form plaques on gD-expressing cells, indicating that foreign viral glycoproteins could be incorporated into the HSV envelope and mediate cell infection, but it did not form plaques on non-gD-expressing cells, indicating a deficit in cell-to-cell spread of infection.

Many of the other commonly used viral vectors have also been modified to increase specificity of infection by conjugating various receptor ligands and specific antibodies to native ligands, as well as by recombinant modification of endogenous viral surface molecules with the specific binding domains for ligands or antibodies [50–52]. In one approach, adeno-associated virus (AAV) vectors were modified by incorporating nonviral antigenic sequences into the AAV capsid and then retargeting infection to specific cellular receptors by using bispecific antibodies that recognized the antigen and a cell surface receptor [53,54]. The Moloney murine leukemia virus gp70 virion envelope protein was modified in a different manner by insertion of the receptor-binding domain of erythropoietin to increase the transduction of cells bearing the receptor [55,56]. A range of tumor-selective ligand epitopes (i.e., EGF, heregulin, and neurotensin) have been utilized in similar systems to retarget retrovirus particles to specific tumor receptors [57]. Modifications have also been introduced into recombinant adenovirus (Ad) fiber (virus attachment) or penton base (virus entry) proteins [58,59]. In one case, a vector was modified to recognize primary tumor cells by incorporating an RGD peptide within the fiber knob domain [60], while another vector was engineered to contain a HS-binding polylysine sequences at the terminus of the Ad hexon fiber [61]. A useful extension of this strategy for use in culture has involved the modification of both vector and target cells. His-tag residues were incorporated into the C-terminal of the Ad fiber protein. The modified Ad vector was able to infect a nonpermissive glioma cell line, U118, displaying an artificial anti-His sFv receptor on the cell surface as measured by luciferase expression [27]. Other specific targeting ligands have been described in the literature [50,52].

The components of this HSV virion targeting system include an amplicon plasmid that allows ready introduction of ligand elements replacing the HS binding domain and packaging with helper viruses either retaining or lacking the wild-type gC gene. This system thus allows a means of targeting HSV vectors to a variety of different cell surface receptors and for increasing virion infectivity. Not only can a specific ligand be incorporated into the virions as a targeting agent, but antigenic ligands can also be used for retargeting vectors using bispecific antibodies, which recognize the antigen on the virion and a cell surface receptor modality. For example, the Fab fragment of an anti-His monoclonal antibody could be conjugated to a monoclonal antibody that binds a receptor of interest. Further, it is envisioned that multiple ligand-binding domains could be incorporated into the same virions to direct infection toward multiple selected cell populations simultaneously. The ongoing identification of high-affinity peptide ligands [31] and antibodies specific for different cell antigens/receptors will increase the number of potential targeting modalities for gene delivery [62]. This amplicon-based virion modification system will allow an easy method to evaluate the effectiveness of various combinations for gene delivery and combine them with current therapeutic vectors.

Materials and Methods

Cell culture. Vero 2-2 (2-2) cells stably transfected with the HSV-1 gene for ICP27 (from Dr. Rozanne Sandri-Goldin, University of California at Irvine, CA, USA [26]), Vero cells (ATCC, Rockville, MD, USA), 293 cells (Microbix, Toronto, Ontario, Canada), and 293 6H cells (bearing His-tag receptor [27]) were maintained in DMEM growth medium supplemented with 200 μML-glutamine (Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA), and 10% fetal bovine serum (Sigma). Geneticin (Invitrogen) was added to the medium for 2-2 and 293 6H cells at 500 μg/ml. To obtain a cell line stably expressing the gC His-tag protein, Vero cells (1.5×10^6 in 60-mm plates) were transfected with pCONGAH. Two days later, at confluency, cells were trypsinized, diluted 10-fold, and maintained in growth medium containing 1 mg/ml G418 for 5 days. G418 concentration was subsequently decreased to 0.5 mg/ml. Individual G418-resistant colonies were picked and analyzed for modified gC containing the His-tag (gC*mut*His) and GFP expression. One high GFP-expressing clone, Vero AH2, was used to generate modified virions.

Helper virus stock. The HSV-1 recombinant viruses used in this study were hrR3 (ICP6$^-$, lacZ$^+$ mutant from Dr. Sandra Weller, University of Connecticut [28]) and gCΔ2-3 (gC$^-$, lacZ$^+$ virus from Dr. Curtis Brandt, University Wisconsin [7]), both derivatives of HSV-1 strain KOS. hrR3 and gCΔ2-3 stocks were prepared by infecting 2-2 cells at a m.o.i. of 1 transducing unit (tu)/cell. Each virus was harvested after approximately 16–24 h, when the cells displayed 100% cytopathic effect, and concentrated by centrifugation through a 25% sucrose gradient at 72,000*g* for 3 h at 4°C, as described [29]. Virus stocks were resuspended in phosphate-buffered saline (PBS), pH 7.4, aliquoted into 0.5-ml microcentrifuge tubes (Fisher, Pittsburgh, PA, USA), and stored at −80°C.

To generate CONGAH virions, Vero AH2 cells were infected with gCΔ2-3 virus (m.o.i. = 2–5) and harvested when 100% cytopathic effect was evident. The resulting CONGAH virions were purified and titered as described below for hrR3 and gCΔ2-3 virions.

Amplicon construction. The full-length HSV-1 gC gene with its own promoter was subcloned between the *Pst*I and *Hin*dIII sites in an amplicon plasmid (pBON) (kindly provided by Dr. Dora Ho, Stanford University, Palo Alto, CA, USA [30]). Site-specific mutagenesis was then performed to insert a unique *Asc*I recognition site prior to amino acid 33 to facilitate modification of gC (pCONGA, Fig. 1A). Next, the HS binding domain of gC was deleted by digestion with *Asc*I and *Eco*NI, corresponding to removal of amino acids 33 to 174 (423 bp), and replaced with a synthetic 42-bp sequence encoding the His-tag, flanked by *Asc*I and *Eco*NI (pCONGAH) (Fig. 1B). In both cases, an expression cassette carrying GFP and neomycin resistance (*neo*^{R}) genes (from pcDNA3.1 (−); Invitrogen) was inserted into a unique *Xho*I site downstream of the gC gene [31].

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Generation and titering of amplicon vector and helper virus. Amplicon plasmid, pCONGAH, was transfected into 2-2 cells (6×10^6 per 100-mm plate) by using LipofectAmine (Invitrogen) according to the manufacturer's protocol. Twenty-four hours posttransfection, the cell monolayers were infected at an m.o.i. of 1 with hrR3 or gCΔ2-3 virus. Virus was harvested when 100% cytopathic effect was evident and concentrated as described above. Titers of amplicon vector and helper virus were determined by transduction assay counting GFP-positive cells for amplicon vectors and lacZ-positive cells for helper viruses, as described. Typical titers were 10^{10} tu helper virus and 10^8 tu amplicon vector per milliliter.

Western blot analysis of modified gC in virions. Amplicon plasmid, pCONGAH, was transfected into 293 cells as described above. Twenty-four hours posttransfection, the cell monolayers were infected at an m.o.i. of 2 with either gC-wild-type (hrR3) or gC-deleted (gCΔ2-3) virus and harvested when cytopathic effect was observed. The modified virions were harvested and purified by banding on a sucrose gradient as described by Handler *et al.* [32]. The band containing virions was then extracted, mixed with PBS, and centrifuged in a Beckman SW28 rotor at 22K (87,275*g*) for 1 h to pellet. The virion pellet was then resuspended in lysis buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 0.02% Na azide, 0.1% SDS, 1% Triton X-100, and 1× Protease Inhibitor cocktail (Roche, Indianapolis, IN, USA). Some samples were treated with N-glycosidase F according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA). Untreated and glycosidase-treated samples were separated electrophoretically in a 12% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with a monoclonal antibody specific for His-tag (1:4000; Amersham Pharmacia Biotech, Piscataway, NJ, USA) or gC (1:10,000; rabbit 47 polyclonal antibody, kindly provided by Drs. G. H. Cohen and R. J. Eisenberg, University of Pennsylvania [33]) in 2% milk, 1× TBS/0.05% Tween 20 (TBS-T) overnight at 4°C. The membranes were then washed three times with TBS-T and incubated for 1 h at room temperature with anti-mouse or anti-rabbit HRP-conjugated antibody (Sigma) in 5% milk/TBS-T solution. After incubation, the blots were washed again and antibody signal was revealed by ECL (Pierce, Rockford, IL, USA). Confirmation of His-pseudo-receptor (which contains hemagglutinin ligand [29]) in 293 6H cells was performed similarly by gel electrophoresis (10 μg of total cellular protein) on a SDS–polyacrylamide gel and probing with a monoclonal anti-hemagglutinin antibody (Roche).

Binding and penetration assay. Monolayers of confluent 293 and 293 6H cells (1.5×10^6 per well in six-well plates) were incubated at 4°C for 30 min, washed twice with cold PBS, and incubated with different virus stocks (CONGAH, hrR3, gCΔ2-3) at identical titers (10^5 tu/well, six-well plate) in duplicate. The viruses were allowed to bind from 30 to 120 min at 4°C, after which the unbound virus was removed by washing the cells 3× with cold PBS. Cells were then shifted to 37°C to allow virus penetration. Twenty-four hours postinfection, cells were stained for lacZ to determine the number of cells infected.

Competition assay. 293 or 293 6H cells (10^5) were infected in suspension with 10^3 tu of CONGAH or hrR3 virions (10 μl), in the absence or presence of 15 μg His-tag control protein (provided by Dr. Rosemary Foster, Massachusetts General Hospital). This His-tag fusion protein consisted of six His residues fused in-frame with amino acids 2616–2811 of neurofibromin (NF1-His), was produced in *Escherichia coli,* and was purified over a nickel agarose column by three successive washes in urea-containing buffers, with decreasing pH. Following incubation with this protein and virions for 1 h at 4°C, the cells were washed three times with PBS, shifted to 37°C, and assayed for lacZ expression.

Virus neutralization assay. hrR3 or CONGAH virus (10^3 tu in 10 μl) was incubated for 30 min at room temperature with either different dilutions of mouse anti-His-tag antibodies (1:50 and 1:100) or mouse monoclonal anti-myoglobin antibody (used at the same concentration as mouse anti-His-tag antibody protein; Sigma). The treated virus preparations were then used to infect 3.5×10^5 293 6H cells/well in 24-well plates for 1 h at 4°C. The infected monolayers were washed twice with cold PBS to remove nonbinding virus, overlaid with medium without serum, and shifted to 37°C to allow virus penetration. Twenty-four hours later cells were washed and stained for lacZ expression.

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