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Cellular expression of gH confers resistance to herpes simplex virus type-1 entry

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Abstract

Entry of herpes simplex virus-1 (HSV-1) into cells requires a concerted action of four viral glycoproteins gB, gD, and gH–gL. Previously, cell surface expression of gD had been shown to confer resistance to HSV-1 entry. To investigate any similar effects caused by other entry glycoproteins, gB and gH–gL were coexpressed with Nectin-1 in Chinese hamster ovary (CHO) cells. Interestingly, cellular expression of gB had no effect on HSV-1(KOS) entry. In contrast, entry was significantly reduced in cells expressing gH–gL. This effect was further analyzed by expressing gH and gL separately. Cells expressing gL were normally susceptible, whereas gH-expressing cells were significantly resistant. Further experiments suggested that the gH-mediated interference phenomenon was not specific to any particular gD receptor and was also observed in gH-expressing HeLa cells. Moreover, contrary to a previous report, gL-independent cell surface expression of gH was detected in stably transfected CHO cells, possibly implicating cell surface gH in the interference phenomenon. Thus, taken together these findings indicate that cellular expression of gH interferes with HSV-1 entry.
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Keywords: Viral entry; HSV; gB; gD; gH; gL; Nectin-1; HVEM; gD interference; gH interference

Introduction

Human and animal representatives of the α-herpesvirus subfamily, exemplified by herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), bovine herpesvirus 1 (BHV-1), and pseudorabies virus (PRV), exhibit similar requirements for entry into cells (Spear, 1993; Spear et al., 2000; Mettenleiter, 1995). The initial interaction of virus with cells is binding of the virion glycoprotein gC, and possibly gB, to cell surface glycosaminoglycans (GAGs), preferentially heparan sulfate (Shukla and Spear, 2001). After the initial attachment, the interaction of a third viral glycoprotein, gD, with one of its cognate cell surface receptors triggers the start of fusion of viral and cellular membranes. Fusion is required for the penetration and the release of viral capsid into the cytoplasm. In case of HSV-1, although gC is dispensable for the infection of many cultured cells, four other glycoproteins, gB, gD, gH, gL, and the presence of one or more cellular gD receptors (and possibly other cellular molecules) are required for mediating fusion (Spear et al., 2000; Fuller and Lee, 1992).

Expression of gD homologs is limited to the α-herpesvirus subfamily of herpesviruses. All α-herpesviruses studied to date, except varicella-zoster virus (VZV), express some form of gD. In contrast, homologs of HSV-1 gB, gH, and gL have been identified in herpesviruses of all subfamilies. Previous reports indicate that gH and gL exist as a heterodimer (Spear, 1993) with gL being necessary for proper folding and surface expression of gH (Hutchinson et al., 1992; Forrester et al., 1992). The significance of these glycoproteins in viral entry and cell fusion appear to vary among herpesviruses. For example, fusion of HSV-1 with cell plasma membrane requires gB, gH, and gL as well as gD (Spear, 1993; Pertel et al., 2001; Browne et al., 2001; Muggeridge, 2000; Cai et al., 1987), whereas Epstein–Barr

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virus and human herpesvirus-8 require only gB, gH, and gL for fusion (Pertel, 2002; Haan et al., 2001). Furthermore, in case of VZV, gH and gL alone can induce fusion (Duus et al., 1995; Maresova et al., 2001). Although, based on the above findings it appears likely that gB, gH, and gL possess fusogenic potential, none of these glycoproteins contain any obvious counterparts to the hydrophobic fusion peptides of influenza virus hemagglutinin (HA) or human immunodeficiency virus (HIV) gp41. Similarly, they do not share significant homologies to any other known fusion proteins. It is also unclear whether these molecules, possibly after some conformational changes, can penetrate directly into the plasma membrane or work in conjunction with some cellular partners/receptors. In this regard, a recent study suggests that human cytomegalovirus gH may function via a putative cellular gH receptor (Baldwin et al., 2000).

In the past few years two human cell surface proteins have been identified as gD receptors that mediate the entry of HSV-1 and HSV-2 into cells (Spear et al., 2000). HVEM, or herpes virus entry mediator (TNFRSF14), is a member of the TNF receptor family (Montgomery et al., 1996). Nectin-1 (CD111), on the other hand, is a member of immunoglobulin (Ig) superfamily closely related to poliovirus receptor (CD155) (Geraghty et al., 1998; Cocchi et al., 1998b). Another member of this family, Nectin-2, mediates entry of some mutant strains of HSV-1 and possibly HSV-2 (Warner et al., 1998). Recently, both Nectin-1 and Nectin-2 were shown to be homophilic cell adhesion molecules that localize to sites of cadherin-based cell junctions (Yoon and Spear, 2002). A newly discovered HSV-1 entry receptor is generated in heparan sulfate by the action of specific glucosaminyl 3-*O*-sulfotransferases (Shukla et al., 1999a; Xia et al., 2002). In the absence of other entry receptors described above, resistant cells can be made susceptible to HSV-1 entry, but not entry of HSV-2, PRV, or BHV-1, by expression of 3-*O*-sulfotransferase-3, provided cell surface heparan sulfate is present (Shukla et al., 1999a).

Regardless of the nature of gD receptors, susceptible cells expressing gD exhibit resistance to viral entry. It was shown that CHO cells transiently cotransfected with plasmids expressing gD receptors and gD (or naturally susceptible cells expressing gD) of HSV, BHV-1, or PRV can be resistant to infection by homologous virus and, in some cases, by heterologous α-herpesviruses (Campadelli-Fiume et al., 1988; Geraghty et al., 2000; Dasika and Letchworth, 1999; Chase et al., 1993). This phenomenon, termed gD-mediated interference, results from the sequestering of gD receptors and blocking of their accessibility by cell-associated gD (Geraghty et al., 2000). It has been suggested that cell surface expression of gD is required for interference to occur (Dasika and Letchworth, 2000).

In order to further understand the mechanism of entry and also to generate evidence in support of any potential cellular receptors/partners for entry glycoproteins other than gD, the present study sought to characterize gB, gH, and gL, for possible interference phenomenon. The hypothesis was that if the cellular expression of these glycoproteins results in resistance or interference to viral entry, then quite possibly they might interact with a cellular component much like gD interacts with a cell surface receptor. This study, therefore, examines the effects of cellular expression of gB, gH, and gL on Nectin-1-mediated HSV-1 entry and describes a new interference phenomenon that we call gH-mediated interference. It is the first preliminary evidence of its kind in support of the possible existence of a cellular receptor for gH. The data presented here also indicates that the surface expression of gH may be more independent of gL than previously reported.

Results

Cells expressing gH–gL exhibit resistance to HSV-1 entry

The Chinese hamster ovary (CHO) cells lack gD receptors and hence are resistant to HSV-1 entry. These cells can be rendered susceptible by transient transfection with gD-receptor-expressing plasmids. Studies have demonstrated a direct correlation between the proportion of CHO cells transfected and the proportion infected (Geraghty et al., 1998; Shukla et al., 1999a). Use of CHO cells, therefore, allows a unique opportunity of studying the properties and the requirements of various gD receptors. At this point, among the known gD receptors, Nectin-1 appears to have the largest host range and is likely to be expressed in several cell types (Geraghty et al., 1998; Cocchi et al., 1998a). Therefore, we began our study by addressing the effects of glycoprotein coexpressions in Nectin-1-mediated HSV-1 entry. To achieve our results, CHO cells were transiently cotransfected with plasmids expressing Nectin-1 and viral glycoproteins or empty vector (pcDNA3) as control. The basic experimental approach for the results described below has been used several times in the past with excellent reproducibility (Shukla et al., 1999a, 1999b, 2000; Geraghty et al., 2000; Dasika and Letchworth, 2000). Basically, after cotransfections with plasmids, cells were incubated for an additional 36 h to allow for the expression of the desired proteins. The cells were then infected with serial dilutions of an HSV-1(KOS) recombinant, HSV-1(KOS)-gL86 (Montgomery et al., 1996) that expresses β-galactosidase upon entry into cells. As shown in Fig. 1A cellular expression of Nectin-1 with HSV-1 gD resulted in reduced entry of HSV-1(KOS) as compared to entry observed in cells cotransfected with Nectin-1 and the empty vector pcDNA3 (as control). This result is very much in accordance with a previous report (Geraghty et al., 2000). It is interesting, however, that instead of using a 1:4 ratio of Nectin-1 to gD plasmids for transfection (Geraghty et al., 2000) we chose a 1:1 ratio for our experiments and were still able to observe a significant gD-mediated interference to viral entry. In parallel experiments, when HSV-1 gB was coexpressed with Nectin-1 (Fig. 1B) viral entry was clearly not affected,

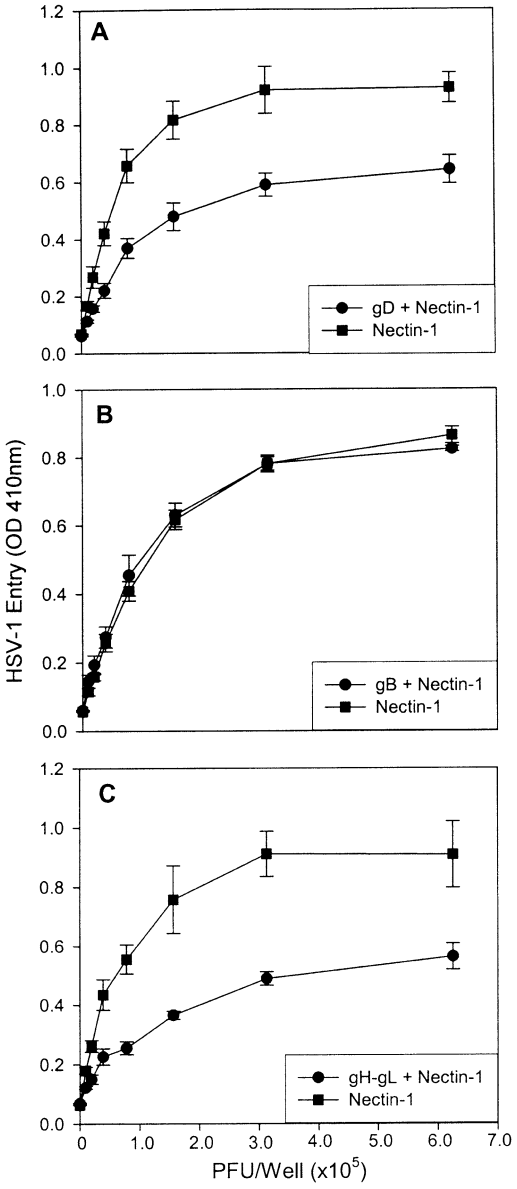

Fig. 1. Effects of HSV-1 glycoproteins coexpression with Nectin-1 on entry of HSV-1. CHO cells were cotransfected with a Nectin-1-expressing plasmid and glycoprotein-expressing plasmids (gD, gB, gH–gL, as indicated in each panel, solid circles) or pcDNA3 as control (solid squares) in a 1:1 ratio. The transfected cells were then exposed to β-galactosidase-expressing HSV-1(KOS) at the doses indicated. Six hours later, the cells were permeabilized and assayed for β-galactosidase activity as described in the text. The values shown (means of triplicate determinations) represent the amount of reaction product detected spectrophotometrically (OD_{410}) at the plaque forming units (PFU) indicated. In this and other figures each value shown is the mean of three or more determinations.

as judged from the viral entry observed in control cells. This illustrates that cellular expression of gB, which was confirmed by flow cytometry (data not shown), does not appear to have any significant effect on HSV-1 entry. Contrary to gB expression, as shown in Fig. 1C, when gH–gL were similarly coexpressed with Nectin-1, a marked reduction in viral entry was observed compared to the Nectin-1 control. These results indicate that the cellular expression gH-gL interferes with viral entry and results in significant resistance to HSV-1 entry.

The gH–gL-mediated interference exhibits dose dependence

It was previously found that significant gD-mediated interference is observed when during transfection the gD-expressing plasmid vs the receptor-expressing plasmid ratio is kept at 4:1 (Geraghty et al., 2000). In other words, it is likely that a higher molar ratio of endogenous gD (assuming that a higher copy number of gD-expressing plasmids indeed results into more gD production) is needed to effectively sequester the membrane-bound receptors from incoming virions. Therefore, in order to study the parallels between gD-mediated interference and gH–gL-mediated interference, we decided to examine if increasing the gH–gL plasmids ratio for transfection would indeed have an effect on viral entry. Since two separate plasmids were used for expression of gH and gL, the receptor to glycoprotein plasmids ratios (Nectin-1:gH:gL) tested were 1:1:1 or 1× gH–gL and 1:2:2 or 2× gH–gL. As shown in Fig. 2, when compared with the Nectin-1 control, the higher dose of gH–gL (2× gH–gL) clearly resulted in stronger resistance to viral entry as compared to the lower dose (1× gH–gL). Although even at the higher dose level (2× gH–gL), the cells were not completely resistant to HSV-1(KOS) entry (pcDNA3 control in Fig. 3 shows the background generated

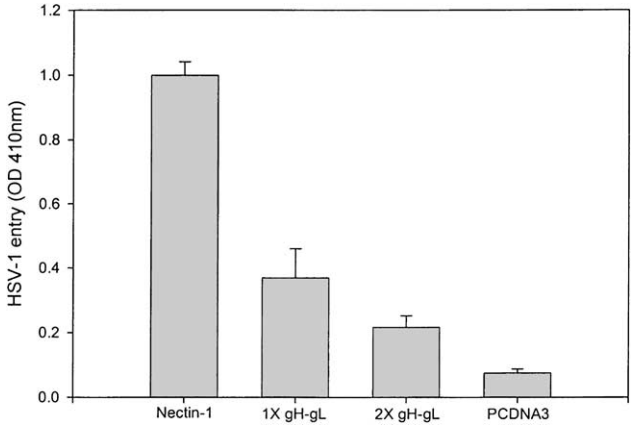

Fig. 2. Dose dependence of gH–gL-mediated interference. CHO cells were cotransfected with Nectin-1-expressing plasmid and gH and gL-expressing plasmids in 1:1:1 (marked 1X gH–gL) or in 1:2:2 (marked 2X gH–gL) ratio or transfected with Nectin-1 plasmid alone (marked Nectin-1) or control vector pcDNA3 (marked pcDNA3) alone, replated in 96-well plates and then inoculated with serial dilutions of HSV-1(KOS)gL86. After 6 h, the β-galactosidase activity was quantitated as described in the text. The values shown (means of triplicate determinations) represent the amount of reaction product detected spectrophotometrically (OD_{410}) at a single input dose of 10^6 PFU/well in the linear range of the dose-response curve for the virus.

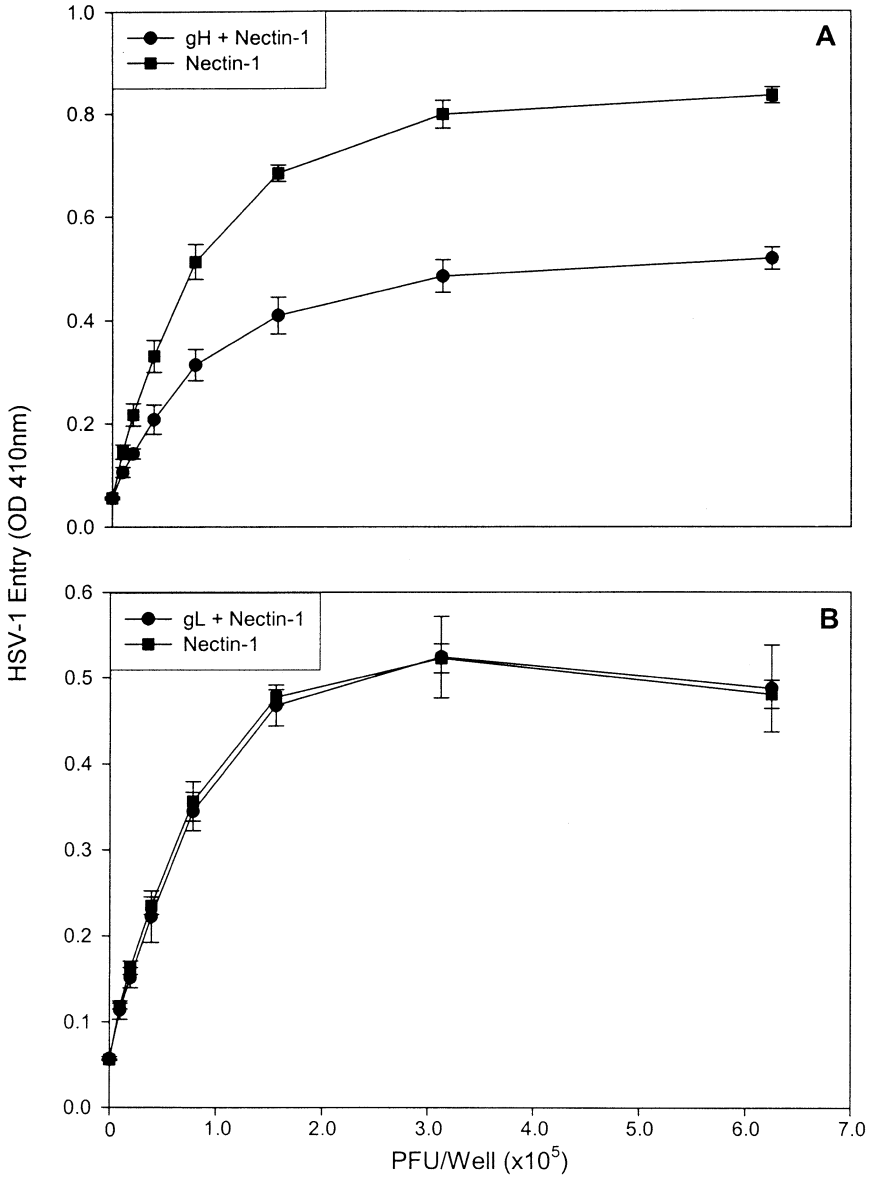

Fig. 3. Expression of gH alone (without gL) is sufficient to confer resistance. CHO cells were cotransfected with a Nectin-1-expressing plasmid and gH- or gL-expressing plasmid (as indicated in each panel, solid circles) or pcDNA3 control (solid squares in both panels) in a 1:1 ratio. The transfected cells were infected with β-galactosidase-expressing HSV-1 (KOS) at the plaque forming units (PFU) indicated. The β-galactosidase activity was assayed as described in the legend to Fig. 1.

by the resistant CHO cells). It is possible that increasing the gH–gL ratio even more could further enhance the observed resistance; however, in order to maintain the transfection efficiency we were limited by the amount of total DNA used in these experiments. Nevertheless, it was clear that very much like gD, a higher dose of gH–gL was more effective in generating the interference phenomenon.

The gH–gL-mediated interference results from gH alone, gL is not significant for this phenomenon

Although it was previously reported (Hutchinson et al., 1992) that cell surface expression of gH requires a simultaneous expression of gL, we attempted to show that gH and gL together were also required for the observance of the interference phenomenon. Surprisingly, we found this not to be the case. As seen in Fig. 3A, CHO cells expressing gH and Nectin-1 were significantly resistant (or less susceptible) to HSV-1(KOS) entry for multiple viral dosages compared to the control cells with Nectin-1 alone (without gH). In a sharp contrast to that, CHO cells expressing gL and Nectin-1 were no different in their susceptibility for HSV-1(KOS) entry compared to the Nectin-1 (no gL) control cells (Fig. 3B). In order to further verify the gH-mediated interference, we also constructed a CHO cell line that stably expresses HSV-1 gH without gL. Once again, when tran-

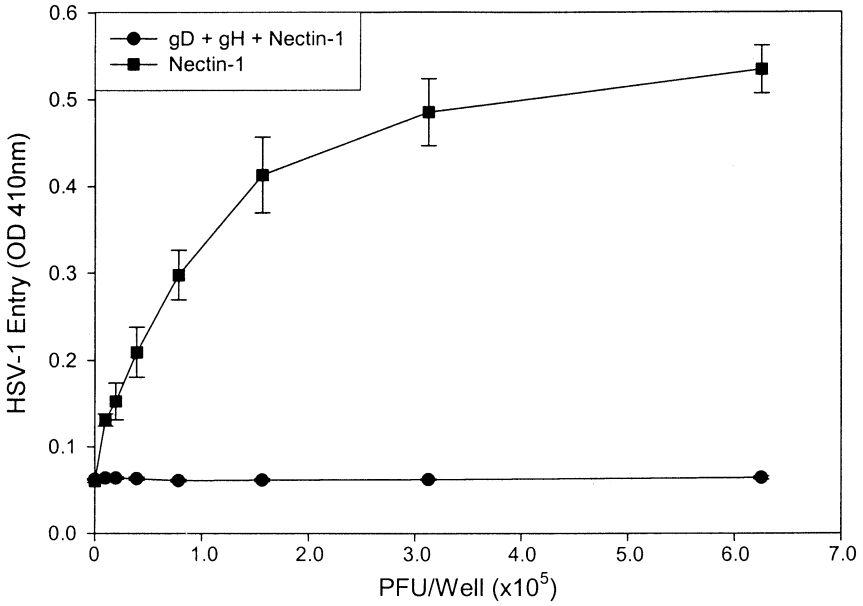

Fig. 4. Expression of gD and gH together renders cells almost completely resistant to HSV-1 entry. CHO cells were cotransfected with a Nectin-1-expressing plasmid and gH-and gD-expressing plasmids (solid circles) or pcDNA3 control (solid squares) in a 1:1:1 ratio. The transfected cells were infected with β-galactosidase-expressing HSV-1(KOS) at the plaque forming units (PFU) indicated. The β-galactosidase activity was assayed as described in the legend to Fig. 1.

siently transfected with a Nectin-1-expressing plasmid and compared with similarly transfected CHO cells (stably transfected with pcDNA3), the entry of HSV-1(KOS) was severely reduced in gH stable cell line (data not shown). It therefore, appears clear that expression of gH itself is enough to cause the interference observed in Nectin-1-expressing CHO cells and that gL appears to have no significant role in this process.

Coexpression of gD and gH together results in a significantly enhanced resistance to HSV-1 entry

Since gD and gH both show interference effects, it was postulated that together they would show an increased effect. This would be true if cellular gD and gH block two separate steps in viral entry; then the effects of their combined expression would probably be more pronounced than either of them expressed alone. Our results seem to prove both hypotheses true. As seen in Fig. 4, HSV-1(KOS) entry was almost completely blocked when the cells were made to express both gD and gH. It is possible that relatively higher expressions of gD and gH combined can result into some form of cytotoxicity; however, under our assay conditions the cells looked healthy and exhibited normal growth and adherence to 96-well culture dishes after being transferred from 6-well dishes (used for transfection) about 20 h posttransfection. Therefore, it is highly unlikely that our results were due to any kind of cytotoxicity effects associated with overexpression of gD and gH.

gH-mediated interference is independent of gD receptor expressed and it also occurs in naturally susceptible HeLa cells

To ensure that gH interference is not just a Nectin-1-receptor-dependent phenomenon, we sought to ascertain whether gH-mediated interference can be observed with other entry receptors as well. The results in Fig. 5A indicate that CHO cells transiently cotransfected with HVEM and gH exhibited reduced susceptibility (less entry) at multiple dosage of HSV-1(KOS) as compared to the control cells expressing HVEM alone. A similar experiment was done with 3-*O*-sulfated heparan sulfate (3-OS-HS) as the gD receptor. The 3-OS-HS is generated by the enzyme 3-*O*-sulfotransferase-3 (3-OST-3), which modifies cell surface heparan sulfate to create an entry receptor for HSV-1 gD (Shukla et al., 1999a). In our experiments, the CHO cells were cotransfected with 3-OST-3-expressing plasmid and either gH-expressing plasmid or empty vector pcDNA3 as control. As seen in Fig. 5B, gH expression definitely resulted in reduced entry, although the observed resistance was relatively less strong than that observed with the protein receptors Nectin-1 and HVEM. Nevertheless, it clearly indicated that gH generates some partial resistance to viral entry with this receptor as well.

To further ensure the gD-receptor independence of this phenomenon, and also to ascertain that the gH-mediated interference can exist in cell types other than CHO cells, we decided to examine gH-mediated interference in HeLa (human epithelioid carcinoma) cells. These cells are naturally

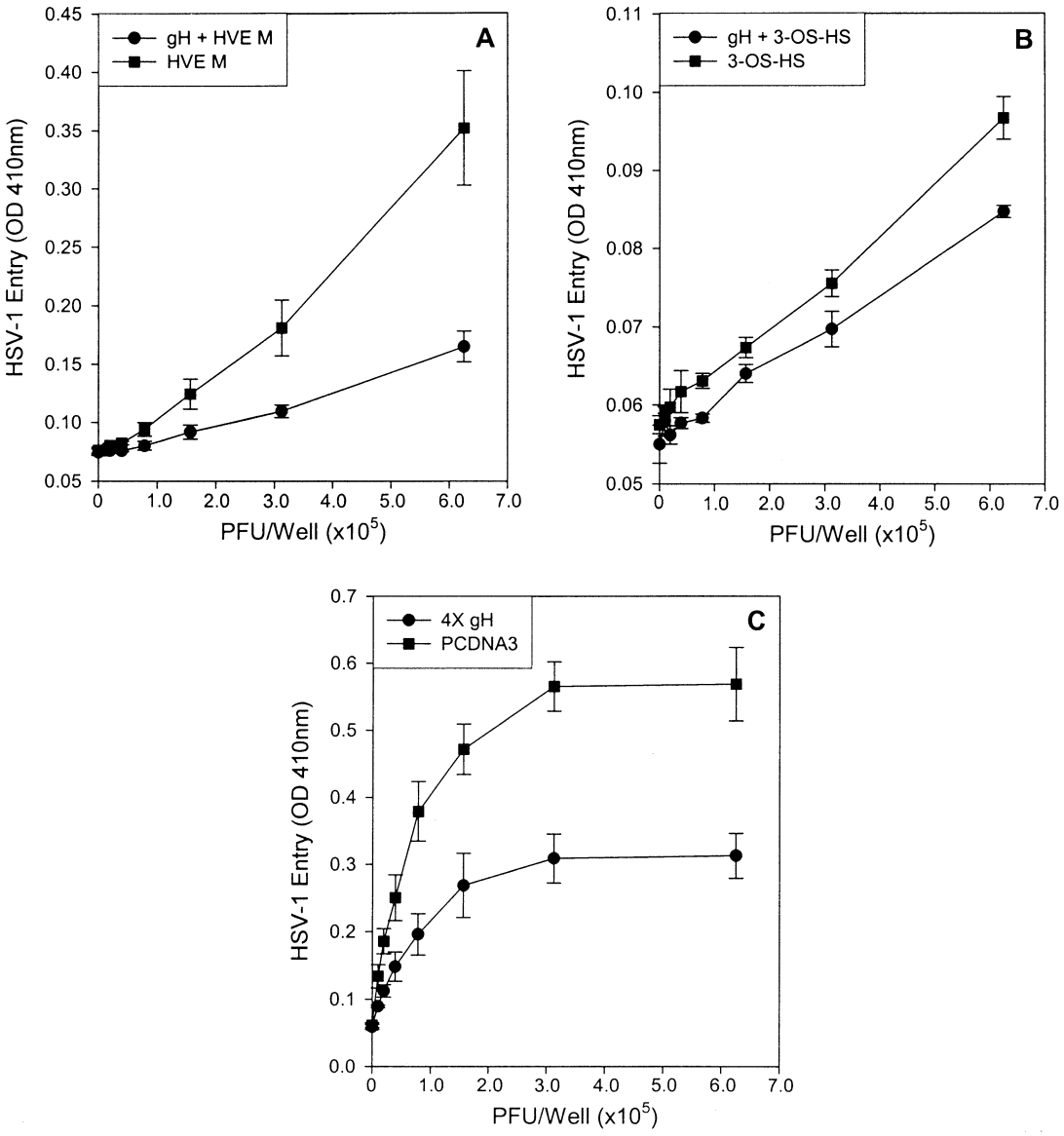

Fig. 5. The gH-mediated interference is observed with other known gD receptors and also in naturally susceptible HeLa cells. CHO cells were cotransfected with HVEM-expressing plasmid and gH plasmid (A, solid circles) or pcDNA3 control (A, solid squares) in 1:1 ratio. (B) CHO cells were cotransfected with a heparan sulfate 3-*O*-sulfotransferase-expressing plasmid and gH plasmid (solid circles) or pcDNA3 control (solid squares) in 1:1 ratio. (C) HeLa cells were transfected with either gH-expressing plasmid (solid circles) or pcDNA3 control (solid squares) with about 4 times more gH (or pcDNA3) plasmid compared to experiments shown in A and B. The transfected cells were infected with β-galactosidase-expressing HSV-1(KOS) at the plaque forming units (PFU) indicated. The β-galactosidase activity was assayed as described in the legend to Fig. 1 and in the text.

susceptible to HSV-1 and are already reported to express multiple gD receptors (Montgomery et al., 1996; Geraghty et al., 1998; Warner et al., 1998). In order to carry out the experiment, HeLa cells were transiently transfected with gH expression plasmid or empty vector (pcDNA3) as control. The transfected cells (transfection efficency approximately 70% as judged by a control transfection with a β-galactosidase-expressing plasmid, pMN84) were then infected with serial dilutions of HSV-1(KOS). Once again, as shown in Fig. 5C, the gH-expressing HeLa cells were significantly reduced in susceptibility to HSV-1 entry. This result clearly confirms that the observed phenomenon is independent of the gD receptors expressed and cell types used.

Cell surface expression of gH can occur without gL

Since gH interference appeared similar to gD interference, it was logical to think that gH must similarly be expressed on the surface of the cell. To test this hypothesis, cell surface expression of gH in two different cell-types was examined using a cell-enzyme-linked immunosorbent assay (Cell-ELISA) (Geraghty et al., 2000; Shukla et al., 1999a)

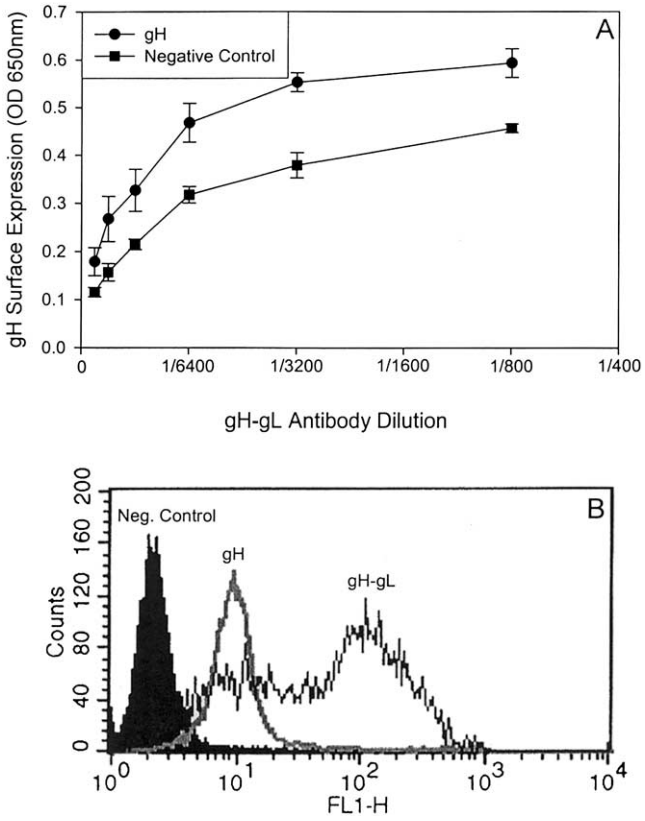

Fig. 6. Cell surface expression of gH as determined by ELISA and flow cytometry. HeLa cells transiently transfected with gH-expressing plasmid (A; solid circles) or pcDNA3 control (A; solid squares) were plated in 96-well plates. Twenty-four hours later, cell-ELISA assays were performed. Live cells were exposed to twofold serial dilutions of primary antibody (R137) with a starting dilution of 1:1000 and then washed and fixed. Binding of the primary antibodies was detected by use of biotinylated secondary antibodies and streptavidin-conjugated horseradish peroxidase. The peroxidase activity was assayed as a measure of gH antigen on the cell surface. (B) Stably transfected gH-expressing CHO cells (open left profile), stable gH-expressing CHO cells transiently transfected with gL (open right profile), and pcDNA3 control CHO cells (solid profile) were incubated with a primary polyclonal antibody against gH–gL (R137), followed by a fluoresceinated secondary antibody and flow cytometry analysis.

and by flow cytometry (FACS) analysis. As seen in Fig. 6A, compared to the mock-transfected HeLa cells, roughly twofold higher binding of anti-gH polyclonal rabbit antibodies was observed with gH-transfected HeLa cells. A similar result was also observed with CHO cells stably transfected with a gH-expressing plasmid (data not shown). The stronger binding to gH expressing cells indicates that the antibodies were recognizing and binding to the cell surface gH. A relatively high background seen with control cells is not uncommon with cell-ELISA. Also, it is highly unlikely that the antibodies bound to intracellular gH, because the primary antibody was incubated with live and healthy cells and the cells were fixed only after several rounds of thorough washing after the removal of the primary antibody.

In order to verify the cell-ELISA data, FACS analysis was also performed on live cells. Once again, as shown in Fig. 6B, the anti-gH antisera clearly differentiated the stable gH-expressing CHO cells from the non-gH-expressing CHO cells. Interestingly, the stable gH cells when transfected with a gL-expressing plasmid show a shift to the right, indicating greater fluorescence. This may be due to the fact that antibody R137 is polyclonal and recognizes both gH and gL (Peng et al., 1998). The antibodies to gL would then cause an increase in fluorescence compared to gH alone; therefore, it may not necessarily indicate an increase in surface expression of gH. In any case, the result of the FACS analysis further confirms the Cell-ELISA result that gH alone was indeed detected on the cell surface.

Cell surface expression of Nectin-1 may change in the presence of gD but not in the presence of gH–gL or gH alone

FACS analysis was conducted to examine the Nectin-1 expression in the presence of gD, gH, and gH–gL glycoproteins. In these experiments a stable Nectin-1-expressing CHO cell line was used so that a uniform amount of Nectin-1 on live cells was expressed for analysis (Geraghty et al., 1998). Using a Nectin-1 (CD111) antibody conjugated to phycoerythrin (PE), Nectin-1 expression was analyzed using FACS. As seen in Fig. 7A, Nectin-1 expression was virtually identical to the Nectin-1 positive control when gH and gH–gL were expressed. In the case of gD there was a left shift, or less Nectin-1 was detected on the surface during FACS analysis. The results of the FACS analyses, therefore, indicate that gH and gH–gL expressions do not downregulate Nectin-1 on CHO cell surfaces. The decreased detection of Nectin-1 in gD-expressing CHO cells might possibly indicate gD's ability to downregulate the surface expression of Nectin-1.

In order to verify the interference phenomena in Nectin-1 stable cells, parallel experiments were performed using Nectin-1 stable CHO cells transiently transfected with plasmids expressing glycoproteins gD, gH, and gH–gL. The bar graph in Fig. 7B shows the level of interference as a percentage of the Nectin-1 stable control cells. Once again, similar gH-mediated interferences were observed in the presence or in the absence of gL, which in turn, confirms that gL does not play a significant role in this phenomenon.

Discussion

The results presented here demonstrate a novel form of entry interference phenomenon through cellular expression of HSV-1 glycoprotein gH. Since gD was already known to cause interference, we focused on gB, gH, and gL, that are also essential for HSV-1 entry. Although the cellular expression of gB caused no interference (Fig. 1B), gH–gL together clearly interfered with HSV-1(KOS) entry (Fig. 1C). Although we attempted to show that gH and gL to-

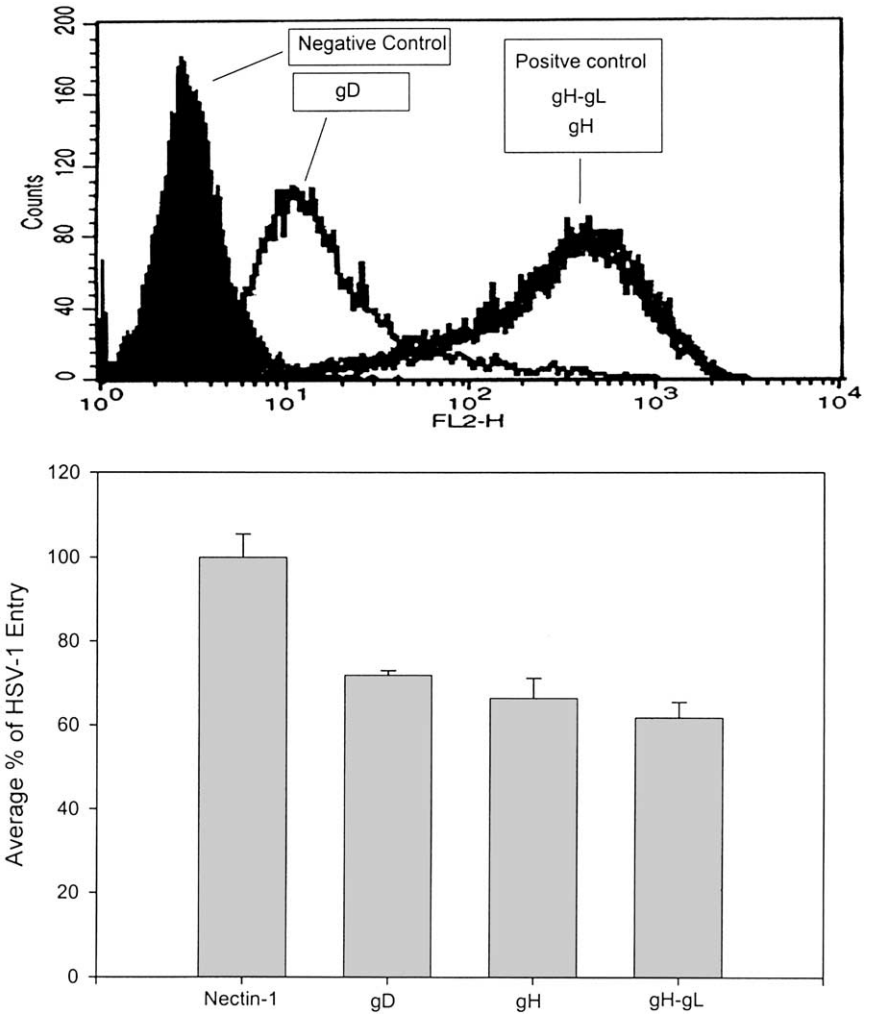

Fig. 7. Cell surface expression of Nectin-1 and gH-mediated interference in cells transfected with gB, gH, and gH–gL. Nectin-1-expressing cells were transfected in 6-well plates as described in the text. The transfection solution was removed and replaced with growth media. After about 20 h cells were loosened and incubated with Nectin-1 antibody conjugated to PE and analyzed by flow cytometry. In parallel, interference assays were performed as described in the legend to Fig. 1. The values shown (means of triplicate determinations) represent the amount of reaction product detected spectrophotometrically (OD_{410}) at a single input dose of 10^6 PFU/well in the linear range of the dose–response curve for the virus.

gether were required for this, we found that the phenomenon was specific to gH alone, although it did occur also in the presence of gL. It is noteworthy that when direct comparisons were made, the interference phenomena seen in the presence or in the absence of gL were not significantly different from each other (data not shown). Therefore, it is reasonable to suggest that the interference seen is not likely to be due to an improperly folded form of gH because an identical phenomenon is also seen in presence of gL as well. In a separate experiment, we collectively examined gB, gH, and gL for interference and the net result of that experiment was no different from what is seen with gH–gL (or gH) expression (data not shown). Therefore, it is suggested that gB and gL likely play a passive role in the interference phenomenon reported here.

However, when gH and gD were expressed together the resistance seen was significantly more pronounced than gD or gH alone (Fig. 4). We are very much tempted to extrapolate this result in suggesting that gD and gH interferences probably represent two separate steps in viral entry and that gH probably has its own receptor (or a cellular partner) that it interacts with during entry. One of the results that substantiate our hypotheses comes from the finding that gH-mediated phenomenon is common to entry mediated by all three gD receptors. Although not impossible, it is highly unlikely that like gD, gH can also bind to three different gD-receptors that belong to three diverse protein and sugar families of receptors. In addition, since the gH-mediated interference does not require gD, it is unlikely that gD provides a bridge between gH and gD receptors to cause this phenomenon. Similarly, the possibility of a cellular receptor for gH appears from not only the observance of interference itself but also from our interesting finding that cell surface expression of gH was detectable in a stable gH-expressing CHO cell line by FACS and in HeLa cells transiently expressing gH by cell-ELISA (Fig. 6). The transfection of

gL in a stable gH-expressing CHO cell line may be showing an increase in gH surface expression as shown in Fig. 6; however, since we used a polyclonal anti-gH–gL antibody to detect surface gH expression it is possible that a subset of the antibody could bind to gL and thus show more fluorescence. In any case, the main point is that gH is detected on the surface and shows interference in the absence of gL expression. In an effort to better understand and insure that gH is causing a novel interference, which is independent of gD-receptor expression, we examined Nectin-1 expression on the surface of Nectin-1 stable CHO cells transfected with gD, gH, and gH–gL (Fig. 7). We saw no downregulation of Nectin-1 in the case of gH and gH–gL. In the case of gD, we see a shift to the left, indicating less Nectin-1 antibody binding. It is possible, therefore, that either gD downregulates the surface expression of Nectin-1 or the monoclonal antibody used for Nectin-1 detection fails to recognize its epitope on Nectin-1 when it is bound to gD.

If we make an analogy to gD-mediated interference, the cell-associated gD has been shown to interact with its receptor in a fashion that renders the receptor inaccessible to virions (Geraghty et al., 2000). It was also noticed by more than one group that cell surface gD was significant in generating this phenomenon (Dasika and Letchworth, 2000; Geraghty et al., 2000). Although our study does not provide strong-enough evidence that cell surface gH is the cause for the observed phenomenon, a strong resemblance to gD-mediated interference, including the cell surface expression of gH definitely points in that direction. Alternatively, if the intracellular gH is the cause for the phenomenon, then it is likely to target a potential but unknown signaling mechanism required for fusion. Studies are beginning to suggest that intracellular signaling pathways might play a role in HSV-1 entry (Qie et al., 1999).

Previous reports indicate some controversy in gH expression and whether gL is required for surface expression. It is widely believed that HSV-1 gH requires gL for its proper expression on the cell surface (Hutchinson et al., 1992; Forrester et al., 1992). However, Ghiasi and coworkers (Ghiasi et al., 1991) showed that gH was present on cell surfaces using a baculovirus system and measuring surface expression by indirect immunofluorescence. The gH product seen was glycosylated and had a molecular weight similar to wild-type HSV-1 gH (Ghiasi et al., 1991). Our results, on the other hand, by no means attempt to refute the common knowledge that gH–gL heterodimers exist and are crucial for herpesvirus entry. Actually our findings raise an interesting possibility that HSV-1 gH might exist in two forms, one in complex with gL and the other gH alone. If true, then it is likely that gH alone binds to a cellular receptor, whereas the complex possibly works via a different, yet unknown, mechanism. Does it mean that gH has some gL independent functions/interactions during entry or gH has an additional function of anchoring gL to the membrane (and possibly hiding a potential fusogenic part of gL until the fusion is triggered by a gD–receptor interaction)? Although our study does not provide an answer to these questions, it rightfully raises them for future studies to answer.

It is possible that envelope glycoprotein-mediated interference may have some physiological relevance associated with it. In case of retroviruses expression of glycoproteins by infected cells help to prevent superinfection. Logically it makes sense since prevention of superinfection could be helpful in avoiding multiple proviral insertions and promoting cell survival. Similarly it is possible, yet unproven, that HSV-1 glycoprotein expression on cell surfaces might be a way of regulating the maximum number of infectious virions entering into a cell during early stages of infection. Obviously, too many virions are likely to kill the host cell. In α-herpesviruses, gD has been reported to be expressed in lytically infected cells and the resultant interference has been suggested to be important for efficient egress and release of infectious virus by preventing newly enveloped virus from fusing with membranes of the virus-producing cells (Campadelli-Fiume et al., 1990; Johnson and Spear, 1989). Future studies will determine if gH also has a similar function in egress.

Materials and methods

Plasmids

The plasmids used in these experiments were pPEP98 (gB), pPEP99 (gD), pPEP100 (gH), and pPEP101 (gL), all expressing HSV-1(KOS) glycoproteins (Pertel and Spear, 1997). Other plasmids used included pBG38 (Nectin-1) (Geraghty et al., 2000) and pBec10 (HVEM) (Montgomery et al., 1996) that express entry receptors, whereas pDS43 (3-OST-3B) (Shukla et al., 1999a) expresses 3-*O*-sulfotransferase that modifies heparan sulfate to generate gD receptors. The plasmid pMN84 expresses β-galactosidase and it has been previously used to determine transfection efficiency (Montgomery et al., 1996). The control plasmid pcDNA3 was from Invitrogen (Carlsbad, CA, USA).

Cells and virus

P.G. Spear (Northwestern University) provided wild-type CHO-K1 cells (designated CHO throughout this manuscript). All CHO cell lines including stable gH cells (described below) were passaged in Ham's F12 medium (Gibco/BRL), Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin (Gibco/BRL). HeLa cells were passaged with Dulbecco's modified Eagle's medium (DMEM). The β-galactosidase-expressing recombinant HSV-1(KOS) gL86 was provided by P.G. Spear (Northwestern University). Since the virus lacks gL, it was grown in a complementing Vero cell line that stably expresses gL. The HeLa cells were obtained from B.P. Prabhakar (University of Illinois at Chicago).

Transient transfection and viral entry assay

CHO cells were transferred to 6-well plates and grown to a subconfluent level. Unless otherwise noted, the amount of transfected plasmid was in a 1:1 ratio for glycoproteins and Nectin-1. In order to achieve consistency, in every transfection experiment the total amount of plasmid DNA was kept constant at 2.5 μg. Whenever needed, the total DNA requirement was met by adding pcDNA3. Subconfluent CHO cells were transfected with 5 μl of LipofectAMINE reagent (Gibco/BRL) and 2.5 μg of plasmid DNA per well of a 6-well dish according to the manufacture's protocol. About 20 h later, the cells were replated into 96-well dishes (approximately 2×10^4 cells/well). The cells were then allowed to recover in growth media for 18 h at 37°C and 5% CO_2. After that cells were washed 2X with phosphate-buffered saline (PBS) and 1×10^7 virus added to the 12th well in 50 μl of PBS with 1% glucose and 0.1% heat-inactivated calf serum. The virus was twofold serially diluted starting from wells 12 through 2. Wells in column 1 were the negative control and contained no virus. Plates were allowed to incubate for 6 h at 37°C. Plate wells were washed with 50 μl PBS 2 times and 50 μl *o*-nitrophenyl-β-D-galactopyranoside (ONPG) reagent (ImmunoPure) was added. The plates were incubated for 1 h at 37°C and the enzymatic activity was measured at 410 nm using a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA). ONPG reagent contains 6 mg ONPG with 20 ml PBS and 120 μl of IGEPAL (Sigma).

Construction of gH-expressing stable CHO cell line

The HSV-1(KOS) gH-expressing plasmid pPEP100 and the control plasmid pcDNA3 were transfected separately into subconfluent CHO cells using LipofectAMINE (Gibco/BRL) as described above. After replating in 96-well plates at a dilution of 10 transfected cells/96-well plate, G418 selection was applied and the cells showing resistance were grown for several generations under selection. Several G418-resistant clones were obtained for both pPEP100 and pcDNA3. Based on comparable growth kinetics under G418 selection, one clone for each (pPEP100 and pcDNA3) was further grown and pursued.

Cell ELISA

The cell-ELISA protocol is described elsewhere (Geraghty et al., 2000). The primary antibody, R137, used in this assay was obtained from Gary Cohen and Roselyn Eisenberg (Univ. of Pennsylvania). It is a rabbit polyclonal antibody to gH–gL (Peng et al., 1998). The primary antibody was diluted 1:1000 with PBS containing 3% bovine serum albumin (BSA), which was further serial diluted to obtain a dose–response curve. All other reagents were used in same amount (no serial dilution). The secondary antibody was an antirabbit IgG antibody produced in mouse and biotinylated (Sigma). It was diluted 1:5,000 in PBS, with 3% BSA. AMDEX streptavidin-conjugated horseradish peroxidase (HRP) (Amersham Arlington Heights, IL, USA) was used to detect the secondary antibody and was diluted 1:10,000 in PBS. The liquid substrate used was 3,3′, 5,5′-tetramethylbenzidine in slow kinetic form (Sigma). Optical density (OD) was read at 650 nm on a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry

Stably transfected CHO cells were grown to subconfluency of approximately one million cells. Cells were loosened with an enzyme-free solution (PBS/4 mM EDTA) and washed 3 times with 1 ml PBS by spinning at 1000*g* for 2 min at 4°C. Primary antibody R137 was used at a 1:50 dilution and of this 50 μl was added to the CHO cells and incubated for 20 min at 4°C. For Nectin-1 expression Nectin-1 stable CHO cells were incubated with primary CD111 (Nectin-1) antibody conjugated to PE (phycoerythrin) (Immunotech, France, Catalog No. PN IM3451) at a 1:50 dilution and of this 50 μl was added to the CHO cells and incubated for 20 min at 4°C. Cells were washed again as outlined above. FITC-labeled goat antirabbit antibody diluted to 1:100 was added as a secondary antibody and incubated at 4°C for 20 min. No secondary antibody was used in the Nectin-1 stable cell experiments. Immediately prior to performance of flow cytometry (Becton–Dickinson FACS-Calibur) propidium iodide was added to a final concentration of 1 μg/ml in PBS to exclude dead cells from analysis. For gB expression, anti-gB monoclonal antibody was obtained from Virusys corporation (Catalog No. HA056-100). The FACS analysis was performed using CHO cells transiently cotransfected with gB (pPEP98) and Nectin-1 (pBG38) plasmids.

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