FUSOGENIC DOMAINS IN HERPES SIMPLEX VIRUS TYPE 1 GLYCOPROTEIN H

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Infection of eukaryotic cells by enveloped viruses requires fusion between the viral envelope and the cellular plasma or endosomal membrane. The actual merging of the two membranes is mediated by viral envelope glycoproteins which generally contain a highly hydrophobic region, named the fusion peptide. The entry of herpesviruses is mediated by three conserved glycoproteins: gB, gH and gL, however, how fusion is executed remains unknown. Herpes simplex virus type 1 (HSV-1) gH exhibits features typical of viral fusion glycoproteins and its ectodomain seems to contain a putative internal fusion peptide. Here we have identified additional internal segments able to interact with membranes and to induce membrane fusion of large unilamellar vesicles. We have applied the hydrophobicity-at-interface scale proposed by Wimley and White to identify six hydrophobic stretches within gH with a tendency to partition into the membrane interface, and four of them were able to induce membrane fusion. Experiments in which equimolar mixtures of gH peptides were used indicated that different fusogenic regions may act in a synergistic way.

The functional and structural characterization of these segments suggests that HSV-1 gH possesses several fusogenic internal peptides that could participate in the actual fusion event.

Membrane fusion occurs in a wide variety of biological processes including the infection of cells by enveloped viruses, and as a result of fusion, the viral capsids are transferred to the cytoplasm of the newly infected cells (1, 2). Energy barriers hinder spontaneous fusion between two apposing membranes, however, during viral infections, these barriers are overcome by specific viral "fusion proteins" present in the viral envelope. Despite the lack of sequence homology between fusion proteins from distantly related viruses, most of them share a common motif, termed the "fusion peptide" comprised mostly of hydrophobic residues, which is thought to interact directly with the target membrane and to trigger events that lead to membrane fusion (3, 4). At least two classes of viral fusion proteins have been identified; in both classes tightly regulated conformational changes are involved in membrane fusion (5). Class I fusion proteins are present in many unrelated virus families such as paramyxoviruses, orthomyxoviruses, retroviruses and filoviruses (6-11). Class II fusion proteins are found in members of the Flaviviridae family, which includes Tick-borne encephalitis virus (TBE) (12, 13), dengue virus (14, 15), yellow fever virus, West Nile virus, and the hepatitis C virus, and in the Togaviridae family, of which the best characterised are Semliki Forest virus and rubella virus (16-18).

Class I fusion proteins (4, 19) are composed of three identical subunits, whose functional form is generated from a precursor that is cleaved into two fragments; the membrane-anchored fragment contains at its N-terminus or proximal to the N-terminus the

fusion peptide. During fusion the three fusion peptides become exposed and are inserted into the target cell membrane, generating an intermediate that is anchored both to the cellular and viral membrane; thus, the refolding of the protein into a trimeric coiled-coil (N and C terminal heptad repeats) relocates the fusion peptides and the transmembrane anchor domains to the same end of the coiled coil bringing viral and cellular membranes together.

Class II fusion proteins do not form spiky projections perpendicular to the viral membrane, but lie parallel to the membrane and possess an internal fusion peptide (13, 15, 20). In particular, they are predominantly non helical, having a β-sheet structure, they are not cleaved, and the fusion peptide corresponds to an internal hydrophobic fusion loop. The proteins are composed of three domains and the fusion peptide is contained in domain II; they are homodimers with the two subunits arranged into a head-to-tail orientation. During fusion they undergo dramatic irreversible structural changes that result in a homotrimeric form lifting up from the virus surface and projecting the internal fusion loop towards the target membrane.

The presence of a fusion peptide is thus a feature of both Class I and Class II viral fusion proteins (21-23); these hydrophobic sequences are involved in driving the initial partitioning of the fusion protein into the target membrane, making the viral envelope glycoprotein an integral component of both viral and cellular membrane. The real interplay between fusion peptides and the membrane is still unknown; fusion peptides are believed to facilitate local dehydration, to help to overcome the energetic barriers associated with the fusion process and to serve as membrane anchors that facilitate partition of other regions of the viral envelope proteins to the membrane, which can subsequently participate in membrane merging. Fusion peptides are hydrophobic stretches of 10-30 amino acid residues, typically rich in alanine and glycine residues that may form sided helices, with bulkier and more hydrophobic residues on one side associating with the membrane and small residues on the other. Although there is conflicting evidence on the active secondary structures of fusion peptides, several studies have shown that fusion peptides can flip between different conformations depending upon their environment. Since membrane fusion is a dynamic process, structural plasticity appears to be crucial for the

fusion process and the orientation of the fusion peptide within lipids is also important (24, 25). Although it was initially thought that viral fusion glycoproteins contained a single fusogenic region responsible for the actual merging of the membranes, over the last few years a more complex view has emerged (26). This is illustrated by recent studies on Sendai virus (27, 28) in addition to the N and C terminal heptad repeats, similar to those found in Class I fusion proteins, an extra leucine zipper is located in the interior of the fusion protein, this leucine zipper together with the N-terminal fusion peptide is involved in membrane fusion. The importance of the second fusion peptide in Sendai virus induced membrane fusion was highlighted by the finding that a synthetic peptide corresponding to the internal fusion peptide inhibits the fusion between the virus and red blood cells (27). Moreover, the two heptad repeats do not induce membrane fusion by themselves, but the N-terminal heptad repeat was shown to assist the N-terminal fusion peptide in the fusion process.

Thus, the actual fusion step of Sendai virus is not mediated solely by the action of an N terminal fusion peptide but rather is the consequence of the concerted action of several regions of the fusion glycoprotein.

Studies using synthetic peptides, which correspond to the sequences of viral fusion peptides, have in many cases been valuable for shedding light on the molecular mechanisms involved in viral mediated fusion. Moreover, synthetic peptides appear to be the most useful for studies on the minimum and precise molecular and structural requirements for membrane destabilization, and represent an alternative method which is not hampered by the difficulties of analysing mutations which could affect the overall folding of the protein. A possible way to elucidate the molecular mechanism of membrane fusion is to study the interaction of synthetic peptides corresponding to specific domains of viral fusion proteins with model membranes. In fact, synthetic peptides corresponding to the N-terminal fusion peptide of several viruses, including influenza virus (29), SIV (30) and HIV-1 (24, 25, 31-33), have been shown to interact with the lipid bilayer and to promote fusion of lipid vesicles.

Herpesviruses are structurally complex enveloped viruses which have at least a dozen glycoproteins on their surface. Unlike orthomyxoviruses, paramyxoviruses, filoviruses, and retroviruses that use a single glycoprotein

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for membrane fusion, herpesviruses employ multicomponent membrane fusion machines that comprise at least three proteins, glycoprotein B (gB), glycoprotein H (gH), and glycoprotein L (gL), all of which are conserved in all members of the Herpesviridae. In addition to this core requirement for fusion (as exemplified by a number of reports on different members of the herpesvirus family (34, 35), other glycoproteins may also be involved. For example, HSV-1 mediated fusion requires gB, gH, gL and gD (36-38), whereas fusion of the gamma-herpesvirus, EBV, is mediated by gB, gH, gL and gp42 (39-41).

While much progress has been made in understanding how membrane fusion is promoted by single-component fusion proteins, little is known on how multiple components mediate fusion. Although it seems likely that multiple component fusion machines require cooperation among the fusion proteins, it remains unclear if and how herpesvirus glycoproteins interact with one another, and the molecular details of the fusion process are not understood.

None of the required four HSV-1 glycoproteins has yet been found to possess all the characteristics of Class I or Class II fusion proteins. Nevertheless, a number of studies have implicated key roles for both gH and gB in the fusion process (42-44). Mutations in the extracellular, transmembrane and cytoplasmic domains of HSV-1 gH have been shown to influence membrane fusion (45, 46) as have mutations in the cytoplasmic domain of gB. Furthermore, a recent study (47) identified a potential α-helical region in the ectodomain of HSV gH which showed some attributes of an internal fusion peptide. Since it is now becoming increasingly clear that the fusion mechanism employed by some viruses involves more than simply the interaction of an N-terminal fusion peptide with membranes, we attempted to identify likely regions of HSV-1 gH which may have this property. We therefore screened the amino acid sequence of gH for regions of highly interfacial hydrophobicity which show homology with other known virus fusion peptides, and synthetic peptides corresponding to those regions were tested for their ability to induce the fusion of large unilamellar vesicles.

Materials and Methods

Materials - Fluorenylmethoxycarbonyl (Fmoc) protected amino acids were purchased from INBIOS (Pozzuoli, NA, Italy), NovaSyn TGA resin from Nova Biochem (Darmstadt, Germany). The reagents (piperidine, pyridine) for the solid phase peptide synthesis were purchased from Fluka (Sigma-Aldrich, Milano, Italy), trifluoroacetic acid (TFA) and acetic anhydride were from Applied Biosystem (Foster City, CA, USA). H_2O, DMF and CH_3CN were supplied by LAB-SCAN (Dublin, Ireland). Egg phosphatidylcholine (PC), egg phosphatidilethanolamine (PE), phosphatidylserine (PS), egg sphingomyelin (SM), cholesterol (Chol), lysophosphatydilcholine (LC) and the fluorescent probes N-(7-nitro-benz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and N-(Lissamine-rhodamine-B-sulfonyl)phosphatidylethanolamine (N-Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, Ala). Triton-X100 was obtained from Sigma (St. Louis, Mo.). All other reagents were of analytical grade.

Proteomics computational methods - Domains with significant propensity to form transmembrane (TM) helices were identified with Tmpred (ExPaSy, Swiss Institute of Bioinformatics) and Membrane Protein eXplorer (MpeX, Stephen White laboratory, http://blanco.biomol.uci.edu/mpex). Tmpred is based on a statistical analysis of Tmbase, a database of naturally occurring TM glycoproteins (48), while MpeX detection of membrane spanning sequences is based on experimentally determined hydrophobicity scales (49, 50). Sequences with a propensity to partition into the lipid bilayer were also identified with MpeX using interfacial settings, with mean values for a window of 11 amino acids. In particular, hydrophaty plots corresponding to the pretrasmembrane domains were obtained using the Kyte-Doolittle (51) (KD) hydrophathy index and the Wimley-White (49) (WW) interfacial hydrophobicity scales for individual residues. Secondary structure predictions were performed using Antheprot software. Allignments were performed using Blast (52) and ClustalW (53). The gH sequence used was taken from SWISS-Prot database, with accession number P08356.

Peptide synthesis - Peptides were synthesised using standard solid-phase-9-fluorenylmethoxycarbonyl (Fmoc) method, on a PSSM8 multispecific peptide synthesiser (Shimadzu Corporation Biotechnology Instruments Department Kyoto JAPAN). The NovaSyn TGA resin (substitution 0.25 mmol/g) was used as the solid-phase support, and

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syntheses were performed on a scale of 100 μmol.

The first amino acid was coupled with 10 equiv. relative to resin loading according to the DIC (N,N'-Dicyclohexylcarbodiimide)/DMAP (4-Dimethylaminopyridine) method: 10 equiv. of Fmoc-amino acid, 5 equiv. of DIC and 0.1 equiv. of DMAP. All successive amino acids, 4 equiv. relative to resin loading, were coupled according to the HOBT (N-hydroxybenzotriazole) /HBTU (2-(1H-Benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate /DIPEA (Di-iso-propylethylamine) method: 1 equiv. of Fmoc-amino acid, 1 equiv. of HBTU, 1 equiv. of HOBT (0.5 mM HOBT in NMP) and 2 equiv. of DIPEA (1 mM DIPEA in NMP). The Fmoc protecting group was removed with 30% piperidine in NMP (v/v).

Peptides were fully deprotected and cleaved from the resin with trifluoroacetic acid (TFA) with 5% thioanisole, 3 % ethandithiol and 2% anisole as scavengers; the crude peptides were precipitated with ice-cold ethyl ether, filtered, dissolved in water and lyophilised. The crude peptides were purified to homogeneity by preparative reverse-phase high-pressure liquid chromatography (HPLC) on a Waters Delta Prep 3000 chromatographic system, equipped with an UV Lambda Max Mod. 481 detector. The samples were injected on a Vydac (The Separation Group, Hesperia, CA) C18 column (22 mm x 25 cm, 5 μm) eluted with a H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) solvent mixture. A linear gradient from 20 to 70% of B over 30 min at a flow rate of 20 mL/min was employed. The collected fractions were lyophilised to dryness and analysed by analytical reverse-phase HPLC on a Shimadzu class-LC10 equipped with a diode array detector SPD-M10AV using a Phenomenex C18 analytical column (4.6 x 250 mm, 5μm); a linear gradient from 20 to 70% of B over 30 min at a flow rate of 1 mL/min was used. The identity of purified peptides was confirmed by Maldi spectrometry.

Liposome preparation - Large unilamellar vesicles (LUV) consisting of PC, PC/Chol (1:1), PC/PE (1:1), PC/PS/Chol (1:1:1) and PC/PE/SM/Chol (1:1:1:3) and when necessary with different amounts of Rho-PE and NBD-PE, were prepared according to the extrusion method of Hope *et al.* (54) in 5mM HEPES, 100mM NaCl, pH 7.4. Briefly, lipids were dried from chloroform solution with a nitrogen gas stream and liophilized overnight. For fluorescence experiments, dry lipid films were suspended in buffer by vortexing to produce large unilamellar vesicles. The lipid suspension was freeze-thawed 8 times and then extruded 20 times through polycarbonate membranes with 0.1 μm diameter pores. Lipid concentrations of liposome suspensions were determined by phosphate analysis (55). Small unilamellar vesicles (SUV) of lysophosphatydilcholine were prepared from LUVs by sonication.

Lipid mixing assay - Membrane lipid mixing was monitored using the resonance energy transfer assay (RET) reported by Struck *et al.* (56). The assay is based on the dilution of the NBD-PE (donor) and Rho-PE (acceptor) groups. Dilution due to membrane mixing results in an increase in NBD-PE fluorescence. Thus, we monitored the change in donor emission as aliquots of peptides were added to vesicles. Vesicles containing 0.6 mol % of each probe were mixed with unlabelled vesicles at a 1:4 ratio (final lipid concentration, 0.1 mM). Small volumes of peptides in dimethylsulfoxide (DMSO) were added; the final concentration of DMSO was no higher than 2%. The NBD emission at 530 nm was followed with the excitation wavelength set at 465 nm. A cut off filter at 515 nm was used between the sample and the emission monochromator to avoid scattering interferences. The fluorescence scale was calibrated such that the zero level corresponded to the initial residual fluorescence of the labelled vesicles and the 100% value corresponding to complete mixing of all lipids in the system was set by the fluorescence intensity of vesicles upon the addition of Triton X-100 (0.05% v/v) at the same total lipid concentrations of the fusion assay. All fluorescence measurements were conducted in 5 different sets of LUVs: PC, PC/Chol (1:1), PC/PE (1:1), PC/PS/Chol (1:1:1) and PC/PE/SM/Chol (1:1:1:3). Lipid mixing experiments were repeated at least three times and results were averaged. Control experiments were performed using scrambled peptides and DMSO.

Triptophan fluorescence measurements - Emission spectra of the peptides containing at least one tryptophan residue (gH220-226, gH579-597 and gH626-644) in the absence or presence of target vesicles (PC/Chol = 1:1) were recorded between 300 and 400 nm with an excitation wavelength of 295 nm. Trp fluorescence measurements were done in the absence and presence of iodide, which acts as an aqueous collisional quencher.

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Circular Dichroism measurements - CD spectra were recorded using a Jasco J-715 spectropolarimeter in a 1.0 cm quartz cell at room temperature. The spectra are an average of 3 consecutive scans from 260 to 195 nm , recorded with a band width of 3 nm, a time constant of 16 second and a scan rate of 10 nm/min. Spectra were recorded and corrected for the blank. Mean residues ellipticities (MRE) were calculated using the equation Obsd/lcn where obsd is the ellipticities measured in millidegrees, l is the length of the cell in centimetres, c is the peptide concentration in moles per litre, and n is the number of amino acid residues in the peptide. The percentage of helix was calculated from measurements of their mean residue ellipticity at 222 nm (57). We used $[\vartheta]_{222}$ values of 0 and $-40.000(1-2.5/n)$ deg cm^2 dmol^{-1} per amino acid residue for 0% and 100% helicity; n is the number of amino acid residues. Solutions of peptides (0.2 μM) were prepared in water and at various percentages of TFE. Peptide samples in SDS were prepared using the following protocol (58): all peptides were first dissolved in TFE; immediately after preparation, the peptide solution was added to an equal volume of an aqueous solution containing the appropriate SDS concentration, and water was added to yield a 16:1 ratio of water to TFE by volume; The samples were vortexed and lyophilized overnight; the dry samples were rehydrated with deionised water to yield a final SDS concentration of 3mM, 6mM and 10mM. Peptide samples in lipids were prepared using the following protocol (59): all peptides were first dissolved in TFE; immediately after preparation, the peptide solution was added to an equal volume of a chloroform solution containing the appropriate lipid concentration; Solutions were dried with a nitrogen gas stream and lyophilized overnight; The dry samples were rehydrated with deionized water to yield a final lipid concentration of 0.05mM and 0.9mM. Small unilamellar vesicles (SUV) of lysophosphatydilcholine (LC) were prepared from LUVs by sonication.

Results

Interfacial hydrophobicity analysis, helical propensity and identification of peptide sequences - In order to identify hydrophobic stretches in the sequence of gH with the potential to interact with target membranes, we applied the hydrophobicity-at-interface scale proposed by Wimley and White (49). The hydrophobicity-at-interface scale together with the Kyte and Doolittle analysis (an estimate of hydrophobicity based on bulk phase partitioning of side chain hydrophobicity alone) have been successfully used to detect putative regions involved in partitioning within sequences of several fusogenic viral proteins (11). As shown in Fig. 1A, the combined use of both plots reveals several interesting features in the sequence of gH. The first peak at the N-terminus corresponds to the signal peptide sequence. Then we identified two hydrophobic regions corresponding to residues gH220-262 and gH381-420, which seem to combine an overall hydrophobic character arising from side chains (Kyte-Doolittle scale) with a high ability to partition at interface. Toward the C-terminus four further significant peaks corresponding to gH468-486, gH493-537, gH579-597 and gH626-644 were detected, even though they show a lower tendency to partition at the interface compared to gH220-262 and gH381-420. Finally, at the C terminus we identified a large peak corresponding to the pre-transmembrane and the transmembrane regions of the glycoprotein.

We also screened the extracellular domain of gH for regions with homology to known fusion peptides. We selected a set of well characterized fusion peptides from Class I fusion proteins of different viruses (orthomyxoviruses, paramyxoviruses and retroviruses) and aligned them with the sequence of HSV-1 gH. As shown in Fig. 2A, we identified two sequences gH402-420 and gH495-513 which showed some similarity to other fusion peptides. Both sequences were contained in peptides found from the analysis of the hydrophobicity at interface plots, namely gH381-420 and gH493-537, indicating that both our strategies are useful to identify membrane interacting regions. Moreover we identified the sequence gH626-644 which aligns with the N-coil of HIV-1 gp41.

When peptides gH579-597 and gH626-644 are in the helical conformation (Fig. 2B), the polar residues are distributed on both sides of the helices, but they concentrate in the portion closer to the C-terminal end, resulting in an extremely hydrophobic N-terminus and a polar C-terminus, giving to the peptides an amphiphilic character. A predominantly helical conformation and an amphiphilic nature are common characteristics of fusion peptides in most fusion glycoproteins of enveloped viruses.

Lipid mixing assay - The fusogenic activity of the peptides was determined by their

ability to cause lipid mixing of large unilamellar vesicles (LUV) composed of PC/PS/Chol (1:1:1), PC/PE (1:1), PC/Chol (1:1), PC and PC/PE/SM/Chol (1:1:1:3). Briefly, a population of LUV labelled with both NBD-PE and Rho-PE was mixed with a population of unlabelled LUV in the presence of increasing concentrations of peptides. Fusion between the labelled and unlabeled vesicles caused by the peptides results in dilution of the labelled lipids and therefore reduced energy transfer between NBD-PE and Rho-PE. This change can be visualised as an increase of NBD fluorescence.

The dependence of both the extent and kinetics of lipid mixing on the peptide to lipid molar ratio were analysed. In separate experiments, increasing amounts of gH220-262, gH381-420, gH468-486, gH493-537, gH579-597 and gH626-644 were added to fixed amounts of vesicles and the percentage of lipid mixing as a function of the peptide to lipid molar ratio was calculated. Fig. 3A shows the results of lipid mixing assays using PC/Chol containing vesicles. gH468-486 is unable to induce lipid mixing under these conditions. However, we observed significant vesicle fusion in the presence of gH220-262, 381-420, 579-597 and 626-644. This latter peptide was the most effective at inducing lipid mixing. Scrambled peptides were also tested in this assay and no fusion was observed. gH626-644 was able to induce lipid mixing at lower peptide-to-lipid ratios than the other peptides we tested, and we detected fusion with this peptide, at a peptide-to-lipid ratio of 0.4, of approximately 50%; at the same peptide-to-lipid ratio the other peptides reached approximately 20% of lipid mixing.

Some viruses show a specific requirement for particular lipids in the target membrane, so to determine whether this is also the case for HSV-1 we generated liposomes of different lipid compositions and measured the ability of the gH peptides to induce lipid mixing. The results are shown in Fig. 4. We found a common trend for the six peptides we tested, and noted a significant increase in fusion of liposomes containing cholesterol. In particular, fusion was highly efficient with liposomes containing phosphatidylcholine and cholesterol in a 1:1 ratio, but in the absence of cholesterol, although fusion still occurred, it was much less efficient and all the peptides analysed were less effective in inducing fusion. Inclusion of cholesterol in the target membrane, although not absolutely

essential, appears to facilitate fusion of HSV-1 virus .

All the peptides show the highest percentage of fusion in PC/Chol except for gH493-537, which seems to fuse better in PC/PE and PC/PE/SM/Chol. The outer leaflet of human cellular membranes is composed mainly of PC, PE, SM and Chol, therefore the testing of peptide-induced fusion of PC/PE/SM/Chol liposomes is more relevant to the physiology of viral infection of human cells. The percentages of lipid mixing obtained for all the peptides are comparable with those obtained with the other liposome preparations except for PC that is always supporting the lowest lipid mixing percentage. gH626-644 shows the highest percentage of lipid mixing in PC/PE/SM/Chol and in PC/Chol.

Assuming that all the sequences would be present at an equimolecular ratio within a potential fusogenic complex during gH mediated fusion, we also analised the membrane fusion abilities of combinations of the most active peptides (Fig. 3B). gH220-262, gH381-420, gH493-537 and gH626-644 were combined together at equimolar amounts and following incubation with liposomes, a clear synergistic effect was observed. In fact, low doses ([Peptide]/[Lipid]=0.2) that alone induce up to 5-15% of fusion of liposomes, caused an approximatively 75% of fusion. We performed this experiment first with the three most active peptides gH220-262, gH381-420, and gH626-644 and we obtained a percentage of fusion of 53% at a peptide/lipid ratio of 0.2. When gH579-597 was added, there was no significant increase of fusion, while the addition of gH493-537 induced an increase of fusion up to 75%. Although the results of fusion experiments performed using single peptides indicated that gH579-597 induced a higher degree of fusion compared to gH493-537, the experiments performed with the combinations showed that gH493-537 is more effective when these peptides are used together to induce fusion. This result may be correlated to the higher conformational flexibility of gH493-537, which, as shown by CD, was able to assume different conformations in different environments.

Tryptophan fluorescence emission − Since the results of the lipid mixing experiments strongly suggested that the gH peptides we have identified can interact with membranes, we compared the fluorescence emission spectra of gH220-226, gH579-597 and gH626-644 in the presence of PC/Chol vesicles with that in PBS.

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The fluorescence emission of tryptophan residues increases when the amino acid enters a more hydrophobic environment, and together with an increase in quantum yield, the maximal spectral position will be shifted toward shorter wavelengths (blue shift). Fig. 5 shows the fluorescence emission spectra of the peptides gH220-262, gH579-597 and gH626-644 upon interaction with PC/Chol vesicles. In all cases changes in the spectral properties of the three peptides were observed, suggesting that the single tryptophan residue of peptides gH579-597 and gH626-644, and the three tryptophans of peptide gH220-262 are located in a less polar environment upon interaction with lipids. Emission intensity was enhanced and the maxima shifted to lower wavelength. These results suggest that all 3 peptides are capable of penetrating a lipid bilayer. We observed that the levels of trp fluorescence of both peptides in solution decreased with time (data not shown), an effect that may be related to self-aggregation due to their hydrophobic character. This effect may compete with membrane association and therefore precludes correct determination of real partition coefficients.

Secondary structure of synthetic peptides - Since structural conformations have been shown to be important for the fusogenic activity of fusion peptides, the secondary structure of peptides in aqueous solution and in membranes was analysed by CD spectroscopy, as measured in water, TFE, SDS and in the presence of lysophosphatydilcholine and lysophosphatydilethanolamine SUV.

Under all conditions tested, the spectra were not reliable below 200 nm because of light-scattering, and therefore are not shown.

The CD spectrum in buffer solution indicated a random coil conformation for the peptide gH626-644. A decrease in peptide environmental polarity occurs when the peptide is transferred from water to membrane interfaces; the effect of polarity on peptide conformation can be studied using aqueous mixtures of TFE (Fig. 6). In the presence of 20%TFE, the peptide gH626-644 already showed two negative bands at about 208 and 222nm, suggesting the adoption of an α-helical conformation; calculations of helix content according to (57) corresponded to a percentage of helix of 27%; increasing concentrations of TFE induced a further helical stabilization (approximatively 35%). This result suggests that

the peptide contains a particular sequence of amino acids that in a non-polar environment can adopt an α-helical conformation. Peptides gH220-262, gH381-420 and gH493-537 show spectra in buffer that indicated the presence of an extended structure with a minimum at approximatively 218 nm. For the three peptides the same β-type conformation is found in 20%TFE; however, spectra were consistent with peptides becoming more structured in low-polarity solvent; in fact increasing amounts of TFE induced stabilization of α-helical structures, with 27% helical content for gH220-262, 57% helical content for gH381-420 and 31% helical content for gH493-537 at 80%TFE. Thus, CD analysis for gH220-262, gH381-420 and gH493-537 would be consistent with the presence of β-sheet structures in buffer that are preserved in low polarity environments. Peptides gH468-486 and gH579-597 showed a spectra typical of a random coil conformation with no considerable increase of α-helical content upon addition of TFE.

At low percentages of TFE a β-form can exist if a segment has the β-forming potential, but excess TFE usually disrupts the β-form and may convert it into a helix if the segment also has the helix-forming potential. As shown in Fig. 6, from our analysis we obtained that peptides gH220-262, gH381-420 and gH493-537 show a strong potential to give β-structures at low percentages of TFE while at higher percentages they convert to α-helices. Only peptide gH626-644 shows a strong potential to give α-helices also at low percentages of TFE.

Therefore it is possible that the secondary structures of peptides gH220-262, gH381-420, gH493-537 and gH626-644 change from a random coil to an α-helix upon membrane binding. To determine whether this is the case, CD spectra of the four peptides in the presence and absence of liposomes and in the presence or absence of SDS were determined. As shown in Fig. 7 peptide gH220-262 is a random coil in buffer solution, as well as in SDS 3mM and 10 mM; however when the peptide was incubated in the presence of LC liposomes, at high lipid/peptide ratios the α-helical content slightly increased. Peptide gH381-420, shows a high helical content (approximately 32%) in SDS 3mM and 10mM as well as in LC liposomes at the highest lipid/peptide ratio tested in our experiments. Peptide gH493-537 shows a high helical content (approximately 28%) but lower

percentages were observed at lower lipid/peptide ratios. Peptide gH626-644 shows the highest helical content in LC (approximatively 38%).

Discussion

Viral fusion proteins interact simultaneously with two membranes during the fusion process; the fusion peptide, a conserved stretch of hydrophobic amino acids, inserts into the target membrane and triggers fusion (2). Hydropathy analysis based on the hydrophobicity-at-interface scale proposed by Wimley and White enabled us to detect six regions of HSV gH that may be involved in the interaction of the virus envelope and host cell membranes. The Wimley-White hydrophathy analysis has been reported to be superior to other methodologies for detecting potential membrane interacting sequences within viral fusion protein ectodomains (11); the main advantage being that it takes into account the effect of membrane interface on partitioning. The interface is composed of a complex mixture of water and chemically heterogeneous phospholipids groups in which significant changes in polarity occur at short range; thus, aromatic residues appear to be the most hydrophobic ones when located at interfaces. According to our results the sequences gH220-262 and gH381-420 showed the highest probability to localize at the bilayer interface; but the other four gH peptides identified also show a certain hydrophobicity that is compatible with membrane interacting sequences.

Here, we have demonstrated that sequences gH220-262, gH381-420, gH579-597 and gH626-644 of HSV-1 gH are able to induce rapid membrane fusion and to act in a synergistic way. Furthermore, these peptides showed characteristics of membrane interacting regions as measured by analysis of tryptophan fluorescence emission analysis and by their tendency to assume particular secondary structures by CD.

Liposomes have been widely used as model systems to understand the molecular mechanism of viral membrane fusion (60, 61). In particular, membrane fusion can be divided in three steps: 1) the two membranes that are supposed to fuse must approach each other closely; 2) there is a transient disruption of stable bilayer structures; 3) the two membranes must mix their components and fuse into one membrane. One widely accepted view is that the fusion peptide is responsible for the fusion of the two membranes inserting into the target membrane and somehow destabilizing the lipid bilayer. It has been recently reported (26-28) that other domains of the fusion glycoproteins are involved in the process and these domains do not insert into the bilayer, but may interact closely with the target membrane thereby promoting the formation of fusion intermediates.

Although much information on the correlation of the fusogenic activity of viral synthetic fusion peptides with peptide secondary structure is available, detailed studies on the nature of specific interactions of fusion peptides with different phospholipids vesicles are still lacking. An interesting feature of membrane fusion is its requirement for specific lipids in the target membrane (62). The zwitterionic phospholipids, mainly PC and PE together comprise the majority of the membrane phospholipids of eukaryotic cells, gram-negative bacteria and many gram-positive bacteria (62). PC and PE have been treated as interchangeable in many experimental designs. Due to the more desirable physical properties of PC in forming vesicles and defined structures in solution, PC has been preferentially used in many in vitro studies. However, there are significant and important differences in chemistry and properties between these two lipids, and it is now clear that they are not functionally exchangeable in supporting biological processes. Therefore, experimental design involving lipids must be governed by the lipid specificity of the system.

Cholesterol is almost entirely non polar, with a single OH group attached to the ring system; thus, when incorporated into the bilayer this very small head group is not enough to shield the non polar part of cholesterol from interfacial water, so cholesterol must use the head groups of the adjacent lipids as umbrellas to shield itself from interfacial water. The main consequence of this is that it enhances cholesterol-lipid lateral interactions and precludes cholesterol-cholesterol interactions within the bilayer, which would increase cholesterol exposure to water. PE has a smaller head group cross-sectional are than PC and is able to accommodate only one cholesterol molecule per PE, whereas PC can accommodate two molecules of cholesterol per PC. It is interesting that the outer leaflet of the plasma membranes of most mammalian cells contains a significant fraction of glycosphingolipids where the head group cross sectional area could be larger than a phospholipids, thus potentially

providing an umbrella not only over a single cholesterol molecule but also over cholesterol multimers (63).

One feature of viral fusion mechanism is the varying requirement for specific lipids in the target cell membrane. For example, for SIV and HIV fusion peptides lipid mixing occurs provided there is PE in the lipid bilayer (32, 64); VSV prefers PS (65) and influenza and Sendai peptides interact preferentially with PC membranes (27, 29, 66). Cholesterol is a major component of mammalian membranes in vivo, and several studies have focussed on the effect on fusion of incorporating cholesterol into liposomes. Some studies report that inclusion of cholesterol reduces fusion, while other report increased activity (67). The increased activity is often coupled to the inclusion also of other lipid molecules, especially sphingomyelin (SM). It has been proposed that the inclusion of cholesterol and sphingomyelin into artificial membranes may mimic the formation of lipid rafts.

Studies with several viruses suggest that plasma membrane microdomains, or lipid rafts, that are highly enriched in cholesterol and sphingolipids play a crucial role in virus entry. Bender *et al.* (68) reported that lipid rafts serve as a platform for HSV entry and cell signalling, in fact, treatment of virions with a cholesterol sequestering drug inhibited entry, therefore the presence of cholesterol could be a necessary aspect of the membrane to fuse (68). Our results further support this model; in fact the highest levels of fusion for all the peptides were obtained using vesicles containing cholesterol.

Although all the peptides gH 220-262, gH381-420, gH468-486, gH493-537, gH579-597, gH626-644 promote fusion of liposomes of different composition, the varied level of fusion observed for the various liposomes, may suggest a different mode of interaction. This possibility may result in a different structure for the peptide vesicle complex. The best results were obtained in PC/Chol for all the peptides except gH493-537 and our experiments showed that omission of cholesterol from liposome preparations strongly reduced the extent of lipid mixing. Liposome fusion experiments showed that peptide gH468-486 is unable to induce lipid mixing; peptide gH493-537 is able to induce only low percentages of lipid mixing while the highest levels of fusion were obtained using gH220-262, gH381-420, gH579-597 and gH626-644.

We investigated the binding of peptides gH220-262, gH579-597 and gH626-644 to phospholipid vesicles containing PC/Chol using fluorescence techniques. The trp residue of this three peptides provides a suitable chromophore which can be used to monitor the lipid-peptide interactions. The fluorescence spectra show that the tryptophan residues would be located in a less polar environment upon interaction with the lipids, on the basis of the blue shift observed in the tryptophan fluorescence emission maximum and the quantum yields.

A decrease in peptide environmental polarity occurs when the peptide is transferred from water to membrane interfaces resulting in an increased α-helical content. Thus, the effect of polarity on peptide conformation was studied by using TFE, SDS and vesicles composed of lysophosphatydilcholine. CD studies showed that the peptides gH220-262, gH381-420, gH493-537 and gH626-644 are able to adopt different conformations when challenged in different environments i.e. TFE, aqueous solution, SDS and phospholipid vesicles.

Beyond the ability to interact with lipid membranes, one of the most important properties of fusion peptides is their flexibility as well as their capability to adopt different secondary structures (64, 69-72). There is good evidence (71) that influenza HA adopts random coil, α-helical and β-sheet conformations in different environments; it may be unstructured in solution on its way to the target membrane; the helical form may prevail at low concentrations in membranes but self-associated β-sheets may be induced at higher concentrations in the membrane interfaces. Analogous conformational transitions have been observed with the fusion peptide of HIV gp41 (64). Since fusion is an extremely rapid and multi-step process, structural flexibility, rather than the rigid adoption of a particular secondary structure may be a key property of fusion peptides. It is possible that a critical feature of fusion peptides is their ability to flip between different secondary structures extremely rapidly, rather than the adoption of any single, well-defined secondary structure. In most fusion peptide studies to date, the fusion peptides appear to have considerable structural plasticity and can exist as either α-helices or β-strands (64, 69-72).

Thus, as reported in the literature (24, 25) for other fusion peptides, the gH peptides we have examined seem to change from an α-helical structure at high lipid/peptide ratios towards a β-strand conformation at low

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lipid/peptide ratios and the same behaviour is observed when moving from high to low percentages of TFE. In particular, at low percentages of TFE we find β-structures for the three peptides gH220-262, gH381-420 and gH493-537 while in 20% TFE they convert to α-helices.

As holds for influenza virus (29), the fusion activity can be modulated by the action of distinct target membrane lipids, suggesting that distinct lipids in target membranes can act as a reversible secondary switch for fusion peptides, providing an elegant means for regulating membrane fusion activity. Peptide gH381-420 partially corresponds to the peptide that has recently been shown (47) to possess some features of an internal fusion peptide in HSV gH; it is indeed able to induce a considerable level of fusion in model membranes and exhibits a highly conformational flexibility forming both α- and β-structures according to different environmental conditions; gH381-420 may correspond to the canonical fusion peptide as also shown from alignments with the fusion peptides of several other viruses (Fig. 1B). Peptide gH626-644 is characterised by a strong propensity to form helices in spite of the conditions used but is the peptide that induced the highest level of liposomal fusion. It is possible that the two regions have to cooperate in order to induce complete fusion. Peptide gH468-486, unable to give any defined structure, is not involved in forming a fusion pore, while the remaining hydrophobic peptides may be involved to a lesser extent, mainly by interfering either in the early stages of fusion pore formation or at a later stage in stabilising the fusion pore. It is of interest that peptide gH468-486 overlaps a domain of gH which has recently been shown to possess the characteristics of a heptad repeat sequence (HR-1), and which was effective at inhibiting viral fusion at a concentration of 500μM (73). gH468-486 is also able to reduce viral infectivity (preliminary unpublished data from our laboratory), supporting the view that this hydrophobic sequence of gH may be directly involved in membrane fusion.

The biophysical observation that peptides derived from gH are capable of interacting with membranes is also consistent with mutagenesis studies since partial deletions of gH381-420, or substitutions (L382P and GLL384WPP) result in decreased fusion activity (47). A previous report (45) characterised a large set of linker insertion mutants of HSV gH and noted that insertions at some sites (ie amino acids 226, 381 and 591) resulted in gross misfolding of the protein and were defective in cell fusion and infectivity complementation activity. Nevertheless, further mutagenesis studies will be required to better define key residues of gH which are involved in membrane destabilisation.

In this study, we have also demonstrated that the combined use of the most effective peptides has a cooperative effect, achieving maximal fusion activity when they are used together, even at low concentration.

We have demonstrated that regions of HSV-1 gH can induce membrane fusion and that gH appears to contain several membrane interacting segments that may play a role in the fusion process. In particular, we propose that gH381-420 may be the canonical fusion peptide, gH626-644 also plays a fundamental role in the fusion process, while the others gH220-262, gH493-537 and gH579-597 may interact with the membrane in later stages of the fusion process. Our results lend some support to a model of fusion by which several domains of the fusion glycoprotein have a high tendency to locate at the interface between the viral and the target membranes. As recently reported by Gianni *et al.* (47), HSV gH presents a fusion peptide exposed on a fusion loop, made by cysteines 2 and 4 (74), rather than at the N-terminus, but it seems that several different fusogenic domains are needed to execute complete fusion.

These results may shed some light on the complex mechanism of HSV entry into host cells. This mechanism involves other viral and cellular components, and considering the virion structural complexity, it remains plausible that gH is not the only glycoprotein to contain a fusion peptide.

Our findings do not demonstrate that gH behaves like a Class I or a Class II fusion protein. In fact, the real mechanism could be somewhere in between the two canonical classes and it is likely also that gB is directly involved in the fusion mechanism by direct insertion in the membrane bilayer. The main conclusion emerging from our studies is that the interaction of gD with one of its receptors may induce conformational modifications of gH leading to the exposure of several regions of the glycoprotein that interact with target membranes, and subsequent virus entry. Further analysis of these regions of gH, both in biochemical assays and in the context of virus infection will be of value in determining in more

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detail the mechanism of HSV-1 mediated membrane fusion.

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Figure Legends

<u>Fig1.</u>   A) Hydrophobicity plots corresponding to the sequence of the gH glycoprotein from HSV-1. The plots were elaborated using the Kyte-Doolittle hydrophaty index (in pink) and the Wimley-White (in blue) interfacial hydrophobicity scales for individual residues B) linear representation of the HSV-1 gH protein. The position of the selected peptides is indicated.

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Fig2. A) Allignments of selected peptides with the N-coil of HIV gp41 and with the fusion peptides of Class I fusion proteins. In bold are reported residues that are similar, polar, aromatic, hydrophobic, charged; residues underlined are conserved. B) Helical wheel projection of gH626-644 and gH579-597.

Fig3. Peptide-promoted membrane fusion of PC/Chol (1:1) LUV as determined by lipid mixing; peptide aliquots were added to 0.1mM LUV, containing 0.6% NBD and 0.6% Rho. The increase in the fluorescence was measured 15 min after the addition of peptide aliquots; reduced Triton-X-100 (0.05% v/v) was referred to as 100% of fusion. A) Dose dependence of lipid mixing; B) combinations of peptides.

Fig4 Peptide-promoted membrane fusion of LUVs composed of PC, PC/PE, PC/Chol, PC/PS/Chol and PC/PE/SM/Chol as determined by lipid mixing. Dose dependence of lipid mixing; peptide aliquots were added to LUV 0.1mM, containing 0.6% NBD and 0.6% Rho. The increase in the fluorescence was measured 15 min after the addition of peptide aliquots; reduced Triton-X-100 (0.05% v/v) was referred to as 100%.

Fig5 Tryptophan fluorescence upon binding of peptides gH220-262, gH579-597 and gH626-644 to phospholipid. Excitation was set at 295 nm and emission spectra were recorded in the range 300-450 nm in PBS and PC/Chol 1:1.

Fig6. Circular dichroism spectra of gH peptides. Spectra were taken at different percentages of TFE

Fig7 Circular dichroism spectra of gH peptides. Spectra were taken in SDS and lysophosphatydilcholine.

Table1: Peptide sequences

PEPTIDES	SEQUENCES
gH220-262	TWLATRGLLRSPGRYVYFSPSASTWPVGIWTTGELVLGCDAAL
gH381-420	RLTGLLATSGFAFVNAAHANGAVCLSDLLGFLAHSRALAG
gH468-486	ALHALGYQLAFVLDSPSAY
gH493-537	AAHLIDALYAEFLGGRVLTTPVVHRALFYASAVLRQPFLAGVPSA
gH579-597	LFWLPDHFSPCAASLRFDL
gH626-644	GLASTLTRWAHYNALIRAF

Figure 1

A

Hydrophobicity Plot for HSV-1 gH

gH220-262 gH381-420 gH579-597
gH493-537
gH468-486 gH626-644

Hydrophobicity

Amino acid residue

Kyte-Doolittle
Wimley-White

B

TWLATRGLLRSPGRYVYFSPSASTWPVGIWTTGELVLGCDAAL
gH220-262

GLASTLTRWAHYNALIRAF
gH626-644

RLTGLLATSGFAFVNAAHANGAVCLSDLLGFLAHSRALAG
gH381-420

TM
804-824

1   100   200   300   400   500   600   700   800

LFWLPDHFSPCAASLRFDL
gH579-597

AAHLIDALYAEFLGGRVLTTPVVHRALFYASAVLRQPFLAGVPSA
gH493-537

ALHALGYQLAFVLDSPSAY
gH468-486

Figure 2

A

Alignment of gH626-644 with the N-coil of HIV gp41

```
GLAST LTR WAH YNALIRAF        gH626-644
LLQ-- LTV WGIKQLQ ARIL        gp41
```

Alignment of gH with known fusion peptides of Class I

```
gH402-420    AVCLSDLLGFLAHSRALAG
HIV-2        --GV-FVLGFLGFLAT---
SIV          --GV-FVLGFLGFLGA---
HIV-1        AVGI-GALL-FLGFLGAA—
HBV          -----GLFGPLLVLQAGF-
WMHV         -----GFLGPLLALQAV—
WHV          -----SLLGLLAGLQVVY-
GSHV         -----LLGLLAGLQVVY-
ReSV         ------FLGFLLGVGSAI-
Influenza    -----GLFGAIAGFIEN—
DHBV         -----GILAGLIGLLVSF-

gH495-513    HLIDALYAEFLGGRVLTTP
HIV-2        ---GVFVLGFLGFLAT---
HIV-1        AV-GIGALFLGFLGAA—
SIV          ---GVFVLGFLGFLGA---
HBV          -----GLFGPLLVLQAGF-
WMHV         -----GFLGPLLALQAV—
GSHV         -----GLLGLLAGLQVVY-
Influenza    -----GLFGAIAGFIEN--
```

B

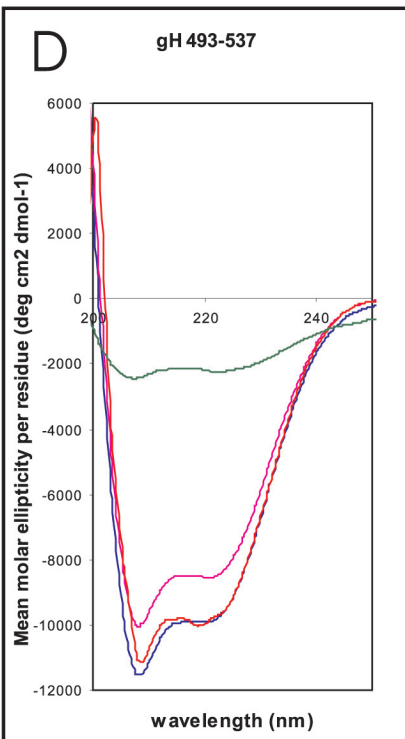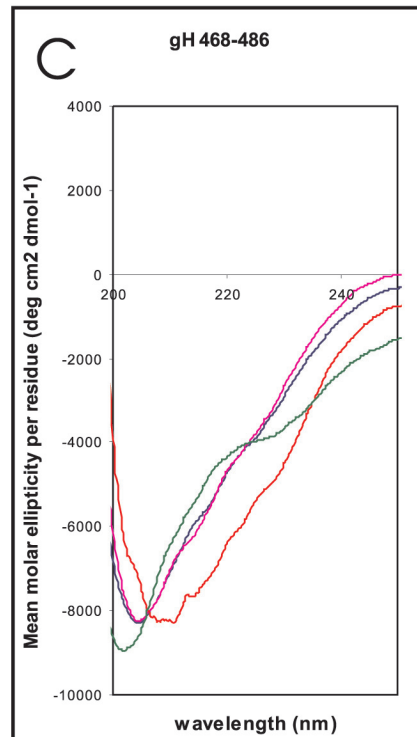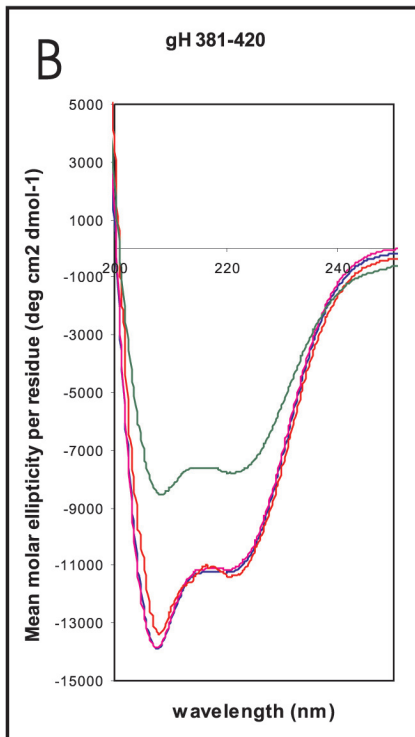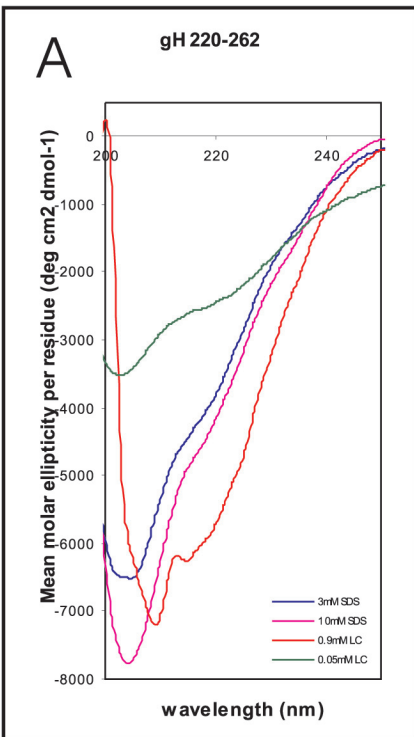Helical wheel diagrams: gH 579-597 and gH 626-644
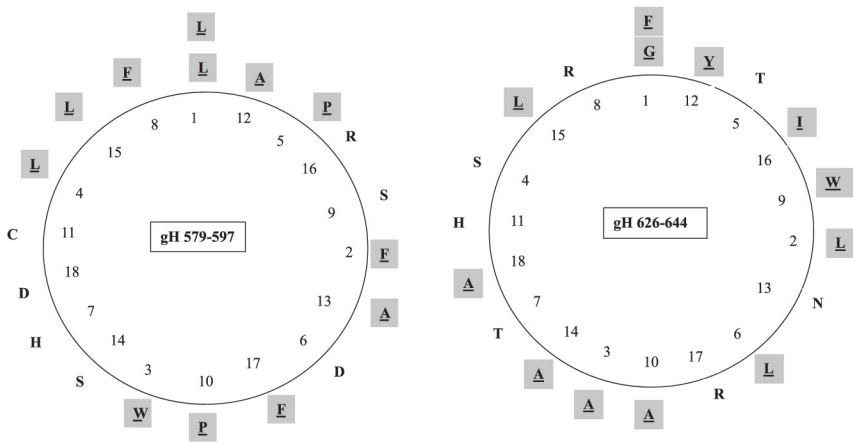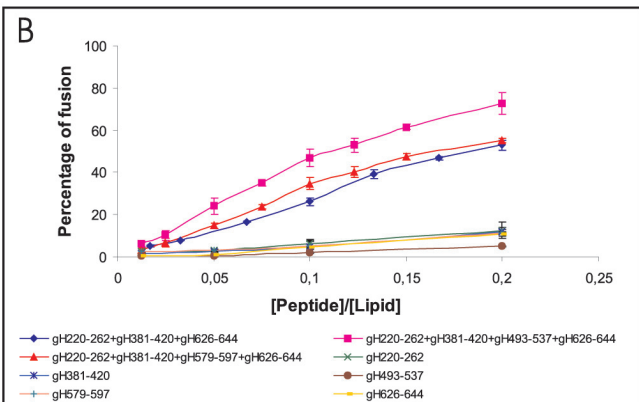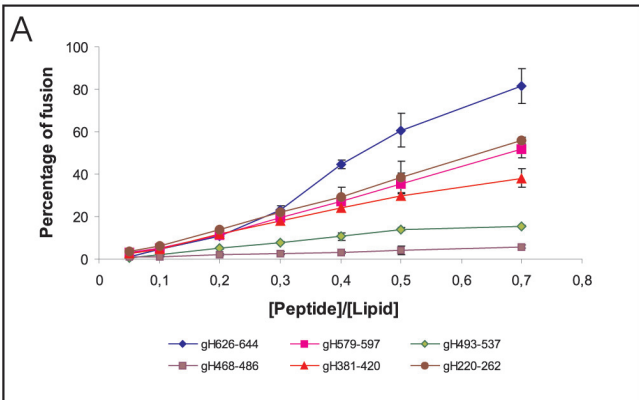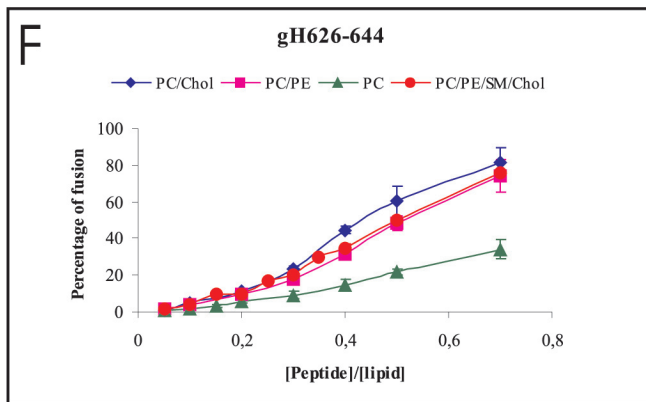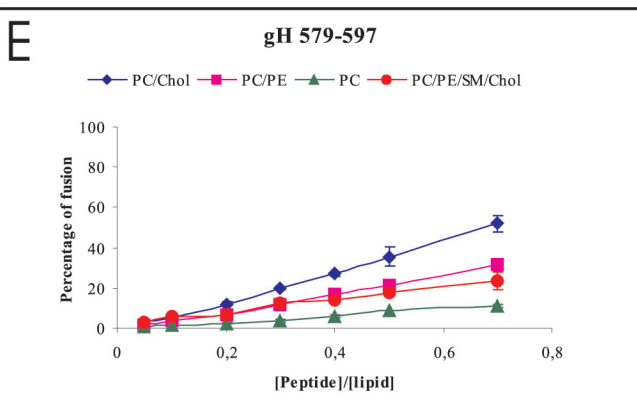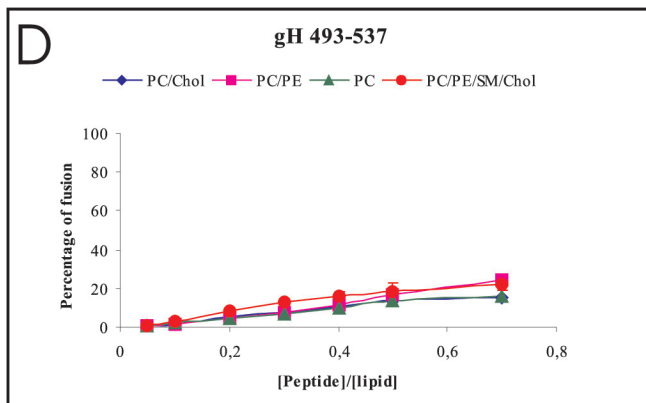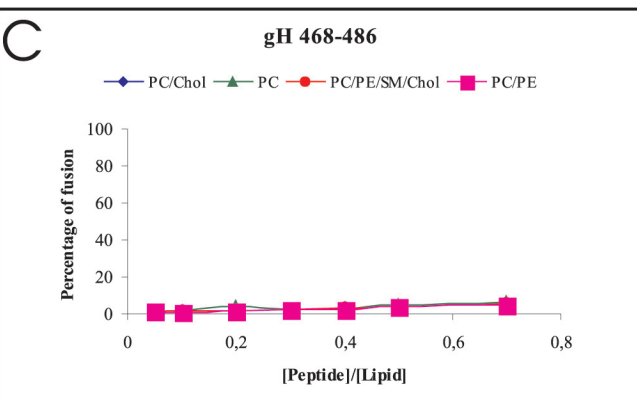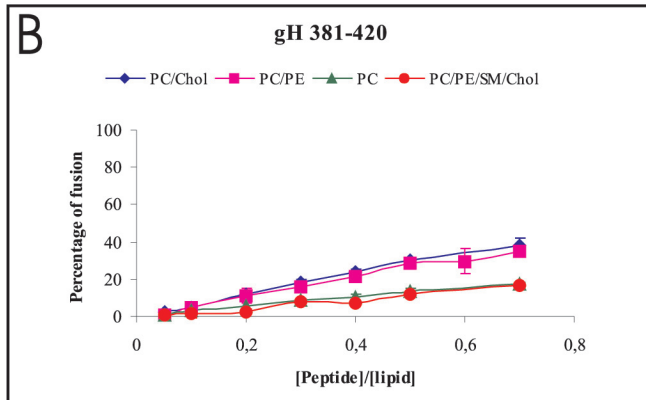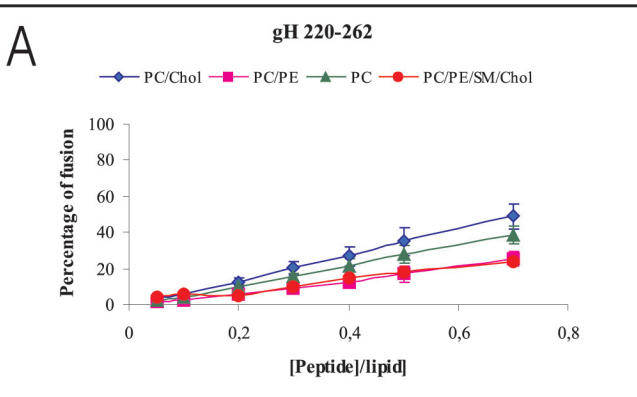
Figure 3

Figure 4

Figure 5
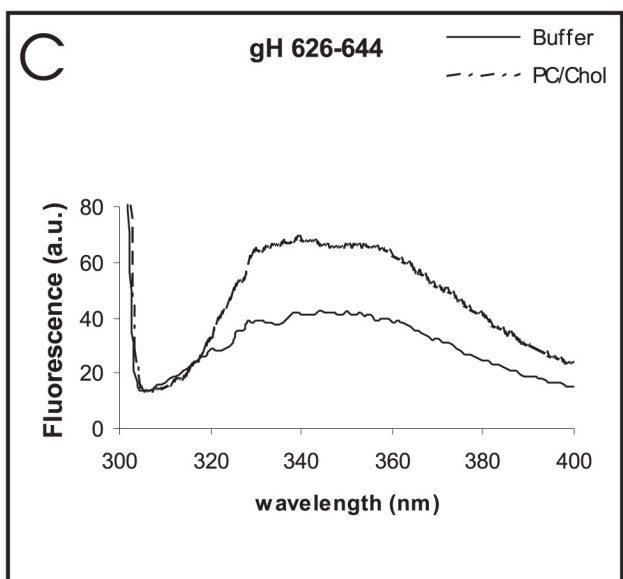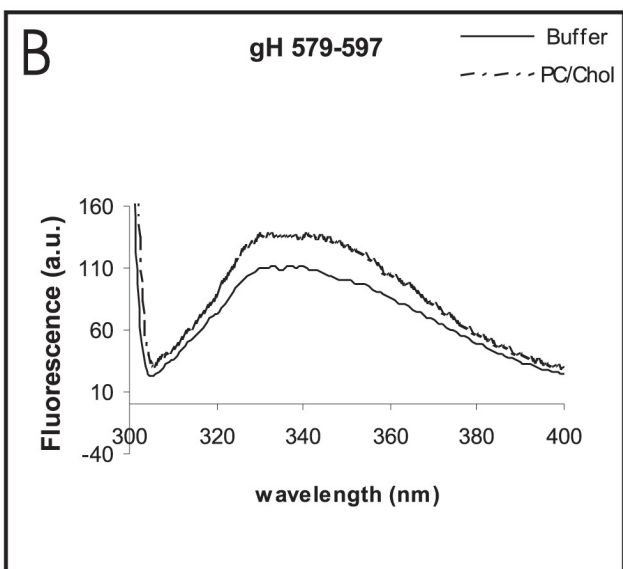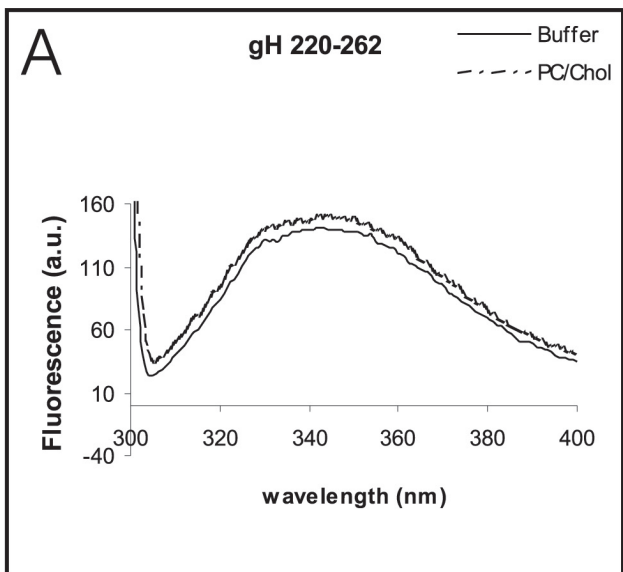
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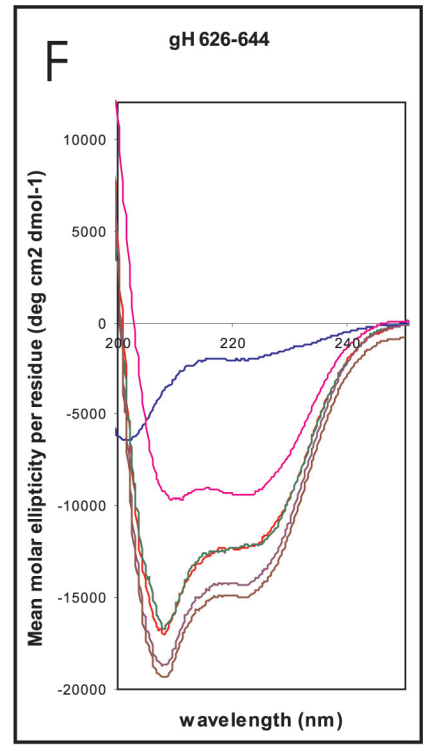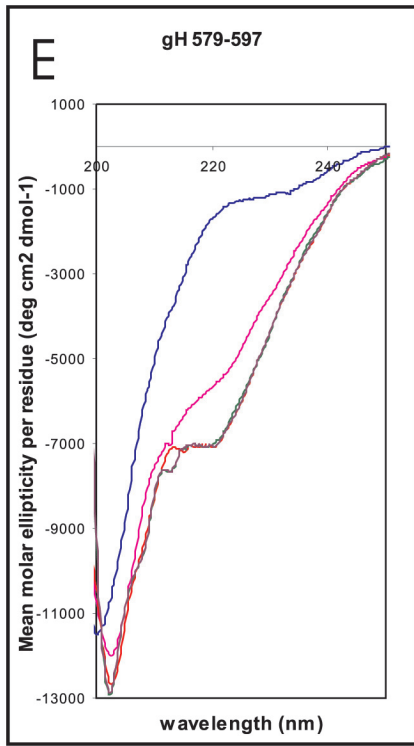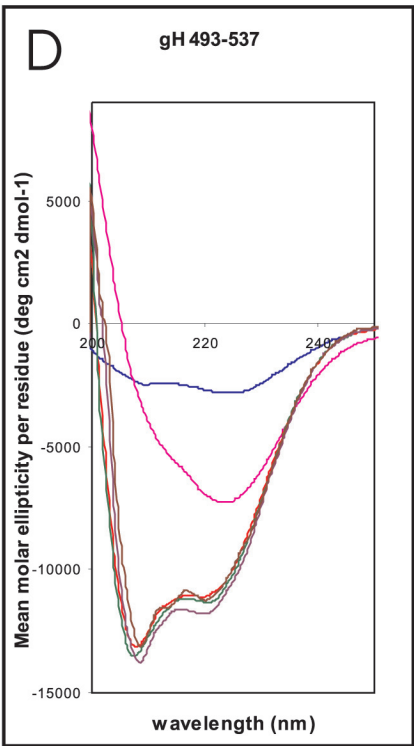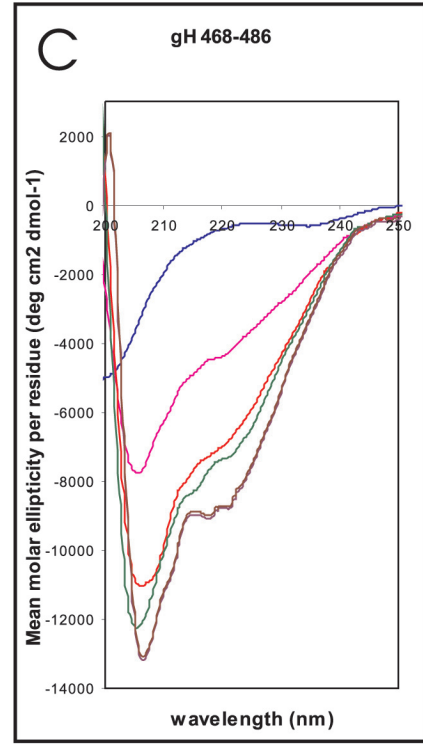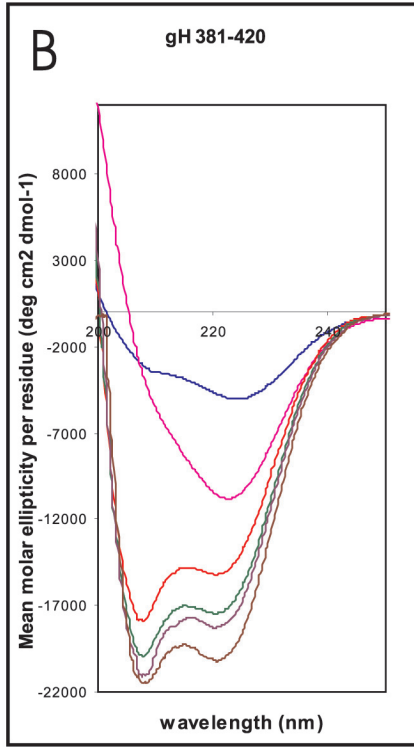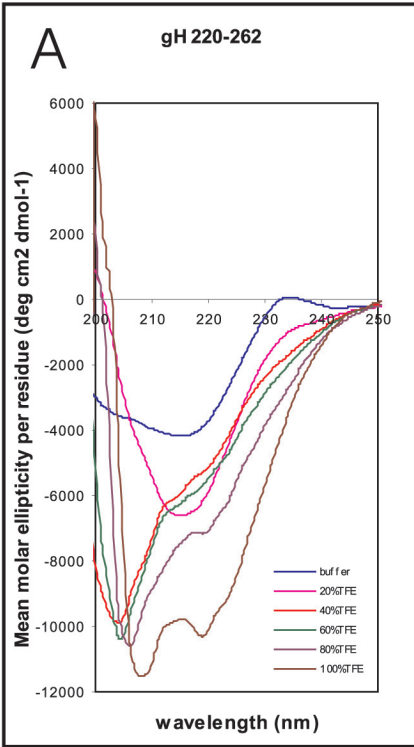
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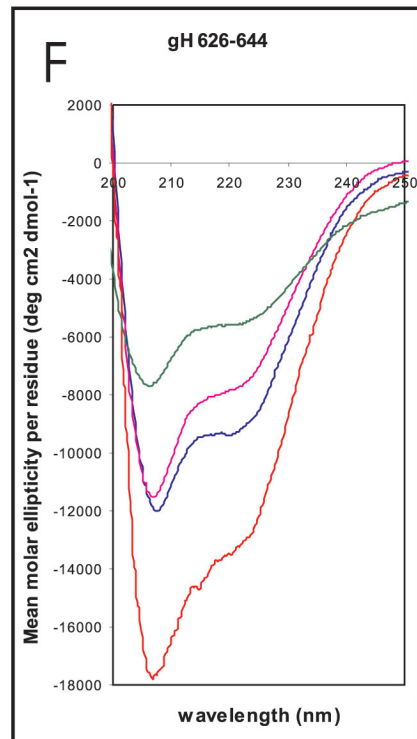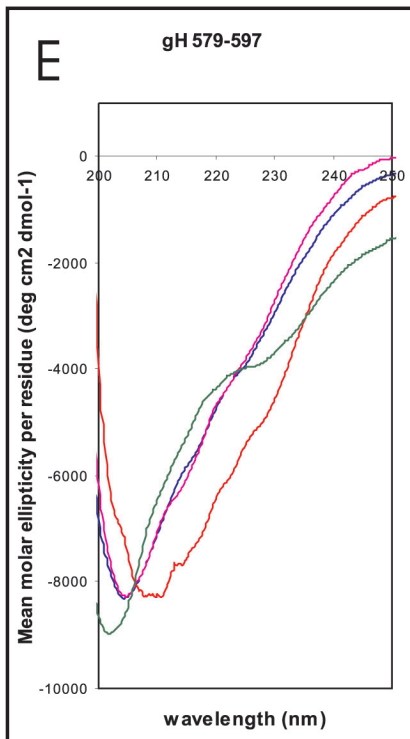
Fusogenic domains in herpes simplex virus 1 glycoprotein H

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