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2 **Herpes Simplex Virus Glycoprotein C Regulates Low pH Entry**

3 **Tri Komala Sari^{a,b}, Katrina A. Gianopulos^{a,b}, Darin J. Weed^{a,b}, Seth M. Schneider^a,**

4 **Suzanne M. Pritchard^a, and Anthony V. Nicola^{a,1}**

5 ^aDepartment of Veterinary Microbiology and Pathology, College of Veterinary Medicine,

6 Washington State University, Pullman, WA, USA; ^bProtein Biotechnology Graduate Training

7 Program, College of Veterinary Medicine, Washington State University, Pullman, WA, USA

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9 Running title: HSV gC regulation of entry

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12 ¹Correspondence: anthony.nicola@wsu.edu

Abstract

Herpes simplex viruses (HSVs) cause significant morbidity and mortality in humans worldwide. Herpesviruses mediate entry by a multi-component, virus-encoded machinery. Herpesviruses enter cells by endosomal low pH and pH-neutral mechanisms in a cell-specific manner. HSV mediates cell entry via envelope glycoproteins gB, gD, and the heterodimer gH/gL regardless of pH or endocytosis requirements. HSV envelope proteins that function selectively in a given entry pathway have been elusive. Here we demonstrate that gC regulates cell entry and infection by a low pH pathway. Conformational changes in the core herpesviral fusogen gB are critical for membrane fusion. The presence of gC conferred a higher pH threshold to acid-induced antigenic changes in gB. Thus, gC may selectively facilitate low pH entry by regulating conformational changes in the fusion protein gB. We propose that gC modulates the HSV fusion machinery during entry into pathophysiologically relevant cells, such as human epidermal keratinocytes.

Keywords: herpesviruses, herpes simplex virus, viral entry, viral glycoproteins

Importance

Herpesviruses are ubiquitous pathogens that cause lifelong latent infections and are characterized by multiple entry pathways. We propose that herpes simplex virus (HSV) gC plays a selective role in modulating HSV entry by a low pH pathway, such as into epithelial cells. gC facilitates conformational change of the main fusogen gB, a class III fusion protein. We propose a model whereby gC functions with gB, gD, and gH/gL to allow low pH entry. In the absence of gC, HSV entry occurs at a lower pH, coincident with trafficking to a lower pH compartment where gB changes occur at more acidic pHs. This study identifies a new function for gC and provides novel insight into the complex mechanism of HSV entry and fusion.

Introduction

Herpesviruses contain multi-component fusion complexes and commandeer diverse entry pathways to enter target cells (1-5). Intracellular low pH facilitates entry of several herpesviruses in a cell-specific manner. The prototype alphaherpesvirus, herpes simplex virus, utilizes an acidic endosomal pathway to enter epithelial cells and a pH-independent, direct penetration pathway to enter neuronal cells (6, 7). The cellular triggers of herpesvirus entry, including intracompartamental pH, remain incompletely understood. HSV entry requires a host cell receptor that binds to viral glycoprotein D, such as HVEM or nectin-1 (8-10), but additional virus-host interactions are likely critical for entry. HSV particles contain at least 12 different virus-encoded envelope proteins. HSV entry into all cells requires gB, gD, and gH/gL. However, the majority of the remaining viral envelope proteins are not thought to be required for entry via either low pH or pH-neutral routes (11). Envelope proteins specific for a given HSV entry pathway have not been identified.

Glycoprotein B is conserved among herpesviruses and is a member of the class III fusion protein family. Unlike other class III fusion proteins such as VSV G and baculovirus gp64, herpesviral gB alone is not sufficient for fusion and requires additional viral proteins, most commonly gH/gL (12). Activation and regulation of the fusion function of gB are incompletely understood. The gH/gL complex is thought to positively regulate gB (13-15). HSV-1 gB undergoes conformational changes during fusion and entry (16, 17). Low pH specifically induces reversible changes in gB domains I and V, which comprise a functional region containing hydrophobic fusion loops (16). Acid-triggered changes in specific gB epitopes correlate with fusion activity: (i) HSV particles entering by endocytosis have reduced reactivity with gB domain I antibody H126, and elevation of endosomal pH blocks this change (16), (ii) irreversible acid-triggered changes in the H126 epitope coincide with irreversible acid-inactivation of HSV

fusion and entry (18), and (iii) a hyperfusogenic form of gB has reduced reactivity with domain I and domain V antibodies, similar to low pH-treated gB (19). Thus, the acidic milieu of endosomes may serve as a host cell trigger of gB function.

HSV-1 gC, a 511 amino acid, type I integral membrane glycoprotein, mediates HSV-1 attachment to host cell surface glycosaminoglycans. This interaction is not essential for HSV entry (20-22). Here we report that gC regulates low pH viral entry independent of its known role in cell attachment. We demonstrate that gC facilitates low-pH-induced antigenic changes in gB and that gC enhances the ability of HSV to enter and infect cells by a low-pH pathway. The results are consistent with a model: in the absence of gC, HSV entry occurs at a lower pH, coincident with trafficking to a lower pH compartment where gB changes occur at more acidic pHs. We propose that gC modulates HSV entry mediated by gB, gD and gH/gL into physiologically relevant cell types, such as human keratinocytes.

Results

HSV entry requires intracellular low pH in a cell-type dependent manner. Herpesviruses commandeer acidic endosomal pathways to enter physiologically relevant host cells, a concept that was first demonstrated for HSV (1, 6, 7, 23-28). HSV is proposed to utilize distinct cellular routes to infect its main target cells in the human host. HSV enters epithelial cells, the site of lytic replication by a low pH mechanism, and neurons, the site of latent infection, via a pH neutral one. HSV entry into human keratinocyte cell lines HaCaT and HEKa and into model CHO-HVEM cells is inhibited by lysosomotropic agents that elevate the normally low pH of endosomes (Table 1). In contrast, entry into human neural cells IMR-32 and SK-N-SH and into model Vero cells are not blocked by lysosomotropic agents (Table 1).

HSV-1 gC facilitates entry and infectivity of cells that support a low pH entry mechanism.

HSV envelope glycoproteins gB, gD, and gH/gL are required for entry regardless of a role for intracellular pH in a particular cell type (23, 29-32). A survey of seven additional viral envelope proteins indicated that HSV gE, gG, gI, gJ, gM, UL45, or Us9 are dispensable for entry by either low pH or pH-neutral pathways (11). In this study, we probed the role of gC in low pH entry by employing an HSV-1 KOS strain with the gC gene deleted (HSV-1 Δ gC2-3 or Δ gC) and a repaired version of this virus containing the wildtype gC gene (HSV-1 gC2-3R or gCR) (33).

HSV-1 gC is widely recognized to initiate the viral entry process by attaching to host cell surface glycosaminoglycans, principally heparin sulfate proteoglycans (22, 34). When gC-negative HSV-1 is added to cells, from 20 to 60 min p.i. there is a delay in entry relative to wild type HSV-1 (20). However, by 90 min p.i., penetration of wild type and gC-null viruses are indistinguishable. HSV-1 lacking gC has a 1 log defect in infectivity (20). Thus, while gC is dispensable in cell culture, it is important for the viral replicative cycle.

The contribution of gC to entry of HSV-1 by a low pH pathway was evaluated. HSV entry into CHO-receptor cells and human keratinocytes proceeds via a low pH endocytic pathway and is well-characterized (Table 1) (6, 7). The efficiency of Δ gC infection of CHO-HVEM cells and primary human keratinocytes (HEKa) was compared to Vero and IMR-32 cells, which support pH-neutral entry via penetration at the plasma membrane (35, 36). To control for attachment, virus was first added to cells at 4°C for 1 hr. Following a shift to 37°C for 6 h, the percentage of viral antigen-positive cells was quantitated. The efficiency of HSV-1gCR entry into each of the four cell types was similar under the conditions tested (Fig. 1A). In contrast, entry of HSV-1 Δ gC into CHO-HVEM and HEKa cells was ~50% and 35% less efficient than into Vero and IMR-32 cells, respectively (Fig. 1B). This suggests that gC contributes to low pH entry of HSV.

gC contributes to HSV plating efficiency on cells that support a low pH entry pathway.

To confirm and extend this conclusion using an alternate approach, the plating efficiency of HSV-1 Δ gC on different human cell lines was tested. The neuroblastoma SK-N-SH line supports pH-neutral entry of HSV, and HaCaT epidermal keratinocytes support low pH entry (Table 1) (6). Identical preparations of HSV-1 Δ gC or gCR were titered. gCR had a similar plating efficiency on SK-N-SH and HaCaT cells (Fig. 2A). Δ gC had ~ 1 log lower plating efficiency on HaCaT cells than on SK-N-SH cells ($P < 0.01$) (Fig. 2B). This suggests that gC is specifically important for HSV infectivity of HaCaT cells, which support a low pH entry pathway.

To investigate a potential mechanism of gC's involvement in low pH entry, we determined whether ectopic expression of gC restored the infectivity defect of Δ gC in HaCaT cells. SK-N-SH or HaCaT cells were transfected with gC or gD plasmids, and then the plating efficiency of gCR or Δ gC was determined. Cellular expression of gC had no effect on the infectivity of gCR or Δ gC (Fig. 2C, D). Cell-expressed gD reduces HSV entry and infectivity by competing with virion gD for receptor binding (37). As expected, ectopic expression of gD in either of the cell types reduced infectivity of both gCR and Δ gC (Fig. 2C, D). These results suggest that gC functions in low pH entry by playing a role other than receptor-binding.

gC has no detectable effect on the protein composition of HSV particles.

To address the possibility that deletion of gC might affect the incorporation of gB or other viral proteins into the HSV particle, the protein composition of Δ gC was compared to gCR. The absence of gC from virions did not measurably alter the protein composition of particles as measured by SDS-PAGE and protein staining (Fig. S1A). Envelope proteins gB, gD, gE, and gH were detected to an equivalent extent in both viruses by Western blot (Fig S1B). As expected, gC was not detected in Δ gC virions. The rescuant gCR contains an equivalent amount of gC as

the wild type KOS parent (33) (data not shown). These results are consistent with gC playing a specific role in low pH entry and infectivity of HSV.

Effect of ammonium chloride on low pH entry of Δ gC HSV.

The quartet of gB, gD, and gH/gL are essential for pH-neutral and low pH entry. gC is dispensable for pH-neutral entry (38). To determine whether gC was essential for low pH-dependent entry of HSV, we tested the effect of ammonium chloride treatment of CHO-HVEM, HaCaT, and HEKa cells on Δ gC entry using a reporter assay for entry. Ammonium chloride blocks wild type HSV entry into these cells (Table 1). Ammonium chloride inhibited Δ gC entry into CHO-HVEM, HaCaT, and HEKa cells in a concentration-dependent manner (Fig. 3D-F). gCR was similarly inhibited. Together the results suggest that gC contributes to low pH entry (Figs. 1, 2), but is not by itself a viral determinant of selection of the low pH pathway (Fig. 3). Ammonium chloride had little to no inhibitory effect on gCR entry into Vero, SK-N-SH or IMR-32 cells (Fig. 3A-C), which is consistent with pH-neutral entry of wild type HSV in these cells (Table 1). Entry of Δ gC into Vero, SK-N-SH or IMR-32 cells was similarly unaffected by ammonium chloride (Fig. 3A-C), consistent with the notion that gC is dispensable for pH-neutral entry of HSV (38).

gC does not contribute to viral attachment under the conditions tested.

We assessed the role of gC in HSV-1 attachment to the cell types used in this study. HSV-1 Δ gC or gCR was added to Vero, CHO-HVEM, SK-N-SH, HaCaT, IMR-32, or HEKa cells on ice for 1 hr at 4°C. Cell-attached HSV-1 was quantitated by qPCR. Δ gC attached to all cells in a manner similar to gCR (Fig. 4A, B). CHOpgs745 cells lack a gene required for heparan sulfate biosynthesis. Both viruses exhibited defective attachment to control CHOpgs745 cells (Fig. 4A, B). These results suggest that the altered entry and infectivity phenotypes of HSV-1 Δ gC cannot be explained by a defect in HSV-1 attachment.

gC drives the kinetics of viral penetration from acidic vesicles following intracellular transport of endocytosed HSV.

To probe further the role of gC in low pH entry, we monitored the kinetics of intracellular transport of HSV-1 (23). Virus was attached to cells at 4°C for 1 hr. Cultures were then shifted to 37°C, and at different times post-infection (p.i.), the remaining extracellular virions were inactivated, and cells were lysed. The detection of infectious HSV in cell lysates reflects enveloped HSV present within cellular endocytic compartments. As expected, HSV uptake into vesicles was rapid. At 10 min p.i. in CHO-HVEM cells, there was a peak of intracellular, infectious gCR virus (Fig. 5A). Following endocytic uptake, HSV fuses rapidly with the endosomal membrane and releases its capsid into the cytosol (23). This was reflected in the sharp decrease in infectious enveloped gCR recovered by 20 min p.i. (Fig. 5A). Interestingly, ~50% of infectious, intracellular ΔgC was recovered as late as 40 min p.i., suggesting a delay in HSV fusion with endocytic compartments in the absence of gC (Fig. 5A). Analysis of HSV-1 gCR and ΔgC trafficking in HEKa cells yielded results similar to CHO-HVEM cells (Fig. 5B). For ΔgC entry into the primary human keratinocytes, there was a ~40 min lag in the intracellular transport and exit of HSV relative to gCR (Fig. 5B). The results from Figure 5 suggest an important role for gC in the first ~ 20 min of wild type infection. The absence of gC appears to be overcome by ~ 60-120 min p.i., perhaps reflecting why gC is not absolutely essential for low pH entry when longer-term assays are employed. Together, the results suggest that gC mediates rapid intracellular transport of enveloped HSV or may aid in rapid exit of HSV from acidic intracellular vesicles.

gC positively regulates low pH-induced antigenic changes in gB.

To further delineate the mechanism underlying gC's role in low pH entry, the effect of gC on low pH-triggered antigenic changes in gB was assessed. The prefusion conformation of gB in the

virion envelope undergoes low pH-triggered changes in gB domains I and V (16). These changes are at least partially reversible (16, 39, 40) and are thought to be important for membrane fusion (16, 18, 19). Domain I of gB contains internal hydrophobic fusion loops that are critical for membrane fusion (41, 42). Δ gC or gCR virions were treated with a range of mildly acidic pHs (5.0 to 7.3) and blotted immediately to nitrocellulose membrane. Blots were probed at neutral pH with nine monoclonal antibodies (MAbs) to distinct epitopes in gB, and antibody reactivity was detected and quantitated (Fig. 6). A single representative dot blot experiment for one antibody from each of six gB structural domain are shown in Figure S2.

As a reference for comparing Δ gC to gCR, the pH treatment that reduced MAb reactivity by > 50% is indicated (Fig. 6). Domain I MAb H126 had reduced reactivity with gB from gCR that had been acid-treated, but interestingly exhibited little reduction with gB from Δ gC that had been similarly treated (Fig. 6). Using 50% reactivity as a reference point, in the absence of gC, the pH at which changes in the accessibility of the H126 gB epitope occurred was reduced by at least 0.6 pH units (Fig. 6). Similar results were obtained with SS55, another MAb to gB Domain I. For SS55, the pH of gB conformational change in Δ gC was reduced by ~ 0.5 pH units relative to gCR (Fig. 6). We have not previously examined the effect of low pH on gB Domain II. Interestingly, MAbs to domain II, H1781 and H1838, had reduced binding to gCR virions that had been treated with mildly acidic pH, suggesting that gB domain II undergoes pH-triggered conformational change (Fig. 6 and S2). The pH of antigenic change in both of the Domain II epitopes tested was decreased in the absence of gC (Fig. 6). H1838 reactivity with gB in the Δ gC virus was particularly resistant to low pH; treatment with pH 5.0, the lowest tested, still resulted in >50% reactivity, suggesting that gC alters the pH of antigenic change in the H1838 epitope of gB by > 0.7 pH units (Fig. 6). Domain III MAb H1359 had > 50% reduced reactivity only with

gB from gCR that had been treated with the most acidic condition tested, pH 5.0. The H1359 epitope in HSV-1 Δ gC was more resistant to pH 5.0-treatment relative to gCR.

Acidic pH triggers specific, not global, changes in gB conformation, as low pH does not induce changes in the SS10 (domain IV) or H1817 (domain VI) epitopes of gB (16). The SS10 and H1817 epitopes in both gCR and Δ gC viruses were not altered by pH (Fig. 6). In contrast, gB Domain V from wild type HSV is known to undergo pH-induced conformational change. Following pH 5.0 treatment, Domain V MAbs SS106 and SS144 had a > 50% reduction in reactivity with gB from both gCR and Δ gC, suggesting that gC has an effect on antigenic change in gB domain V (Fig. 6).

Control deletion of a viral envelope glycoprotein gene other than gC did not alter the pH of gB antigenic change of a representative epitope (Fig. S3). The gB H126 epitope (domain I) in HSV that lacks gE (HSV-1 F-gE/GFP), underwent similar pH-induced changes relative to gB from wild type virus strain F. As a control, the H1817 (domain VI) epitope was unaffected by low pH regardless of the presence of gE (Fig. S3). Together, the results suggest that HSV-1 gC specifically increases the pH threshold of gB conformational change, particularly in Domains I and II. This is consistent with gC facilitating low pH entry and infectivity of HSV (Fig. 1, 2, 5), possibly at the level of fusion with an endosomal membrane. Cargo transiting the host lysosome-terminal endocytosis pathway is subjected to decreasing pH, from ~ 6.5 to 4.5. HSV co-localizes with endocytosis markers, but the specific fusion compartment has not been identified (6, 43). Intracellular transport of gC-negative HSV may be delayed because transit to a lower pH compartment is necessary for fusion-associated changes in gB.

The pH threshold of reversibility of conformational changes in gB.

Reversibility of pH-triggered changes is a hallmark of gB and other class III fusion proteins (16, 44, 45). Pre-fusion and post-fusion forms of class III proteins are proposed to exist in a pH

equilibrium that is shifted to the post-fusion state by acidic pH (46). During HSV egress, reversibility may allow gB on progeny virions to avoid nonproductive activation during transport through low-pH secretory vesicles. The pH threshold of initiation of gB conformational change is ~ 6.2 to 6.4 (16). The pH at which acid-treated gB reverts to a pH-neutral conformation is not known. To determine this, virions were treated with pH 5 or maintained at pH 7.3 for 10 min at 37°C. The pH 5-treated samples were increased to different target pHs for 10 min at 37°C to determine the pH at which reversibility occurs. Samples were then blotted to membrane and probed with representative MAbs to gB (Fig. 7). Upon treatment with a low pH of 5, there was a reduced reactivity of MAb H126 (domain I) (Fig. 7A), H1781 (domain II) (Fig. 7B), and SS144 (domain V) (Fig. 7C), but not H1817 (Domain VI) (Fig. 7D). Consistent with Figure 6, the reduction of gB antibody reactivity with Δ gC was less pronounced. Upon increasing the pH of gCR from 5.0 to 5.6, there was a partial restoration of gB antibody reactivity. Increasing to pH 6.2-6.6 resulted in restoration of >80% of the reactivity measured at pH 7.3. This suggests that the pH threshold of reversibility of HSV gB conformational change is between pH 5.0 and 5.6. gB appears to be conformationally labile in this pH range.

To assess the influence of gC on the reversibility of gB conformational changes, Δ gC virions were tested for the reversibility of pH-triggered changes in the H126, H1781, SS144, and H1817 epitopes of gB. The results suggest that gC had little influence on the reversibility of gB conformational changes as measured here (Fig. 7A-C), rather gC has greater impact on the initial low pH-induced antigenic changes in gB (Fig. 6 and S2).

Mildly acidic pH changes in gB oligomeric conformation are independent of gC.

An independent indicator of pH-induced alterations in gB is a shift to a lower density gB oligomer in response to acid treatment (16). The role of gC in gB oligomeric changes was tested with MAb DL16, which recognizes an oligomer-specific epitope within gB domain V (47).

When either Δ gC or gCR were pretreated with low pH, DL16 reactivity was similarly reduced (Fig. 8A), distinguishing the DL16 epitope on gB from the other acid-sensitive epitopes (Fig. 6). This result signifies acid-triggered change in the oligomeric conformation of gB (Fig. 8A) regardless of gC's presence. This outcome was confirmed by an independent measure of gB oligomeric conformation. When HSV is first treated with low pH and then subjected to 1% SDS and native PAGE, the slower migrating, higher molecular weight (HMW) species of gB oligomer disappears, suggesting a change in gB oligomeric conformation (16). Using this approach, the HMW gB oligomer from Δ gC disappeared in a manner similar to gB from the control rescuant virus gCR (Fig. 8B). Although gC regulates low-pH induced antigenic changes in gB (Fig. 6), it does not appear to affect acid-triggered alterations in the gB oligomer. This is consistent with the notion that a similar low pH triggers both antigenic and oligomeric alterations in gB, but these changes are experimentally separable and not identical (18).

Since the changes in gB oligomeric conformation did not require gC, we expected that their reversibility would also be gC-independent. This was indeed the case. Acid-induced reduction of reactivity with the oligomer-specific MAb DL16 was partially reversible in both Δ gC and gCR viruses (Fig. 8C). Likewise, the susceptibility of low-pH treated gB oligomer to disruption by 1% SDS was also reversible, regardless of the presence of gC (Fig. 8D). Thus, the reversibility of low pH-triggered conformational changes in the gB oligomer is independent of gC.

Discussion

The mechanisms underlying how herpesviruses traverse distinct cellular entry pathways is paramount to our understanding of these important pathogens. The experiments described here reveal a selective role for HSV-1 gC in low pH entry. HSV-1 gC confers an infectivity advantage in cells that support low pH entry of HSV-1, such as human keratinocytes. There is a

lag in the exit of enveloped gC-negative particles from endocytic compartments, which may reflect a role for gC in optimal virus trafficking or low pH fusion. Low pH-triggered antigenic changes in gB domains I, II, and V are thought to be critical for fusion (16, 39, 40, 48-50); (Fig. 6 and S2). gC facilitates pH-induced gB conformational changes, increasing the pH of antigenic change by as much as 0.4 – 0.7 pH units. The reduced entry of gC-negative HSV may be explained by gC's role in facilitating fusion-associated conformational changes in gB, which result in optimal penetration from an endocytic compartment. Importantly, in the absence of gC, HSV still uses a low-pH pathway to enter and infect cells, likely mediated in part by changes in gB that occur in the absence of gC, albeit at a lower pH. HSV-1 gC also binds to complement component C3b and inhibits complement-mediated immunity (51). When HSV particles are treated with soluble heparin in a cell-free assay, the UL16 tegument protein is rearranged in a gC-dependent manner (52). The role of gC in entry by an acidic endosomal pathway described here is independent of its role in attachment to cell surface heparan sulfate.

Viral envelope glycoprotein B (gB) is highly conserved among all subfamilies of the *Herpesviridae*. Current models of HSV-1 entry posit that 1) gC, and to a lesser extent gB, mediates viral attachment to host cell surface glycosaminoglycans; 2) gD binds to a cognate host cell receptor such as HVEM or nectin-1, resulting in pH-independent conformational change in gD; 3) This is thought to transmit a signal to gH/gL; and 4) culminate in the execution of membrane fusion by gB. Thus, gB, unlike other members of the class III fusion protein family, does not mediate fusion on its own. In addition to pH-triggered changes in gB domains I and V, we show here that acid-induced antigenic changes also occur in domain II (Fig. 6 and S2).

There is structure-based evidence that gB can exist in multiple conformations. The postfusion structure of gB is known, and distinct, membrane-associated non-postfusion forms, that may reflect the prefusion conformation, have also been resolved (53-55). MAbs that bind

specifically to either pre-fusion or post-fusion gB have not been identified. Antibody binding to pre-fusion gB present in virions that are pretreated with low pH does not disappear completely; instead there are decreases in antibody reactivity. Low pH causes gB to assume a non-prefusion form, but is likely not sufficient to shift gB to the post-fusion form. In the absence of gC, the pH threshold for gB conformational changes, particularly in domain I and II is lower by 0.4-0.7 pH units (Fig 6). As a comparison, variants and mutants of influenza HA exhibit a ~ 0.2 -0.6 shift in the pH of both conformational change and fusion (56, 57). The H126 epitope in the fusion domain of gB might be particularly important for the pH-activation of fusion (18). In the absence of gC, a more acidic pH is required to trigger changes in the accessibility of the H126 epitope.

The cell tropism of herpesvirus entry and infection is influenced by subfamily-specific viral proteins. EBV gp42 is required for fusion and entry in B cells but not epithelial cells (58-62). The HCMV pentamer complex of envelope proteins is necessary for endosomal entry into epithelial and endothelial cells but not for pH-neutral entry into fibroblasts (25, 26, 63-65). However, a detailed mechanism underlying EBV gp42 or the HCMV pentamer role in selection of entry pathway is not known. The alphaherpesvirus-specific protein gC is the first HSV envelope protein reported to selectively participate in endocytic entry. Here we suggest that gC is important for HSV-1 epithelial infection but less so for neuronal entry. The results are consistent with a mechanism whereby gC acts to ensure that gB undergoes optimal conformational change to mediate fusion with an appropriate endosomal compartment.

The results suggest a functional interaction between gC and gB. Direct interaction between gC and gB has not been detected by co-immunoprecipitation approaches at different pHs (data not shown). Low affinity or transient interactions may not be captured, or gC may exert an indirect effect on gB through another viral or host factor. Physical interactions between

HSV-1 gB and gH have also been difficult to detect, despite demonstration of functional interactions (14, 15, 66). We propose a model whereby gC aids gB, and together with gD and gH/gL, allows rapid entry of HSV-1 into epithelial cells.

Materials and Methods

Cells and viruses

Human HaCaT epithelial keratinocytes and Vero cells were propagated in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals). Non-differentiated human SK-N-SH and IMR-32 neuroblastoma cells (ATCC) were propagated in Eagle's minimal essential medium supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and Earle's salts (Invitrogen). CHO-HVEM (M1A) cells (67) (provided by R. Eisenberg and G. Cohen, University of Pennsylvania) are stably transformed with the human HVEM gene and contain the *E. coli lacZ* gene under the control of the HSV-1 ICP4 gene promoter. CHO-HVEM cells were propagated in Ham's F-12 nutrient mixture (Gibco/Life Technologies) supplemented with 10% FBS, 150 µg of puromycin (Sigma-Aldrich, St. Louis, MO, United States)/ml, and 250 µg of G418 sulfate (Thermo Fisher Scientific, Fair Lawn, NJ, United States)/ml. Cells were subcultured in non-selective medium prior to use in all experiments. Primary human epidermal keratinocytes (HEKa) (ATCC) up to passage 8 were maintained in dermal cell basal medium (ATCC) supplemented with keratinocyte growth kit (ATCC) and penicillin-streptomycin-amphotericin B solution (ATCC). CHOpgs745 cells (ATCC), which lack a gene required for heparan sulfate biosynthesis, were propagated in Ham's F-12 nutrient mixture supplemented with 10% FBS. HSV-1 strain KOS and all viruses in this study were propagated and titered on Vero cells. HSV-1 (KOS) ΔgC2-3 virus or ΔgC is HSV-1 KOS in which most of the gC gene is deleted and

replaced by *lacZ* (33); this virus is considered a gC-negative HSV-1. HSV-1 (KOS) gC2-3Rev virus or gCR is a recombinant in which HSV-1 (KOS) Δ gC2-3 was rescued by insertion of the wild-type gC gene (33). Both viruses were obtained from C. Brandt, University of Wisconsin, Madison. The viral genomes have not been sequenced. However, HSV-1 gCR and the parental KOS are indistinguishable in terms of specific infectivity, cell attachment, heparan sulfate binding, and infectivity. Their genomes are similar as measured by restriction enzyme analysis and Southern blotting (33). In addition, the phenotypes attributed to HSV-1 gCR in this study, are similar to those previously reported for HSV-1 wild type strain KOS (6, 7, 16, 18, 23, 68, 69). HSV-1 F-gE/GFP lacks the gE gene (70) (obtained from D. Johnson, Oregon Health Sciences University).

Antibodies

Anti-gB mouse monoclonal antibodies H126 (domain I), H1359 (domain III), and H1817 (domain VI) were from Virusys. Anti-gB monoclonal antibodies DL16 (oligomer-specific; domain V), SS10 (domain IV), SS55 (domain I) (71), SS106 (domain V), and SS144 (domain V) (47) were provided by G. Cohen and R. Eisenberg, University of Pennsylvania. Anti-gB monoclonal antibodies H1838 and H1781 (domain II) were provided by L. Pereira, University of California, San Francisco (72).

Immunofluorescence assay of HSV entry and infectivity

Equivalent amounts of HSV-1 Δ gC or gCR were added to cells grown on coverslips in 24-well plates in triplicate. Cells were incubated at 4°C for 1 hr then washed twice with cold PBS. Cultures were then shifted to 37°C for 6 hr then fixed with 100% ice-cold methanol. Primary antibody to HSV-1 ICP4 was then added, followed by Alexa Fluor-488-labeled secondary antibody. Nuclei were counterstained with 12.5 ng/ml DAPI. Approximately 500 cells per well were counted and scored for successful infection.

HSV-1 plaque assay

HSV-1 titers were determined by limiting dilution plaque assay. At 18-20 hr p.i., cells were fixed with ice-cold methanol and acetone (2:1 ratio) for 20 min at -20°C and air-dried. Titers were determined by immunoperoxidase staining with rabbit polyclonal antibody to HSV, HR50 (Fitzgerald Industries, Concord, Mass.).

Ectopic expression of HSV-1 gC

Lipofectamine 3000 (ThermoFisherScientific) was used to transfect cells with plasmids encoding gC (pSH140 obtained from G. Cohen and R. Eisenberg), gD (pPEP99; obtained from P. Spear), or empty vector. At 48 hr post-transfection, cells were infected with HSV-1, and infectivity was measured by plaque assay.

SDS-PAGE and Western blotting

HSV-1 in Laemmli buffer with 200 mM dithiotheriol was boiled for 5 min. Proteins were separated by SDS-PAGE on Tris-glycine gels (Thermo Fisher Scientific). For protein staining, gels were then fixed and stained with 0.025% Coomassie brilliant blue (J.T. Baker Chemical Co., Philipsburg, NJ), 40% methanol (Baker Chemical), and 10% glacial acetic acid (Baker Chemical), followed by destaining with 30% methanol and 7% glacial acetic acid (69). Gels were dried and imaged with a Gel Doc XR imager (Bio-Rad, Hercules, CA). For Western blotting, following transfer to nitrocellulose, membranes were blocked and incubated with HSV polyclonal antibodies R68 (α -gB), R47 (α -gC) (73), R2 (α -gD) (74), R137 (α -gH), anti-gD monoclonal antibody DL6 (75), which were gifts from Gary Cohen and Roselyn Eisenberg; HSV-1 gE monoclonal antibody H1A054-100 (Virusys); or monoclonal antibody H1A021 to VP5 (Santa Cruz Biotechnology, Dallas, TX). After incubation with horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescent substrate (Pierce) was added, and membranes were exposed to X-ray film (Kodak).

Effect of ammonium chloride on HSV entry and infectivity

Cells grown in 24-well plates were treated with medium containing ammonium chloride for 1 hr at 37°C. Virus was added in the continued presence of agent for 6 h. Medium was then removed and replaced with complete DMEM. At 16 h p.i., plaque assay was performed to measure infectivity. CHO-HVEM cells grown in 96-well plates were treated with medium containing ammonium chloride for 20 min at 37°C. Virus was added (MOI of 5) in the continued presence of agent, and beta-galactosidase activity of cell lysates was measured at 6 h p.i.

Attachment of HSV-1 to the cell surface

Cells grown in 96-well plates were pre-chilled in carbonate-free, serum-free medium supplemented with 20 mM HEPES and 0.2% bovine serum albumin (binding medium) at 4°C on ice for 20 min. Approximately 10⁶ particles of HSV-1 ΔgC or gCR in binding medium were added to cells at 4°C on ice for 1 hr. Cultures were washed twice with ice-cold PBS. Cells were trypsinized, and cell-associated HSV-1 DNA was isolated with the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Attached HSV-1 was quantitated by qPCR as previously described (11, 76).

Intracellular tracking of enveloped, infectious HSV

HSV-1 ΔgC or gCR was added to confluent cell monolayers (MOI of 8) on ice at 4°C for 2 hr. Cultures were washed with PBS and shifted to 37°C. At the indicated times p.i., extracellular virus was inactivated by adding sodium citrate buffer (pH 3.0) for 1 min at 37°C (7). Monolayers were immediately put on ice and washed with cold PBS. One milliliter of Ham's F12 medium with 20 mM HEPES and 1% FBS was added, and cells were lysed by two cycles of freezing and thawing. Titers of lysates were determined on Vero cells. Each data point is the mean of triplicate samples.

Dot blot analysis of HSV-1 gB

Extracellular HSV preparations were diluted in serum-free, bicarbonate-free DMEM with 0.2% bovine serum albumin (BSA) and 5 mM each of HEPES, MES, and sodium succinate. Virions were adjusted with HCl to achieve a range of different pHs from 5.0 to 7.3. Samples were incubated at 37°C for 10 min, and then either blotted directly to nitrocellulose membrane using a Minifold dot blot system (Whatman), or were first neutralized by addition of pretitrated amounts of 0.05 N NaOH (77). Virus-dotted nitrocellulose membranes were blocked, and then incubated with antibodies to gB at neutral pH. After incubation with horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescent substrate (Thermo Fisher Scientific) was added, and blots were exposed to X-ray film (Genesee Scientific). Densitometry was performed with ImageJ.

Analysis of gB oligomeric structure by PAGE

Extracellular preparations of HSV were diluted in the same medium used for dot blot analysis. Virus samples were adjusted to the indicated pHs with pretitrated amounts of 0.05 N HCl for 10 min at 37°C. To test for reversibility of conformational change, samples were then neutralized by addition of pretitrated amounts of 0.05 N NaOH for 10 min at 37°C. 1% SDS was then added. Laemmli sample buffer containing 0.2% sodium dodecyl sulfate (SDS) with no reducing agent (78) was added. Samples were not heated, and proteins were resolved by PAGE. After transfer to nitrocellulose, membranes were blocked and incubated with gB monoclonal antibody H1359. After incubation with horseradish peroxidase-conjugated secondary antibody, enhanced chemiluminescent substrate (Thermo Fisher) was added and membranes were exposed to X-ray film (Genesee Scientific).

Acknowledgments

We thank Ryan Manglona, Erik Walker, George Wudiri, and Youki Yamasaki for early contributions to this work. We are grateful to Curtis Brandt, Gary Cohen, Roselyn Eisenberg, David Johnson, Lenore Pereira, and Patricia Spear for providing reagents. This study was supported by National Institutes of Health (NIH) grant R01 AI119159 (A.V.N) and NIH Training Grant T32 GM008336 (T.K., D.J.W., and K.A.G.).

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TABLE 1 Cells used in this study and the role of low pH in HSV entry

Cell	Type	Low pH-dependent entry of HSV ^a	Reference
CHO-HVEM	Chinese hamster ovary cell expressing HVEM	yes	(7)
HaCaT	Human epidermal keratinocyte	yes	(6)
HEKa	Primary adult human epidermal keratinocyte	yes	(6);This study
IMR-32	Human neuroblastoma	no	(6)
SK-N-SH	Human neuroblastoma	no	(6)
Vero	African green monkey kidney	no	(35)

^aInhibited by ammonium chloride, bafilomycin A1, or monensin

Fig. 1. Reduced low pH entry and infectivity of HSV-1 lacking gC. Equivalent inocula of HSV-1 gCR (A) or ΔgC (B) was bound to Vero, CHO-HVEM, IMR-32, or HEKa cells for 1 h at 4°C. Following shift to 37°C for 6 hr, infected cells (MOI < 1) were quantitated by immunofluorescence. Infectivity is measured as percent HSV antigen-positive cells of ~ 500 total cells. Student's *t* test (*, *P* < 0.05).

Fig. 2. Efficiency of gC-negative HSV-1 infection of human cells that support pH-neutral or low pH entry.

Equivalent inocula of HSV-1 gCR (A) or ΔgC (B) were added to SK-N-SH or HaCaT cells at 37°C. At 18-24 h p.i., titers were determined by plaque assay. (C, D) Attempt to restore infectivity of HSV-1 lacking gC by providing gC in the cell. Equivalent inocula of HSV-1 gCR (C) or ΔgC (D) were added to SK-N-SH or HaCaT cells transfected with empty vector, gC, or gD plasmids at 37°C. At 18-24 h p.i., titers were determined by plaque assay. Student's *t* test (**, *P* < 0.01).

Fig. 3. HSV-1 ΔgC enters cells via a low pH-dependent pathway. Vero (A), SK-N-SH (B), IMR-32 (C), HaCaT (E), or HEKa (F) cells were treated with ammonium chloride for 1 h at 37°C. Cells were infected with 100 PFU of ΔgC or gCR for 6 h in the continued presence of drug. Normal medium was added, and at a total of 22 h p.i., infectivity was determined by plaque assay. The infectivity of no-drug samples was set to 100%. CHO-HVEM cells (D) were treated with ammonium chloride for 20 min at 37°C. HSV-1gCR or ΔgC was added to cells (MOI of 5) at 37°C in the continued presence of agent. At 6 h p.i., entry was measured as a percent of beta-galactosidase activity obtained in the absence of ammonium chloride.

Fig. 4. HSV-1 Δ gC attachment to cells. (A, B) 10^6 genome copies of HSV-1 gCR (A) or Δ gC (B) were added to the indicated cell monolayers on ice at 4°C for 1 hr. Following PBS washes, cell-associated HSV-1 was quantitated by qPCR. CHOPgs745 cells lack heparan sulfate receptors for HSV attachment and serve as controls. One way-ANOVA (*, $P < 0.01$).

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Fig. 5. gC contributes to rapid HSV penetration following endocytosis. HSV-1 Δ gC or gCR was bound to CHO-HVEM (A) or HEK293 (B) cells for 1 h at 4°C (MOI of 8). Following shift to 37°C, at the indicated times p.i., extracellular virions were inactivated, cell lysates were titrated as an indication of infectious, enveloped, intracellular particles. This allows monitoring of viral trafficking and penetration over time. Peak recovery was set to 100%.

MAb	Virus	pH									
		7.3	6.6	6.4	6.2	5.9	5.7	5.6	5.4	5.2	5.0
H126 (I)	gCR	100±0	99.7±6.2	97.5±10.5	91.2±10.7	88.6±14.7	78.7±21.8	47.5±32.0	8.2±10.4	1.8±2.5	1.2±1.7
	ΔgC	100±0	98.3±13.8	95.0±12.9	94.8±18.5	98.9±19.6	92.8±22.3	93.3±22.3	80.7±24.5	88.4±24.7	71.8±22.0
SS55 (I)	gCR	100±0	83.5±7.0	71.7±5.1	62.4±5.2	39.6±8.1	29.3±9.6	27.8±17.1	22.3±1.3	8.7±4.4	4.7±0.1
	ΔgC	100±0	104±17.6	75.5±10.8	75.2±3.8	80.1±16.4	66.5±33.8	53.0±21.6	32.9±2.3	52.2±3.0	18.3±20.9
H1781 (II)	gCR	100±0	78.0±2.8	81.6±13.1	82.2±6.1	60.0±4.0	52.6±5.6	46.5±9.0	50.8±14.3	39.0±23.8	14.2±0.1
	ΔgC	100±0	92.9±3.0	82.6±3.0	64.6±20.6	71.0±8.7	66.9±6.5	61.0±11.5	50.3±13.6	33.3±16.0	16.0±11.0
H1838 (II)	gCR	100±0	67.6±11.4	54.7±34.2	72.7±12.3	60.5±17.2	45.1±42.9	38.9±27.4	36.2±31.9	4.8±6.7	10.2±14.4
	ΔgC	100±0	91.2±4.8	95.6±5.6	79.1±28.5	99.8±5.6	82.3±24.9	81.9±20.8	69.4±10.4	71.3±18.0	67.2±16.2
H1359 (III)	gCR	100±0	81.3±5.4	55.6±4.8	73.3±10.6	83.2±16.6	77.5±29.0	75.2±30.4	76.4±31.7	68.3±13.7	44.3±8.1
	ΔgC	100±0	98.4±4.8	93.8±7.3	95.1±5.8	93.1±13.1	84.7±14.6	81.5±16.7	77.2±7.0	69.3±11.5	61.8±15.8
SS10 (IV)	gCR	100±0	98.3±2.7	95.7±3.5	93.6±0.3	97.5±0.3	88.0±0.8	89.8±0.4	90.4±4.1	82.1±6.7	80.8±0.3
	ΔgC	100±0	103.4±0.5	102.7±1.4	102.3±3.3	97.3±7.7	91.6±10.6	91.6±10.4	88.7±12.5	93.7±1.3	95.1±6.2
SS106 (V)	gCR	100±0	100.7±3.7	105.5±2.1	105.1±0.2	102.8±3.4	101.9±3.5	101.7±2.6	84.6±3.6	75.3±4.3	22.6±30.1
	ΔgC	100±0	104.5±9.4	117.5±4.6	117.7±3.2	103.8±9.9	105.0±2.4	97.4±5.3	72.0±23.1	77.4±24.4	48.6±31.6
SS144 (V)	gCR	100±0	96.4±5.1	91.1±2.5	76.0±9.5	71.3±9.5	57.3±24.3	65.5±4.9	63.6±2.7	55.6±7.7	27.8±11.6
	ΔgC	100±0	97.5±3.5	96.5±6.9	93.7±5.4	90.0±1.9	79.3±12.4	75.0±9.0	68.1±13.2	64.7±8.3	43.3±14.0
H1817 (VI)	gCR	100±0	98.8±1.6	98.3±5.5	96.4±7.6	95.0±8.7	100.3±2.9	96.9±6.4	99.6±2.8	90.1±9.4	80.8±3.7
	ΔgC	100±0	98.4±2.3	99.2±3.4	102.5±4.0	102.0±6.7	100.7±3.5	94.4±4.0	90.6±5.8	94.6±7.2	86.2±9.8

Fig. 6. HSV-1 gC influences the pH of conformational changes in the fusion protein gB. Extracellular preparations of HSV-1 gCR or ΔgC (~ 10⁷ genome copy numbers) were treated with pHs ranging from 7.3 to 5.0 and blotted directly to nitrocellulose. Blots were probed with gB MAbs at neutral pH. Antibody reactivity was quantitated with ImageJ. Reactivity of pH 7.3 samples were set to 100%. The pH treatment that reduced reactivity by > 50% is shaded grey (gCR) or outlined in red (ΔgC). After each MAb, the gB domain (I to VI) containing each MAb epitope is indicated (see Fig. S4).

Fig. 7. The pH threshold of reversibility of gB conformational changes. HSV-1ΔgC or gCR was treated with pH 7.3 or 5.0 and incubated for 10 min. The pHs of the pH 5-treated samples were increased to the indicated pHs for an additional 10 min. Samples were directly blotted to nitrocellulose and probed at neutral pH with gB MAb H126 (A), H1781 (B), SS144 (C), or H1817 (D). MAb reactivity was quantitated, with the pH 7.3-treated sample set to 100%.

Fig. 8. Low-pH-induced changes in gB oligomer are independent of gC as are their reversibility. (A) HSV-1 gCR or ΔgC was treated with a range of pHs for 10 min. Samples were directly blotted to nitrocellulose and probed at neutral pH with gB oligomer-specific MAb DL16. Reactivity was quantitated and the pH 7.3 sample was set as 100%. (B) gCR or ΔgC was treated with a range of pHs for 10 min. 1% SDS was added, and reactions were added to “native” PAGE sample buffer. Unheated samples were resolved by 8% SDS-PAGE and Western blot for gB. (C) gCR or ΔgC was treated with pH 5 for 10 min then blotted directly to nitrocellulose or first neutralized back to pH 7.3 for 10 min. Blots were probed at neutral pH with anti-gB MAb DL16. (D) gCR or ΔgC was treated with pH 5 for 10 min. One set was neutralized back to pH 7.3. 1% SDS was added and samples were processed as in panel B. Molecular size markers in kilodaltons are indicated at the right.

Fig. S1 Protein composition of gC-negative HSV-1 Δ gC. Equivalent VP5 units of HSV-1 gCR or Δ gC in Laemmli buffer were separated by SDS-PAGE followed by (A) protein staining with Coomassie blue or (B) Western blotting for the indicated viral proteins. Molecular weight standards in kilodaltons are indicated to the left.

Fig. S2. HSV-1 gC facilitates acid-induced conformational changes in the fusion protein gB. Extracellular preparations of HSV-1 gCR or ΔgC ($\sim 10^7$ genome copy numbers) were treated with pHs ranging from 7.3 to 5.0 and blotted directly to nitrocellulose. Blots were probed with the indicated gB MAbs H126, H1838, SS144, or H1359 at neutral pH followed by HRP-conjugated anti-mouse secondary antibody. The antibody name is shown at the left and the gB domain to which each MAb is directed is indicated in parenthesis. These are individual examples of experiments that were quantitated and averaged together with multiple similar independent determinations. Summarized quantitative results are depicted in Figure 6.

Fig. S3. HSV-1 gE does not influence acid-induced conformational change in the H126 epitope of gB. (A) HSV-1 strain wild type F or its derivative gE-GFP were treated with indicated pHs then directly blotted to nitrocellulose membrane. Blot was probed with representative gB MAbs H126 or H1817 at neutral pH. (B) Antibody reactivity was quantitated and treatment with pH 7.4 was set as 100%.

Fig. S4. Domain structure of HSV-1 gB and location of MAb epitopes. (A) gB ectodomain trimer representing a post-fusion conformation. (B) Location of monoclonal antibody-binding sites. Monoclonal antibody-resistant mutations in Domain I, which contains bipartite hydrophobic fusion loops, map to amino acid residue 303 for H126 and residues 203, 335, and 199 for SS55 (79, 80). The MAb H1781 epitope in Domain II maps to 454-473, and H1838 maps to 391-410 (47). The H1359 epitope in Domain III maps to 487-505 (72). SS10 in Domain IV maps to 640-670 (47). SS106 and SS144 to Domain V both bind to 697-725 (53). The MAb H1817 epitope in Domain VI (not resolved in the structure) maps to 31-43 (47).

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MAb	Virus	pH									
		7.3	6.6	6.4	6.2	5.9	5.7	5.6	5.4	5.2	5.0
H126 (I)	gCR	100±0	99.7±6.2	97.5±10.5	91.2±10.7	88.6±14.7	78.7±21.8	47.5±32.0	8.2±10.4	1.8±2.5	1.2±1.7
	ΔgC	100±0	98.3±13.8	95.0±12.9	94.8±18.5	98.9±19.6	92.8±22.3	93.3±22.3	80.7±24.5	88.4±24.7	71.8±22.0
SS55 (I)	gCR	100±0	83.5±7.0	71.7±5.1	62.4±5.2	39.6±8.1	29.3±9.6	27.8±17.1	22.3±1.3	8.7±4.4	4.7±0.1
	ΔgC	100±0	104±17.6	75.5±10.8	75.2±3.8	80.1±16.4	66.5±33.8	53.0±21.6	32.9±2.3	52.2±3.0	18.3±20.9
H1781 (II)	gCR	100±0	78.0±2.8	81.6±13.1	82.2±6.1	60.0±4.0	52.6±5.6	46.5±9.0	50.8±14.3	39.0±23.8	14.2±0.1
	ΔgC	100±0	92.9±3.0	82.6±3.0	64.6±20.6	71.0±8.7	66.9±6.5	61.0±11.5	50.3±13.6	33.3±16.0	16.0±11.0
H1838 (II)	gCR	100±0	67.6±11.4	54.7±34.2	72.7±12.3	60.5±17.2	45.1±42.9	38.9±27.4	36.2±31.9	4.8±6.7	10.2±14.4
	ΔgC	100±0	91.2±4.8	95.6±5.6	79.1±28.5	99.8±5.6	82.3±24.9	81.9±20.8	69.4±10.4	71.3±18.0	67.2±16.2
H1359 (III)	gCR	100±0	81.3±5.4	55.6±4.8	73.3±10.6	83.2±16.6	77.5±29.0	75.2±30.4	76.4±31.7	68.3±13.7	44.3±8.1
	ΔgC	100±0	98.4±4.8	93.8±7.3	95.1±5.8	93.1±13.1	84.7±14.6	81.5±16.7	77.2±7.0	69.3±11.5	61.8±15.8
SS10 (IV)	gCR	100±0	98.3±2.7	95.7±3.5	93.6±0.3	97.5±0.3	88.0±0.8	89.8±0.4	90.4±4.1	82.1±6.7	80.8±0.3
	ΔgC	100±0	103.4±0.5	102.7±1.4	102.3±3.3	97.3±7.7	91.6±10.6	91.6±10.4	88.7±12.5	93.7±1.3	95.1±6.2
SS106 (V)	gCR	100±0	100.7±3.7	105.5±2.1	105.1±0.2	102.8±3.4	101.9±3.5	101.7±2.6	84.6±3.6	75.3±4.3	22.6±30.1
	ΔgC	100±0	104.5±9.4	117.5±4.6	117.7±3.2	103.8±9.9	105.0±2.4	97.4±5.3	72.0±23.1	77.4±24.4	48.6±31.6
SS144 (V)	gCR	100±0	96.4±5.1	91.1±2.5	76.0±9.5	71.3±9.5	57.3±24.3	65.5±4.9	63.6±2.7	55.6±7.7	27.8±11.6
	ΔgC	100±0	97.5±3.5	96.5±6.9	93.7±5.4	90.0±1.9	79.3±12.4	75.0±9.0	68.1±13.2	64.7±8.3	43.3±14.0
H1817 (VI)	gCR	100±0	98.8±1.6	98.3±5.5	96.4±7.6	95.0±8.7	100.3±2.9	96.9±6.4	99.6±2.8	90.1±9.4	80.8±3.7
	ΔgC	100±0	98.4±2.3	99.2±3.4	102.5±4.0	102.0±6.7	100.7±3.5	94.4±4.0	90.6±5.8	94.6±7.2	86.2±9.8