Analysis of synthetic peptides from heptad-repeat domains of herpes simplex virus type 1 glycoproteins H and B

Stefania Galdiero,1,2,3 Mariateresa Vitiello,2,4 Marina D'Isanto,4 Annarita Falanga,4 Craig Collins,5 Katia Raieta,4 Carlo Pedone,1,2,3 Helena Browne5 and Massimiliano Galdiero2,4

Correspondence
Massimiliano Galdiero
massimiliano.galdiero@unina2.it

1,2Department of Biological Sciences, Division of Biostructures1 and Centro Interuniversitario di Ricerca sui Peptidi Bioattivi2, University of Naples Federico II, Via Mezzocannone 16, 80134 Naples, Italy
3Istituto di Biostrutture e Bioimmagini, CNR, Via Mezzocannone 16, 80134 Naples, Italy
4Department of Experimental Medicine, II University of Naples, Via De Crecchio 7, 80138 Naples, Italy
5Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK

Human herpesviruses enter cells by fusion of their own membrane with a cellular membrane through the concerted action of multiple viral proteins and cellular receptors. Two conserved viral glycoproteins, gB and gH, are required for herpes simplex virus type 1 (HSV-1)-mediated membrane fusion, but little is known of how these proteins cooperate during entry. Both glycoproteins were shown to contain heptad repeat (HR) sequences predicted to form α-helical coiled coils, and the inhibitory activity against infection of four sets of synthetic peptides corresponding to HR1 and HR2 of gB and gH was tested. The interactions between these HR peptides were also investigated by circular dichroism, native polyacrylamide-gel electrophoresis and size exclusion high-performance liquid chromatography. gH coiled-coil peptides were more effective than gB coiled-coils peptides in inhibiting virus infectivity. The peptides did not impair fusion when added to cells immediately after infection. In contrast, inhibition of infection was observed, albeit to various extents, when peptides were added to virus before or during inoculation. The results of biophysical analyses were indicative of the existence of an interaction between HR1 and HR2 of gH and suggest that the HRs of gB and gH do not interact with each other.

INTRODUCTION

Infection of a target cell by enveloped viruses requires a virally mediated mechanism to promote membrane fusion (Weissenhorn et al., 1999; Earp et al., 2005). This mechanism seems surprisingly conserved across several diverse viral families, which makes the fusion process a potentially attractive target for antiviral drugs for a number of human and animal viruses (Dimitrov, 2004).

Enveloped viruses use transmembrane (TM) viral proteins to mediate fusion with host cell membranes (Hernandez et al., 1996; Eckert & Kim, 2001; Blumenthal et al., 2003; Jahn et al., 2003). Key structural elements shared by most viral fusion glycoproteins are a fusion peptide and a coiled-coil bundle of helices. The fusion peptide is a hydrophobic α-helix able to penetrate the membrane of the target cell in order to destabilize the lipid bilayer organization. By means of the fusion peptide, inserting in the host cell membrane, and the TM domain, which anchors the protein on the viral envelope, a bridge is formed between the two membranes. Subsequent rearrangements in the conformation of these proteins lead to the close juxtaposition of the viral and cellular membranes, culminating in the merging of two bilayers. In addition to the fusion peptide domain, many fusion proteins also contain one or more heptad-repeat (HR) regions, which are often adjacent to the fusion peptide sequence and to the TM anchor domain (Chambers et al., 1990; Skehel & Wiley, 1998). There are usually two of these regions: an N-terminal HR region (HR1) adjacent to the fusion peptide and a C-terminal HR region (HR2) close to the TM anchor. Structural studies of several viral fusion proteins revealed that the HR regions form a six-helix bundle structure implicated in viral entry (Eckert & Kim, 2001). The HR1 and HR2 domains associate to form a six-helix bundle, with a central trimeric coiled-coil domain formed by HR1 helices; the three HR2 helices pack obliquely in an anti-parallel configuration into the highly hydrophobic grooves on the surface of the central coiled coil. This conformation
brings the N-terminal fusion peptide into close proximity to the TM anchor. Recent evidence suggests that the actual six-helix bundle formation is directly coupled to the merging of the membranes (Melikyan et al., 2000; Russell et al., 2001). The similarities in the structures of the six-helix bundle complexes elucidated for influenza virus haemagglutinin (HA) (Bullough et al., 1994; Chen et al., 1999), Human immunodeficiency virus type 1 (HIV-1) and Simian immunodeficiency virus (SIV) (Weissenhorn et al., 1997; Caffrey et al., 1998; Chan & Kim, 1998; Malashkevich et al., 1998), Moloney Murine leukemia virus type 1 gp21 (Fass et al., 1996), Ebola virus GP2 (Weissenhorn et al., 1998; Malashkevich et al., 1999), human T-cell leukemia virus type I gp21 (Kobe et al., 1999), Visna virus TM (Malashkevich et al., 2001), simian parainfluenza virus 5 (SV5) F1 (Baker et al., 1999) and human respiratory syncytial virus (HRSV) F1 (Zhao et al., 2000), all point to a common fusion mechanism for these viruses. Based on structural similarities, two classes of viral fusion proteins have been distinguished (Jardetzky & Lamb, 2004). Proteins containing HR regions and an N-terminal or N-proximal fusion peptide are classified as class I viral fusion proteins. Class II viral fusion proteins (e.g. the alphavirus E1 and the flavivirus E fusion proteins) lack HR regions and have an internal fusion peptide. Their fusion protein is folded in tight association with a second protein as a heterodimer. Here, fusion activation takes place upon cleavage of the second protein.

Therefore, the role of coiled coils in the entry of several viruses has been widely investigated, demonstrating that α-helical coiled coils form the basis for critical protein–protein interactions within the fusogenic glycoprotein and play a pivotal role in membrane fusion. In fact, when soluble peptides comprising the HR sequences are presented to virus during inoculation into cells, entry of the virus is significantly impaired (Wild et al., 1994; Rapaport et al., 1995; Lambert et al., 1996; Wild & Buckland, 1997; Joshi et al., 1998; Kilby et al., 1998).

Fusogenic machines of most viruses are composed of one or two glycoproteins, which, in the majority of cases, can perform both receptor recognition and binding as well as viral penetration. On the other hand, herpesviruses specify multiple envelope glycoproteins, a subset of which are known to play a role in membrane fusion (Mettenleiter 2002a, b; Spear & Longnecker, 2003; Spear, 2004). Herpesviruses infect their target cells by fusion of the viral envelope with the cellular plasma membrane at neutral pH. For penetration, a set of envelope glycoproteins that are conserved throughout the subfamilies of the family Herpesviridae is required. This includes glycoproteins B (gB), H (gH) and L (gL). In addition, each subfamily employs different receptor-binding proteins. Herpes simplex virus type 1 (HSV-1) attachment requires gB, gC and gD. The first two bind to cell surface proteoglycans, whereas gD interacts with cellular surface receptors from the tumour necrosis factor receptor or immunoglobulin superfamily (Montgomery et al., 1996; Cocchi et al., 1998; Geraghty et al., 1998; Warner et al., 2001; Lopez et al., 2000). Interaction of gD with its receptor then triggers the fusion machinery, which results in the formation of a continuous membrane consisting of the plasma membrane of the infected cell and the viral envelope (Cocchi et al., 2004). Fusion of the HSV-1 envelope with the plasma membrane is mediated by four glycoproteins, gB, gD, gH and gL (Turner et al., 1998).

The main candidate proteins for performing fusion are gH and gB. Recently, Gianni et al. (2005a) reported that gH possesses a fusion peptide, although this does not rule out the possibility that other glycoproteins, such as gB, may also play an active role in the fusion process. Furthermore, it has also been shown that, besides this region, additional hydrophobic domains of gH may be required for efficient induction of fusion (Galdiero et al., 2005).

gH shares features typical of both class I and class II virus fusion proteins. In analogy to type I membrane proteins, it contains two HR sequences, one located near the putative fusion peptide and one in close proximity to the TM region. However, in common with many class II fusion proteins, gH lacks an N-terminal or even N-proximal fusion peptide in the membrane-anchored subunit; there is no evidence that it exists as a trimer and the cleavage of gH is not required for membrane fusion.

The role of HSV-1 gB in fusion is less well defined. However, HR regions have been identified in the human cytomegalovirus (HCMV) gB homologue (Lopper & Compton, 2004). gB may also be a type I membrane glycoprotein and recent crystallographical evidence suggests that HSV-1 gB may exist as a trimer (Heldwein et al., 2005).

To understand the molecular events in the early steps of HSV-1 infection, we sought to determine the functional roles of different regions of gH and gB α-helical coiled coils in membrane fusion and virus entry.

A recent report by Gianni et al. (2005b) identified a predicted coil region in HSV-1 gH. In addition, synthetic peptides modelled on coiled coils derived from glycoproteins of other members of the herpesvirus family, namely BHV-1 (Okazaki & Kida, 2004) and HCMV (Lopper & Compton, 2004), have recently been described.

Using an algorithm to detect potential coiled coils, we identified two HR regions in gH and two in gB and generated peptides of different lengths that correspond to the predicted coiled-coil regions. We demonstrated that the gH coiled-coil peptides were more effective than gB coiled-coil peptides in inhibiting virus infectivity. The peptides did not impair fusion when added to cells immediately following infection. In contrast, when peptides were added to virus before or during inoculation into cells, inhibition of infection was observed, albeit to various extents. In the present study, we also applied biophysical methods to determine whether the HR regions present in HSV-1 gH and gB were able to establish intramolecular or intermolecular interactions.
METHODS

Materials. Fluorenylmethoxycarbonyl-protected amino acids were purchased from INBIOS; NovaSyn TGA resin from Nova Biochem. The reagents (piperezine and pyridine) for the solid phase peptide synthesis were purchased from Fluka (Sigma-Aldrich); trifluoroacetic acid (TFA) and acetic anhydride were purchased from Applied Biosystem. H2O, dimethylformamide and CH3CN were supplied by LAB-SCAN.

Proteomics computational methods. The bioinformatic search for coils in HSV gH (protein Swiss-Prot accession no. P08356) and gB (protein Swiss-Prot accession no. P10211) was performed with the program Coils (Lupas et al., 1991), with window widths of 14, 21 and 28.

Peptide synthesis. Peptides were synthesized using a standard solid-phase-9-fluorenylmethoxycarbonyl method, on a PSSM8 multi-specific peptide synthesizer (Shimadzu) as described previously (Galdiero et al., 2005).

Cells and virus. Vero cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. HSV-1 carrying a lacZ gene driven by the CMV IE-1 promoter to express β-galactosidase was propagated as described previously (Forrester et al., 1992).

Virus entry assays. Peptides were dissolved in DMEM without serum and used at a range of concentrations. All experiments were conducted in parallel with scrambled peptides (peptides with the same amino acids but in a different order) and no-peptide controls.

To assess the effect of peptides on inhibition of HSV infectivity, four different ways of treating cell monolayers were performed.

Virus pre-treatment. Approximately 2 x 10^6 p.f.u. HSV-1 were incubated in the presence of different concentrations of peptides (10, 100, 250 and 500 μM) for 45 min at 37°C, then titrated on Vero cell monolayers.

Cell pre-treatment. Vero cells were incubated with peptides (10, 100, 250 and 500 μM) for 30 min at 4°C. Peptides were removed and cells were washed with PBS before being infected with serial dilutions of HSV-1 and incubated for 45 min at 37°C.

Co-treatment. Cells were incubated with increasing concentrations of the peptides (10, 100, 250 and 500 μM) in the presence of the viral inoculum for 45 min at 37°C.

Post-treatment. Vero cells monolayers were challenged with HSV-1 for 45 min at 37°C. Different concentrations of peptides (10, 100, 250 and 500 μM) were then added to the inoculum, followed by a further 30 min incubation at 37°C.

For all treatments, non-penetrated viruses were inactivated by citrate buffer at pH 3-0 after the 45 min incubation with cells at 37°C. Cells were then incubated for 24 h at 37°C in DMEM supplemented with carboxymethylcellulose (CMC). Monolayers were fixed, stained with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) and plaque numbers were scored. Experiments were performed in triplicate and the percentage of inhibition was calculated with respect to no-peptide control experiments.

Circular dichroism (CD) measurements. CD experiments at different concentrations of H-HR1 and H-HR2 (5, 10, 20, 40 and 50 μM) were performed in 5 mM phosphate buffer pH 7.4 as described by Galdiero et al. (2005). For mixing experiments, 10 μM of each peptide was prepared.

Toxicity. Peptide cytotoxicity was measured by a lactate dehydrogenase (LDH) assay that was carried out according to manufacturer’s instructions using a cytotoxicity detection kit (Roche).

SDS-PAGE. Isolated peptides (7.5 mM in phosphate buffer, pH 7.3) were incubated at 100°C for 5 min (final volume of 5 μl) and analysed by SDS-PAGE in denaturing conditions on 18% polyacrylamide gel.

Native-PAGE (N-PAGE). Isolated peptides and equimolar mixtures of peptides (7.5 mM in phosphate buffer, pH 7-3) were incubated at 37°C for 30 min (final volume of 5 μl). After addition of an equal volume of Tricine sample buffer [0·0625 M Tris/HCl, pH 6·8, 40% (v/v) glycerol, 0·001 g bromophenol blue] the mixture was analysed by PAGE on 12% Tricine gel with a Tricine/glycine running buffer (pH 8·3). Gel electrophoresis was carried out with 25 A constant amperages at room temperature for 2·5 h. The gel was then stained with Coomassie blue.

Analytical size exclusion (SE) chromatography. A BioSEP-SEC-S2000 column (300 x 7·8 mm; Phenomenex) was used for SE chromatography (SE-HPLC) to assess oligomer formation. Peptides were injected into the column connected to an HPLC system (class LC10 equipped with a diode array detector SPD-M10AV; Shimadzu), HEPES/Na buffer (pH 7-4) was used as a mobile phase with a flow rate of 0·5 ml min⁻¹. Peptide mixtures were prepared in the elution buffer and injected into the column to assess the formation of peptide-complexes. Standards, consisting of ovalbumin (44 kDa), cytochrome C (12 kDa), antp (1·5 kDa) asy (1·0 kDa) and RPK (0·4 kDa), were used to define the molecular masses of the peptides and their complex. Antp (Ac-EQQIKIAAQNRR-COOH), asy (Ac-RKARKAAAAR-COOH) and RPK (NH₂-RPK-COOH) are peptides of known molecular mass.

RESULTS

Bioinformatic analysis

By using the Coils program (Lupas et al., 1991), two HR regions were identified in HSV-1 gH and one in gB, with probability scores higher than 0·1 (Fig. 1a); moreover a third coil was identified in the cytoplasmatic tail of gB but was not considered in this study. The two HR motifs in gH were designated HR-1 and HR-2; HR-1, presents a very high probability of forming a coiled-coil structure (with a score of 0-9) and as recently reported by Gianni et al. (2005b) it also corresponds to residues 444-476 of HSV-1 gH; HR-2 was predicted at residues 542-582 with a lower probability of forming a coiled coil (0·4 with the weighted option and 0·1 with the unweighted option). To avoid false-positives due to the presence of highly charged sequences, which although presenting high coiled-coil probabilities do not really have heptad periodicity and coiled-coil forming potential, we used the weighted option. It has been recently reported (Gianni et al., 2005b) that HR-1 (peptide 444-468), corresponding to the smallest region of gH with the highest probability to form coils, plays a critical role in the fusion mechanism, so we analysed further the fusion activity of HR-1 of HSV-1 gH using peptides of different length and, in addition, verified the activity of HR-2.

To elucidate the potential role played by gB in fusion, we performed a similar analysis on the gB amino acid sequence. A first coil with a probability higher than 0·3 was found
between residues 92 and 112, and a region with a lower probability was observed from residues 618 to 631. We used peptides corresponding to these regions of gB to determine whether these domains of gB were important in fusion.

To study the sequence dependence and to optimize the efficacy of the inhibition, we synthesized peptides derived from each of these predicted coils together with a shorter and scrambled form.

**Effect of HR sequences of gH and gB on virus infectivity**

Nine peptides modelled on HRs were synthesized (Table 1) together with four scrambled peptides. To confirm that these peptides did not exert toxic effect on Vero cells, monolayers were exposed to different concentrations (100, 250 and 500 \( \mu \)M) of each peptide for 24 h and cell viability was assayed by an LDH assay. No statistical difference was observed between the viability of untreated cells and that of cells exposed to the peptides (data not shown). To test whether the coiled-coil regions of gH and gB could affect HSV infectivity, we inoculated HSV-1 into Vero cells at 37 °C in the presence or absence of each peptide under a range of different conditions as described in Methods. These results are shown in Fig. 1(b).

As previously reported by Gianni *et al.* (2005b), peptide H-HR1 showed a dose-dependent inhibition of HSV entry when present on the monolayers together with the virus inoculum for the 45 min period prior to low pH treatment, resulting in approximately 65% inhibition at the highest dose tested (500 \( \mu \)M). A shorter peptide derived from the same region, H-HR1-SN, showed no inhibition at any of the concentrations tested, while a hydrophobic peptide (H-HR1-SC) that overlaps and extends toward the C terminus of the gH protein retained a small amount of inhibition (25–30% inhibition) (Fig. 1b). This sequence has previously been analysed in a model system of membrane fusion with liposomes and although possessing characteristics of a membrane interacting segment failed to induce any fusion (Galdiero *et al.*, 2005). gH HR2 peptides showed reduced efficacy in infectivity inhibition assay, with the shorter version (H-HR2-S) showing no significant effect, while H-HR2 reached about 40% inhibition of infectivity at the highest concentration used (500 \( \mu \)M) (Fig. 1b).
The effects of gB peptides were much less pronounced: the shorter and longer versions of B-HR1 showed minor inhibitory effects at concentrations of 500 μM and both B-HR2 and B-HR2-S peptides failed to have any effect on inhibiting HSV-1 infectivity (Fig. 1b). The inhibitory effects of gH and gB peptides were specific in that scrambled versions of these peptides exerted no inhibition (Fig. 1b).

Further experiments were carried out to identify the step in the entry process, which was inhibited by the three peptides, that showed the greatest inhibitory effect on infection as described above. These peptides were: H-HR1, H-HR2 and B-HR1. We chose a peptide concentration (500 μM) that gave between 30 and 65 % inhibition in the previous experiment, with the co-exposure treatment, and compared the effect of three different methods of exposure of the cells and/or virus to peptide (Fig. 2a). Controls included incubation with scrambled versions of the three active peptides and samples that had not been incubated with any peptide. These results are shown in Fig. 2(a), which shows that all peptides only inhibited HSV infection when present during the period of virus attachment-entry into cells. We also pre-treated the virus with peptide for 45 min at 37 °C before infection and the results showed that the peptides were also effective in inhibiting infection to various extents using this approach (Fig. 2a).

**Table 1.** Sequence of synthetic peptides derived from HR1 and HR2 regions of HSV-1 gH and gB glycoproteins

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Protein region</th>
<th>Sequence</th>
<th>Molecular mass</th>
<th>Net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-HR1</td>
<td>gH 444–479</td>
<td>TARLQEARLQHLVAE1EREGQSLALHALGYQLFV</td>
<td>4102</td>
<td>−1</td>
</tr>
<tr>
<td>H-HR1-SN</td>
<td>gH 447–463</td>
<td>LQLEARLQHLVAE1ER</td>
<td>2031</td>
<td>−1</td>
</tr>
<tr>
<td>H-HR1-SC</td>
<td>gH 468–486</td>
<td>ALHALGYQLFVLDSPAY</td>
<td>2036</td>
<td>−1</td>
</tr>
<tr>
<td>H-HR1-scrambled</td>
<td>gH 444–479</td>
<td>YQFLVLHE1RALRQUALSRLQILGREL1QELV1ELAT</td>
<td>4102</td>
<td>−1</td>
</tr>
<tr>
<td>H-HR2</td>
<td>gH 542–582</td>
<td>RARRSLIASALCISDVA1ATNLNARAD1HQRKD1FLW</td>
<td>4469</td>
<td>+3</td>
</tr>
<tr>
<td>H-HR2-S</td>
<td>gH 555–582</td>
<td>TSDVAA1ATNLNARAD1HQRKD1FLW</td>
<td>3057</td>
<td>0</td>
</tr>
<tr>
<td>H-HR2-scrambled</td>
<td>gH 542–582</td>
<td>HATC1SLAF1ALS1VALTR1ND1LL1RWAA1RD1Q1T1LSKR1D</td>
<td>4469</td>
<td>+3</td>
</tr>
<tr>
<td>B-HR1</td>
<td>gB 84–112</td>
<td>AGDN1A1VAAGH1ATLRE1RLDK1A1ENT1DN</td>
<td>3032</td>
<td>−2</td>
</tr>
<tr>
<td>B-HR1-S</td>
<td>gB 92–112</td>
<td>AGH1ATLRE1RLDK1A1ENT1DN</td>
<td>2332</td>
<td>0</td>
</tr>
<tr>
<td>B-HR1-scrambled</td>
<td>gB 84–112</td>
<td>TAAG1DAR1ANVAR1AGL1HD1L11E1TD1TER1NH</td>
<td>3032</td>
<td>−2</td>
</tr>
<tr>
<td>B-HR2</td>
<td>gB 613–631</td>
<td>VEG1QL1GEN1EL1R1LTR1DA1E</td>
<td>2156</td>
<td>−3</td>
</tr>
<tr>
<td>B-HR2-S</td>
<td>gB 618–631</td>
<td>GEN1NEL1R1TRDA1</td>
<td>1630</td>
<td>−1</td>
</tr>
<tr>
<td>B-HR2-scrambled</td>
<td>gB 613–631</td>
<td>DVREE1QL1GER1AT1GL1NL11NI</td>
<td>2156</td>
<td>−3</td>
</tr>
</tbody>
</table>

**Fig. 2.** Peptide inhibition of HSV infectivity. (a) Cells were exposed to peptides (H-HR1, H-HR2 and B-HR1) at a concentration of 500 μM either prior to infection (Cell pre-exposure), during attachment and entry (Co-exposure), after virus penetration (Post-exposure) or, alternatively, the virus was pre-incubated with peptides for 1 h at 37 °C before addition to the cells. Control included incubation with scrambled versions of the three active peptides and samples that had not been incubated with any peptide. These results are shown in Fig. 2(a), which shows that all peptides only inhibited HSV infection when present during the period of virus attachment-entry into cells. We also pre-treated the virus with peptide for 45 min at 37 °C before infection and the results showed that the peptides were also effective in inhibiting infection to various extents using this approach (Fig. 2a).
We also analysed the possible interaction between different HSV-1 gB and gH HRs by co-exposure of virus and cells with combinations of different peptides to determine whether the inhibitory effect seen with H-HR1 could be reduced in the presence of other HR regions. The rationale behind this experiment was that, if an inhibitory peptide aggregates with a peptide from another HR domain such that they create a structure that could resemble the six-helix bundle of HIV gp41, it would be less efficient at exerting an inhibitory effect on infectivity. The combinations of peptides tested and the results obtained are shown in Fig. 2(b). None of the peptides derived from gB affected the inhibitory effect exerted by H-HR1 alone. However, when H-HR2 was incubated together with H-HR1, we observed a significant reduction of the inhibitory activity to approximately 30% from values of approximately 65% inhibition. This observation lends weight to the view that HR1 and HR2 regions of HSV-1 gH may interact with each other during the fusion process.

Structural analysis of HR peptides by CD

Biophysical approaches were employed to characterize further the structure of these peptides and to determine whether any evidence of interaction could be observed.

Fig. 3 shows the individual CD spectra of all peptides in buffer solution and in 60% trifluoroethanol (TFE). The CD spectra of peptides H-HR1 and H-HR2 indicate a percentage of α-helical content in buffer of 15 and 8%, respectively. Peptides H-HR1-SN, H-HR1-SC, H-HR2-S and B-HR1, B-HR1-S, B-HR2, B-HR2-S all gave spectra indicative of random coil conformations in buffer. In order to determine their potential to form helical structures, titrations of the peptides in TFE (the spectra at 0 and 60% TFE are only shown in Fig. 3 for clarity) were also performed. These data show that all peptides, except H-HR1-SC, have a high capability to adopt a helical conformation at 60% TFE (Fig. 3).

CD spectra were collected for H-HR1 and H-HR2 at increasing peptide concentration in the range 5–50 μM in order to determine their maximum helical potential; higher concentrations could not be analysed using our experimental conditions (Fig. 4). H-HR1 presents the highest helical potential and the percentage of helix reaches a maximum at 20 μM, while at higher concentrations there was evidence of aggregation phenomena as shown by the two almost superimposable spectra at 40 and 50 μM (Fig. 4a). H-HR2 presents a much lower helical potential; the percentage of helix increases and at 50 μM it does not reach a plateau (Fig. 4b).

Further experiments (Fig. 5) were performed to determine whether structural changes resulted from peptide interactions, by measuring the CD spectra of different peptide combinations. If two peptides do not interact with each other and no structural change occurs, the theoretical (the sum of the two spectra of the non-interacting peptides) and experimental (the spectrum of the mix of the two peptides) spectra will be identical; however, if two peptides do interact, resulting in a structural change of the components, the
were compared and showed that both peptides exhibited an increased helical content in the presence of TFE. However, mixing experiments with B-HR1 and B-HR2 showed no evidence for any structural change, suggesting that gB domains do not interact with each other (Fig. 5g).

The results of the CD experiments correlate well with the results of the infectivity inhibition experiments. Those peptides (H-HR1, H-HR2 and H-HR1-SC) whose CD spectra in mixing experiments showed a change corresponding to an increase of helicity showed the highest degree of antiviral activity when tested in infectivity.

**Analysis of coil interactions by SDS-PAGE, N-PAGE and SE chromatography**

It has been previously shown by N-PAGE that peptides corresponding to the N-terminal and the C-terminal HRs of several viruses can interact with each other to form a six-helix bundle (Liu et al., 2003, 2005; Ingallinella et al., 2004). Since the migration properties of a molecule or a complex in N-PAGE depend on both the size and the net charge of the molecule or complex, this method cannot be used alone to measure exactly the molecular mass of complexes in the gel or to determine whether the single peptides are present as monomers or oligomers (Fig. 6); thus, analytical SE chromatography experiments were also performed. Using an SE column BioSEP-SEC-S2000, five molecules with different molecular sizes could be effectively separated (Fig. 7d). The plot of logarithmic molecular mass against retention time (Fig. 7d, insert) allowed the calculation of the molecular masses of single peptides and complexes.

N-PAGE and SE-HPLC results are shown in Figs 6 and 7 and were, thus, considered in conjunction. All the peptides migrated as single bands in SDS-PAGE (Fig. 6f), confirming that they were all highly pure.

H-HR1 gave three species in native gels (Fig. 6a and d) and also showed three peaks in SE-HPLC corresponding to the monomer, dimer and probably tetramer, with the major peak corresponding to the dimer (Fig. 7a). H-HR2 migrated as a single band in N-PAGE (Fig. 6a–c), while it showed two peaks, the monomer and the dimer, with a predominance of the monomer in SE-HPLC (Fig. 7a); this result, may indicate that H-HR2 is present as both a monomer and a dimer, but only the dimer can be detected by N-PAGE because the H-HR2 monomer carries a net positive-charge (+3) and may have migrated off the gel.

Peptide H-HR1-SN (from which SE-HPLC data are present mostly as a dimer or higher-order oligomers; Fig. 7c), migrated as a single band in N-PAGE (Fig. 6c) and to a similar position in the gel to H-HR2, while H-HR1-SC migrated as a single band both in N-PAGE and SE-HPLC (Figs 6b and 7b). H-HR2-S (which has a net charge of zero), migrated as single bands in SDS-PAGE (Fig. 6f), confirming that they were all highly pure.

The gB peptides were also analysed by CD spectroscopy (Fig. 3). Their spectra in buffer solution and in 60 % TFE
Fig. 5. Analysis of interactions between gH or gB peptides by CD. Mixing experiments were performed by comparing the spectrum of the two peptides mixed together at the desired concentrations (experimental spectrum; solid trace) to the sum of the individual spectra of the peptides (theoretical spectrum; dashed trace). Spectra were measured with 10 μM concentration peptide solution in 5 mM phosphate buffer pH 7.4.
detected for B-HR1, and a single species was detected for B-HR1-S; B-HR2 and B-HR2-S are not visible on the gel, probably because they are very small and present as monomers (Fig. 6d).

The migration patterns of combinations of peptides were also analysed by N-PAGE. No interaction could be observed between H-HR2-S and any of H-HR1 (Fig. 6a), H-HR1-SC (Fig. 6b) or H-HR1-SN (Fig. 6c). No interaction could be detected between the two gB HR peptides or between H-HR1 and B-HR1 (Fig. 6d). However, the combination of equimolar amounts of H-HR1/H-HR2 or H-HR1-SC/H-HR2 showed a different migration profile from that observed when these peptides were analysed individually (Fig. 6a and b). When H-HR1/H-HR2 were mixed together, the species corresponding to monomeric H-HR1 was no longer detected. In addition, a notable decrease of the intensity of the bands corresponding to higher orders oligomers of H-HR1 and a net increase of the intensity of a band close to that of the H-HR2 oligomer was seen, indicating that the complex migrates similarly to the H-HR2 oligomer. In the complex H-HR1-SC/H-HR2 mixed at equimolar concentrations, we also noted the complete disappearance of the band corresponding to monomeric H-HR1-SC and an increase of intensity of the band which migrates in a similar fashion to the H-HR2 oligomer. We performed a titration with decreasing amounts of H-HR2 (7.5, 3.75 and 1.9 nmol) and kept the concentration of H-HR1-SC (15 nmol) constant. As the concentration of H-HR2 was decreased, the intensity of the band corresponding to the H-HR1-SC monomer form increased (Fig. 6e). The disappearance of H-HR1-SC is, therefore, due to the interaction with either form of H-HR2 (monomer and oligomer) and the complex with the monomer is probably running off the gel as the H-HR2 monomer itself.

The SE-HPLC profiles of the mixture H-HR1/H-HR2 showed a great change with the appearance of several new peaks, even if the interpretation of the HPLC profile is complicated by the fact that the heterocomplex has a molecular mass...
similar to that of the homodimer. We were able to detect the formation of a complex between both H-HR1 and H-HR1-SC with H-HR2 (Fig. 7a and b). The same analysis performed on the other mixtures and on the gB peptides gave HPLC profiles that could be obtained by the simple sum of the single chromatographic profiles, indicating the absence of any heterocomplex formation between these peptides. Therefore, the two sets of data, N-PAGE and SE-HPLC, agree in supporting the notion that H-HR1/H-HR2 and H-HR1-SC/H-HR2 are capable of interacting with each other.

**DISCUSSION**

No detailed information is currently available on the fusion mechanism of herpes viruses, although a number of recent advances in the understanding of this process have been reported (Lopper & Compton, 2004; Gianni et al., 2005a; Galdiero et al., 2005). Interestingly, the proteins gH and gB of HSV-1 both contain HR regions in the sequences of their ectodomain, which clearly play a role in the fusion process. Our results suggest that the HR1 and HR2 regions of HSV-1 gH can indeed form a complex typical of other type 1 fusion
proteins and they function as inhibitors of infection, while the HR1 and HR2 regions of gB show lower or absent inhibitory activity and no evidence of complex formation, at least with the techniques used in this study, namely SE-HPLC, CD analysis and N-PAGE.

Predicted coiled-coil regions were identified in both gH and gB, and peptides of different lengths were generated and tested for their effects on virus entry. gH coiled-coil peptides inhibited HSV-1 infection in a dose-dependent, sequence-specific manner. H-HR1 showed the strongest activity and we also observed that longer coiled-coil peptides were more potent inhibitors of virus entry, probably due to increased ability to form more stable secondary structures than the shorter peptides. This observation was confirmed by CD data that indicated that H-HR1 has a significant helical content in 5 μM buffer solution, which increased upon increasing peptide concentrations or upon addition of TFE (Figs 3 and 4). Treatment of cells with peptides (H-HR1, H-HR2 and B-HR1) following incubation with virus had no effect on entry, indicating that the inhibitory peptides act at a step during virus entry and have no effect once the virus has penetrated the cell membrane. Pre-treatment of cells with peptides, followed by their removal by acid wash, also did not impair the ability of HSV-1 to enter cells, suggesting that the peptides do not irreversibly condition the cell susceptibility to the virus. Efficient inhibition was observed only when the gH or gB peptides were incubated with virus at the same time as inoculation into Vero cells, or when the virus was pre-incubated with peptides for 45 min before being inoculated into the cells. These results are consistent with previous reports (Lopper & Compton, 2004; Gianni et al., 2005b) and show that the domain with the highest probability score to form coils, namely the HR1 of gH, is the peptide which exerts the strongest inhibitory effect.

The interesting observation resulting from these studies was that not only was H-HR1 (which showed a high probability of forming coiled-coils) active in inhibiting virus entry, but H-HR2 and B-HR1 (which have a low probability score) also were able to inhibit virus entry, albeit with lower efficacy.

This finding, together with the fact that co-exposure of cells with a mix of H-HR1 and H-HR2 was able to reduce the level of inhibition obtained with H-HR1 alone, prompted us to analyse in further detail the nature of any interactions between coiled-coil sequences of both gH and gB. While most enveloped viruses need a single fusion protein and the two interacting HRs are present on the same glycoprotein, we do not know if herpes viruses, which require four different glycoproteins (Turner et al., 1998) to mediate fusion, established intramolecular contacts between different regions of the same protein or perhaps intermolecular interactions between gH and gB. The first line of evidence pointing towards an intramolecular interaction between the two HRs of gH was the reduction of the inhibitory effect when we performed infectivity assays with the mix of peptides. In fact, the only combination of peptides able to reduce the effect of H-HR1 on infection was that of H-HR1/H-HR2. No reduction was observed with any of the gB peptides. This finding can be explained by the possible sequestration of H-HR1 peptides. H-HR1 is able to inhibit infection when free in the suspension, but unable to inhibit once H-HR2 is present.

The results of biophysical analyses were consistent with the existence of an intramolecular interaction between H-HR1 and H-HR2 of gH and would suggest that the HRs of gB and gH do not interact with each other to form intermolecular interactions. However, it remains a possibility that gB and gH do interact during fusion, but that these interactions involve other domains of these glycoproteins.

It is interesting to note that, in most viral fusion models, synthetic peptides corresponding to the C-terminal HR are generally more active in inhibition assays if compared with peptides corresponding to the N-terminal HR; this is in contrast with our results on gH and gB of HSV-1 and with previous data on herpesvirus glycoproteins, which only analysed N-terminal HRs due to the higher probability of those regions to form coiled-coils. Nevertheless, our results indicate that C-terminal HRs in HSV-1 gH can also slightly inhibit fusion and can interact with the N-terminal part of the protein. The only other known case of HR1 domains being more potent than HR2 regions is that of SARS-CoV (Yuan et al., 2004).

In summary, the presence in HSV-1 gH of a putative canonical fusion peptide (Gianni et al., 2005a), several membrane interacting domains capable of fusing artificial membranes (Galdiero et al., 2005), and both an N-terminal and a C-terminal HR region potentially capable of forming coiled-coils that clearly interact with each other, demonstrates that gH plays a critical role in membrane fusion mediated by HSV-1. The finding of domains in gH and gB HR1 and HR2 supports the assumption that the HSV fusion mechanism may employ a strategy similar to other class I envelope fusion proteins. Further studies are needed to unravel the role of gB, since HR regions are present in this protein as well, and the N-terminal HR domain is also partially effective in reducing HSV-1 infectivity.

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