Herpes simplex virus type 1 glycoprotein H binds to \( \alpha v \beta 3 \) integrins

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Glycoprotein H (gH) homologues are found in all members of the herpes virus family, and gH is one of the virion envelope glycoproteins that is essential for virus entry. In this study, a recombinant soluble form of Herpes simplex virus type 1 (HSV-1) gH, in which the ectodomain is fused to the Fc-binding region of IgG, has been generated. This was expressed in mammalian cells together with gL and the resulting gHFc–gL heterodimer was purified using Protein A Sepharose. Low-affinity cell binding assays showed that gHFc–gL bound specifically to Vero cells and mutation of a potential integrin-binding motif, Arg-Gly-Asp (RGD), in gH abolished binding. CHO cells failed to bind in this assay. However, CHO cells expressing the human \( \alpha v \beta 3 \) integrin bound efficiently to gHFc–gL, suggesting that HSV-1 gH can bind to cells using \( \alpha v \beta 3 \) integrins and that this binding is mediated by the RGD motif in the gH ectodomain.

Although a number of molecules which bind to gD have been identified, there are as yet no reports of cellular receptors for either HSV-1 gB or gH. Nevertheless, the notion that a receptor for HSV-1 gH exists is supported by recent observations by Scanlan et al. (2003) who demonstrated that cells expressing gHL were resistant to virus infection. There is also evidence that gB and gH homologues in other herpesviruses interact with a number of different cellular proteins; gB molecules of human cytomegalovirus (HCMV) bind to the EGF receptor (Wang et al., 2003b) and annexin II (Pietropaolo & Compton, 1997), and human herpesvirus 8 (HHV8) gB binds to \( \alpha 3 \beta 1 \) integrins (Akula et al., 2003). gH of human herpesvirus 6 binds to CD46 (Mori et al., 2003; Santoro et al., 2003), HCMV gH has been reported to bind to a 92.5 kDa cellular protein (Keay & Baldwin, 1991) and in Epstein–Barr virus the gHL complex is associated with a further polypeptide, gp42, which reacts with HLA class II (Li et al., 1997).

The predicted amino acid sequence of both the HSV-1 and HSV-2 gH ectodomains contains a potential integrin-binding motif, Arg-Gly-Asp (RGD), so to address the question as to whether HSV-1 gH can bind to cells, and if so, whether binding is mediated by an RGD-integrin interaction, we generated recombinant soluble forms of both wild-type gHL and mutated gHL, in which the RGD sequence was Arg-Gly-Glu (RGE), a mutation which ablates integrin-binding, and tested these molecules in cell binding assays.

The sequence encoding the predicted ectodomain of HSV-1 strain HFEM gH (aa 1–803) was fused in-frame at the N terminus of the Fc portion of human IgG1 under control of the \( \beta \)-actin promoter. A 6 aa residue linker connects the gH sequence to the natural hinge between the CH1 and CH2 domains of IgG, and the construct continues with the two immunoglobulin constant domains, CH2-CH3, which comprise the Fc-binding region of IgG. This hybrid molecule was expressed in Chinese hamster ovary (CHO) cells together with HSV-1 gL. The gL coding region was also cloned under the control of the \( \beta \)-actin promoter, and the gHFc–gL fusion protein was purified from cell supernatants by affinity chromatography on a Protein A
Sepharose column. SDS-PAGE analysis of the purified protein confirmed that the molecule was expressed as a heterodimer of the gHFc fusion polypeptide and gL (Fig. 1a). The recombinant fusion protein was also recognized by the conformation-dependent neutralizing monoclonal antibody to gH, LP11 (Buckmaster et al., 1984), as determined by ELISA (Fig. 1b). Recognition of the LP11 epitope on HSV-1 gH is dependent on the presence of gL (Hutchinson et al., 1992), so LP11 reactivity was considered to be a good indication that the recombinant molecules were conformationally authentic. A modified form of this protein, in which the RGD motif was mutated by site-directed mutagenesis to the non-integrin binding sequence, RGE was also produced and could also be detected in ELISA by LP11 (Fig. 1c). To determine whether the gHFc–gL fusion protein bound to cells, we carried out binding assays as described in Diamond et al. (1990). This is a method that is used to detect low-affinity binding interactions. A 50 μg ml⁻¹ solution of protein was coated as 50 μl spots onto a 20 cm² plastic Petri dish and incubated at room temperature for 90 min. After blocking non-specific binding sites with 1% BSA/PBS, the dishes were overlaid with 5 x 10⁶ trypsinised Vero cells or CHO cells, and incubated at 37 °C for 1 h, after which time unbound cells were gently removed and the dishes were fixed in formal saline and stained with toluidine blue. Binding was quantified by counting the number of cells, per field of view, bound to spots of gHFc–gL protein as compared with the number bound to spots of human IgG. As shown in Fig. 2(a), Vero cells bound specifically to gHFc–gL and not to the IgG control, while CHO cells failed to bind in this assay. This observation suggests that there may be a gH receptor on Vero cells which is resistant to trypsin digestion, while any gH-binding proteins on CHO cells must be sensitive to this treatment. If an interaction between gH and a cellular receptor is required for entry, then such receptors must be present on CHO cells, since CHO cells which express gD receptors can be infected with HSV (Montgomery et al., 1996). Nevertheless, it remains a possibility that if gH binding to cells has additional functions, then CHO cells may not express the appropriate receptor.

Since HSV-1 gH contains an RGD motif it was of interest to test whether the binding of gHLFc to Vero cells was mediated by an interaction with cell surface integrins, so we repeated binding assays using soluble gH RGE Fc–gL protein coated onto plastic dishes. As shown in Fig. 2(b), Vero cells failed to bind to this molecule, and no binding of either wild-type or mutated gH–gL fusion proteins to CHO cells was observed. These findings are consistent with the view that the binding of gHFc–gL to Vero cells occurs via an interaction between the RGD motif and a cellular integrin molecule.

Integrin molecules exist as heterodimers comprised of α and β subunits. Both subunits are type 1 transmembrane proteins and there are eight β and 18 α subunits in mammalian cells, which are known to combine to form 24 distinct integrins. In an attempt to identify the nature

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**Fig. 1.** (a) Purified gHFc–gL (1 μg) was analysed by SDS-PAGE under reducing conditions followed by staining with Coomassie blue. Molecular mass standards are indicated in kDa, and the gHFc and gL polypeptides are indicated with arrows. (b) Serial double dilutions of purified gHFc–gL or a control protein gDFc (starting at 1 μg ml⁻¹) were incubated on plates coated with anti-human IgG. Protein was detected with monoclonal antibody LP11 and anti-mouse alkaline phosphatase, followed by incubation with Sigmafast pNPP substrate and measurement of OD₄₀₅. (c) ELISA assays (as described for Fig. 1b) were carried out on serial twofold dilutions of either gHFc–gL or gHRGEFc–gL. The first well in each case contained neat supernatant from cells expressing each molecule.
of the integrin to which gH binds, binding assays were carried out using Vero cells that had been pre-treated with serial twofold dilutions of a panel of monoclonal antibodies to several different α- and β-integrin subunits, and the ability of these antibodies to inhibit binding was measured by counting the number of cells that adhered to spots of the gHFc–gL protein. Fig. 3(a) shows that antibodies to β1, β2 and αIIb subunits failed to inhibit binding except when used at concentrations between 10–20 μg ml⁻¹ where binding was reduced by approximately twofold. However, anti-β3 antibodies at concentrations as low as 1 μg ml⁻¹ reduced binding by 100-fold, and similarly pronounced inhibitory effects were seen with anti-αv antibodies at concentrations as low as 0·15 μg ml⁻¹. Although these data do not formally prove the nature of the integrin that mediates binding of gH to Vero cells, they suggest that it may be comprised of an αv and a β3 subunit, and this is a well-established integrin subunit combination. To confirm this, CHO cells expressing human αvβ3 integrin molecules (Mekrache et al., 2002) were tested in binding experiments with both wild-type gHFc–gL and the gH RGE mutant protein. These cells bound efficiently to gHFc–gL, but did not bind to the form of gH in which the integrin-binding motif had been mutated to RGE (Fig. 3b).

The observation that HSV-1 gH binds to αvβ3 integrins raises a number of questions as to its functional significance. Many viruses, including adenovirus, rotavirus, human parechovirus 1 and hantavirus use integrin molecules as entry receptors, and this is also true for HHV8 which binds to αvβ1 integrins via an RGD sequence present in gB (Akula et al., 1998). This does not appear to be the case for HSV-1 since a recombinant virus in which the RGD motif in gH was mutated to RGE (Galdiero et al., 1997) showed no growth deficit in vitro, exhibited wild-type particle infectivity ratios, and entered cells at equivalent rates to wild-type virus. Furthermore, antibodies to β3 and αv integrins failed to inhibit HSV-1 plaque formation (data not shown). This observation is consistent with data in the literature (Israel et al., 1998), which showed that RGD peptides also had no effect on inhibiting infection.
Nevertheless, we cannot discount the possibility that there may be some elements of redundancy in terms of the receptor usage of HSV-1, nor can we rule out the fact that integrin binding by gH may be important for efficient entry into specific cell types in vivo. Furthermore, a consequence of the binding of many ligands to integrins is the induction of intracellular signalling pathways which lead to a wide spectrum of downstream events. These include activation of gene expression, promotion of cell survival and cell growth, alterations in calcium levels and cytoskeletal rearrangements (reviewed by Giancotti & Ruoslahti, 1999). It has recently been reported (Cheshenko et al., 2003) that HSV triggers activation of Ca\(^2+\) signalling pathways and phosphorylation of focal adhesion kinase (FAK), both of which are known downstream events that may occur as a result of the binding of a number of ligands to \(\alpha v\beta 3\) integrins. However, the phosphorylation of FAK was also induced when cells were incubated with virions containing the \(gH^{RGE}\) mutation (B. Herold and N. Cheshenko, personal communication) and this may imply that different signalling pathways are triggered by the binding of HSV-1 gH to \(\alpha v\beta 3\) integrins.

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References


