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## Interaction of Glycoproteins H/L from human α-herpesviruses with αvβ integrins

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# ABSTRACT

Herpes Simplex Virus (HSV) and Varicella-Zoster Virus (VZV) belong to the viral subfamily Alphaherpesvirinae, and are important neurotropic human pathogens. HSV and VZV fuse their envelope with the membrane of the host cell, a process termed cell entry, accomplished by a conserved trio of viral glycoproteins (gB, gH and gL). Host cell receptors mediate viral entry, i.e. nectin1 is recognized by the viral tropism factor gD in HSV, while the host receptor is still elusive in VZV. Integrins are widely distributed adhesion membrane proteins, and give critical contribution to the entry of most human Herpesviruses. αvβ6 or αvβ8 integrins are interchangeable receptors for HSV gH/gL, and they could synchronize fusion exerted by gB with the virion endocytosis they mediate. αvβ6 or αvβ8 integrins modify the glycoprotein machinery of HSV, but the modification they induce on gH/gL heterodimer was never investigated. The results presented here argue in favor of an integrin dependent mechanism which involves the conserved glycoprotein apparatus of HSV and seems to be essential to HSV entry. This mechanism, which also depends on the presence of gB and nectin1-bound gD, entails the dissociation of gL from gH/gL heterodimer, and its release in the media of culture cells upon binding of αvβ6 or αvβ8 integrins receptors. Recent findings disclose both the resemblance between HSV and VZV gH/gL heterodimer structures and the role of integrins of the alpha V family as receptors for VZV entry, a role probably shared with Myelin Associated Glycoprotein (MAG). However, while MAG function in fusion was associated with VZV gB, it was not clarified which glycoprotein(s) was(were) involved in integrin-mediated VZV entry. Data presented here argue in favor of integrin direct binding with a part of the conserved VZV entry machinery, while VZV fusion seems to be the result of more complex interactions.

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## 1 INTRODUCTION

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## 1.1.1 Brief introduction to Herpesviruses

The Herpesviridae family includes viruses from reptiles, birds and mammals, which all share a similar architecture of the virion.[1] The Human species hosts nine herpesviruses: herpes simplex virus 1 (HSV-1, HHV-1), herpes simplex virus 2 (HSV-2, HHV-2), varicella-zoster virus (VZV, HHV-3), Epstein-Barr virus (EBV, HHV-4), human cytomegalovirus (HCMV, HHV-5), Human herpesviruses 6A, 6B, and 7 (HHV-6A, HHV-6B, HHV-7), and Kaposi's sarcoma–associated herpesvirus (KSHV or HHV-8). Epidemiology varies among viruses, as infection might result in a specific disease. Herpesviruses have a double strand DNA genome, the replication cycle is characterized by a nuclear viral gene transcription, a nuclear viral DNA synthesis and a nuclear assembly of the viral nucleocapsid. Another shared feature among herpesviruses is latency, that allows a lifelong persistence of the virus in specific tissues inside the host. Herpesviruses produce viral progeny through lytic infection, namely, the destruction of the host cell. Herpesviruses could infect different kinds of hosts, i.e. HSV has the capability to infect also murine cells. Another important hallmark is the duration of their replicative cycle, with important implication for *in vitro* studies. Common to all herpesviruses is the ability to encode a variable set of enzymes, which allows the virus to speed up its own nucleic acid metabolism once inside a susceptible and permissive host. This way, the virus favors its replication at the expense of that of the host cell. Examples include: DNA polymerase, DNA helicase, DNA primase and thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase.

## 1.1.2 Brief History of HSV and VZV

Hippocrates was the first to ever document herpesviruses lesions, in 400 BCE. They were referred to as herpes, because of their ability to crawl inside the skin.[2]

In the 18th century, Bateman described herpes as a “restricted group of localized vesicles with a short, self-limiting course”, while in 1768 Herberden operated distinctions among varicella and smallpox, whose etiology was previously thought to be the same. In 1863, Von Barensprung identified a correspondence between dermatomes and rash distribution of Zoster. The 19th and 20th centuries were characterized by the first experiments on viral transmission involving human volunteers.

In particular, in 1873, Vidal showed that herpes simplex was caused by an infectious agent, inasmuch it could be passed between humans. In 1892, Von Bokay demonstrated the common infectious etiology between Zoster and Varicella by means of inoculating children with Zoster derived essudates.[3] In 1924, Gruter showed that the isolated HSV agent could be experimentally transmitted among rabbits. Garland and Hope Simpson proposed that Zoster originated when latent Varicella reactivated.[4,5]

Concurrently, in the 1930s, Burnett and William described the lifelong persistency of HSV and observed its reactivation during stress conditions. Importantly, these reactivations happened only in individuals with HSV neutralizing antibodies, as suggested by Andrews and Carmichael. In 1953, Weller replicated the same hallmarks of VZV infection in tissue culture by using both Varicella and Zoster starting material.[6]

In the 1970s, it was demonstrated that a human fetus could be seriously threatened by encephalitis induced by genital herpes. For this purpose, antiviral Vidarabine was used to prevent replication of HSV-2. Eliot et al. subsequently discovered Acyclovir, whose derivatives are still administered nowadays to control herpesvirus infections. In 1974, Takahashi developed the first vaccine against a herpesvirus, starting from VZV Oka parental strain, making Varicella and Zoster preventable diseases.[7,8] In 1986, Davison and Scott completed the DNA sequencing of VZV genome (strain Dumas), the smallest genome among that of herpesviruses.[9]

### 1.1.3 Herpesvirus Classification

In the 1970s, before the advent of DNA sequencing, known herpesviruses were classified as Herpesviridae by the International Committee on the Taxonomy of Viruses (ICTV). Whitin this family, three subfamilies were identified, resuming species-specific biological characteristics: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae.[10] In particular, alphaherpesviruses are characterized by a short life cycle and are thus easily and rapidly spread in culture.

Alphaherpesviruses establish latency in the sensoria ganglia of the host and destroy infected cells very quickly. Genomic data analysis and the comparison of gene distribution provide the basis for a deeper organization of the virus subfamily into genera, i.e. both HSV-1 and HSV-2 belongs to the genus Simplexvirus. VZV belong to the genus Varicellovirus, together with Pseudorabies Virus (PrV), which, in turn, affect swine. HSV-1, HSV-2, VZV and PrV belong to the alphaherpesivirinae subfamily. HCMV belongs to Betaherpesviridae and is characterized by a long life cycle which is performed in a restricted number of host cells. *In vitro* culture of HCMV requires more time to proceed and host cells are peculiarly modified in their structure, exhibiting dilatation referred to as cytomegalia. Betaherpesviruses establish latency in secretory glands, lymphoreticular cells and kidneys. Other human betaherpesviruses are Roseolovirus (HHV-6, HHV-7). Gammaherpesviruses are divided into genera Lymphocryptovirus (EBV) and Rhadinovirus (KSHV). They infect prevalently T or B cells, i.e. EBV infects only human cells from limphoid tissues, where it also establishes its latency. The availability of specific receptors alone is not sufficient to explain the possibility to infect a wide range of hosts.

It was recently demonstrated that degenerated primers for viral hallmark genes (as DNA polimerase) could be annealed by a polimerase chain reaction (PCR) technique onto genomic samples from mollusks to mice. This result discloses an unexpectedly wide spread during evolution of herpesviruses.[11] Viral DNA polimerase gene was conserved during evolution as it performed an essential function in the viral infection cycle.

### 1.1.4 Epidemiology of HSV

Only an infected human transmits HSV to susceptible humans, and no animal vector has ever been recognized for HSV. HSV is distributed in the worldwide population without distinction between developed and less developed countries; HSV infection is observed with no seasonal influence.[12] 50% of the human population could have been infected by HSV; HSV is life long infection, as the virus became latent inside neurons of the infected individual. This mechanism raises the possibility for subsequent reactivations and consequent spreading in the rest of the population. HSV causes very little fatality cases, most of them are recorded after severe infection of newborns. On the contrary, when HSV infects children (termed primary infection, often located in the mouth or ocular area), symptoms are usually subclinical or absent. Infection requires infectious agent penetration through abraded skin or

mucosa. The encounter with axonal termini of sensory neurons leads to retrograde transportation of the capsid to the neuron nucleus, where virus latency is established.[13]

HSV becomes latent inside the sensoria ganglia of the host, where it is maintained as an episomal DNA until various environmental stimuli -connected with psychophysical stress or absorption of UV radiations- induce its reactivation. After a limited lytic cycle, which allows to maintain a reserve of viral genome in infected ganglia, reactivated HSV undergoes anterograde transportation to the innervated skin site (that usually corresponds to the site of primary infection) originating typical fever blisters. The recurrent cold sore are sites of active replication, functional for virus spreading among susceptible individuals and the lesion produced might affect skin sites near the original one.[13]

HSV incubation period is commonly 4 days long but it could last up to 12 days. During the incubation period, the DNA of the virus is detectable by PCR assays. HSV-1 symptomatology includes oropharyngeal disease which could last for up to 3 weeks. Symptoms involve ulcerations on the lips (typical of recurrent infection) and in the gingival mucosa (the typical site of primary infection).

HSV-2 symptomatology includes genital macules, vesicles and ulcers, that are productive sites of infection; vaginal delivery in these conditions allows spreading to the newborn, with encephalitis or disseminated infection as possible complications. In immunodeficient individuals, HSV might cause severe infection, leading to progressive diseases of the respiratory and gastrointestinal tracts.

In the cases where HSV infection may lead to complications, i.e. aseptic meningitis, the prodrug acyclovir must be administered intravenously; otherwise, topical or oral administration is used to mitigate symptoms of infection, while it remains not possible to eradicate the latent form of the virus.

### 1.1.5 Epidemiology of VZV

Only an infected human transmits VZV to susceptible human and no animal vector has ever been recognized. VZV tropism includes T cells, epithelial cells and neuron cells, but dissemination to other organs as lung, liver or the central nervous system might happen when viremia and viral replication escape the control of the adaptive immune response.[14]

Varicella is the clinical manifestation of the VZV primary infection, which is presumed to start with inhalation of the airborne virus, because VZV might spread by means of respiratory droplets.[15] Among

susceptible individuals, VZV infections may originate also from the inoculation of mucosal epithelium with vaccine strain preparation or vesicular fluids from VZV skin lesions.

Initial VZV replication involves the respiratory mucosa; primary infection causes varicella and occur after 10 to 21 days of incubation. During this period, VZV infects host T cells and it spreads throughout the body into the skin. The incubation period is prolonged, inasmuch VZV skin infection is counteracted by epithelial innate barriers, i.e. epithelial IFN constitutive expression.[16] From the respiratory mucosa, VZV might gain access to the tonsils, where the virus spreads to T cells with skin homing markers. [17] In 10 to 21 days these cells disseminate in the skin, where VZV creates a site of productive replication, termed varicella vesicles, that allow spreading in the rest of the population by contact or by airborne transportation via desquamation.

48 h before the onset of varicella, the individuals are contagious, and prodromal symptoms include fever up to 40°C and headache, while lethargy and anorexia appear after the erythematous macules (rash). VZV associated rash evolves rapidly into skin vesicular lesions, with intense itching. Subsequently, these lesions start to form crusts. The number of lesions may range from 10 to 2000 units, initially distributed throughout the face or the trunk. As T cell associated VZV is transported throughout the blood torrent, VZV can easily reach the liver or other target organs, whose infection typically causes complications in susceptible individuals. To establish latency, VZV might reach sensoria ganglia both by centripetal migration from axons termini of sensory neurons, or directly by hematogenous trasportation.

The prevalence of VZV in the human population was determined by means of autopsy studies up to 94%, and up to 99% by means of IgG antibodies detection in the population living in temperate climate zone.[18,19] In tropical regions, VZV has a less wide distribution, as only half of the individuals under 24 had developed varicella.[20,21]

Reactivation of latent VZV follows undetermined factors, progressively reaching a 50% risk in people older than 85, and may originate Zoster. Zoster is a vesicular rash with dermatomal distribution, whose manifestation is due to the loosening of innate and adaptive immunity.[22] Zoster is associated with pain, referred to as post herpetic neuralgia (PHN), since the VZV reactivation phenomenon induces neuronal damages, with inflammation, necrosis and consequent neuritis, myelitis and degeneration.[23,24] The onset of complications associated with VZV depends prevalently on the immune state of the host, and may facilitate bacterial infections in children, varicella pneumonia in adults and the already mentioned neurologic syndromes in the elderly.[25-27]

VZV prevalently establishes latency in sensoria ganglia from T3 to L2 of the trunk and, as it infects neurons but also their satellite cells, it may expand to other neurons inside the ganglion, contributing to PHN.[28,29] T cells are important to terminate initial varicella viremia; in immunodeficient individuals, VZV reactivation might lead to a generalized Zoster. Generalized Zoster does not have a dermatomal distribution; the atypical pattern results from direct reinfection of circulating T cells, which had encountered the reactivating ganglia, originating another varicella like viremia.[30]

Memory B cell pool is maintained active in the individual to prevent subsequent reinfection upon repeated environmental contacts with the pathogen. VZV replication is neutralized by means of circulating IgG antibodies, while memory T cells hamper VZV reactivation.[31,32] Immune response mediated by T cells could be monitored at skin level by analysis of delayed type hypersensitivity (DTH) response to VZV antigens, indicating if one individual is subjected to VZV reactivation due to waning immunity.[33]

Zoster and varicella persist for 1-2 weeks, while PHN may precede or follow Zoster manifestation by months, or even occur without skin manifestation; in this latter case, the chronic radicular pain produced was termed zoster sine herpete. VZV adverse symptoms, as well as those generated by severe HSV infections, are controlled by administration of the prodrug Acyclovir, which results especially important to terminate VZV associated viremia in immunocompromised individuals.[34]

VZV vaccine is a mixture of different live attenuated virion particles derived from Oka strain, that was obtained by VZV propagation in guinea pig and human fibroblast; it is typically administered to children at 17000 plaque forming units (pfu) per dose.[7,35] Genome variability between the parental and vaccine Oka strains affects prevalently 63 sites, whose main target is the gene regulator open reading frame (ORF) 62.[36] Interestingly, only 5% of vaccinated children develop varicella, and the vaccine Oka strain retains the ability to infect other individuals.[37] VZV vaccine induces long term (up to 10 years) specific cellular immunity against the wild type VZV strains, and vaccination of elderly individuals was associated with diminished PHN onsets. Two vaccine administrations are required to gain high VZV seroconversion rate.[39,40]

Both varicella and zoster skin lesions are site of productive infection yielding cell-free virions *in vivo*, while *in vitro* VZV remains associated with culture cell membranes, and cell free virions have a particle to infectivity ratio of 40,000:1, instead of 60:1 typical of HSV-2.[40] Cell-free VZV virions, derived *in vivo* from keratinocytes, have higher titers as compared to that harvested *in vitro*. VZV can infect also Schwann cells and astrocytes *in vitro*.[41,42]

As VZV vaccine has proved to be safe, it is now tempting to convey foreign antigens from other pathogens for vaccination purpose, so as to allow engineering of a multiplex vaccination platform.[43]

## 1.2.1 Herpes Virion Structure

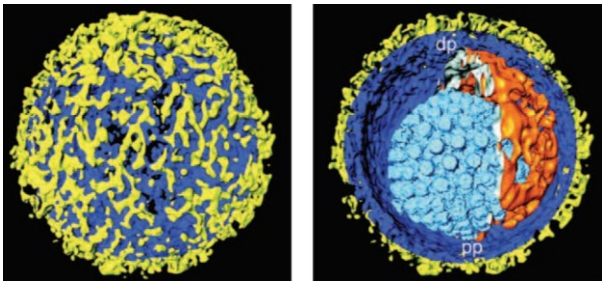



**Figure A. Rendering of the HSV-1 virion by cryoelectron tomography**. (H) Envelope surface (blue, membrane) with spikes (yellow, glycoproteins). Spliced view of the HSV virion: capsid (light blue), with its thread-like surface structures; tegument (orange), which is asymmetrically arranged, envelope (blue and yellow). pp, proximal pole; dp, distal pole. Images are adapted from ref 60.

The genetic material of the herpesviruses resides in the virion core as a molecule of dsDNA which ranges from 124 to 295 kilo base pairs (kbp) in length throughout the family. Virion capsid is made of 161 capsomers; these latter form an icosahedron of 100 nm in diameter. The core and the capsid form the nucleocapsid. The genetic material is released in the host cell or inserted during virion assembly through a unique portal structure, which is found in the assembled capsid. The nucleocapsid is absorbed into a proteinaceous material, termed tegument, which appears to be amorphous and asymmetrical. The tegument is bordered by a lipid bilayer, decorated with integral viral proteins. The lipid bilayer is termed envelope.

The mature virion diameter ranges between 120 to 260 nm and depends prevalently on the thickness of the tegument layer (figure A).[44] As estimated by Sodium dodecyl sulphate poliacrilamide gel electrophoresis (SDS-PAGE) or proteomic analyses, the Herpes virion contains up to 7 viral proteins in the nucleocapid, up to 20 viral proteins in the tegument and up to 19 viral proteins in the envelope.[46-48] The Herpes virion encases about 10000 protein units, some viral proteins were not addressed to any virion compartment. The copy number of proteins of viral origin is variable from 1 to 1000 units. The envelope glycoprotein B is present on Herpes virion in 800 copies on average. The herpes virion also

encases proteins of host origin, with structural or catalytic function, but without an obvious significance for virus infection. The Herpes virion also carries mRNAs into the host cell, both from viral and host origin, whose transcription might be inherent to infection.[49,50]

## The Core

Herpes virion core encases the genetic material as a single molecule of dsDNA, which is tightly packed and presents a toroidal shape, and whose negative charges are neutralized by means of spermine and no histones.[51-53] As the volume occupied by the buffered DNA molecule perfectly fits the capsid capacity, ATP is required to perform core filling. Pressure facilitates the subsequent ejection of the genetic material during host infection.[54]

## The Capsid

The herpes virion capsid presents threefold symmetry and is made of 161 capsomers. 150 hexons form the faces, whose longitudinal section is 9.5 × 12.5 nm, 11 pentons form the edges and DNA portal. The four constituent proteins are evolutionarily conserved.[55,56] Three forms of capsid coexist inside infected cells: A form is the capsid devoid of the core, B capsid is capsid without DNA, still depending on a mounting scaffold, and C capsid is the mature capsid without scaffold, already filled with viral DNA.[57] Conserved capsid proteins are distributed as follows:

- major capsid protein (MCP), 6 copies per hexon, 5 copies per penton and 955 copies per capsid;
- monomer and dimer proteins of the triplex (one triplex is formed by 1 TRI1 and 2 TRI2), 320 triplex per capsid;
- capsomere-interacting protein (SCP)

MCP protein folding showed resemblance to that of tailed DNA bacteriophages capsid proteins, implying a common ancestry between these classes.[58] At the end of a vertical axis of the capsid, 12 copies of the portal protein (PORT) form the capsid portal, which is crossed by dsDNA during

packaging or after infection in the opposite direction. The capsid portal is occluded by the portal capping protein (PCP), only associated with type C capsids.

In HSV VP5 (UL19), VP26 (UL35), VP23 (UL18), and VP19C (UL38) form the capsid, whose faces are made of layered lattices of polymerized capsid proteins; these layers are concentric, to allow the passage of the DNA molecule.[59] In the HSV virion, the nucleocapsid occupies 30% of the total virion volume and is found in an eccentric position. The nucleocapsid is indeed tethered to the so termed proximal pole of the envelope, while the distal pole stands 30 nm apart, separated by a thick tegument layer.[60]

In VZV, structural studies were impeded by difficulties in harvesting cell free virus at high titer in culture. Moreover, virion harvested in culture lacked the capsid dense core associated with DNA filling, which is in accordance with the typical low infectivity of cultured VZV.[61] In VZV the capsid is formed by ORFs 20, 21, 23, 33, 40, and 41, as inferred by analogy among other herpesviruses homologs.

## The Tegument

The tegument is a proteinaceous structure included between the nucleocapsid and the envelope, showing a fibrous appearance upon negative staining.[62,63] The thickness of the tegument around the nucleocapsid is not constant and is controlled by the virus.[64] In fact, the 20 viral proteins that form the tegument show polarity, as the tegument is an ordered structure. Specific interaction of tegument proteins with nucleocapsid proteins is documented.[65,66] In particular, the inner tegument contacts the nucleocapsid and the outer tegument contacts integral proteins of the viral envelope. The polarity of the tegument is acquired neatly during virion maturation. In fact, the virion sequentially crosses the cytoplasmic compartment, characterized by the presence of a particular selection of viral and host proteins.[67] These proteins, once inside the newly infected host, represent a stock of ready-to-use enzymatic tools that help conquering the host by inhibiting host innate immunity or blocking host protein synthesis.

Notable proteins included in the tegument of HSV virion are virion host shut-off (VHS) protein (UL41), virion transactivator protein VP16 (UL48) and VP1-2 (UL36), which is a very large protein. VZV includes three different transactivators in its tegument, namely ORFs 4, 62, and 63. VZV

tegument also includes proteins identified by genes ORF 9, ORF 10, ORF 11 and ORF 12 (that represent a conserved gene cluster) and two kinases encoded by ORF 47 and ORF 66.[68-71] To determinate whether a protein derives from the tegument, infection should be carried out in the presence of cycloheximide and then actinomycin D.[72]

## The Envelope

The envelope is the lipid bilayer derived from host cellular membrane that surrounds the nucleocapsid and the tegument, it presents a trilaminar appearance when investigated through an electron microscope.[73] The fact that herpes virions are encircled by lipids and that the envelope is essential for infection, implies that herpes virions are sensitive to detergents. The virus hijacks the biosynthetic pathway and modifies the host cell membranes with the insertion of viral membrane glycoproteins, which are subsequently acquired during virion maturation, when the nucleocapsid packaging is complete. Viral glycoproteins are essential both to perform egress from host membrane compartments and to infect another cell, i.e. to accomplish fusion and cell entry. The importance of glycoproteins is attested by their conspicuous presence on the virion envelope surface. For example, HSV expresses 11 different envelope glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM), with up to 1000 individual copy number per virion, while only four glycoproteins are essential to mediate the entry of susceptible cells.

In HSV, glycoprotein spikes increase the virion diameter up to 225 nm locally, while the average diameter measures 186 nm. Glycoprotein spikes do not follow a homogeneous distribution, but are organized forming a dense cluster at the distal pole.

The VZV envelope membrane, contrary to what happens with HSV, does not have a single source, as it includes markers from the endoplasmic reticulum (ER) membrane, from cytoplasmic vesicles, and from the surface of host cell.[75]

## The Genome

The Herpes virion dsDNA is linear, but it circularizes upon release from the nucleocapsid to the nucleus of an infected cell. In the herpesviradae family, genome length is comprised between 124 kbp and 295 kbp, while genome variability among cultured viruses of the same species may account for up to 10kbp additional variability. This additional variability is due to repeated passaging in cell culture and often results in genome modification, like gene duplication, gene deletion and copy number variant at reiterated sequence level. *In vitro* virus fitness does not necessarily overlap with the one required for an efficient *in vivo* infection. Strains with a high number of cell passages present mutations that eliminate needless gene expression to allow a better spread. For example, HCMV strain used for *in vitro* cultivation carries the deletion of an array including 19 genes. Besides, mutations in a restricted group of genes in HCMV clinical isolates allow for an efficient spread in culture cells.[75,76]

In the herpesviradae family, the total G+C content in the genome is comprised between 31% and 77%, while the G+C content varies inside the same genome, typically leading to a higher G+C content in genome repeated terminal ends. Repeated terminal ends in herpesviruses genomes are generally 100bp long. In VZV, the right terminal end is repeated and inverted inside the genome, but not inside the left terminal end. The biological behavior of inverted repeats allows for inversion and gene expansion of the part of the genome they limit. In herpesviruses, allow the small part, termed S, of the genome to switch with the large part of the genome, termed L. The switching of the S part and the L part of the VZV genome leads to the recovery of two equimolar species of genome from purified VZV virion.[77] In HSV, the inverted terminal repeats surround both the L part and the S part of the genome, leading to the recovery of four equimolar species of genome from purified HSV virion.[78] There are no relations between L and S termini sequences.

HSV genome is complex and characterized by two unique sequences, referred to as long ($U_L$) and short ($U_S$), surrounded both by inverted repeats, termed a, b and c, and by direct repeats, termed $a_L$ and $a_S$. $U_L$ is comprised between repeats ab and b'a', while $U_S$ is comprised between repeats a'c and c'a; the number of repeats varies at both $U_L$ termini, namely at the L-S junction and at the left $U_L$ termini. Hence HSV genome could be represented by the following array: $a_L\ a_n\ b - U_L - b'\ a'_m\ c' - U_S - c\ a\ a_S$.

HSV genome has three origins of replication: one, termed $ori_L$, resides is in the $U_L$ sequence, while the others, $ori_S$, are located in the $U_S$ flanking regions. HSV genome is 152,261 bp long, with a G+C

content of 68%.[79,80] VZV genome is 124,784 bp with 46% of G+C content, with an unpaired cytosine at its left end and an unpaired guanine at the right end that allow circularization.[81]

In VZV there are two genome regions, termed short terminal repeat (TRS) and short inverted repeat (IRS), that account for genome isomerization, namely the $U_S$ termini orientation switching. These repeats encode a series of three conserved duplicated genes (ORF 62/71, ORF 63/70 or ORF 64/69).[82] VZV genome encases five small repeated regions, termed R1, R2, R3, R4 and R5, whose length varies depending on the number of repetitions of the same short sequence. Intergenic regions between ORFs 60 and 61, 61 and 62, and 62 and 63 originate non coding transcripts in VZV.[83] Terminal ends of herpesvirus genomes contain *pac* sequence, which serves both for dsDNA packaging inside the capsid and for cleavage of the concatemeric genomes, generated by rolling circle DNA replication.[84]

### 1.3.1 Characteristics of Herpes Genes

A typical herpes gene presents cis-acting regulatory sequences comprised between 50bp and 200bp upstream the TATA box, while the transcription start site is comprised between 20bp and 25bp downstream. Translation takes place after a non-translated leader sequence extended for 30bp to 300bp upstream the ORF. The ORF terminates before the non translated trailer sequence, that is 10 to 30 bp long, and a regular polyadenylation signal, which can be shared by co-terminal transcripts. Both HSV and VZV made use of GU rich sequences GTCTGTGT and TGGTGGTA after polyadenilation sites, that are important for termination of transcription.[85] The obvious requirement for genome space economy and the strong selective pressure, typical of viruses, generated aberrant genes. For example, the expression of this genes depends on a degenerated TATA box, i.e. HSV-1 gamma 34.5. Other examples are HSV UL26 and UL26.5 genes that originate proteins with different functions but are translated from the same mRNA (from different starting codons).[86,87] Genes whose promoters reside after the TATA box and promoters shared by head-to-tail genes were also described.[88] HSV genome contains overlapping genes (gamma 34.5 and ORF P and O), with antisense coding ORFs.[89] In general, viral transcripts are not subjected to splicing, and their expression depends only on host RNA polymerase II transcription and subsequent mRNA polyadenilation. At most, 10% of herpes transcripts are spliced. To accomplish the sequential expression program typical of herpesviruses (further detailed below), a spliced isoform needs to be transcribed from more than one promoter, increasing genome

space requirements.[90] Herpesviruses codify also for miRNA and non coding mRNA, i.e. the HSV Latency Associated Transcript (LATs).[91]

In herpesviruses, the number of genes is comprised between 70 (VZV) and 200 (HCMV). These data were inferred by herpesviral mutants, transcriptional mapping and in silico analysis. HSV encodes for at least 84 proteins, while 90 genes are transcribed including non coding RNA, miRNAs or mRNAs, that originate more than one protein (UL3, UL26).[92] The gene numbers should be underestimated as compared to the total amount of herpesvirus expressed genes: detection techniques skip very short ORFs, alternatively spliced mRNA or translationally frameshifted proteins. Large portions of VZV and HSV genome are colinear and proteins exhibit similar regions; 56 of the 62 genes in the VZV UL region and all the 4 genes in the VZV US region are homolog to those encoded by HSV. VZV ORFs 1, 2, 13, 32, 57, and S/L have no homolog in HSV and are not essential for VZV replication in cell culture, while ORF 13 has a KSHV homolog.[93-97]

### 1.3.2 Gene Functions in Herpesviruses

To determine the functionality of various herpesvirus genes experimental systems were employed. However, these experimental systems lack the essential protein determinants proper of the host cell. To overcome biases originated by restricted systems, the whole herpes genome could be modified by recombineering technology, i.e. ablating one gene at time, by means of the bacterial artificial chromosome (BAC) system. The mutant genome is subsequently reconstituted directly into its natural host by DNA transfection. Promoter and gene dissection in the VZV genome was established by Arvin et al. They employed VZV recombinants for *in vivo* infection of human tissues (T cells, fibroblast or neurons) xenografted in SCID mice.[98-102] Herpes gene products have multiple functions, which could be triggered by their subcellular location, by a specific post-translational modification or by the presence or absence of a specific host or viral interactor. For example, HSV ICP0 works as a specific transactivator of viral genes in the nucleus, but it subsequently localizes in the host cytoplasm, to interact with specific proteins.[103,104]

To investigate interactions between viral and host proteins, analysis in the context of infection is required as reference. In fact, inside the infected host, viral proteins exert their natural influence on themselves, and encounter all their natural host targets, together with their post translational modifiers. 50% of the proteins encoded by HSV is dispensable during cell culture infection, but not for viral

replication or reactivation from latency in animal models. To establish a latent infection, all the viral encoded genes are dispensable one at time. Not dispensable genes for herpesvirus infection in cell culture are termed essential genes, with notable intermediate cases as, i.e. HSV ICP0. ICP0 is an essential gene, but its expression is dispensable during infection in cell culture at high multiplicity of infection (MOI).

### 1.3.3 Interferences with the Host Cell

Proteins encoded by herpesviruses allow for the conquest of the host; they firstly promote host cell entry, then contribute to develop the cascade of protein expression ,which finally leads to the hijacking of biosynthesis machineries and to the production of a new viral progeny. In particular, viral protein expression operates in regulation of viral gene expression, as well as in the nucleid acids metabolism, in the formation of the virion capsid or to counteract host cell defenses. Alphaherpesviruses share a conserved array of homolog regulatory genes, namely ICP0, ICP4 and ICP27, which serve to transactivate viral genetic material, following an established pattern of protein expression. Examples of proteins involved in managing host homeostasis are conserved in alphaherpesviruses.

VZV ORF 10 forms a ternary complex with host Oct1 and HCF on TAATGARAT sequences to allow transcription of VZV ORF 62. VZV ORF 10 is the tegumental homolog of HSV VP16 and complements HSV VP16 mutants, but it is not essential for VZV infection.[105-107] VZV ORF 36, viral thymidine kinase (TK), is not essential for VZV replication and, if compared to that of HSV, has a prevalent deoxycytidine activity.[108] VZV ORF 18 and ORF 19 are homolog to HSV ribonucleotide reductase, respectively to the small and large subunits, that are insensitive to concentration of deoxyribonucleotides.[109] VZV ORF 47 and ORF 66 are tegument serine threonine kinases; VZV ORF 66 shares homology in its catalytic domain with HSV US3.[110] VZV ORF 17 is the homolog of HSV UL41 viral host shutoff (VHS), a RNAse that degrades host mRNAs in the cytosol, but has lower activity than its HSV counterpart.[111]

Modifications in cell homeostasis upon herpesvirus infection include shutting off host anabolic reactions to facilitate the onset of viral biosynthesis, i.e. seizure of protein synthesis machinery to build capsids or activation of DNA replication in these host cells, whose cell cycle is arrested in G0 state. Moreover, modifications in the cell exerted by some herpesviruses are known also to promote the

immortalization of the host cell. Herpesviruses have genetically invested in proteins whose main function is to react to the host cell innate defenses and host immunity, in order to pursue viral survival in an hostile environment.[112-114] For example, herpesviruses block apoptosis or induction of interferons at cellular level, while in the host organism they could interfere with antigen presentation, sequester antibodies (gE/gI, HSV) or even mimic immuno modulator proteins (IL10, HCMV). These viral strategies delay the development of an immune or cellular response in the host. Herpesviruses take advantage of this delay to efficiently infect the host and to shortly establish life-long latency. The fact that herpesviruses are hardly lethal testifies that they establish fine interactions with their hosts (i.e. latency), in order to gain the maximum spreading capability inside the host population. In particular, alphaherpesviruses are different from other herpesviruses, because they sequester and modify cellular proteins instead of expressing cellular orthologs, whose aim is to mislead the host. In the case of HSV, the host protein phosphatase 1 α is sequestered by the viral protein gamma 34.5.

Herpes DNA resides in the cell nucleus of infected cells as an episome, and its transcription depends on its state of chromatinization, namely if promoters are accessible to RNA polymerase II. The state of chromatinization depends on the cells, which sequester viral DNA in PML bodies to prevent viral transcription; to counteract this function, HSV encodes the ICP0 transactivator, whose manifold functions include an E3 Ubiquitin ligase activity specifically directed to PML (see below).

Promyelocytic Leukemia Protein Nuclear Bodies (PML-NBS), termed also ND10 bodies, are upregulated by IFNs and exert a cytoprotective function in the host, sequestering aberrant proteins or newly assembled viral nucleocapsids.[115] HSV ICP0 targets PML for degradation to prevent restriction of gene expression, while VZV ORF61, the ICP0 homolog, employs small ubiquitin like modifier (SUMO) to disperse PML-NBS.[116]

In the case of HSV, the onset of lytic infection encompasses a sequential derepression of specific heterochromatinized alpha genes that, in turn, favor the transcription of beta genes, while in the case of reactivation from latency, the genome derepression process seems to be not specific.[117]

All herpesviruses encase a group of 41 evolutionarily conserved genes, termed core genes, that are found into six blocks. These gene blocks show conserved order and polarity, but are distributed in manifold combinations of order and polarity among herpes genomes.[118,119] The pattern of these conserved gene blocks is similar at subfamily level (i.e. between alphaherpesviruses), while gene sequence conservation is generally poor. Inside alphaherpesviruses, evolutionarily conserved genes are the tropism factor gD (except for VZV), the tegument trans inducing factor (alpha-TIF) -which induces

transcription of immediate early genes-, and the transcriptional regulator ICP4. The origin binding protein (OBP) is conserved only in alpha and partly in beta families of herpesviruses.[120] OBP function is to allow aggregation of the viral replication machinery around its binding site on viral DNA. During evolution, herpesviruses acquired at least one gene from host source, that is thymidylate synthase in the case of alphaherpesviruses, while KSHV inherited 12 host genes, with obvious implications for oncogenicity in this latter case.[121,122] To acquire genes, retroviruses should coinfect an herpervirus host; indeed, acquired genes are encoded by what resembles a single ORFs cDNA. Acquired genes localize in the genomes termini or between blocks of conserved genes. Herpesviruses might acquire these pieces of host DNA after speciation, namely by independent retrotrasposition events. In the case of KSHV, the viral encoded type D cyclin gene had undergone modifications in contrast to the one encoded by the host, being constitutively activated and promoting transformation of the infected cells.[123]

### 1.4.1 Biological cycle of Herpesviruses: Initiation of Infection

The biological cycle of Herpesviruses starts with initiation of infection and continues either with lytic replication or with latency. In those cells, where the virus is already latent, it can only proceed in lytic infection. These processes are influenced only by the virus and depend on the availability of specific factors inside the host cell. Initiation of infection is, for the virus, the period between the encounter with viral cognate receptor on the host cell surface and the onset of viral transcription. Virion envelope glycoproteins orchestrate the cell entry, sequentially interacting with each other and with host surface receptors. Initial tethering is provided by gC and gB binding to host heparan sulphate (HS) moieties. HS is a glycosaminoglycan related to heparin, a sulfated polymer of both disaccharides glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc), that is present on the membrane surface of mammal cells. Heparan sulphate increases HSV infectivity in a reversible and non-essential manner, as acidic detached virions maintain their infectivity. The state of the art of the alphaherpesvirus cell entry is described in detail in the dedicated section. Briefly, the conserved herpesvirus glycoprotein trio is composed by gB, which provides first attachment, and gH/gL, which activates gB to exert its fusogenic capabilities. Host cellular receptors respond to virion binding. Namely, they could promote endocytosis (i.e. integrins) or trigger molecular pathways, which alter the host cellular homeostasis (i.e. TLRs). The host cytoskeleton represents the first barrier for the viral capsid, preventing viral transition to the

nuclear pore. Cytoplasm macromolecular density constitutes a second barrier to viral capsid diffusion. Nucleocapsids tether themselves to dynein microtubular motor with centripetal direction to translocate near the nucleus. Once in proximity of the nuclear pore, intrinsic pressure and ATP consumption drive the ejection of nucleocapsid genetic material inside the nucleus.[54] The genome becomes immediately chromatinized inside the nucleus; at this level, the availability of host factors lets the virus enter the lytic or latency cycle.[124] Tegument proteins released in the cytoplasm contribute to the host defense blockade and activation of immediate early genes.

### 1.4.2 HSV Lytic Replication

Lytic replication consists in the synthesis of viral DNA, DNA encapsidation and virion envelopment; namely, the production of viral infectious particles, exhaustion and destruction of the host cell. Lytic replication encompasses a cascade expression of viral genes, synthesis of viral DNA, assembly of viral capsids and egress. HSV induces compartmentalization of existing host cellular districts.

Herpesvirus protein expression follows a precisely timed program, made of immediate early (alpha), early (beta) and late (gamma) genes, which is necessarily synchronized with viral DNA replication. In particular, alpha genes require no viral protein synthesis once inside the host, beta genes require no DNA synthesis to be expressed while gamma genes expression depends on DNA synthesis. In particular, gamma 1 (leaky-late) gene transcription increases proportionally to viral DNA synthesis while gamma 2 (true-late) genes totally depends on viral DNA synthesis for transcription.

Transcription, genome replication and capsid assembly take place in separate zones inside the nucleus. Immediate early genes encode for regulatory proteins, capable of activating transcription of early genes, shuttling their RNA to the host cytoplasm, altering the cell cycle and the chromatin structure. Early genes encode for viral DNA synthesis machinery, namely the genes that constitute the replicative fork, and enzymes that enrich the host nucleotide pool, as i.e. tymidilate synthase (HSV). Late genes encode for capsid, tegument and envelope proteins.

HSV alpha genes are generally transcribed from near the termini of the genome, while ICP0 and ICP4 immediate early genes are encoded into genome inverted repeats (thus are present in more than one copy). The tegument protein VP16 (alpha TIF) promotes gene transcription in the nucleus and it is detectable between 2 and 4 h post infection. The role of VP16 in the host nucleus is to promote the

onset of transcription from immediate early promoters, characterized by the GnTAATGArTTC response element. VP16 interacts with octamer binding protein 1 (Oct1) and host cell factor (HCF1). The ternary complex VP16/Oct1/HCF1 recruits lysine specific demethylase 1 (LSD1), which is part of the CoREST/REST complex, to modify the epigenetic state of the viral genome, thus allowing ensuing of the viral transcription.[125]

In HSV there are six immediate early proteins, US1.5 and those termed infected cell protein (ICP) 0, ICP4, ICP22, ICP27, ICP47. These alpha proteins regulate their own expression, promote transcription of beta genes and could be important to drive the transition between beta and gamma gene expression.

In particular, alpha proteins provide inhibition of various host cell functions, i.e., transcription, mRNA splicing, mRNA transportation, protein synthesis and immune response. ICP34.5 is located in the repeated $U_L$ termini, while the other beta and gamma genes are located throughout the HSV genome.

The Alpha protein ICP4 is required for HSV beta gene expression, and these genes present an onset of 4 to 8 h post infection. Beta genes encode for enzymes involved in the nucleic acid metabolism, important for genome replication and nucleotide pool maintenance. Beta genes are divided into two groups: genes which belong to the first group, as ICP8, UL29, ICP6 and UL39, are transcribed together with alpha genes. Beta genes belonging to the second group, as UL23 (the viral tymidine kinase), are transcribed later (these include UL30, UL42, UL52, UL9, UL8 and UL5). Alpha and beta genes allow transcription of gamma genes, which in turn require genome synthesis for expression; late gamma genes instead repress transcription of alpha and beta genes. Gamma genes encode structural proteins, i.e. the constituents of the capsid or the tegument; examples are ICP5, gB, gD, ICP34.5 and UL44, gC, UL41, UL36, UL38 and UL11.

### 1.4.3 VZV Lytic Replication

VZV accomplishes its replicative cycle and could be detected into the extracellular matrix at 12 h post infection *in vitro*, while mature enveloped virions are formerly detectable at 9 h post infection. VZV *in vitro* and *in vivo* replication in skin fibroblasts leads to the formation of syncytia, namely multinucleated cells that result from the fusion of multiple cells. Syncytia formation is not essential for infection. Syncytia induced by VZV infection appear at 9 h post infection in melanoma cell culture; within 48 h the cell fusion involves 75% of the cell monolayer, while after 60 h syncytia detach from

the growing surface.[126] VZV gB/gH/gL are essential for syncytia formation, a process that also depends on a functional gE/gI heterodimer *in vivo*, and requires gE N-terminus.[127-129] Syncytia show enlarged nuclei with a modified structure, with marginated chromatin and abnormal nucleoli. VZV deals with the formation of syncytia by setting up "viral highways" on the host cell membrane, that involve filopodia, used by the cell associated virion to circulate efficiently towards other susceptible cells, increasing the overall spread.[130-132]

Upon entry in the host cell, VZV tegument proteins reach the cytoplasm, where they exert modulatory roles on the host (ORF 10) and they subvert protein synthesis machinery (ORF 47 and ORF 66 kinases). VZV tegument proteins might enter into the nucleus together with the viral DNA, so as to activate a specific transcriptional program (ORFs 4, 62 and 63).[68,69] In particular, ORF 62 activates the transcription of its own gene IE 62 and of the most other viral genes, including ORF 61, which is not a tegument protein.[126] The transcriptional cascade in VZV was not further established because of the difficulties in synchronizing the infection, due to the low titer of cell free VZV virions harvested from *in vitro* replication .

VZV ORF 28 (DNA polymerase) and VZV ORF 29 (ssDNA binding protein) are transcribed from a bidirectional promoter with shared regulatory elements; these elements are transactivated by VZV IE62 interaction with host transcription factors as upstream stimulatory factor (USF).[133-135] Also, VZV ORFs 62 and 63 transcription is mediated by a bidirectional promoter.[136]

VZV promoters are influenced by host transcription factors as Sp1, USF and activating transcription factor (ATF). These cellular proteins interact with viral modulatory genes as ORF 61, ORF 62, ORF 63 and IE4. ORF 62 is the VZV transactivator required for DNA replication. The HSV homolog is ICP4; ICP4 HSV mutant can be complemented by VZV ORF 62 although only ICP4 can induce DNA bending.[137-139] VZV ORF 61 is expressed upon VZV entry and immediately localizes into infected cell nuclei; VZV ORF61 is the homolog of HSV ICP0. Both HSV ICP0 and VZV ORF 61 carry a RING finger domain to exert transactivation and dispersion of promyelocytic leukemia (PML) nuclear bodies.[140-143] VZV IE4 homolog in HSV is ICP27, but their functions do not overlap as VZV IE4 cannot complement an HSV1 mutant. Both HSV ICP27 and VZV IE4 carry putative ZINC finger domain to exert transactivation function, which in turn is dependent on dimerization. VZV IE4 can shuttle mRNA and its transactivating activity is modulated by ORF 61.[144-146] VZV IE63 localizes during infection in the nucleus where it colocalizes with IE62, and it is the homolog of HSV ICP22 (HSV US1.5 and US1 genes). IE63 binds to RNA polymerase II and to antisilencing protein 1.[147,148]

## 1.4.4 Genome Replication in Herpesviruses

Herpesviruses genome replication must be correctly timed with capsid assembly and rely on *cis* acting elements interspersed in the viral genome and on *trans* acting factors. The aim of *trans* acting factors is managing genome synthesis from a single viral template. Herpesviruses constantly manage nucleotide pools inside host cells, to provide substrate for their genome synthesis, typically by enhancing pyrimidine biosynthesis.[149] Other examples of host metabolism modifications include the introduction by HCMV of tricarboxylic acid (TCA) cycle shunts, whose aim is to provide a sufficient amount of reserve lipids for subsequent infection stages.[149-151] The genome of any herpesvirus has at least one *cis* acting site, termed oriLyt, employed for aggregation of the DNA replication machinery. Alphaherpesviruses use an origin binding protein (OBP), which provides modification to the DNA structure to allow nucleation of the other proteins involved in the replicative fork assembly. All the viral fork components belong to core genes and are thus evolutionarily conserved, they consist of a six gene array including a viral DNA polymerase (POL), a DNA polymerase processivity subunit (PPS), a single stranded DNA binding protein (SSB) and the helicase primase complex (HP1, HP2, and HP3). Herpesviruses genome replication is based on the rolling circle, that facilitates synchronization of continuous genome production by DNA replication with continuous capsid assembly and filling. As nucleocapsid is devoid of nucleosomes, the genome replication process should include mechanisms to prevent chromatinization.[124]

## 1.4.5 Genome Replication of HSV

Beta proteins are involved in replicating the viral DNA and their aggregation to the viral genome rapidly follows their translocation in the host nucleus. Beta proteins are also involved in pre-replicative sites formation, whose function is to provide a complete subset of enzymes to support DNA replication, as coactivators, corepressors histones and related modifying enzymes.[125]

HSV replication starts after alpha and beta gene expression and in particular with the migration of UL9 into the nucleus, together with early proteins. UL9 binds to $ori_L$ and $ori_S$ sequence in the viral genome

and unwinds the dsDNA. UL9 aggregates also the single strand DNA binding protein ICP8, whose presence finally recruits the complete HSV replication machinery on DNA replication forks. Genes involved in HSV DNA replication are encoded by UL5, UL8, UL52, UL30 and UL42. Initial theta replication switches to the rolling circle, that continuously forms concatemeric genomes. These head to tail linked genomes need to be cleaved into monomers, deprived of histones and packaged into the capsid.

### 1.4.6 Genome Replication of VZV

VZV DNA replication starts at 4h post infection, when ssDNA binding protein ORF29 and IE62 aggregate onto VZV DNA[126] VZV DNA polymerase is encoded by ORF28 while ORF16 is the VZV polimerase processivity factor.[9] VZV genome has no Ori sequence similar to HSV OriL, and contains duplicate OriS in the IRS and TRS.[152] VZV ORF51 is the Ori binding protein, homolog to HSV UL9; both bind to CGC triplet in the Oris sequence.[153] VZV ORF29 is the homolog of HSV ICP8, though it cannot substitute for HSV ICP8; VZV ORF29 exerts negative regulation on DNA replication by binding to Oris.[154]

### 1.4.7 Capsid Assembly in Herpesviruses

The translation of herpesvirus mRNA takes place inside defined areas of the host cell, i.e. viral glycoproteins ought to be translated in ribosomes associated with the endoplasmic reticulum (ER) and with trafficking from the ER to the Golgi *cisternae*, and finally decorate the trans Golgi network membranes. Other examples are capsid proteins, that need to be transported through nuclear pores to reach the capsid assembly location in the nucleus, while tegument proteins should be translated or routed to cytoplasm areas where maturating capsid would traverse. Capsid assembly takes advantage of viral encoded scaffold proteins, whose aim is to catalyze capsid formation and thus are not retained at the end of the process. Insertion of newly-synthetized viral DNA inside the capsids is an energy demanding process.[155] Ancillary proteins, contributing to the capsid assembly process are synthetized as a single precursor polyprotein (prePR) that contains an assembly protein precursor (pAP) and the

assembly protease (PR), which excises itself and contributes to maturation of the assembly protein (AP). Viral DNA fills the capsid until a sensing mechanism induce the cleavage of the genome in correspondence with the conserved *pac* sequence in genome termini. The sensing mechanism is provided by the portal capping protein (PCP) and other components of the portal like PORT, the terminases TER1 and TER2 and the TER binding protein (TERbp).

In HSV, VP5 is the major capsid protein, VP23 and VP26 form the triplex, pre VP22 is the scaffolding protein, and VP19C contributes to the process. Empty capsid is assembled onto a scaffolding protein that is removed simultaneously with genome insertion and cleavage. The intranuclear capsid measures 125 nm in diameter, which is not compatible for passage in an intact nuclear pore. VZV ORF40 is the major capsid protein, it is homolog to HSV UL19 and detectable in infected cell nuclei. VZV ORF 33 gene encode the precursor of a serine protease which is necessary for maturation of the gene product of ORF 33.5, the VZV scaffold protein; ORF33.5 sequence is included into ORF 33 gene.[156,157]

### 1.4.8 Egress of Herpesviruses

Three hypothesis are available to explain filled capsid nuclear egress. The luminal transport hypothesis envisions that the capsid is enveloped in the nuclear inner membrane and proceeds throughout host cellular membrane compartments, finally reaching the cell surface.[158-160] The luminal transport hypothesis collides with the fact that the nuclear inner membrane has a different composition in integral proteins as compared to virion mature envelope, and that viral tegument proteins are available prevalently only in the host cytoplasm. Another postulated mechanism best explains the acquirement of a stratified tegument and was termed envelopment-de-envelopement model. This latter envisions that nucleocapsid reaches the cytoplasm naked and then proceeds towards the cell membrane, sequentially acquiring soluble tegument proteins, which reside in the cytoplasm. In particular, this model suggests the envelopment of the capsid at the inner nuclear membrane and its subsequent de-envelopment at the outer nuclear membrane to reach cytoplasm. Another hypothesis implies a direct release from an enlarged nuclear pore or a destroyed nuclear membrane.[161] Nevertheless, the envelopment-de-envelopement model is the only model supported by ultrastructural data and genetic or biochemical proofs. The envelopment-de-envelopement model states that, inside the nucleus, nuclear egress membrane protein (NEMP) and nuclear egress lamina protein (NELP) are associated to the nucleocapsid, constituting a nuclear tegument. NEMP and NELP contribute, together with host kinases,

to the disaggregation of the nuclear lamina that fence the inner nuclear membrane, which, in the meanwhile, has received a limited subset of viral glycoproteins (presumably consisting in gB,gH/gL). Budding virion in intermembrane nuclear space thus acquires both an immature tegument and an immature envelope, but it carries functional NEMP and NELP, whose presence is restricted to virions budding from the inner nuclear membrane. Finally, these immature virions fuse their temporary (primary or nuclear) envelope with outer nuclear membrane to gain access to the host cell cytoplasm. Once inside the cytoplasm, non-enveloped nucleocapsids with a nascent tegument layer harvest other viral tegument proteins and mRNAs, and are finally targeted, via tethering to microtubules motors, to a cell membrane for release. In particular, target membrane belongs to an exit organelle, decorated with patches of viral encoded membrane proteins. Nascent virions bud into the organelle to gain their secondary envelopment and to gain access to the host cell exterior (virion release).[160] The interaction of tegument proteins with cytoplasmic tails of viral membrane proteins, distributed on target organelles surface, might be necessary to proceed with budding into the organelle. Budding organelles share the common feature of being targeted to the host cell membrane to release their content. The budding organelle is Trans Golgi Network (TGN) for HSV and the recycling endosomes for HCMV while HHV-6A buds into multivesicular bodies. In contrast, *in vitro* modeling of VZV egress is incomplete as VZV replication *in vivo* seems to differ from the one tested in cell culture. Principal VZV proteins involved in egress are ORF24 and ORF27, with similar functions to HSV UL31 and UL34. In general, glycoproteins are inserted into TGN membranes, with an inverted topology; this organization might recall tegument proteins from the cytoplasm, that finally tether the capsid for budding. VZV virions remain usually surrounded by vacuoles in the cell cytoplasm.

### 1.4.9 Latency

Latency is a mechanism typical of all the herpesviruses already discovered that allows for a lifelong persistence in particular tissues of the host which, in turn, might experience no protein expression until reactivation happens (i.e. HSV).

Herpes genome circularizes during latency, and expression in the host cell is restricted to a limited set of viral genes until reactivation takes place. During latency no herpesvirus progeny is produced, in contrast to other viral mechanisms like chronical, persistent or abortive infections. Reactivation from latency awaits a weakness period of the host immunological defenses, since in the immune host an

incoming lytic replication typically ends shortly, counteracted by an immune response onset. During HSV latency, epigenetic factors control latency associated transcription, and suppression of lytic infection inducing factors.[124] The host tissue where latent infection is established varies among herpesviruses; HSV resides in neurons of the dorsal root ganglia, while EBV resides in B lymphocytes. As the latent infection is a lifelong one, it might happen that the same herpervirus carry out both lytic and latent infection in the same host. In contrast, after the clearance of primary VZV or HSV infection, all the infected cells of the entire host present only latent infection. When herpesviruses manifest their pathological effect on an individual, due to the lytic replication, the virus remains latent in another host district to allow for subsequent reactivations.

VZV, in contrast to HSV, may access sensoria ganglia during primary infection by hematogenous route in addition to centripetal axonal transportation; molecular mechanisms of VZV latency are not well established yet.

During latency, HSV maintains its genome inside the neuron nucleus as a chromatinized and repressed episome, with no detectable viral protein expression and no VP16 transactivation. HSV encodes the latency associated transcripts (LATs) gene in its $U_L$ termini; the processing of this gene transcript originates a series of microRNAs that contribute to latency maintenance. In particular, LAT low copy transcript, termed minor LAT, is 8.3 kb long and is subjected to splicing, generating major LATs of 1,5kb or 2kb that accumulate inside the nucleus.[162]

### 1.5.1 *α*-herpesviruses entry summary

Herpesviruses use a wide range of host surface molecules to determine their tropism, that is the attitude to preferentially target specific hosts, which is typically improved in its accuracy by means of a sequential exploitation of different receptors on the same cells.[163] This is possible because herpesviruses evolved an exclusive multipartite entry apparatus, which is composed by a conserved glycoproteins trio, glycoprotein B (gB) and gH/gL, and which eventually include additional species-specific tropism factors proteins or domains.[164,165] Glycoproteins activation follows an interprotein signalling cascade prevalently among their ectodomains, that encase functions allowing entry into host cell. Fusion's functions are unevenly distributed among the entry glycoproteins, whose multifunctionality is conserved accross the herpesviruses family.[164]

At the beginning of infection, Herpesviruses generally establish aspecific tethering to host cell membrane by gB and gC that bind to heparan sulphate moieties. Aspecific membrane binding serves to concentrate virions to the host cell surface where entry receptors could be found.[164] gC is not essential *in vitro*, as is not the gB tethering activity, while *in vivo* HSV virion without gC suffers of an impaired binding.[166,167] Heparan sulphate and syndecans are involved in the HSV virion surfing phenomenon, since HSV virions drive the formation of filopodia before gD encounters its receptors.[168,169] The second step in infection is performed by the species-specific tropism factor, a viral membrane protein acquired throughout evolution, which allows the targeting of specific tissues. In α-herpesviruses, this passage is accomplished by gD binding to nectins, in the case of Herpes Simplex Virus (HSV), and Pseudorabies Virus (PrV, of swine origin) while in the case of Varicella Zoster Virus (VZV) the tropism factor is still elusive. HSV uses also Herpes Virus Entry Mediator (HVEM) to enter host cells. The third event in infection is the choice of a pathway of entry, which was recently elucidated in HSV; this step is not influenced by virion composition but is only dictated by the expression of specific routing factors on the membrane of host cells, i.e. integrins. Options for herpesviruses pathway of entry include the fusion of the virion envelope with plasma membrane or with endosome membrane.[170,171] In particular, fusion with the endosome membrane involves neutral or acidic endosomes, or macropinocytosis.[172-174] Integrins usually interact with gH/gL (HSV, HCMV, EBV) but also with gB (KSHV), and are widely used by Herpesviruses implying conserved functions. Integrins may be recruited as receptors or coreceptors either to trigger endocytosis or to better define tropism or even to exploit activation of integrin's downstream effectors. Finally, the fourth event is the merging of both virion and host cell membrane, referred to as fusion, which is undoubtedly an exclusively gB-driven process in HSV and in a gammaherpesvirus, Epstein-Barr Virus (EBV).[164,175] Integrins bind HSV gH/gL, promoting virion endocytosis.[176] gH/gL might limit the step rate fusion driven by gB to synchronize membrane fusion with endocytosis, which is a structured process.[176] Hence, HSV gH/gL are thought to act as an intermediate in the stepwise process that leads to fusion, while in PrV gH seems to act as a co-fusogen with gB.[164,176-180] PrV gH has a conserved mobile element, termed flap, which masks an important hydrophobic surface in the gH membrane proximal region; the flap could contribute to membrane destabilization when dislocated by a disulfide exchange in flap structural cysteines.[178]

Herpesviruses spread among different susceptible hosts and their success during evolution could be ultimately due to their promiscuous use of an adaptable protein like gH/gL. gH/gL seems to be the smart hub where a combination of signals (molecular interactions) converge and are fairly processed (eventually at a slow rate) to accurately trigger gB to execute fusion. gB is instead the conserved fusogen across the family. gB structure might be under evolutionary pressure prevalently to perform

fusion, but not to allow tropism expansion.[177] The evolution of herpesviruses might have invested in separating the functions needed for entry in different glycoproteins to gain the best result in terms of spreading and targeting efficiency. HSV is a well-fitting example, as function separation here reaches an higher level, with a distinct role for gD, as a tropism factor, and gH/gL as the regulator of gB fusogenic activity.[180]

### 1.5.2 Overview of HSV fusion

The current model for HSV glycoproteins activation cascade, which culminates in fusion and host cell entry, envisions that the tropism factor gD binds Herpes Virus Entry Mediator (HVEM) or nectin1. These receptors dislocate the so called profusion domain in gD, allowing fusion triggering and execution, functions subsequently exerted respectively by gH/gL and gB.[181] In particular, gH/gL conformation is supposed to change in order to trigger fusion via gB, which is the structurally conserved fusion executor.[164,179,182-185] The step accomplished by gD is species specific and is the best established part of HSV entry mechanism while subsequent steps involving HSV gH/gL and gB are still elusive. In fact all human herpesviruses employ tropism factors different from HSV gD, while their core fusion machinery, formed by gB and gH/gL, strikingly resembles the same. gB and gH/gL should have evolved both to perform similar fusion functions and to respond to different host receptors, depending on the type of herpesvirus.

In HSV, it was demonstrated that if gD is present and bound to one of its receptors, gH/gL interacts with gB only.[186,187] Neutralizing MAbs were used to investigate the time course of the interaction cascade among HSV glycoproteins. In particular, MAbs that block gH/gL interaction with gB, as for example LP11, also block fusion.[188-193] Few MAbs to gB, targeting gB fusion loops or whose epitopes reside in gB N-terminus, blocked only fusion without blocking gH/gL interaction with gB.[194,195] These results support the view that HSV gH/gL would not function in fusion but instead will serve as an upregulator of gB fusion activity. This implies a sequential recruitment and activation of the HSV membrane glycoproteins.[180]

To further establish the role of gH/gL as a fusion positive regulator of gB, *in trans* fusion was demonstrated, proving that gH/gL does not act as a co-fusogen in Cyto Megalo Virus (CMV), a β-herpesvirus.[196] In fact, the combination of glycoprotein expression in a co-seeding approach

demonstrates that gH/gL does not have to be in the same membrane of gB to carry out fusion via gB. Cell-cell fusion *in trans* (yet at a lower level than *in cis*) was observed also in HSV using receptor-positive cells transiently expressing gH/gL, co-seeded with receptor negative cells expressing gD and gB[87]. Fusion was measured also if cells positive for HSV receptor expressing gH/gL were challenged with soluble gD and mixed to gB bearing receptor negative cells. On the contrary, no fusion was observed if receptor positive cells expressing HSV gB were incubated with soluble gD and co-seeded with receptor negative gH/gL bearing cells. This demonstrates that HSV gB is the only glycoprotein that must be inserted into a membrane to induce HSV membrane fusion mediated by gB, gH/gL and gD.[197]

Complexes of gB-gH/gL were isolated from lysates of cells infected with HSV or cells expressing HSV glycoproteins.[181,198] Interaction between gD and gH/gL is not stable, if a gD-gH/gL complex is stabilized it does not allow gB mediated fusion unless it is further dissociated.[186] If gD and gH/gL C-terminal tails were fused with splitted EYFP to gain a gD-gH/gL stable complex, fusion is hampered when gB and a host receptor become present together.[187] Another parameter further affecting HSV fusion/entry is the sequential engagement of cellular receptors by gB and gH/gL. In particular, investigations are needed to identify changes in conformation happening to activated HSV gH/gL upon integrin binding. These modifications in gH/gL allow interaction of activated gH/gL with gB, triggering membrane fusion.

### 1.5.3 HSV gD and its Host receptors

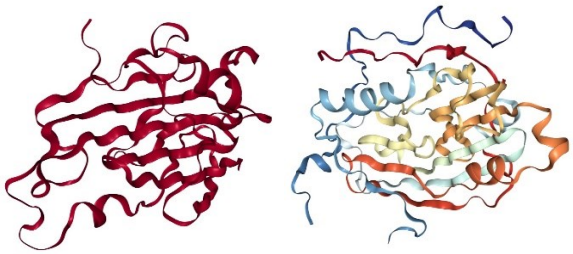


**Figure B. Protein structure of HSV gD ectodomain.** On the left HSV gD is oriented to enlight its IgV shaped core. On the right gD is depicted in rainbow colours and oriented to enlight the proximity between its N-(blue) and C-(red) terminus. Crystal structurea are from PDB (4MYV).

HSV gD has three different disulfide bonds in its structure and 3 glycosylation sites. The unbound gD has 21 N-terminal flexible residues and the transmembrane region (TMR) is located at amino acid position 341 – 361.[199] gD encompasses a V-like immunoglobulin (IgV) core which acts as a scaffold for N- and C-terminal extensions (figure B).[199] . A soluble form of gD, or a full length gD transiently expressed in cells permissive for HSV, blocked HSV entry. This phenomenon, termed "restriction", is typical of receptor proteins, and implies that gD is the HSV glycoprotein which binds host entry receptor.

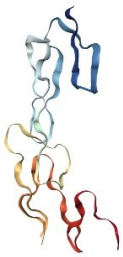


**Figure C. Protein structure of Herpes Virus Entry Mediator (HVEM)**. HVEM ectodomain is depicted in rainbow colours. HVEM can mediate the entry of HSV via gD. Crystal structure is from PDB (4FHQ).

One of the established host receptors for HSV gD is Herpes Virus Entry Mediator (HVEM), a Tumor Necrosis Factor Receptor (TNFR)-like molecule, typically expressed by immune cells (as lymphocytes), originally known as TNFRSF14 (figure C).[179] HSV cannot infect T cells *in vivo*, but it may modify their response to infection via HVEM to gain a better spread. Indeed, gD binds HVEM in the same site for B and T Lymphocyte Attenuator (BTLA), a ligand shown to reduce HVEM surface expression. Another established function of gD is to prevent host monocytic cell apoptosis by interfering with FAS receptor activity in an HVEM dependent manner. The structure of the first 285 of 394 residues of gD bound to HVEM show site-specific protein interactions.[200] The gD N- terminus is not structured when gD is unbound, but if gD binds to HVEM, the gD N-terminus becomes stable with an hairpin-shape.[199-201] The gD/HVEM hairpin accommodates Tyr23 of HVEM in a pocket, and is bent at position 21 of gD. gD even forms an intermolecular β-sheet with two cysteine rich domains (CRD) of HVEM.[201,202]

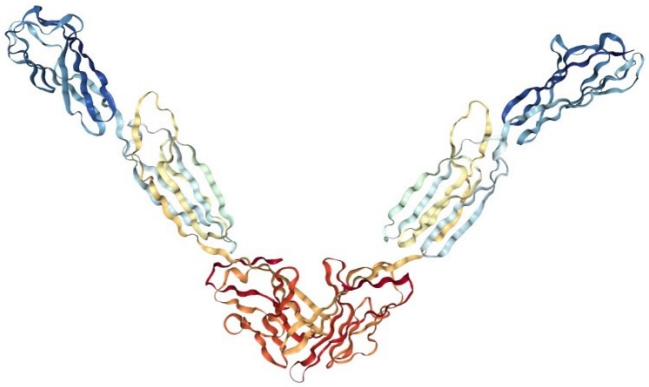


**Figure D. Protein structure of a nectin1 dimer.** Nectin1 dimer is depicted in rainbow colours. Immunoglobulin-like modular structures proper of the nectin1 ectodomain are noticeable. nectin1 can mediate the entry of HSV via gD. Crystal structure is from PDB (4FMF).

The principal receptor for HSV is nectin1, as shown by means of MAbs alternatively directed to nectin1 or HVEM.[203] Nectin1 is a membrane protein belonging to the immunoglobulin superfamily, which is expressed in neurons and epithelia.[204] Nectins contribute to form adherens junction. Mutagenesis and epitope mapping show that nectin1 contacts HSV gD via its distal V-domain, which encompasses Ig-like structure (figure D).[204-206] The binding of this nectin1 domain to gD leads to the same affinity of the full nectin1 ectodomain (figure E).[207] Epitope mapping also shows that nectin1 binds gD to a different site in respect to HVEM. To confirm these data, the mutant gD (A3C-Y38C) was engineered in a way to form a hairpin at gD N-terminus with a disulfide bond.[208-210] the N-terminus flexible domain of gD (A3C-Y38C) mutant is blocked; this mutation avoids binding to HVEM, but not nectin1.[209] Other mutations in gD 285t ectodomain, which affect only Nectin binding, excluded the existence of a common conformational change in gD exerted by HVEM or nectin1. It was shown that gD accommodates nectin1 Phe129 into a pocket of its IgV-like core.[211,212]

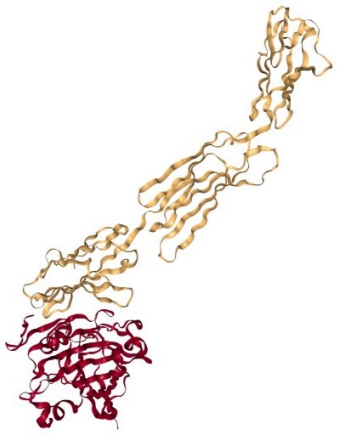


**Figure E. Ectodomain of a nectin1 monomer (yellow) is depicted while interacting with HSV gD (red).** Nectin1 contacts HSV gD by only the terminal part of its ectopic domain. Crystal structures are from PDB (4MYW).

Crystal structure of unbound gD, until amino acid 306, shows that residues Pro 291 and Trp 291 in gD C-terminal domain are inserted in a pocket made of N-terminal residue and gD Ig-like core.[199] $gD306_t$ has a lower affinity for its receptors, arguing that residues between 285 and 306 hinder binding to HVEM or nectin1, accomplishing gD auto inhibition.[205,213,214] It was already known that gD C- and N-terminals were close to each other, before binding to receptors, as proven by MAb AP7 conformational epitope (figure B).[215,216] After receptor binding, the C-terminal gD domain is dislodged form the N-terminal. For example, upon nectin1 binding to gD, Phe129 of nectin1 inserts inside gD pocket and dislodges the gD C-terminal, probably because it has a higher affinity; a similar dislocation might happen when gD binds HVEM. Mutation of nectin1 Phe129 to Leu or Ala prevents binding to gD, while mutation of gD Trp294 enhance gD affinity for its receptors but prevents fusion triggering.[217,218] Finally, another region can be found in gD ectodomain (termed profusion domain), which is important in driving fusion and virus entry, rather than receptor recognition.[219] Soluble mutant form of gD, carrying ablated cytoplasmic tail and this particular domain, were capable of rescuing $gD^{-/-}$ virion infectivity.[219-221] Also $gD260_t$ has a higher affinity for receptor in respect to $gD306_t$, but $gD285_t$ exhibits the highest affinity for receptors ($10^{-8}$M).[205,213,214]

These differences in receptors affinity could be explained as follows: to interact with its receptor, $gD306_t$ needs to dislodge the C-terminal residues (269-307), which are partially absent in $gD285_t$.[222] In this section, C-terminal domain is referred to as the C-terminal part of gD ectodomain, as the real gD C-terminal domain leans inside the virion. Crystal structure of the gD dimer shows that 269-306 C-

terminal residues wrap around the core of the gD monomer, reaching its N-terminal region (1-22), which forms the flexible stretch. As it happens for residues 1-22, also residues 260-268 are not totally solved due to their intrinsic mobility, constituting a flexible hinge. In order to better understand gD C-terminal region, a soluble mutant gD306$_t$307Cys was engineered. In this gD mutant, the C-terminal is locked by an intermolecular disuphide bond that allows the formation of a gD dimer.[199] As gD C-terminal hampers binding of its receptors, also the locked C-terminal in gD306t307Cys mutant cannot bind any receptor. The hiding of gD binding sites to its receptors may aid the virus in saving its fusogenic potential inside the host, until the correct receptor is recognized. The gD mutant in K190C-A277C is neither functional in cell-cell fusion, nor in gD $^{-/-}$ virion rescue, but it still bind receptors.[223] Features of this latter gD mutant imply that the ability of gD to trigger fusion is separable from its receptor interacting function. This aspect of HSV gD tropism factor allowed the creation of a retargeting strategy for oncolytics purpose. MAb MC2 mimics mutant gD (K190C-A277C) in the fact that it is a neutralizing MAb, which blocks infection, but it still allows (and even increases) interaction of gD with its receptors.[224]

gD has four Functional Regions (FR) identified by generation of linker insertion mutants and targeted by neutralizing MAbs. FR1, residues 27-43, is at the N-terminus of gD and it is involved in HVEM induced hairpin. FR2, residues 125-161 and FR3, residues 225-246, correspond to the gD Ig-like core. FR4, residues 277-310, contains the pro fusion domain. FR1 and FR3 of gD elicit also neutralizing MAb, which prevent binding of gD with its receptors.[216,225,226] FR4 spans gD C-terminal and bears some epitope targeted by non-neutralizing MAbs, which promote gD C-terminal dislocation, increasing affinity for receptors. The region between FR3 and FR4 is targeted by MC2 MAb (epitope 234-250), and it is thought to contain residues capable of triggering fusion via gH/gL or gB.[224] Again, by exploiting HSV gD mutation it was possible to define a gH/gL interacting region on gD, located between residues 260-310 (the profusion domain), while both 240-260 and 304-305 of HSV gD were demonstrated to interact with gB.[164,181] Finally, gD residues 7–32 are required to promote gD activation by the host receptor 3-O-sulphated heparan sulphate, whose role in HSV infection seems to be marginal in respect to nectin1 or HVEM.[226-228]

gD mechanism of action is interesting in oncolytic-HSV (o-HSV). In fact, the finely tuned model described so far seems to be less strict in defining structural requirement for fusion triggering and targeted tumor cell entry. In particular, o-HSVs, genetically modified to interact with specific receptors overexpressed on tumor surfaces, behave like the wild-type HSV during infection whereas their tropism factor structure is totally unrestricted[3]. For example, it is possible to introduce in position 61–

218 of gD a heterologous ligand for oncolytic purpose with comparable dimensions to that of the same gD. Other examples carry a single-chain Antibody (ScFv) between position 24 and 25 or inside the deletion 1–32 or 6–38, which allows detargeting from natural receptors.[165] These chimeric glycoproteins retain activities in spread and infection that are comparable to that of the wild type HSV, while this result strikingly contrasts the structural data so far available. In particular, insertion in the structure of gD should interfere with gD auto-inhibition mechanism, as gD C-terminal domain might constitutively fail to find its auto-inhibiting grove on gD.[165] This leads to the hypothesis that oncolytic mutant form of gD would be able to activate fusion independently from profusion domain dislocation. Thus, it seems as if the profusion domain continuously alternates interactions with gH/gL or gB, until the encounter with a gD receptor stimulates fusion. Fusion might occur as elucidated in measles fusion machinery (constituted by Hemoagglutinin-Neuroaminidase and Fusion glycoproteins), i.e., gD would be triggered by an artificial or natural receptor and thus become capable of signal transmission to gB, only if the receptor binds gD when gD profusion domain is transiently bound to gH/gL.[165] The tropism factor function ascribed to gD will be then bring together all the entry components, continuously alternating interactions among them to maintain the glycoproteins ready to release their fusogenic potential upon receptor binding. Subsequent events triggered by gD might encompass stabilization or dissociation of a glycoprotein complex, or change in host interactors engagements.[165]

### 1.5.4 HSV gH/gL

All the herpesviruses so far identified made use of an evolutionarily conserved glycoproteins trio made of the trimer gB and the heterodimer gH/gL as fusion machinery. HSV gH has 838 residues and bears 7 glycosylation sites, its single TMR is located at 804–824 positions. HSV gL is a soluble protein of 224 residues, with only one glycosylation site. HSV-2 gH has seven cysteines, while HSV-1 has eight cysteines. The numbering scheme presented in the HSV-2 gH structure derives from that of HSV-1 gH, but lacks cystein 3 (C3). HSV-2 gH displays three disulfide bonds, in particular cysteines are paired as C2-C4, C5-C6 and C7-C8, which is in agreement with previous genetic studies.[229] α-herpesvirus share conserved C2-C4 and C5-C6 disulfide bonds; mutations in these cysteines cause incorrect conformation of gH/gL.[229] In general, in α-herpesviruses C8 is conserved while C7 has a different position in PrV gH. HSV-2 gH N-terminal appears to be less structured than that of EBV (a

gammaherpesvirus), because of its higher Pro/Gly content (18% in respect to 13%), as Pro/Gly contribute to the formation of disordered regions.[178]

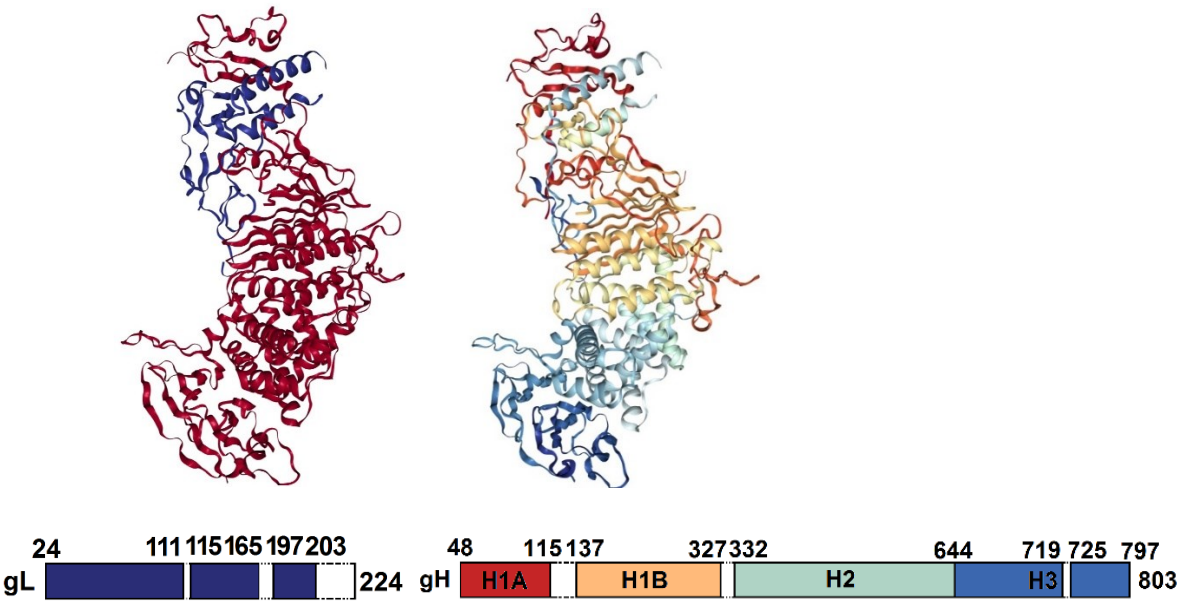



**Figure F. Protein structures of HSV gH ectodomain and gL.** Left: gH ectodomain (red) interacting with gL (blue) is depicted, forming the HSV gH/gL heterodimer. Right: HSV gH/gL heterodimer is depicted in rainbow colors, gH H3 domain (blue) forms membrane proximal domain. Crystal structures are from PDB (3M1C). Schematics provide an overview of ectopic domain arrangements.

HSV gH and gL are found in virion or infected cells at 1:1 stoichiometry ratio and they elicit neutralizing antibodies.[230] Both gH and gL have signal peptide, gL remains tethered to gH to facilitate folding and trafficking of gH in the secretory pathway and to decorate virion envelope.[231] gL reaches the secretion pathway if expressed alone, partly retained and partly secreted from the cell.[232] If gL is absent gH is retained in the endoplasmic reticulum with an improper conformation.[233-235] gH interacts with gL through residues 259 – 323, while gL interacts with gH through residues 20-161, which represents almost the whole gL protein.[188,236]

Unexpectedly, HSV-2 gH/gL crystal structure shows that this conserved heterodimer does not resemble any known structure (figure F).[188] As gH neither resembles any known structure nor forms a trimer, it is not classifiable into any class of fusion proteins. Moreover, gH/gL has no structural homolog in any of the fusion domains described before.[188,237] In particular, the refined model was provided for residues Arg49–Pro797 of gH and Thr24–Asn203 of gL, based on structure from Gly48 to Pro803 of gH in

complex with residues Gly20-Asn224 of gL. Positions His19–Thr47 of gH were ablated to obtain diffraction-quality crystals, these residues did not seem to be involved in viral entry or cell-cell fusion.[238] Disordered loops in gH (Gly116–Pro136, Thr328–Asp331 and Arg720–Arg724) and in gL (Phe112–Ala114 and Leu166–Pro196) were missing from this structure.

Crystal structure shows that HSV-2 gH/gL heterodimer is 8 nm height and 7 nm length and boot-shaped; hence, the gH/gL heterodimer is larger than gD but smaller than the gB trimer. Cryo-electron tomography showed angled glycoprotein spikes emerging from virion surface, attributable to gH/gL.[60]

In HSV gH three different structural domains were identified: H1, H2 and H3.[188] These domains were divided into five distinct domains, considering their intrinsic properties; H1A: 19–120, H1B: 121–331, H2A: 332–441, H2B: 442–645, H3: 646–797.[188] Sequence conservation among herpesviruses gH increases from domain H1 to domain H3, as only 30% of H1 residues display secondary structure. H2 and H3 are made of hydrophobic sequences that seem to be folded independently from H1 and are well conserved across herpesviruses. These domains are speculated to give support in fusion.[164] In particular, domain H3 is very sensitive to changes; most of the insertions or point mutations identified so far in this domain disrupt cell-cell fusion, interfering with gH structure.[229,239]

Several anti-HSV neutralizing MAb target H1 domain, part of these also inhibit spreading; mutations in H1 domain often abolish cell-cell fusion.[233,236,240] The N-terminal part of gH is clamped by gL with extensive interactions to gain proper stability of either the components of the heterodimer. N-terminus of gH lacks a stable folded core, in the absence of gL only a six-stranded β-sheet in gH may account for the improper residual folding of HSV gH N-terminus.[235,238]

HSV gH residues Arg49–Leu115, from domain H1A, form a β-hairpin including a six-stranded mixed β-sheet plus three short α-helices (α1-α3). The mixed β-sheet is contributed by both gH with β2-1 strands and gL with four strands (Lβ4-5-6-2). A linker made of residues Gly116–Pro136 in gH connects subdomains H1A and H1B. Residues Ala137–Pro327 belong to H1B domain, arranged into a six-stranded antiparallel β-sheet (β12-6-7-8-9-10), where strand β-12 belongs to domain H2. This β-sheet climbs the central bundle of helices of domain H2 and acts as a diaphragm separating a bulk made by H2-H3 domains to the above domain H1 and gL. H1B C-terminus contains also two short α-helices, α4 and α5 as well as both a two-stranded and a three-stranded antiparallel β-sheet (β4-11 and β3-5-6); this ensemble resembled a fence.[241] Residues Arg176–Thr230 wrap around the bottom of gH–gL to constrain domain H1 to domain H2 without contacting other residues in H1. H2 forms the central part of gH and is a globular domain, made of 13 α-helices (α6–α21), which also contribute with its

strand β-12 to β-sheets within H1B domain. H2A domain encloses a helical bundle subdomain consisting of three antiparallel helices displaying a Syntaxin-Like Bundle (SLB) folding. The helical disposition in domain H2B resembling that of HEAT repeats, a kind of helix-turn-helix folding.[178,242]

The HSV-2 diaphragm is forced to assume a peculiar planar conformation instead of the typical β-sheets right-handed twist by the SLB.[178] The SLB includes helices α7–α9 in HSV-2 gH and α2–α4 in PrV gH, and both structures resembled the N-terminal domain of syntaxins 1 and 6. Syntaxin 1 mediates intracellular fusion as part of the SNARE complex, its Habc domain is adjacent to a helical bundle which resembled the helical bundle inside gH, next to the SLB subdomain.[178] Conversely to gH, upon triggering, Habc domain oligomerizes to exert SNARE mediated membrane fusion. The fence and the SLB are separated in distinct domains in HSV-2 gH/gL structure.[188] A helix termed α1 impinges both on the SLB and the fence, and it is thought to impose specific angles to the whole gH molecules. α1 helix length might contribute to shape a toe-like gH/gL in HSV-2 or VZV. Interestingly, the SLB domain in HSV-2 gH contains an RGD integrin-interacting motif and it was speculated that integrin interaction might trigger a conformational modification in gH through the SLB, leading gH to the release of the energy needed for gB destabilization.[178,243] In HSV-2, the long α11 helix forms the "heel" of gH/gL, again altering the overall packaging of gH domains towards a boot-shaped conformation, in contrast with PrV gH whose α11 helix is 20 residues shorter.[178,188]

The C-terminal domain H3 forms the membrane proximal base of the ectodomain and is made of 5-stranded β-sheets in a 10-stranded β-sandwich configuration in HSV-2. H3 domain represents the tip of the boot-shaped heterodimer and gH TMR originates from one loop, which protrudes from the margins of the β-sandwich configuration. One β-sheet interacts with domain H2 of HSV-2 gH/gL. The five β-sheets in terminal H3 domain of HSV-2 gH/gL are not perpendicular to the membrane because of the toe-shape of HSV-2 gH/gL. H3 domain of HSV-2 gH exposes extended loops on its edges, two of them are connected by a disulfide bond (C7-C8). In PRV one loop was termed flap, as it masks a hydrophobic patch on the lower surface of gH.[178] The flap connects the two β-sandwich edges of the PRV gH domain IV, leaning on a charged surface spanning from Asn542 to Cys573 between strands β9 and β10. In PrV, two conserved disulfide bonds constrain both the ends of the flap, giving structural contributions to domain IV, eventually supporting a change in PrV flap orientation through disulfide bond reshuffling. The surface created by the membrane proximal domain of α-herpesvirus gH is highly hydrophobic but shielded by the flap, which is an evolutionarily conserved element. Flap dislocation, due to conformational changes that ensue in PrV gH during its activation, was speculated to promote

fusion in PrV.[178] A similar process might happen also in HSV-2 gH/gL, as it displays a similar surface.[178]

The presence of the flap and the SLB as conserved elements of herpesviral gH leads to speculations based on analogies with synaptotagmins interaction with SNARE host fusion proteins.[244-246] Synaptotagmins contain a mobile element, "C2" domain, that upon $Ca^{2+}$ triggering impinges on membranes. This event gives energy contribution to SNARE mediated fusion, providing the necessary membrane curvature, a role which might be exerted by the PrV flap. In particular, flap displacement might induce membrane destabilization, when the unmasked hydrophobic patch underneath the flap exerts repulsive interaction with the surroundings. Alternatively, the hydrophobic surface buried by the flap would interact directly with membranes when the flap dislocates. To this purpose, in VZV, increasing hydrophobic score of the surface underneath the flap rescued fusion impairment due to mutations in neighboring residues.[129,247]

HSV gL is required for thecorrect folding of gH, its trafficking and surface expression.[231,235,236] Anyway, as it remains associated to gH and is necessary for gH to function, gL role resembles the one of a scaffolding protein.[188,231,236] Structurally, gL does not have a stable core, as only 30% of residues have a secondary structure. gL is neither conserved among herpesviruses nor swappable among its members apart for HSV-1 and HSV-2.[229,248]

HSV gL forms three α-helices (Lα1, Lα2 and Lα3) and two β-sheets on its own and contributes with four strands to a six-stranded antiparallel β-sheet in gH domain H1A, forming a V-shaped groove.[188] Together with gH, gL forms also a short three-stranded mixed β-sheet including Lβ3-Lβ1-Lβ7. Both HSV-1 and HSV-2 gL display two disulfide bonds, encompassing four cysteines termed LC1- LC4, implicated in correct folding and function of the heterodimer.[229] Crystal structure of gH/gL is lacking gL residues Leu166–Asn224, a sequence known to reduce HSV-2 or HSV-1 gH/gL surface expression but not cell-cell fusion.[249] These residues are also absent from other α-herpesviruses as PrV and VZV. Crystal structure shows that whole gL interacts exclusively with gH domain H1, covering a hydrophobic surface area of 7,326.8 $Å^2$.[188,233,250] The interacting surfaces between gL and gH integrate seamlessly, such that they clearly need each other to co-fold.

Once bound to gH, gL displays a chemokine fold typical of CC and CXC chemokines, formed by a three-stranded β-sheet and an α-helix, stabilized by a single disulfide bond.[251] Similarities with chemokines and gH/gL include the fact that chemokines dimerization is often achieved by formation

of an intermolecular β-sheet, i.e. as it happens with IL8 homodimer. Provided that mammalian cells secrete HSV gL in the absence of gH, it is possible that gL could function as a chemokine.[232]

In fusion proteins, a lipophilic sequence of residue could take part in fusion peptide formation, but it may also constitute the hydrophobic core of the same protein. Before crystal structure of gH/gL became available, it was speculated that gH/gL carried an intrinsic fusion function.[247,252-254] A fusion peptide could be a loop with hydrophobic residues located in its tip, capable of insertion into membranes. In HSV gH hypothetical fusion peptides were recognized to bind artificial membrane liposomes at low affinity, due to their intrinsic hydrophobicity.[255-257] In gH/gL structure these putative peptides are buried helices (residues 377-397) or β-hairpins found in multistranded sheets. In gH crystal structure, peptides, which mimic putative gH heptad-repeat (residues 444–479 and 542–582, which inhibited cell fusion), form helical hairpins required for domain H2 stability.[258] Coiled coils and helical bundles are often formed by helices bearing heptad repeats.[259-262] In particular, residues 766–802 take part in formation of a five-strand β-sheet in domain H3, arguing against a possible excision and insertion into a target membrane. It was also demonstrated that a synthetic peptide mimicking gH regions 626-644 inhibited cell-cell fusion. These peptides could have interfered with the interaction among fusion proteins. Nevertheless, postfusion trimeric hairpin bundle, characteristic of class I and III fusogens, or internal fusion loops, typical of class II and III, are absent from the gH–gL structure.[263]

HSV gH/gL induces virus-neutralizing antibodies and its functional regions (FR) were defined by epitope mapping of neutralizing MAbs[94]. MAb LP11 and 52S to gH/gL are two potent neutralizing antibodies that identify FR1 and FR2 (H2-H3), respectively.[192,264,265] . Other neutralizing antibodies which block cell-cell fusion and spreading are MAb CHL17 and CHL32 that identified FR3.[236,266]

FR3 is situated in gH N-terminus at H1 domain, in position 19-38, and it also includes gL C-terminal residue as identified by MAb CHL18 and CHL39.[236] Ablation of N-terminal region (FR3) of gH HSV-2 at residues 19–47 or 19–28 induced a constitutive low level of cell-cell fusion by gB even when gD was absent.[267] This was speculated to be due to the resemblance of an N-terminal truncated gH to an activated conformation assumed by gH/gL to trigger fusion by gB.[267]

FR1 is identified by MAb LP11, a neutralizing MAb against gH which hampers cell-cell fusion and binds to the N-terminal half of gH; its epitope encompasses residues 86, 168, 300, 315-317, 325.[188,192,239] MAb LP11 recognizes a conformational epitope, which is present only in correctly folded gH/gL. Mutant HSV-1 with monoclonal antibody resistance (MAR) overwhelmed LP11 neutralization. Sequencing of gH from MAR HSV-1 mutants shows the following mutations: E86K, D168G, D168N,

R329W or R329Q[192] The mutations provided surface charge exchange and impeded LP11 binding to gH/gL, while wild-type HSV-2 gH already has residue substitutions E86A and R329P. In HSV-2 gH/gL structure, Asp168 is close to Pro329, but Ala86 is exposed on the opposite surface of gH. The insertion mutations hampering LP11 binding, partly map on the surface identified by residues Asp168 and Pro329.[239] Most probable positions eligible for LP11 binding are thus gH residues 168 and 329 while position 86 must imply a long-range steric hindrance exerted by LP11.[188]

MAb 52S, which identifies FR2, inhibits cell-cell fusion and neutralizes infection.[188,192] MAR mutant HSV-1 S536L and A537V escape MAb 52S binding to gH/gL and consequent neutralization.[192] On HSV-2 gH/gL, these residues are located on the opposite surface in respect to LP11 MAR residues, in agreement with data suggesting that epitopes for LP11 and 52S do not overlap.[192]

In Bi-Molecular Complementation (BiMC) technique, a fluorescent reporter is splitted in two halves and cloned connected to cytoplasmic tails of investigated membrane interactors.[186] Upon ectodomains binding between the investigated membrane proteins, the fluorescent reporter is reconstituted in a non-reversible, manner testifying that a molecular interaction had happened. In particular, to reveal interactions among these tagged glycoproteins, combinations thereof are expressed on cell surface and challenged with soluble proteins or even antibodies. Modifying these parameters allows to display specific time-course of interactions besides highlighting the interactors themselves.

gH/gL and gB were separately tagged with split halves of EYFP, if gD was omitted only background fluorescence was revealed and no syncytia formation (resulted from HSV fusion) was reported. When gD was expressed, EYFP was reconstituted and emitted fluorescence as gB and gH/gL had interacted.[186,189] Neither the non-neutralizing antibody 53S to gH/gL, nor neutralizing antibody 52S hampered EYFP reconstitution, while neutralizing antibody LP11 did.[188] As expected, only neutralizing LP11 and 52S MAb inhibited cell fusion, decreasing the amount of induced syncytia, while MAb 53S did not.[188]

These observations suggest that neutralizing MAb LP11 blocks gB interaction with gH/gL and cell-cell fusion, while 52S only inhibits fusion. Thus, events in FR1 that lead to gH activation and gB interaction must take place before the ones involving FR2 that promote cell-cell fusion in the HSV glycoprotein's interaction cascade.[189] Since mutants in gB fusion loops hampered both interaction with gH/gL and fusion, it was speculated that gB should insert its fusion loops before interacting with gH/gL.[180,189] Bimolecular complementation showed also association between gD and gH/gL.[186,187] It was speculated that gH/gL serves as a positive regulator for gB to exert fusion, as gH/gL is subjected to

co-activation by a receptor-bound gD.[180] gB–gH/gL complex formation should happen before cell-cell fusion.[188]

The surface eligible for gB binding contains a large groove, which is highly conserved in HSV-1 and HSV-2 gH/gL.[188] As both HSV-1 and HSV-2 gB were shown to be interchangeable in fusion assays, the gB binding site on gH/gL might be conserved in other herpesviruses.[177,248] Mutations engineered in VZV gH/gL to destabilize interactions among helices were proven to hamper cell-cell fusion.[129] Fusion inhibition exerted by MAb 52S could be explained if upon gB binding to gH/gL, gB displaces a series of aligned α-helices in gH domains H2A-H2B changing configuration of the gH α-helix carrying 52S epitope.[177] Structural flexibility typical of gH/gL might provide response to gB interaction with another similar conformational change in gH/gL that could consequently activate gB for fusion. After gB interaction with gH/gL, neutralizing MAb 52S may act on gH/gL preventing the delivering of a response signal to gB, i.e. a conformational modification in gH/gL. These speculated molecular mechanisms imply strong structural flexibility inside gH/gL. Internal mobility of gH/gL structural elements is in accordance with viable mutants accommodating insertion in buried regions of H2A and H2B domains.[239]

It was recently demonstrated by sequentially exchanging HSV glycoproteins serotype that HSV gH/gL defines fusion kinetics. Taking advantage of an improved cell-cell fusion assay, it was shown that gH of HSV-2 ($gH_2$) leads to an increased level of fusion than gH of HSV-1($gH_1$).[180] This was not due to a different expression of gH glycoproteins on cell surface, but was an intrinsic HSV-2 gH property.[180] gH/gL from HSV-2 could have a higher affinity for gB than gH/gL from HSV-1; hence gH/gL from HSV-2 might activate a greater number of gB trimers, thereby enhancing the fusion levels. In fact, mutant gB that could interact with gH/gL, induced enhanced fusion by $gH_2/gL_2$ as compared to $gH_1/gL_1$, regardless of the phenotype imposed by the gB mutation.[180] The maximum fusion activities of all the mutant forms of gB tested were dictated only by changes in its structure, while the fusogenic capacity of any gB molecule was finally regulated by gH/gL.

Other portions of gH/gL which are essential to perform standard fusion, are located both in the cytoplasmic tail and between the non-solved membrane proximal domain and the TMR of the protein. Insertions in position 791-799 impair gH/gL function, while fusion is performed at low efficiency with a truncated gH mutant.[239,268,269]

### 1.5.5 HSV gB

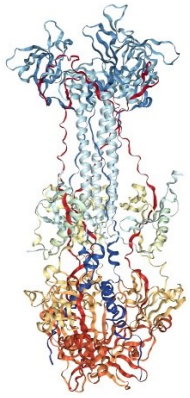


**Figure G. Protein structure of HSV gB.** Post-Fusion gB (POgB) trimer is depicted in rainbow colors, the crown (lighter blue) forms membrane distal domain. Crystal structure is from PDB (2GUM).

gB of HSV is a trimer, and belongs to class III fusion protein, together with VSV G and baculovirus gp64, as it accounts for more than class I and class II features.[175] In particular HSV gB has three long central α-helices, typical of class I fusion protein, and bears a bipartite fusion peptide per protomer, typical of class II fusion protein.[175,263]

N-terminal residues 30 – 773 of a gB protomer represent the gB ectodomain, while TMR encompasses residues 774 – 794; a soluble form of gB, $gB730_t$, was used for crystalization (figure G). The cytoplasmic tail spans residues 795 – 903 and contains Golgi targeting sequence at 848 – 851 and an internalization motif at 888 – 891. gB has 6 N-glycosilation sites and its structure shows 5 disulfide bonds. gB membrane trafficking is dependent on its cytoplasmic domain, gB reaches host membrane surface where it is subsequently internalized in the Golgi apparatus for envelopment of HSV virions.[175] Electron density for Pre-fusion form of HSV gB (referred as PRgB) and crystal structure for stable Post-fusion form of HSV gB (referred as POgB) are available.[175,270]

POgB trimer forms rod-like structures perpendicular to the membrane of 8 nm radius and 16nm height.[175,270,271] The crystal structure shows that the POgB homotrimer is the HSV fusion glycoprotein; syncytial mutations causing hyperfusogenic phenotype are found in gB.[175,272] Nonetheless, gB executes fusion only if the gH/gL heterodimer is present, a feature typical of all herpesviruses.[188]

gB protomer structure consists of five domains, differently distributed between PRgB and POgB. POgB residues 730-773 constitute the essential, but not yet resolved, hydrophobic membrane proximal region.[175]

HSV gB domain I corresponds to the fusion domain, it contains fusion loops characterized by lipophilic residue near charged hydrophilic ones.[175] Mutations in gB domain I cause altered virus entry, in addition to hampered cell-cell fusion.[190] Domain I would be found juxtaposed or inserted into the membrane in POgB structure, while in full-length PRgB electron density map (EM) it stands apart, pointing away from the membrane (figure H).[270] Conformation of PRgB, with domain I in the most distal part, could aid interaction via fusion loops with a target membrane. This is in accordance with the mapping of gH/gL binding site on gB, that identifies domain I or domain II as likely interacting sites.[189]

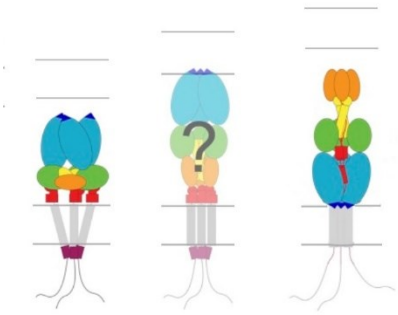


**Figure H. Cartoon of metastable conformation transition in HSV gB trimer.** From left to right: in Pre-Fusion gB (PRgB) trimer, the crown (orange) stands delocalized at the equator of the trimer while the Fusion Loops (blue) occupy membrane distal domain; in Post-Fusion gB (POgB) trimer, the crown (orange) forms membrane distal domain and fusion loops are located at membrane proximal domain. Pictures adapted from ref. 270.

Four different FRs were identified in gB by function blocking analysis with MAbs. FR1 is targeted by MAbs SS55/SS106/SS144 and correspond to gB fusion domain I containing the bipartite fusion loop. In POgB, the fusion domain is located at the bottom of ectodomain, proximal to the membrane.[190,194] FR2 was defined by C226/H1838/H1781 MAbs and it is the interaction domain with gH/gL. Adaptors involved in protein-lipid interactions, Pleckstrin-homology domains (PH), were identified in both FR1 and FR2. FR3 carries epitopes for MAbs SS10/SS67-69 and was mapped on the distal part of POgB, the "crown".[175] A linear epitope in FR4 is recognized by MAb H1817 and resides in the not resolved

flexible N-terminus. FR4 binds heparan sulfate moieties and other surface molecules, identified as gB receptors, as paired immunoglobulin-like type 2 receptor-α (PILR-α) (figure I).[273,274]

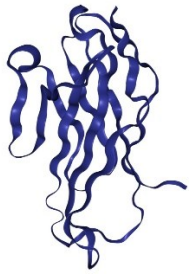


**Figure I. Protein structure of Paired immunoglobulin-like type 2 receptor-α (PILR-α).** PILR-α is depicted in in full color. Crystal structure is from PDB (4NFB).

PILR-α modulate host immune system and is recruited by gB to perform fusion together with gH/gL and gD, at least in several cell lines, i.e. human retinal pigment epithelial cells.[273,274] PILR-α promotes HSV infection in Chinese Hamster Ovary (CHO) cells which bear no gD receptor. PILR-α behaves also as a routing factor, in that it promotes HSV entry at the plasma membrane in CHO, while HVEM or nectin1 expressed in CHO lead to an entry pathway which is dependent on acidic endosomes. In addition to providing initial binding to heparan sulphate together with gC, gB also interacts with CD209, a molecular marker typical of the dendritic cell surface, promoting tethering and infection.[164,275] Myosin 9 or non-muscle myosin heavy chain IIA (NMMHCIIA) is another cellular receptor which seems to interact directly with gB to drive HSV fusion.[164] The role of these receptors in HSV entry remains elusive.

Wild-type HSV gB has an innate fusogenic capacity that can be influenced by structural restrictions imposed by various mutations; specific mutations in the fusion loops of gB which prevented the interaction between gB and gH/gL, were not enhanced by gH/gL serotype swapping, which was shown to improve cell-cell fusion in an HSV-1 glycoprotein context. This indicates that these single-amino-acid changes truly destroyed the ability of gB to function in cell-cell fusion.[180]

Mutations in gB FR3, the crown of gB or domain 4, (F641Y, Y649A, and Q584A) are functional but exhibit slow initiation time and diminished overall extent of fusion.[180] Conversely another mutation in the crown (H657R) lead to an hyperfusogenic gB phenotype (gB fast mutant). Non related mutation in cytoplasmic tail dileucine loop (LL871AA) which enhances gB membrane retention, showed the

highest levels of fusion.[180] gB fast mutants were postulated to represent unlocked forms of gB (further along in the transition path from pre to post fusion conformation) that were inherently in a higher energy state than wild-type (wt) gB.[180]

To provide data on pre-fusion states, full-length gB was expressed to gain proper membrane anchoring and native topology. gB trimer was then analyzed on extracellular cell-derived vesicles, by electron cryotomography (cryoET).[276] Membrane inserted gB trimer conformation was inferred by fitting with restraints POgB domains inside the PRgB volume identified by EM. This approach leads investigators to capture a transitional/metastable state of gB, conformationally foregoing to POgB (figure J).[175,263]

Expressing soluble herpesvirus gB ectodomain lets gB to stabilize in its postfusion conformation (POgB).[175,277-280] A gB pre-fusion conformation exists as gB is synthetized with an intrinsic instability and the tendency, if triggered, to adopt a more stable configuration while merging membranes. In particular, PRgB releases its own structural energy upon conformation modifications to allow the energetically unfavorable process of fusing lipid bilayers.

3D reconstruction of gB showed two kinds of spikes with threefold symmetry, whose axis was perpendicular to the membrane.[270] Spikes of 16 nm height, corresponding to POgB, were found only on small vesicles, since POgB prefers high membrane curvature.[281] Roughly globular spikes with a height of 10 nm and 8 nm in width were also found and represented PRgB, which was thus two-thirds of POgB in height, a condition that might aid in interfacing with the other fusion glycoproteins.

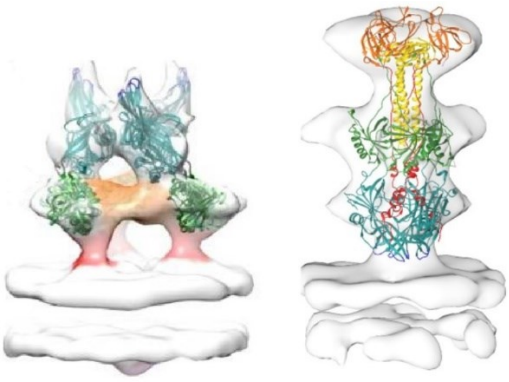


**Figure J. Cryo Electron Microscopy (Cryo EM) volume of HSV gB trimer.** Left, Pre-Fusion gB (PRgB): the crown domain (orange) stands delocalized at the equator of the trimer while the fusion loop domain (blue) occupies membrane distal domain; Right, Post-Fusion gB (POgB): POgB trimer is depicted in rainbow colors encased in its Cryo EM Volume, the crown (orange) forms membrane distal domain and fusion loops are located at membran proximal domain. Pictures adapted from ref. 270.

Remarkable structural rearrangements were expected for PRgB, as in POgB structure TMR would be next to the fusion domain in the protomers, with a “hairpin” conformation.[282] PRgB expands on its equator at 4 nm height from membrane surface, allowing neighboring gB trimers to form a net or contact interactors, via glycosylation site N398. In respect to POgB, PRgB shows a central cavity circumscribed by globular domain IV (the crown of POgB) and domain II; these domains are in part delocalized at the equator together with the flexible domain III. Contrariwise, POgB domain III structures the core of the gB trimer, forming the trimeric coiled coil; in POgB domain V, stalks run conversely and adherent to domain III until reaching the membrane proximal region.[270,282]

PRgB trimer is membrane-anchored by a tripod made by protomer domain V C-terminal arms, the highly hydrophobic membrane proximal region and TMRs. TMRs stand 6 nm apart from each other and converge on the opposite side of the membrane (the cytoplasmic face), forming a central density of 1,5 nm x 3 nm. Fusion loops in the PRgB trimer are 5 nm apart among protomers and different surfaces of domains I and II are exposed in respect to POgB (figure K) .[270]

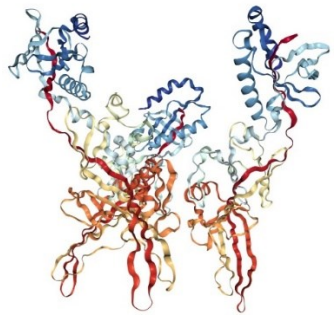


**Figure K. HSV Pre-Fusion gB (PRgB) structure.** PRgB trimer is depicted in rainbow colors, in native and membrane anchored conformation (full length gB). PRgB crown (orange) stands at the equator of the trimer while the Fusion Loops (blue) occupy membrane distal domain. Structure is from PDB (5FZ2).

It was speculated that upon triggering, the rotation of domain I brings the fusion loops of the three protomers together, causing formation of a "hydrophobic fusion patch". Subsequent coiled coil formation let the "hydrophobic fusion patch" to puncture the facing membrane, which is being juxtaposed by other glycoproteins tethering to their receptors. Target and virion membrane are then crushed along gB axis when gB opposed ends are joined by an intrinsic energy release, with domains of gB acting like a spring. The result is the rearrangement of gB domains with the formation of the crown

(domain IV) and the localization of gB fusion loops (domain I) next to TMRs, meaning inside the membrane.[175,270]

Neutralizing MAbs, random linker-insertion mutagenesis and fluorescence protein insertion mutagenesis revealed unexpected exposure of some buried surface in POgB structure.[194,283-285] For example, both MAb H1781 (residues 454–473) and H1838 (residues 390–410), are neutralizing antibodies, but their epitopes could not be fitted on POgB surface. Conversely, MAb H1781 and H1838 epitopes are exposed on the surface of PRgB domain II.

### 1.6.1 VZV fusion/entry

The highly cell-associated nature of Varicella Zoster Virus (VZV) and its human-restricted tropism delayed research on its entry mechanism as compared to other herpesviruses, for which a cell-free high-titer virion could be harvested in culture.[126,286,287] VZV fusion, with a peculiar syncytia formation, is presumed to be different from that of other α-herpesviruses because of the absence of established tropism factors, as i.e. HSV gD.[288] Deeply investigated examples of cellular receptors could be found, both for VZV gB or gE.[289] Recently, integrin implication in VZV entry and cell-cell fusion was proven without discerning a glycoprotein candidate between gB and gH/gL.[290] As already seen for other herpesviruses, thanks to its evolutive plasticity and multifunctionality, gH/gL might encase also the elusive tropism factor function needed for VZV cell entry. MAb 206 to VZV gH/gL hampers *in vitro* virion entry, while it inhibits virion egress or syncytia formation when it is internalized by infected cells. gH N-terminus is also involved in VZV *in vivo* replication and might be involved in receptor recognition. Further investigation in this direction might fill the gap among VZV and other herpesviruses in cell-entry knowledge.

It was earlier proposed that cation-independent mannose 6-phosphate (M6P) receptor [MPRci] was involved in cell-free VZV infection.[291-293] This was supported by finding that M6P inhibits VZV infection and that VZV glycoproteins contain M6P groups in phosphorylated N-linked complex oligosaccharides. MPRci is a membrane protein that functions as a molecular chaperone and is expressed ubiquitously. MPRci transports modified proteins with N-linked oligosaccharides from the trans-Golgi network (TGN) to the early endosomes.[294] The MPRci model for VZV entry should have explained the high VZV virion-cell-association as follows: in culture, as well as *in vivo*, newly

produced VZV virions emerging from the TGN remain linked through the M6P on their glycoproteins to the membrane MPRci, whose recycling is continuative in the cell.[291] Moreover, as also lysosomal enzymes are equipped with M6P, both VZV virion tethered with its glycoproteins and lysosomal enzymes eventually shared the same pathway ending, with constitutive negative outcomes for VZV progeny.[295]

This mechanism apparently self-limited VZV virulence; but the purpose of VZV should be that of conquering and not killing an individual, leaving the possibility for subsequent spreading in the population over many years. Similar mechanisms might be exploited by herpesviruses in general, whose establishment of a life-long latency is due to an extremely fine control of their own virulence.[296]

Access to population ought to be granted to VZV only at skin level, where suprabasal epidermis cells downmodulate MPRci throughout their maturation, allowing dispersion of virions via desquamation even in the air.[293] Nevertheless, as association with MPRci on cell surface is already described for other proteins, it is also possible that the role of MPRci is only that of enhancing adsorption of VZV virion to the membrane.[294] In fact, though MPRci is required for cell-free VZV infection of MeWo cells, cells knockdown for MPRci are susceptible to cell-associated VZV infection, with syncytia formation.[293] Furthermore, cell surface expression of MPRci does not induce cell-cell fusion by VZV glycoproteins, whose physical interaction with MPRci was never demonstrated.[291] It was thus suggested that MPRci and heparin compete in cell-free VZV infection, whereas the insulin-degrading enzyme (IDE) is exploited by VZV gE to spread among cells while mantaining its cell-associated status.[297-300]

## gE/gI

VZV gE is the most represented VZV glycoprotein and it was thought to replace HSV-gD function in VZV cell-entry, since gE is essential for VZV infection and *in vivo* replication, as demonstrated by using gE mutant viruses.[301] VZV gE ectodomain mutation D150N, retrievable in the VZV-MSP natural variant, leads to an increase in cell-cell *in vivo and in vitro* spread, while S31A substitution leads to decreased *in vivo* infection. gE residues 51–187 constitute an ectodomain region which is not conserved but essential for both skin and T cell *in vivo* tropism. gE is required to form the gE/gI heterodimer which is needed for viral replication and VZV virion maturation through TGN *in vitro*.[127,302-304]. gE should participate in fusion and was shown to drive cell-cell fusion if paired with gB, as reported for VZV-gH alone.[127,302-304] However, these latter results were affected by the use of a

recombinant vaccinia virus system to express VZV glycoproteins leading to expression of also interfering vaccinia membrane proteins.[289]

In cells infected with VZV, gE was also proven to interact with Insulin Degrading Enzyme (IDE).[299,300] IDE is ubiquitously expressed and IDE-expressing non-human cells became susceptible to an increased VZV infection, which is instead down modulated by IDE inhibition.[299] Nonetheless, IDE seemed to bind to an immature form of gE in the cytosol and it was also reported that gE was not required for VZV membrane fusion.[127,305] In fact, preventing IDE binding by ablating gE from VZV virion led to infection both in melanoma cells or fibroblasts *in vitro.*[128] Furthermore, when the same mutant was used *in vivo*, it infected both neuronal cells and T cells, even if these latter are cell-cell fusion recalcitrant and do not form syncytia.[306] Flow cytofluorimetry revealed that a gE-Ig fusion protein do not bind to cells expressing a surface form of IDE, and these latter cells are neither infected by VZV. Nevertheless, as cell-cell fusion had slightly increased in presence of IDE, its role in VZV fusion remained elusive.[289]

VZV gE was also shown to bind myelin-associated glycoprotein (MAG) expressed on cell surfaces by flow cytometry and immunoprecipitation.[289,307] MAG expression is restricted to the neuronal tissue and probably represents a VZV receptor only during the neurotropic pathway.[290,308] VZV gE interaction with MAG did not result in membrane fusion and the efficiency of cell-cell fusion driven by MAG and VZV glycoproteins was even enhanced in the absence of VZV-gE. These results were not due to an interference by gE on surface expression of the other VZV glycoprotein, and cell-cell fusion was not induced by a combination of gE, gH, and gL or gE and gB.[289] Moreover, cell-cell fusion efficiency is not altered by the formation of the heterodimer gE/gI.[303] However, VZV gE and gI carry at least one Lys-Gly-Asp (KGD) integrin binding domain, while MAG has an Arg-Gly-Asp (RGD) integrin binding domain.[309-311] Their role in VZV fusion need to be further addressed.

## VZV gB

VZV gB is 931 amino acids in lenght and it is the only α-herpesvirus gB cleaved by host furins, it is essential for VZV infection and shares 48% sequence homology with its HSV-1 homolog.[312] Furin are protein convertases that recognize and cleave the motif [R]-X-[KR]-R-"CLEAVAGE"-X, which is conserved in the gB of all herpesviruses, except for HSV-1. The ablation of this motif leads to an

aberrant *in vitro* phenotype in PrV, while in VZV, the deletion or substitution of gB 491RSRR494 to 491GSGG494 leads to the same gB distribution in transfected melanoma cells, as documented by conformation-dependent anti-gB MAb SG2.[312] Disruption of the furin motif leads to decreased VZV *in vivo* infectivity in skin xenographts. VZV gB cytoplasmic tail carries an immunoreceptor tyrosine-based inhibition motif (ITIM), whose tyrosine 881 phosphorylation was demonstrated to limit VZV fusion and syncytia formation.[313] When Y881 phosphorylation is prevented, the consequent hyperfusogenic phenotype leads to a limited VZV virion production *in vivo and in vitro*. It was demonstrated that HSV-1 gB interacts with paired inhibitory and activating receptor-α (PILR-α), enhancing HSV-1 entry into promyelocytes and monocytes even if the interaction alone is not sufficient to mediate membrane fusion.[273] As PILR family of receptors modulate host-pathogen interaction, PILR-α might also interact with VZV gB.[314] PILR-α -Ig fusion protein did not bind cell surfaces expressing VZV glycoproteins. Inside the Sialic-acid-binding Ig-like lectin (Siglec) family of receptors, PILR-α showed a 5 to 12% homology to Siglec-1, 4, 5.[315] In particular cells expressing Siglec-4, termed also Myelin Associated Glycoprotein (MAG), were stained by VZV-gB-Ig on their surfaces, and their association was documented also by immunoprecipitation of lysates of VZV infected cells by means of a MAG-Ig.[289]

MAG is expressed exclusively on the surface of myelin sheath of neural tissues, in particular it is retrievable on glial cells as oligodendrocytes or Schwann cells *in vivo*.[307] MAG is involved in neural development and exerts regulation of axonal growth interacting with factors as Nogo-66 receptor or paired Ig-like receptor B.[308,316-318] It mediates sialic-acid dependent cell-cell interactions to favor myelinating process on neuron cells, but MAG expression in glial primary culture cells was not detectable.[289] No human cell line expresses endogenous MAG *in vitro* and this might account for *in vitro* restriction of infection by cell-free VZV, as *in vivo* both VZV and HSV-1 infect glial cells during the acute phase of infection.[40,319,320]

MAG was showed to drive fusion in 293T cells with formation of polykaryons when two populations were mixed: one carried VZV-gB, gE, gH, gL and the other MAG on their membrane. This result was replicated with a quantitative luciferase reporter system, showing that VZV gB and gH/gL expression represents the minimal requirement for fusion mediated by VZV glycoproteins of MAG expressing cells.[289] In human oligodendroglial (OL) cells, only the stable expression of MAG allows VZV infection, which was prevented by anti-MAG MAb administration.

### 1.6.1 VZV gH/gL

Further investigations revealed syncytia formation even in absence of MAG, when effector cells bearing VZV gB and gH/gL were co-cultured with melanoma target cells.[288,290] In susceptible cells, VZV gB and gH/gL trio expression is sufficient to induce cell-cell fusion, which should represent the essential fusion machinery mediating VZV entry.[129,289,321] In fact, mutant VZV virion bearing recombinant gH/gL or gB were showed to have impairments in viral replication and cell-cell fusion *in vitro* and in an *in vivo* models.[129,288,313]

It was demonstrated that administering high-titer of Ig directed to VZV envelope glycoproteins in human subjects just exposed to VZV decreases the incidence of varicella, preventing cell-to-cell spread and even the infection from starting.[322] MAb206 directed against VZV gH/gL was proven effective in hampering viral entry and spread in an *in vitro* and in an *in vivo* model.[321,323,324] Recombinant MAb were obtained also from humans, in independent studies, with neutralizing activity against the whole gH/gL heterodimer.[325,326] IgG-24 and IgG-94 derived from a library of pooled B cells coming from naturally immune individuals, while rec-RC IgG was isolated from plasma blasts recovered after a zoster vaccine immunization.[327]

Fragment of antigen-binding (Fab) from these MAbs were used in structural studies to restrict gH/gL conformational flexibility as to gain diffraction quality crystals. Nevertheless, either VZV gH/gL/Fab-RC or gH/gL/Fab-94 complexes display flexible N-terminal residues, thus modeled structures started from amino acid A36 (figure L).[327]

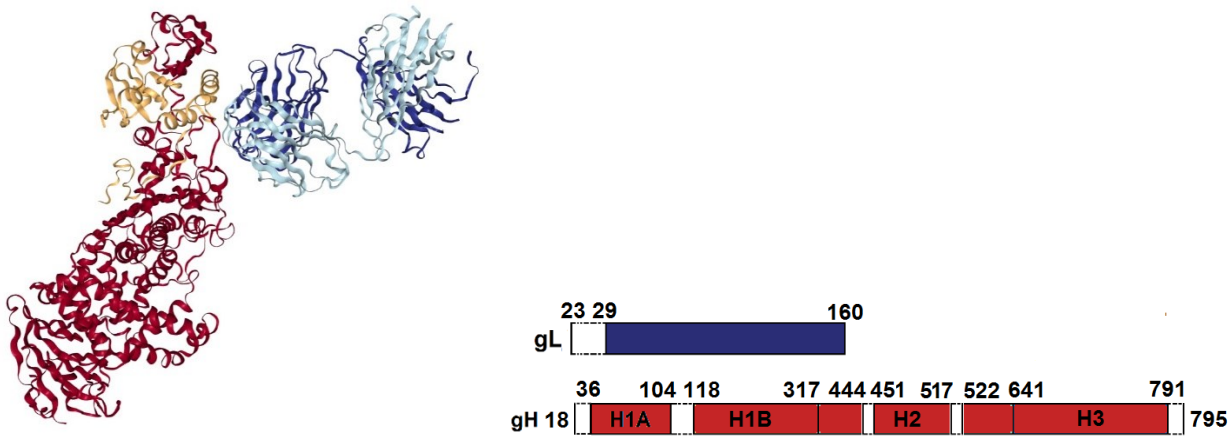



**Figure L. Protein Sturucture of VZV gH/gL, in complex with Fab-RC.** VZV gH (red) complexed with gL (yellow) was co-crystallized with Fab-RC heavy and light chains (blue and light blue). Crystal structure is from PDB (4XHJ). Schematics provide an overview of ectopic domain arrangements.

VZV gH/gL is boot shaped and closely resembles the structure of HSV-2 gH/gL, also in that gH and gL are clearly dependent on each other to fold.[178,188,241,327] VZV gL contains a chemokine fold, which is composed of two α-helices (α2-α3) and a three-stranded β-sheet (β4-β5-β6).[328] Chemokine fold is structured by the conserved disulfide bond between Cys49 and Cys80.[328]

VZV gH/gL domains are divided as in HSV gH/gL; the gH H1 domain is the most divergent domain. In particular, H1A domain in VZV gH/gL has more β-strands than that of HSV; an eight-stranded antiparallel β-sheet is formed by an equal strand contribution by VZV gL and gH. In contrast to HSV gH, whose N-terminus is coiled, in VZV gH the N-terminal strands β1 (aa 36–45) and β2 contributed to the mixed antiparallel β-sheet.[327]

In contrast to HSV gH/gL, the crystal structure of VZV gH/gL shows extended interactions between α-helices Lα2/Lα3 of gL and multiple loops, that belong the gH H1 domain. In details, loop A (with the structural resemblance of a "thumb") of gH H1B domain (aa 285–298) is located on the surface created by gL α-helices Lα2/Lα3. Among gH loops positioned beneath, there is loop B (aa 154–161) which was designated as "the palm", because it gives structural support to the ensemble, including gH loop A and gL Lα2/Lα3 helices.[327]

The first four α-helices of H2 domain contribute to the formation of the syntaxin-like helical bundle (SLB); these helices display extensive interactions with the eight stranded β-sheet in H1B domain.[178,241,327] As expected, gH domains H2 and H3 are conserved in structure in α-herpesviruses,

thus these domains are supposed to perform conserved functions.[327] In VZV gH, the α-helix at position aa 600–609 replaces a disordered loop present in gH homologs.

The location of IG-RC and IG-94 epitope seems to represent a critical target for MAbs in general, since it overlaps with that of LP11, as shown by comparing VZV and HSV-2 structures.[188,192,239,327] The LP11 epitope on HSV-2 gH/gL spans an area that in VZV gH/gL matches loop A and loop B regions that clamp VZV gL α2-α3 helices. By homology, it was suggested that the gB activating site on VZV gH probably resides near IG-RC and IG-94 epitopes.[327]

Fab-24, Fab-94 and Fab-RC interfere by 50–75% with cell-cell fusion; this result highlights the contributions of different gH subdomains to fusion blockade (exerted by different Fabs). An increased fusion inhibition, driven by IgG instead of corresponding Fab-94 and Fab-RC, might be explained by cross-linking gH to gL. Surface plasmon resonance (SPR) evidenced that Fab-RC and Fab-94 competed with each other for gH/gL binding, with IgG-RC having the highest affinity. VZV gH/gL structure shows that Fab-RC and Fab-94 bind a common epitope on VZV gH/gL, with different orientations, and Fab-RC covers a larger surface in respect to Fab-94 (1036 $Å^2$ and 822 $Å^2$, respectively).[327]

Both Fab-RC and Fab-94 interact with a surface groove delimited by loop A 294LNPP297 and loop B 156PLVW159. Fab-94 contacts E114 in a loop between gL Lα2-Lα3 helices and R121 at the end of Lα3 helix of gL. Fab-RC interacts with D122 and D126 of gL Lα3 helix. VZV gH mutated in loop A behaves in fusion assay as the wild-type, arguing that residues side chains interactions are not determinant.[327] Loop A residues mutations 288DTTWFQL294 to 288AGGAAQD294 hampered both Fab-RC and Fab-94 binding of gH, while in the same condition gH retained its ability to heterodimerize with gL and to interact with Fab-24. Indeed, Fab-24 binds both gH domains H1 and H2; Fab-24 epitope seems to exert long range interactions with loop A and loop B.[327]

VZV and PrV share a conserved Protein Disulfide Isomerase (PDI) domain (CXXC) in their gH, whereas this domain is not conserved in HSV-2 gH/gL.[178] CXXC PDI domain in gH might contribute to proper gH folding in the endoplasmic reticulum.[129] However, provided that the CXXC PDI domain is not at the core of gH but near the "flap" conserved structure, it was speculated that the PDI domain would contribute to fusion.[178] In particular, an exchange in disulfide bridge asset, insisting on the flap C-terminal end, might unveil the underlying hydrophobic surface and stimulate fusion.[178]

Residues 18–45 in VZV gH are targeted by the murine neutralizing MAb 206; mutation of 38LREY41 to 38GRGG41 reduced the affinity of MAb 206 for VZV gH . Indeed, a recombinant VZV carrying

38GRGG41 mutation was not neutralized by MAb 206; the same mutant displayed weakened replication in human skin.[129] Mutation in the sequence 38LREY41 in gH may destabilize the first two β-strands of gH (in the mixed antiparallel β-sheet) with a straight impact on overall stability of gH/gL.[327] These data support a role for these species-specific gH β-sheet in receptor binding and skin tropism.[129] The structure of HSV-2 gH/gL was stabilized to gain diffraction quality crystal exactly ablating residues 19–38.[188] This way, HSV-2 gH/gL might have suffered of an irreversible conformational change, such that the HSV-2 gH/gL structure displayed one of gH/gL metastable states instead of the native gH/gL conformation.

### 1.7.1 Integrins and Human α-Herpes Viruses

Integrins serve as adhesion molecules and are heterodimeric membrane integral proteins.[329] Integrin heterodimers are formed by the combination of 18 α-subunits and 8 β-subunits, specifically found in different anatomic districts.[330] Integrins connect the cytoskeleton to the extracellular matrix and are capable of both inside-out and outside-in signal transmission.[331] Integrins are ubiquitous surface molecules, that activate various cellular functions.[332-335] Different viruses evolved entry strategies taking advantage of integrin widespread distribution.[336] Examples include DNA viruses as Adenoviruses (which are not enveloped viruses) or Herpesviruses. Integrin β3 is found on various types of tumors and endothelial tissues, while β6 is typical of proliferating epithelia and melanoma. Integrin β8 is retrievable in fibroblasts, keratinocytes and neural tissues. αVβ3, αVβ6 and αVβ8 integrins are present in epithelial and endotelial tissues and all have similar cellular and tissue distribution due to their shared α-subunit (figures M and N).[329-331,334]

Herpesviruses might take advantage of integrin signaling in order to deliver their capsid to the nucleus, through modification of cytoskeleton or actin filaments. Activated αVβ3 integrin might induce $Ca^{2+}$ release from intracellular stores and activate focal adhesion kinase (FAK). The silencing of αV or β3 integrin subunits or the adsorption of an HSV-2 $gH^{-/-}$ HSV prevented FAK activation or $Ca^{2+}$ release.[337] Downstream events triggered by FAK activation are also hampered by β3 integrin silencing, i.e., phosphorylation of c-Src and Akt kinases (also involved in cytoskeleton modification).[338] Integrin-dependent rearrangements of actin filaments are mediated also by Rho GTPases as Cdc42, Rac1, and RhoA. These small GTPases alter stress fibers, lamellipodia and filopodia formation. These GTPases could contribute to HSV entry by loosening actin physical barrier to virion endocytosis, disassembling

cortical actin.[339,340] HSV drives another mechanism, termed surfing, that facilitates its own entry, namely it induces filopodia formation on cell surface by enhancing Cdc42 activation; dominant negative forms of Cdc42 and Rac1 block virus entry.[341]

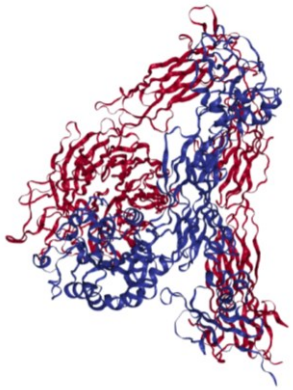


**Figure M. Protein structure of αV(red) and β3 (blue) integrin.** Integrin αV back folds towards membrane. Integrin modular Ig folding is noticeable. Crystal structure is from PDB (4G1M).

Integrins are capable of activating various host signaling pathways, that could be activated also in association with other receptors as epidermal growth factor receptor (EGFR) and Toll-like receptors (TLRs). In particular, integrin αVβ3 is implicated in innate immunity response, as it may trigger nuclear factor κB (NF-κB) pathway, while αvβ6 and αvβ8 integrins do not participate.[342,343] αVβ3 integrin, in association with TLR2 receptor, contributes to sensing pathogen by binding to HSV gH/gL or bacterial lipopolysaccharide (LPS).[338,344] In different cell types, integrin and TLR interaction elicits an innate response via NF-κB activation, inducing inflammatory cytokines including IL-2 and IL-10 or interferon (IFN) induction.[342,345,346] Nevertheless, HSV has evolved molecular strategies to evade the innate response triggered by αVβ3. HSV exploits its immediate early proteins ICP0 and ICP27 to counteract NF-κB response or virion host shutoff RNAse (VHS) that degrades host mRNAs in the cytoplasm.[347-350] In particular, ICP0 protein readily promotes degradation of the myeloid differentiation factor 88 (MYD88) in the cytosol and interferes with the activity of IFN regulatory factor 3 (IRF3) and IRF7.[338,342,351] It is reported that αvβ3 integrin cooperates with TLR2 pathway: the silencing of β3 integrin blocks recruitment of MYD 88 to TLR2. HSV ICP0$^{-/-}$ mutant, whose entry is hampered at low multiplicity of infection (MOI), behaves as the wild type virion in cells deprived of αvβ3 integrin and TLR2.[342]

## 1.7.2 HSV and αVβ6 and αVβ8 integrins

αVβ3 integrin on host membrane acts as a routing factor for HSV-1, relocating HSV receptor nectin1 to lipid rafts and thus defining the HSV-1 pathway of entry. In particular αVβ3 enables an HSV-1 entry pathway which starts at lipid rafts and is dependent on acidic endosomes and dynamin2 and independent of caveolin1.[171,352,353] αVβ3 binds to a soluble form of HSV-1 gH/gL.[176,243,354] αVβ3 is not an entry receptor or coreceptor for HSV-1 as αVβ3 silencing does not impair HSV-1 entry but modifies HSV-1 entry pathway.[352] Furthermore, a soluble form of gH/gL binds different types of cells in an αVβ3 independent manner. HSV *in vitro* infection was prevented by the expression of full lenght gH sequestering host interactors for HSV-1 glycoproteins.[354] Moreover, it was shown that the soluble form of HSV gH/gL is capable of aggregating cells expressing various integrins, including αVβ3.[243]

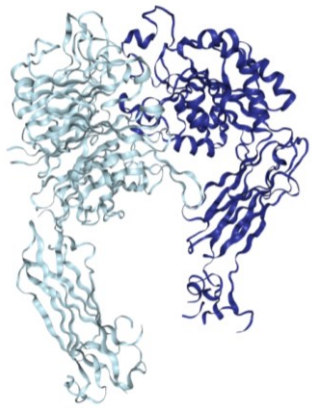


**Figure N. Protein structure of integrin αvβ6 heterodimer ectodomain.** αV(gray) β6 (blue) integrin structure is depicted. Crystal structure is from PDB (4UM8).

HSV further exploits available host integrins to maximize its spread, using integrins as entry receptors. In fact, αvβ6 and αvβ8 integrins serve as receptors for HSV gH/gL, and mediate endocytosis. HSV gH carries a non-conserved integrin interacting motif in domain H1B, positions 176-178 RGD but mutation D178E does not affect entry of HSV.[239,243] Hence, integrins implication in HSV entry was excluded[239]. It was afterwards demonstrated that HSV infection required gH RGD motif only when integrin αvβ6 was used for entry, but not αvβ8. Recently, αVβ6 and αVβ8 integrins were shown to bind

a soluble form of gH/gL ($gH_t$/gL) at high affinity ($10^{-8}$ M). Conversely, αVβ5 did not bind $gH_t$/gL.[176] Function-blocking antibodies against αVβ6 and αVβ8 heterodimers, MAb 2077 and 37E1 respectively, hampered HSV infection. The silencing of αVβ6 and αVβ8 integrins reduced HSV infection in either cases, but maximum effect occurred when both integrins were silenced. Depletion of both αVβ6 and αVβ8 integrins reduced HSV infection to a lower level than a single integrin heterodimer depletion did, thus αVβ6 and αVβ8 were interchangeable.[176]

A gain-of-function experiment was performed in K562αvβ6 myelocytic cell line, that expressed only αVβ6 integrin and a lower amount of α5β1 integrin.[176] α5β1 is supposed not to interfere with αV integrin subunit.[355] Even if K562 are recalcitrant to HSV infection, K562αvβ6 showed an increase in HSV infection, in respect to the parental K562.[356] J cells are baby hamster kidney derivatives, artifically deprived of HSV gD receptors, expressing low level of endogenous hamster integrins. Expression of high level of αVβ6 and αVβ8 human integrins together with low level of the entry receptor nectin1 allowed an increased HSV infection in respect to J cells expressing only nectin1 at the same level. However, the expression of αVβ6 or αVβ8 integrin alone does not allow HSV to infect cells, hence integrins do not substitute for an HSV receptor.[176] It is known that mutations in RGD domain should prevent interactions with αV integrin. To this purpose, a gH/gL with substitution of RGD domain in ADA ($gH_{ADA}$/gL) was used in both cell-cell fusion and virus entry assays. An HSV $gH^{-/-}$ (ScgHZ) was grown in a complementing cell line, expressing $gH_{ADA}$/gL, and was used to infect J cells overexpressing human integrin αVβ6 or αVβ8.[354,357] This pseudotyped HSV failed to infect J cells expressing αVβ6 integrin plus an entry receptor, while the RGD domain was dispensable for infection of J cell expressing an entry receptor and αVβ8 or no integrin. Thus, the RGD domain in HSV gH is exploited for interaction with αVβ6 integrin only, while αVβ8 interaction with HSV gH/gL relies on a different mechanism. Moreover, cell-cell fusion was inhibited by RGD to ADA substitution in gH only when J cells expressed αVβ6 human integrin.[176] J cell expressing nectin1 and αVβ6 or αVβ8 human integrins allowed a deep analysis of HSV infection with entry inhibitors. These cells, upon expression of an HSV entry receptor, enable an entry pathway independent of lipid raft and acidic endosomes.[171] As well as for αvβ3 integrin, also αvβ6 and αvβ8 integrins are involved in the choice of HSV entry pathway. αVβ6 or αVβ8 integrins allowed an entry pathway dependent on acid endosomes, but only entry mediated by αVβ8 depended on lipid rafts and Dynamin 2.[176] These various entry pathways exploited by HSV allow infection to proceed through alternative routes in different cells, facilitating HSV infection and spread, in accordance with HSV broad tropism.[164]

### 1.7.3 VZV and αV integrins

Human melanoma cells (Mel) are permissive to VZV infection, and actually lead to the same peculiar syncytia formations retrieved in infected skin.[288] Mel cells are expected to express cellular components needed for VZV fusion via gB and gH/gL. Since almost five of the eight human herpesviruses exploit integrins as entry receptors or coreceptors, the role of integrins in VZV infection was recently investigated.[176,337,290,358,359] Trascriptome analysis revealed that Mel cells potentially express twelve integrin combinations among the possible twentyfour found in human cell lines.

To gain reproducibility, a new kind of cell-cell fusion assay was engineered, leading to lower background level and higher sensibility, in contrast with a conventional transfection approach. Lentiviruses were used to generate target reporter cell lines, allowing an absolute quantification of fusion, without the experimental variability proper of T7polimerase-T7luciferase systems, where the expression of the reporter gene is typically heterogeneous among target cells.[289,290] In particular, recently described dual split proteins (DSP) were implemented in the so called stable reporter fusion assay (SRFA).[360] SRFA system employs single lentiviral vectors to express both DSP and a shRNA to respectively detect the amount of fusion and to interfere with the expression of cellular proteins relevant for fusion of target cells. Relevant proteins were inferred by transcriptome data. Splitted GFP and Renilla luciferase are constitutively expressed in the cytosol of different cell populations. The whole reporter reconstitution, and thus signal, is immediately revealed upon a cell-cell fusion event involving cytoplasms of different cell populations.[290]

Knocking down αV integrin, α6 integrin and β1 integrin suffice to recapitulate all the possible integrin combinations in Mel cells. shRNA to αV integrin reduced fusion mediated by gB and gH/gL to 60%, implicating integrins in VZV tropism for the first time, namely as receptors for VZV. In accordance, integrin αV is retrievable on surfaces of T cell membrane, epithelial cells, keratinocytes, and dermal fibroblasts, which all are VZV permissive cells.[41, 361-366]

Knocking down exerted by other integrin shRNA instead resulted in 30% fusion increase, due to an enhanced expression of αV integrin, which was documented with WB and depended onto intrinsic balancing mechanisms proper of integrins. shRNAs against β3 integrin, β5 integrin or β8 integrin were tested to discriminate the paired β-subunits further involved in VZV fusion. Only cells knocked down for β3 integrin showed 15% reduction in gB and gH/gL mediated fusion, which, however, was not statistically significant.[290]

Furthermore, administering a MAb anti αV integrin to fusing cocultures of CHO cells, that express gB and gH/gL, and to Mel cells results in a 63% fusion inhibition. The addition of an anti-gI MAb, used as a negative control, did not decrease fusion while the neutralizing anti-gH MAb 206, used as a positive control, did.[290,321,323]

Cell-associated VZV pOka was used to infect Mel cells silenced for αV integrin, β1 integrin, and β3 integrin. VZV spread was measured by averaging respective plaque size displayed after 72 h.[290] Cell-associated infection was reduced to 60% in αV integrin silenced cells as compared to control or other integrin silenced cells.[290] αV Integrin shRNA even causes a statistically significant reduction of virus propagation between 24 h to 72 h post infection, decreasing the viral titer up to ten times in respect to control or other integrins shRNA.[290] Integrins contribution at 96 h post infection was lower, as cell-associated VZV pOka infection of the other integrin-silenced cells led to similar viral titers[147]. In contrast, in cell-free VZV infection, both αV integrin and β3 integrin led to a statistically significant reduction in the mean number of plaque at 96 h post infection, while β1 integrin or non-correlated control shRNAs did not.[290]

Therefore, in VZV entry, all the trascriptionally represented integrin β subunits may act as interchangeable partners for the essential αV integrin subunit, as also occurred for other herpesviruses[176,367] VZV might take advantage for its infection of the membrane modifications driven by an interaction with integrin αV, since αV integrins affect cell adhesion migration and proliferation.[368,369] Another point is that integrins usually recognize in their ligand the RGD integrin interacting motif, which has already been found other herpesviruses entry glycoproteins.[243,370] However, neither RGD domains nor disintegrin-like domains (DLD) were found in VZV gB and gH/gL, leaving the questions open on the VZV glycoprotein involved in integrin binding, and on the kind of integrin interaction exploited by these VZV glycoproteins.[290,370,371] It was speculated that, as for HSV-1 (even if VZV has no gD homolog), integrins could relocalize VZV entry receptor to lipid rafts, were integrin resides.[353] This should facilitate VZV entry process and might account for the recovery in VZV titers seen at 4 days post infection.[290]

Finally, while it was demonstrated that VZV gE and gI were not essential for VZV *in vitro* infection, together they carry three KGD integrin interacting motifs and may play a role in VZV-integrin interactions awaiting to be further investigated.[289,290]

# 2 PUBLICATION

Results reported about HSV were published in:

**Gianni T, Massaro R, Campadelli-Fiume G. 2015. Dissociation of HSV gL from gH by αvβ6- or αvβ8-integrin promotes gH activation and virus entry. Proc Natl Acad Sci U S A 112(29):E3901-10**

Results presented about VZV are unpublished and are should be considered as confidential communication

# 3.1 HSV RESULTS

## 3.1.1 The extracellular domain of integrin β6 increases HSV entry

J cells are baby hamster kidney (BHK) derivatives, deprived of HSV entry receptors: they allow HSV infection only if they express an HSV receptor.[204] J cells surface has a low amount of hamster integrins, which might originate only low interference with human integrin overexpression.[176] Earlier, this laboratory reported that integrin αvβ6 and αvβ8 serve as receptors for HSV, and they also allow an entry pathway depending on acidic endosomes.[176] To discriminate which part of the integrin exerts integrin double role on HSV entry, the mutant integrin β6N1 chimera was engineered. β6N1 chimera contains the extracellular domain of β6 integrin, whereas the transmembrane and cytoplasmic tail belong to nectin1. The integrin β subunit was chosen as it is the signaling part of the integrin heterodimer and nectin1 is a non-signaling receptor. Expression of β6N1 chimera in J cells, together with αv integrin subunit and nectin1, allowed an increased entry upon HSV infection, similar to the one seen with wild type β6 integrin (figure 1).[176] The increase in HSV infection upon αvβ6 or αvβ6N1 integrin expression implies a specific molecular interaction at high affinity among αvβ6 ectodomains and HSV gH/gL.[176] This interaction enhances the tethering between HSV and the target cell surface via gH/gL, interacting with integrin heterodimers, increasing HSV infection.[176]

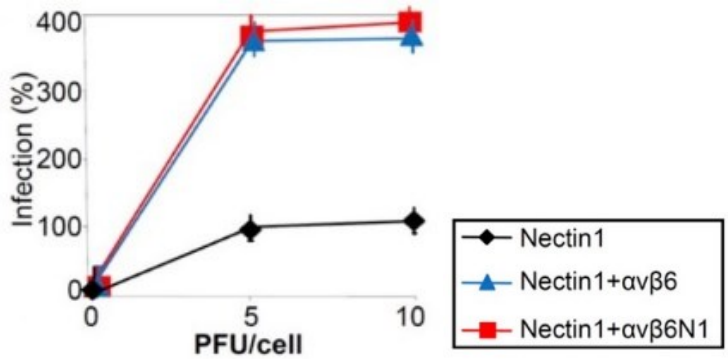



**Figure 1.** The graphic shows a similar increase in HSV infection due to overexpression of integrin αvβ6 or αvβ6N1 chimera in J cells already expressing nectin1. The extracellular domain of β6 integrin determinates HSV increase in infection upon encounter of both αvβ6 and nectin1.[176]

These results indicate that integrins are critical for HSV infection.

### 3.1.2 Integrin αvβ6 cytoplasmic tail determinates the HSV entry pathway

The HSV virion endocytosis in acid endosomes driven by integrins does not seem to be essential as it was also documented HSV entry at neutral endosome compartment or at cell surface.[170,171,174] Two inhibitors were used to interfere with integrin endocytosis mechanism: Bafilomycin A and Wortmannin. Bafilomycin A (BFLA) inhibits specifically vacuolar ATPase and prevents acidification of endosomes, whereas Wortmannin (WM) inhibits phosphoinositide-3-Kinase (PI3K), which is generally involved in endocytosis. BFLA and WM hampered infection in J cells, expressing αvβ6 integrin: once present, this integrin drives HSV to enter through the acidic endosome pathway of entry (figure 2). Endocytosis function essentially maps in the cytoplasmic tail of the integrin β subunit, as HSV entry was not inhibited by BFLA or WM in cells expressing the wt integrin but not the αvβ6N1 chimera.

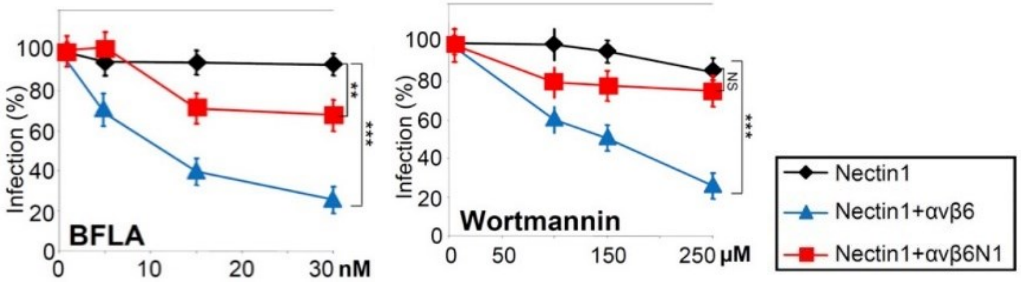



**Figure 2.** The graphics show the dependence of HSV infection to Bafilomycin A (BFLA) (inhibitor of endosome acidification) and Wortmannin (PI-3K inhibitor) when J cells expressing nectin1 and integrin αvβ6 or αvβ6N1 chimera are infected. Replacing the cytoplasmic tail of β6 integrin rendered the HSV entry not dependent on endocytosis.

### 3.1.3 HSV gL is dissociated from gH in HSV glycoprotein complex harvested on STREP-tagged αv β6 or β6N1 or β8 integrins, but only if nectin1 gD, gH/gL and gB were also present *in cis*.

An immunoprecipitation (IP) assay followed by western blot (WB) was used to investigate changes in the glycoproteins constituting the HSV complexes which mediate entry.[179] At first, the overexpression of HSV glycoproteins, together with their receptors and coreceptors on the same cell membrane (*in cis*), was chosen to provide evidence of conformational modification triggered by the interplay between integrin αvβ6 or αvb8 and HSV glycoproteins. In particular, combinations of stable complexes were harvested from protein extracts by pull-down experiments from STREP tagged αv integrin, nectin1 and HSV gD (respectively $\alpha v_{STREP}$, $nectin1_{STREP}$ and $gD_{STREP}$) .$\alpha v_{STREP}$ integrin was paired with non-tagged β6 or β8.[338] Results show that gL of HSV was dissociated from gH/gL heterodimer upon binding to αvβ6 or αvβ8 integrins, and only in the presence of all the HSV glycoproteins and the HSV receptor nectin1 (figure 3, lane 1).

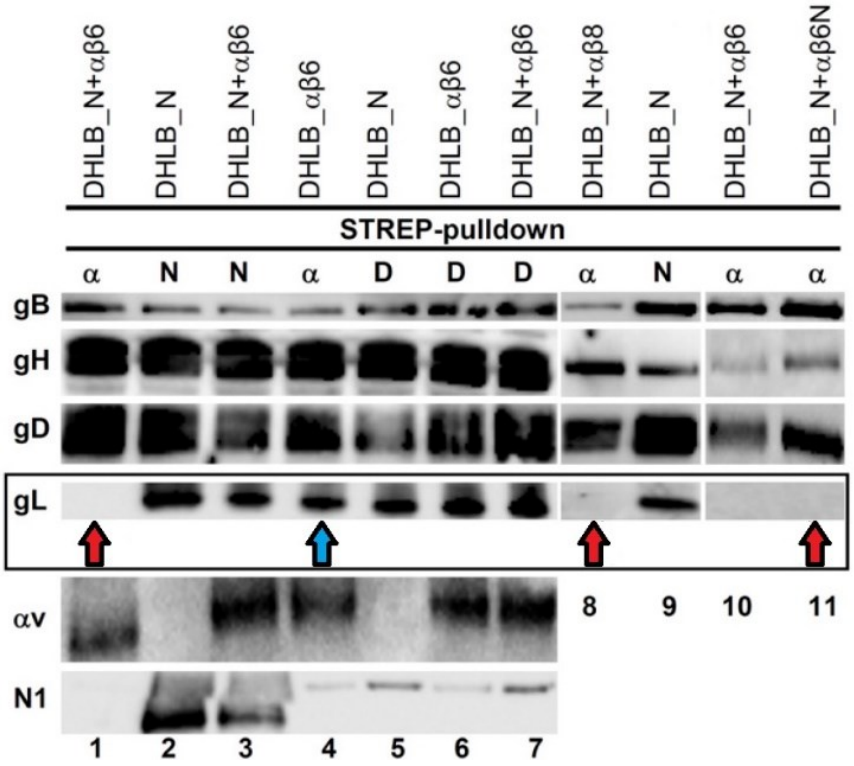



**Figure 3.** Western blot analysis of glycoprotein complexes assembled on STREP tagged αv, nectin1 or gD. Pull-downs were assayed from extracts of J cells expressing various *in cis* combinations of nectin1, integrin αvβ6, αvβ8 or αvβ6N1 chimera and HSV glycoproteins. gL dissociation is indicated with red arrows.

All the combinations including or not integrins were probed, combining all the HSV essential entry glycoproteins and nectin1, using different STREP tagged interactors. Accordingly, gD, gH/gL, gB were expressed in suitable combinations, together with or without nectin1 and/or integrins αvβ6 or αvβ8. The only *in cis* combination in which gL dissociation is detected is the one which better mimicks the composition of the complex that forms upon host HSV infection, or the one which comprises all the essential *in vivo* molecular interactors. If $\alpha v_{STREP}\beta 6$ or $\alpha v_{STREP}\beta 8$ integrins were independently expressed with wild type nectin1, the complex assembled onto integrin contained nectin1 and gD, gH and gB. gL was absent (red arrow, figure 3). When nectin1 was omitted, the combination set on $\alpha v_{STREP}\beta 6$ or $\alpha v_{STREP}$b8 integrins contained again gH/gL and gD or gB (blue arrow, figure 3). To match all the possible cases, an extended comparison was made among complex gathered from $nectin1_{STREP}$ or HSV $gD_{STREP}$. From $nectin1_{STREP}$ all the glycoproteins were collected together, irrespective of the presence of integrins, and only when gD was present. Integrin was not detected in this kind of complex. $gD_{STREP}$ was able to precipitate nectin1 and all the other HSV glycoproteins when present, while it was not capable of pulling down integrins, as expected. $\alpha v_{STREP}\beta 6$ integrin interacted with HSV gH/gL in the absence of nectin1, as demonstrated by surface plasmon resonance.[176] If β6 integrin was replaced with β8 or β6N1 chimera subunits, and assayed in the presence of the complete range of interactors, this condition led to similar results as αvβ6, namely gL dissociation (red arrow, figure 3). Hence, β8 behaves similarly to β6 in promoting gL dissociation from the HSV entry glycoprotein complex in an assay which mimicks HSV entry. Moreover, only the extracellular domain of β6 is involved in gL release from gH/gL. Lysates were analyzed by WB to verify the presence of all the HSV glycoproteins and their host interactors, and of gL also in condition of gL dissociation (figure 4).

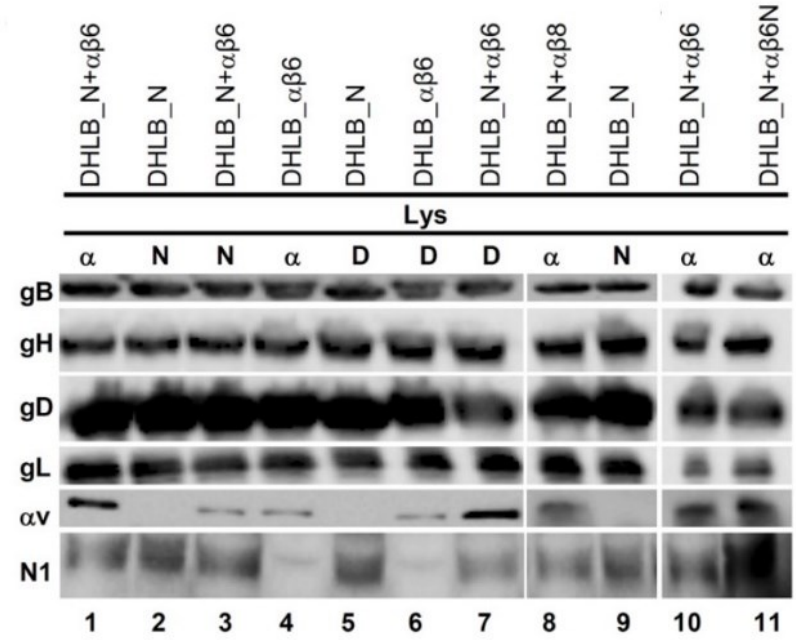



**Figure 4.** Western Blot analysis of lysates from extracts of J cells expressing *in cis* various combinations of nectin1, integrin αvβ6, αvβ8 or αvβ6N1 chimera and HSV glycoproteins, showing glycoprotein complexes and host interactors assayed in figure 3.

### 3.1.4 gL dissociation is not a consequence of gH degradation

Integrin αvβ6 might act in combination with nectin1 to degrade HSV gH N-terminus and thus freeing gL from the gH/gL heterodimer. Therefore, gL dissociation might only be the outcome of a mechanism eventually induced by integrin or an aspecific effect. To rule out this possibility, an aliquot of the same pull-down protein extracts described before probed with H12 MAb, which specifically recognizes gH (figure 5).

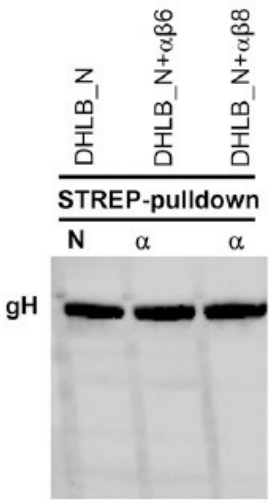



**Figure 5.** Western Blot analysis of three lysates from the extracts assayed in figure 3 showing all the same molecular weight, as detected by Mab H12 to gH. gL dissociation upon gH degradation is excluded.

If gH had been degraded, its apparent molecular weight would have been decreased, in those samples which exibited gL dissociation. Another possibility is that, if gH had been degraded, the whole amount of gH would have been smaller in the sample where gL dissociation took place. The results show that neither is the case.

### 3.1.5 HSV gL is dissociated from gH in HSV glycoprotein complex harvested on STREP-tagged αv plus β6, β6N1 or β8 integrins, but only if nectin1 gD, gH/gL and gB were also present *in trans*.

After HSV virion attaches to heparan sulphate on the host cell and interacts via its glycoproteins, specifically with host receptors, integrins and nectin1 are *vis-a-vis* or *in trans* to HSV glycoproteins. This situation could be easily mimicked by co-culturing two cell populations, one called “target”, which expresses HSV receptors, while the other population, termed “donor”, expresses cognate HSV glycoproteins or combination thereof. Target J cells population expressed combination of αvβ6 integrin and nectin1, while donor J cells expressed combinations of HSV gD, gH/gL and gB. A pull-down from $\alpha v_{STREP}\beta 6$ or $nectin1_{STREP}$ was performed when integrin and nectin1 were present both together and individually as control. All the HSV glycoproteins were pulled down on $nectin1_{STREP}$ irrespective of the

simultaneous presence of αvβ6 integrin (figure 6, blue arrow). In contrast, yet accordingly with previous co-transfection data, the glycoproteins' complex assembled onto $\alpha v_{STREP}\beta 6$ integrin, lacked gL when nectin1 was present (figure 6, red arrow).

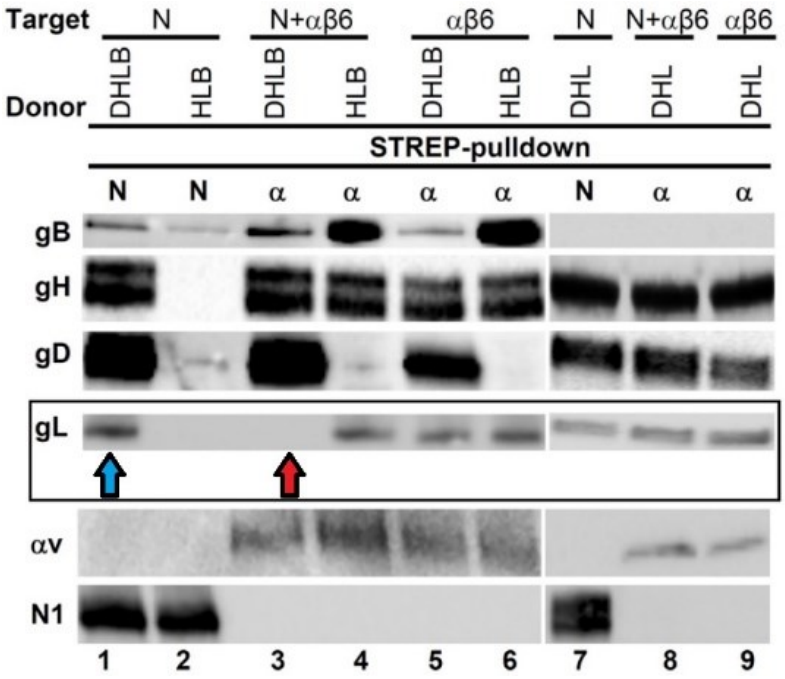



**Figure 6.** Western Blot analysis of glycoprotein complexes assembled *in trans* on $\alpha v_{STREP}\beta 6$ or $nectin1_{STREP}$. Pull-downs assayed from extracts of J cells expressing various combinations of nectin1 and integrin αvβ6 (target) and HSV glycoproteins (donor). gL dissociation is indicated with a red arrow.

Again, the glycoproteins' complex assembled onto $\alpha v_{STREP}\beta 6$ integrin contained gL when nectin1 was absent (figure 6, lane 5). Interestingly, if gD was omitted, $\alpha v_{STREP}\beta 6$ integrin was still capable of collecting gB and gH/gL, confirming that these HSV glycoproteins interact in their steady-state, as already demonstrated (figure 6, lane 6).[179] Moreover, even when gB was not included, $\alpha v_{STREP}\beta 6$ integrin precipitated gH/gL together with gD, arguing in favor of the presence of HSV glycoproteins' pre-assembled complexes in resting conditions (figure 6, lanes 8 and 9).[179] The amount of gD precipitated by $nectin1_{STREP}$ was higher than the one precipitated in the same conditions by $\alpha v_{STREP}\beta 6$ integrin (figure 6, lane 5), while the whole lysate quantification confirmed homogeneous expression of the different glycoproteins and receptors (figure 7). Lysates were analyzed by WB in order to verify the presence of HSV glycoproteins and their host interactors (as described for every combination), also in conditions that led to gL dissociation (figure 7).

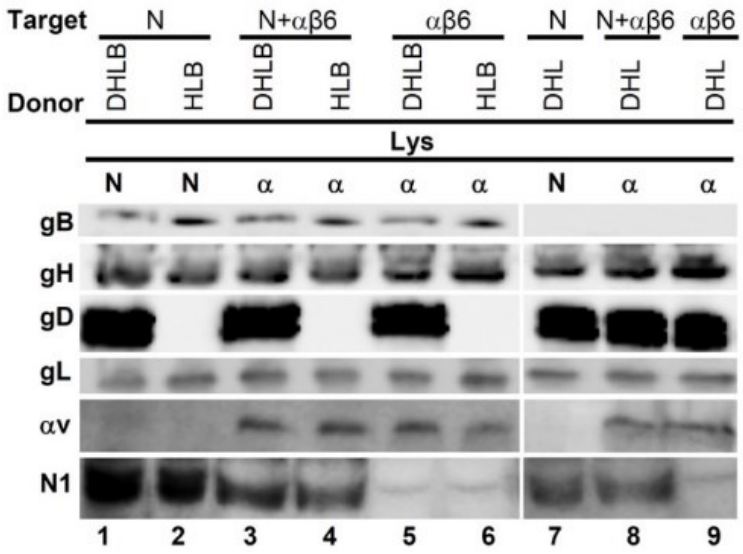



**Figure 7.** Western Blot analysis of lysates from extracts of J cells expressing *in trans* various combination of nectin1 and integrin αvβ6 and HSV glycoproteins, showing glycoprotein complexes and host interactors assayed in figure 6.

## 3.1.6 The RGD domain of HSV gH is essential for gL dissociation

HSV gH 176RGD178 domain is an integrin interacting motif located at the beginning of the H1B domain. HSV gH mutant 176ADA178 ($gH_{ADA}$) is capable of infecting susceptible cells via an αvβ6 integrin dependent entry, although at a low level of infection.[176] This suggests that RGD domain is exploited by HSV, but it might not represent the only domain interacting with integrins. Figure 8 shows that the HSV glycoprotein complex assembled onto integrin αvβ6 contained gL when $gH_{ADA}$ mutant was used instead of the wild type gH and nectin1 was present (blue arrow, figure 8) . Since gL did not dissociate from $gH_{ADA}$ mutant, the conclusion is thatthe RGD domain in wild type gH is essential for gL dissociation.

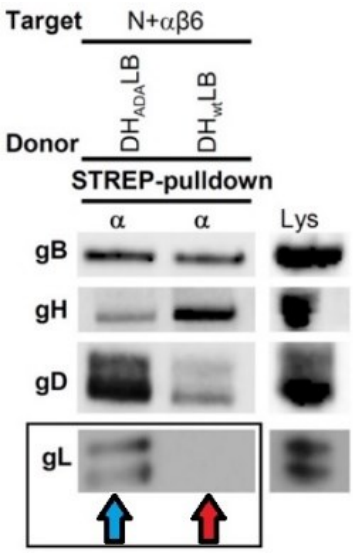



**Figure 8.** Western Blot analysis of glycoprotein complexes assembled *in trans* on αv$_{STREP}$β6 integrin. Pull-downs assayed from extracts of J cells expressing nectin1 and αvβ6 integrin (target cells) and HSV glycoproteins (donor cells) with or without gH mutated in its RGD domain (gH$_{ADA}$). gL dissociation is indicated with a red arrow. Reference lysates of wild type HSV glycoproteins are shown on the right.

### 3.1.7 HSV gH mutant RGD to ADA is still precipitated by αv$_{STREP}$β6 integrin

The absence of gL dissociation from gH$_{ADA}$ was not due to the lack of gH$_{ADA}$/gL heterodimer formation. Since αv$_{STREP}$β6 integrin was able to pull-down HSV gH/gL on its own, as previously demonstrated here and in ref.176, this feature was used to investigate whether also gH$_{ADA}$/gL could have been pulled down by αv$_{STREP}$β6 integrin, implying that mutant gH$_{ADA}$ is folded correctly. Pull-downs from αv$_{STREP}$β6 integrin analyzed by WB revealed that gH$_{ADA}$/gL were precipitated in a similar fashion to gH/gL wild type, but at a lower level (figure 9). Again, as HSV gH$_{ADA}$ still interacts with αv$_{STREP}$β6 integrin, HSV gH RGD domain contribution might depend on nearby residues or domains.[176]

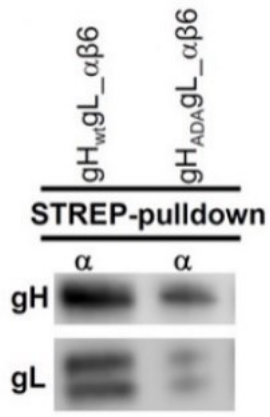



**Figure 9.** Western Blot analysis of HSV gH/gL assembled on $\alpha v_{STREP}\beta 6$. Pull-downs assayed from extracts of J cells expressing only integrin αvβ6 and HSV gL plus wild type or gH mutated in its RGD domain ($gH_{ADA}$). A lower amount of gH/gL heterodimer is pulled down by integrin αvβ6 alone if $gH_{ADA}$ is used instead of wild type HSV gH.

## 3.1.8 gL dissociates from gH of immobilized HSV virions (VIR ELISA)

To detect gL release directly in HSV virions, the properties of two different MAbs to HSV gH, Mab 52S and MAb 53S were exploited. MAb 52S is a neutralizing MAb while MAb 53S is non-neutralizing.[265] MAb 52S binds a gL independent epitope on gH and allowed the detection of the total amount of gH present on HSV virions, either bound or not bound to gL. MAb 53S allows the detection of the gH, which is in complex with gL, as MAb 53S binds a gH epitope, which is gL dependent. gL dissociation should correlate with a decreased reactivity of MAb 53S on HSV virions which had interacted with cells bearing αvβ6 and nectin1. No decrease in MAb 53S reactivity should be detected if only nectin1 is present on the cells. MAb 52S reactivity should remain constant during HSV virions interaction with susceptible cells, irrespective of the receptors they express. A comparison was made between MAbs reactivity during HSV virions absorption of susceptible cells bearing αvβ6 plus nectin1 or nectin1 only. In the VIR-ELISA approach, HSV virions were immobilized on the multiwells surface, a suspension of J cells expressing nectin1 or nectin1 plus αvβ6 integrin was overlaid. J cells were removed and MAb 53S reactivity of the tethered HSV virions was probed, resulting in a 76% reduction in HSV virions which had interacted with J cells carrying αvβ6-integrin plus nectin1, as compared to HSV virions which had interacted with J cells carrying only nectin1 (figure 10). Since MAb 52S reactivity was instead at least the same between J cells expressing nectin1 plus or minus αvβ6 integrin,

the decrease in MAb 53S detected in gL dissociation conditions implied no HSV virion degradation or detachment from the plate. MAb 52S reactivity level was even higher in HSV virions subjected to J cells bearing αvβ6 integrin and nectin1; this could be due to unknown structural modifications in gH during αvβ6 integrin interaction, which further increases epitope reactivity. The fast kinetics of VIR-ELISA test implies that gL dissociation, detected via MAbs, is likely to take place during HSV virion absorption or entry.

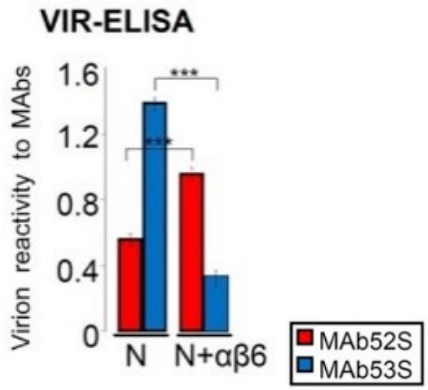



**Figure 10.** VIR-ELISA: MAb 52S recognizes an epitope in gH that is gL independent, while 53S epitope in gH is gL dependent. HSV virion reactivity to MAb 52S and 53S was measured after absorption and removal of J cells expressing nectin1 plus or minus αvβ6 integrin to tethered HSV virion. gL release corresponded to the decrease in 53S reactivity detected when HSV virions absorbed to J cells expressing nectin1 plus αvβ6 integrin. Increase in Mab 52S reactivity to HSV gH might be due to an unknown conformational modification ensued in gH upon gL dissociation.

### 3.1.9 gL dissociates from gH of HSV virion only when virions encounter nectin1 together with αvβ6 integrin

To further investigate gL dissociation from HSV virions, an opposite layout between HSV and target J cells was adopted. The reactivity of MAb 52S and MAb 53S to HSV virions absorbed to J cells was assayed by means of flow cytofluorimetry. HSV virions were absorbed for 1h at 37°C to J cell monolayers, and then removed and immediately analyzed by flow cytofluorimetry for MAb 52S and MAb 53S reactivity. As mentioned above, MAb 52S reactivity reflects the total amount of HSV virions. Again, the decrease in MAb 53S reactivity reflected the gL dissociation from gH/gL,

happening in HSV virions absorbed to J cells expressing nectin1 plus αvβ6 integrin but not nectin1 only (figure 11). In particular, MAb 52S reactivity of absorbed HSV virions was comparable among J cells expressing nectin1 plus αvβ6 integrin (62.5%) and nectin1 alone (56%). MAb 52S reactivity of HSV virions absorbed to J cells expressing only αvβ6 integrin without nectin1 was 40% (figure 11, quantification). MAb 53S reactivity was 58% when HSV virions absorbed to J cells bearing nectin1 only, whilst it was diminished to 13% upon absorption to J cells expressing αvβ6 integrin together with nectin1. MAb 53S reactivity decrease implies that, upon interaction with J cells carrying integrin αvβ6 and nectin1, gH/gL heterodimer changes its conformation as the gL dependent epitope identified on gH by MAb 53S disappears (gL dissociation). Decrease in both MAbs reactivity when HSV virions absorbed to J cells expressing αvβ6 integrin might only be due to decreased HSV virions absorption.

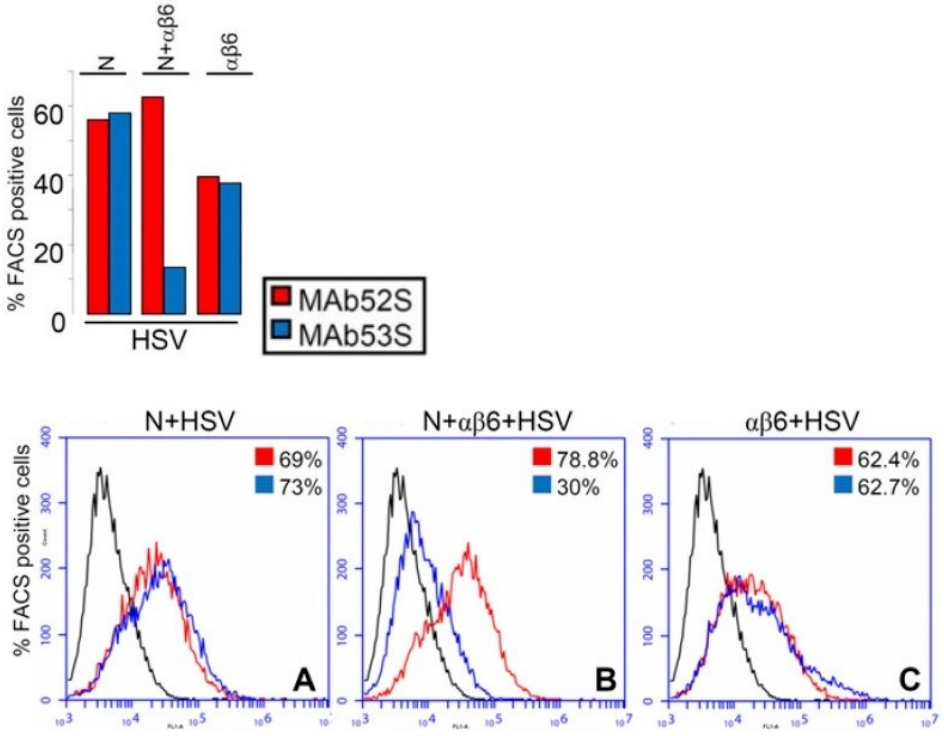



**Figure 11.** Quantification and flow cytofluorimetry analysis of HSV virions absorbed to J cells. MAb 52S recognizes an epitope in gH, while 53S epitope in gH is gL dependent. HSV virion reactivity to MAb 52S and 53S was measured after absorption to immobilized J cells expressing nectin1 plus or minus αvβ6 integrin or αvβ6 integrin only. gL was dissociated as documented by the decrease in MAb 53S reactivity when HSV virions absorbed to J cells expressing nectin1 plus αvβ6 integrin.

### 3.1.10 HSV virion, virion deleted in gB or HSV virion neutralized with PAb to gD prevent gL dissociation from gH upon binding of virions to J cell expressing nectin1 and integrin αvβ6.

Here, the question was if gL dissociation took place under conditions preventing virus entry, exemplified by non-infectious virions or virions preincubated with neutralizing Mabs. In particular, as it happened for the pull-down experiments, here it was tempting again to investigate gL detachment requirements eliminating once at a time one or the other essential glycoprotein, but acting directly on the infective particle. Notably, as gL detached only if all the actors of the entry apparatus were present, the condition tested would have nonetheless resulted in HSV entry blockade, either due to the interference in gD-nectin1 interaction mediated by the neutralizing PAb R8, or to the lack of gB fusogen as for the entry defective HSV mutant $gB^{-/-}$. HSV in presence of PAb R8 or mutant $gB^{-/-}$ was used to infect J cells carrying various combinations of αvβ6 integrin and nectin1. A lack in required glycoproteins or receptors mediating entry may hamper the conformational changes to the gH/gL heterodimer, in addition to the obvious neutralization effect on virus infectivity. Infection performed with both virion pre-incubated with PAb R8 and $gB^{-/-}$ virion, led to the retention of gL, as no decrease on MAb 53S reactivity was detected compared to MAb 52S reactivity (figure 12, quantification for $gB^{-/-}$, plots for PAb R8 pre-incubation). Thus, virion gL dissociation took place when gD, gH/gL and gB were present on the HSV virion and the host cell expresses together receptors nectin1 and αvβ6 integrin.

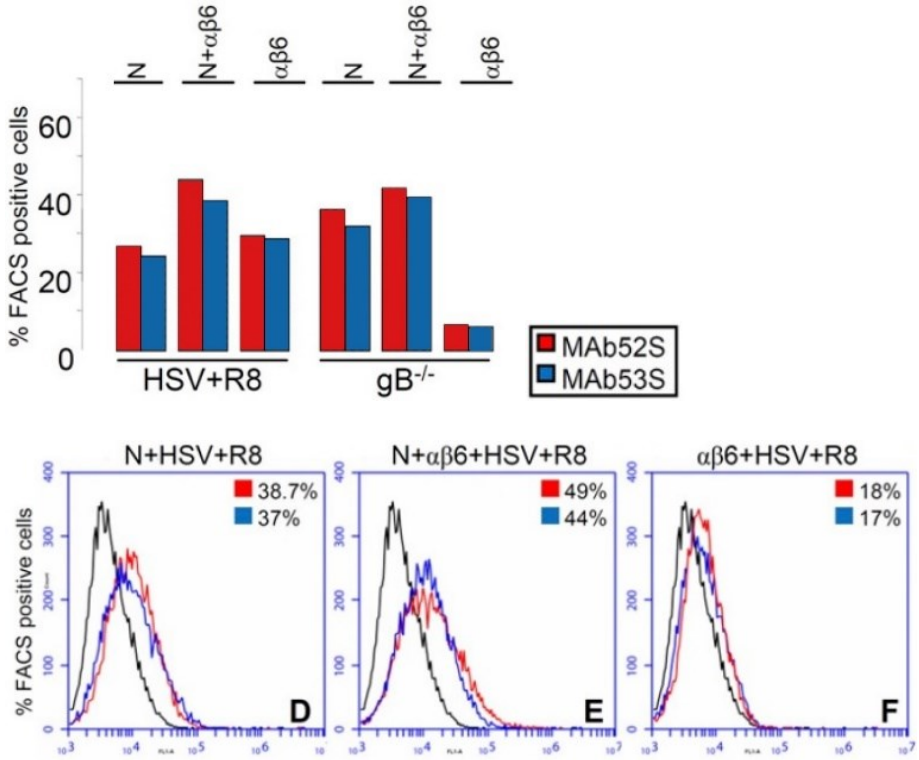



**Figure 12.** Quantification and flow cytofluorimetry analysis of HSV virions preincubated with Neutralizing PAb to gD (R8) absorbed to J cells and quantification of $gB^{-/-}$ HSV virions absorbed to J cells. Mab 52S recognizes an epitope in gH, while 53S epitope in gH is gL dependent. HSV virion reactivity to MAb 52S and 53S was measured after absorption to immobilized J cells expressing nectin1 plus or minus αvβ6 integrin or αvβ6 integrin only. gL was never dissociated as documented by MAb 52S and 53S reactivity when HSV virions absorbed to J cells expressing nectin1 plus αvβ6 integrin.

## 3.1.11 gL dissociation is not affected by presence of BFLA

αvβ6 integrin dependent decrease in MAb 53S reactivity (up to 12%) upon infection of J cells bearing both integrin and nectin1 took place irrespective of the presence of the endocytosis inhibitor BFLA (figure 13). Thus, virion gL dissociation took place when gD, gH/gL and gB were present on the HSV virion, and the host cell expresses receptors nectin1 and αvβ6 integrin together. gL dissociation was either not dependent on, or took place before, endocytosis of the HSV virion.

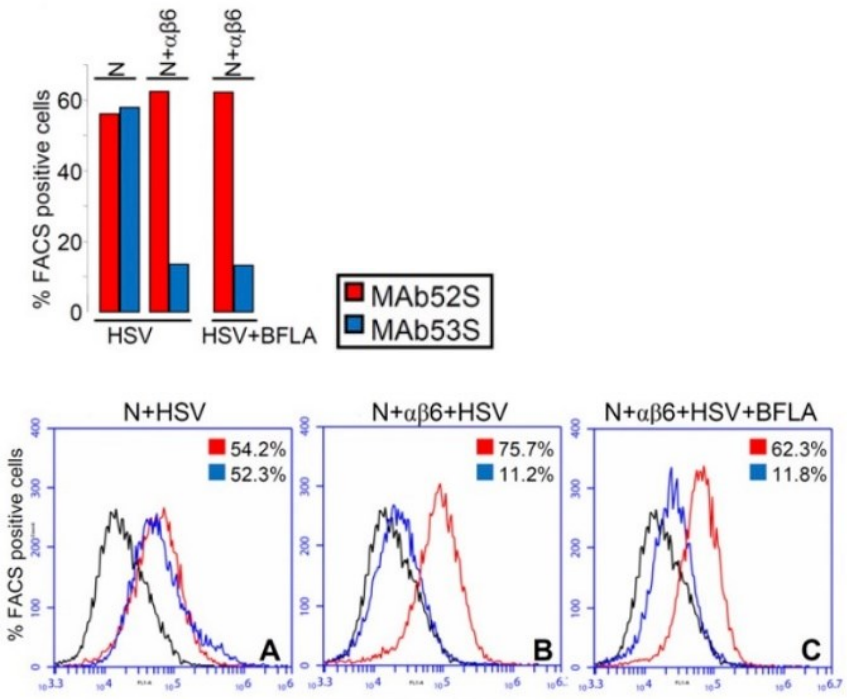



**Figure 13.** Quantification and flow cytofluorimetry analysis of HSV virions absorbed to J cells. MAb 52S recognizes an epitope in gH, while 53S epitope in gH is gL dependent. HSV virion reactivity to MAb 52S and 53S was measured after absorption to immobilized J cells expressing nectin1 or nectin1 plus αvβ6 integrin in absence or presence of the endocytosis inhibitor BFLA. gL was dissociated irrespective of the presence of BFLA, as documented by the decrease in MAb 53S reactivity when HSV virions absorbed to J cells expressing nectin1 plus αvβ6 integrin.

## 3.1.12 gL dissociation coincides with its release in the media of HSV virion and host cell mixture

When gL is detached from the gH heterodimer, it should be found in the medium of virions absorbed to J cells expressing nectin1 and αvβ6 integrin. Media from the experiments described above (figure 11-13) were harvested after virion absorption to J cells, and assayed by WB. gL was detected in the media of virion-cell mixtures only if nectin1, αvβ6 integrin and gD, gH/gL, gB were present, and irrespectively of the presence of the endocytosis inhibitor BFLA (figure 14, red arrows). Neither J cells expressing only nectin1 (figure 14, blue arrow) nor integrin αvβ6 alone caused dissociation of gL during HSV infection and its detection in the media. Moreover, the pre-incubation with neutralizing PAb R8 to gD or the use of $gB^{-/-}$ virions hampered gL dissociation and its presence in the media.

Importantly, only gL was found in the media of virions-J cells mixtures, as WB showed no gB, gH or gD release or shedding in the media (figure 14).

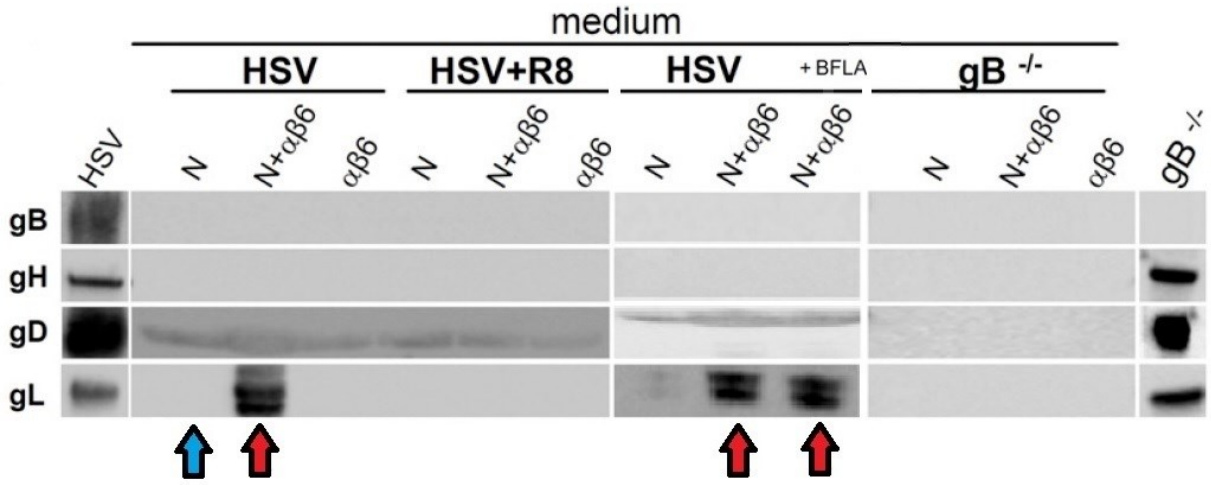



**Figure 14.** Western Blot analysis of culture media harvested from HSV virion- J cell mixtures. J cells expressed nectin1 plus or minus integrin αvβ6 or integrin αvβ6 alone. Red arrows indicate gL release in the media due to its dissociation from gH/gL upon virion binding to J cells expressing nectin1 together with αvβ6. Reference lysates of wild type HSV glycoproteins are shown on the left, while reference lysates of HSV $gB^{-/-}$ virions glycoproteins are shown on the right. BFLA was added only in the sample indicated.

## 3.1.13 gL dissociation and its release in the media of HSV virion - J cells mixtures is not induced by gH degradation

As for the pull-down in the J cell transfection system, it is necessary to exclude that gL detected in the media, where gL dissociation took place, was instead the result of an αvβ6 integrin dependent degradation of gH. To this end WB analysis of virion- J cell mixtures showed that, in every case examined, gH molecular mass had not changed (figure 15). The decrease in gH reactivity seen after HSV virion absorption to J cells expressing only integrin αvβ6 and no nectin1 might be due to a less efficient absorption of HSV virions mediated by integrin αvβ6 in the absence of nectin1.

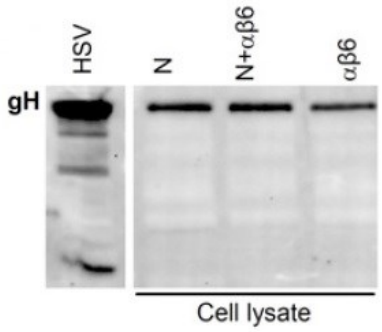



**Figure 15.** Western Blot analysis with MAb H12 to gH of three cell lysates from of HSV virion - J cells mixtures showing all the same molecular weight. gL dissociation upon gH degradation is excluded. MAb H12 reactivity to a reference lysate of wild type HSV is shown on the left.

### 3.1.14 αvβ8 integrin promotes gL dissociation, similarly to αvβ6 integrin

To ascertain if the results obtained with HSV virions and αvβ6 integrin extended also to integrin αvβ8, an experiment was performed, where HSV virions were absorbed to J cells expressing nectin1 and integrin αvβ8 or nectin1 alone or integrin αvβ8 alone. Apart from a lower expression on J cells of integrin αvβ8 (33%) as compared to integrin αvβ6 (60%), results showed again a consistent decrease in MAb 53S reactivity compared to MAb 52S reactivity only upon HSV virions absorption to J cells expressing both nectin1 and integrin αvβ8. Hence, also integrin αvβ8 mediated gL release from gH/gL heterodimer upon entry of HSV virion in susceptible J cells (transiently expressing nectin1). Moreover, in the case gL was dissociated and MAb 53S reactivity was decreased as compared to that of MAb 52S, gL was also detected in the media. In those cases where gL was not dissociated, it was not detected in the media (figure 16, western blot).

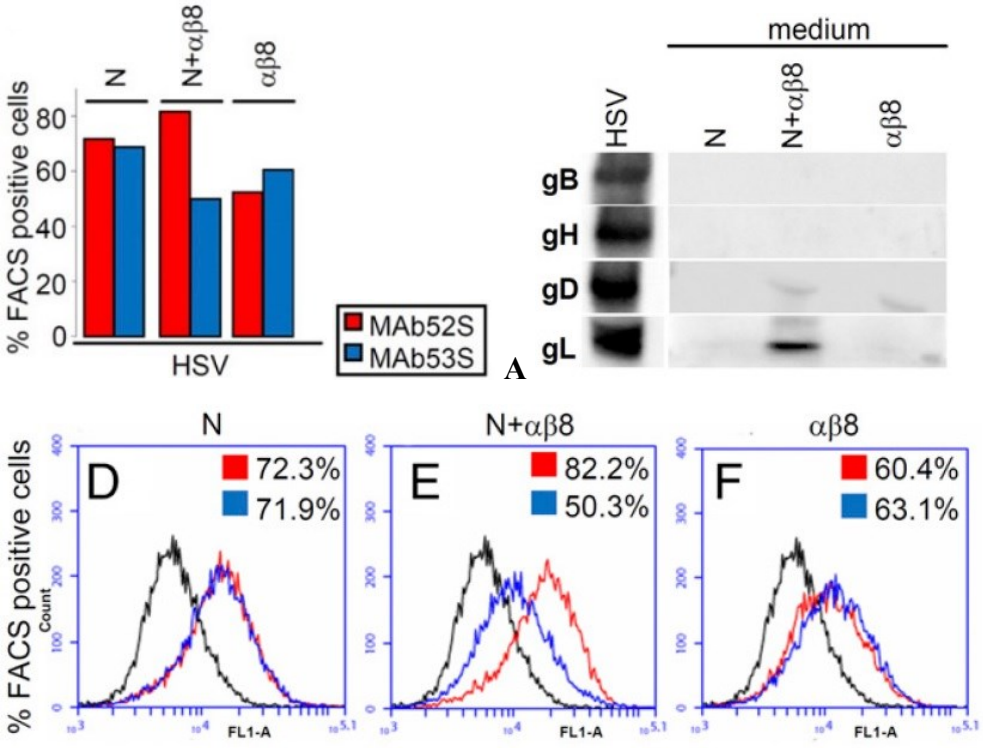



**Figure 16.** From left to right: Quantification of flow cytofluorimetry analysis (left panel), western blot culture media analysis (A) and flow cytofluorimetry analysis (panel d, e, f below) of HSV virions absorbed to J cells expressing combination of αvβ8 integrin and nectin1. MAb 52S recognize an epitope in gH while 53S epitope in gH is gL dependent. HSV virion reactivity to MAb 52S and 53S was measured after absorption to immobilized J cells expressing nectin1 or nectin1 plus αvβ8 integrin or αvβ8 integrin alone. gL was dissociated in the same conditions as for αvβ6 integrin. gL dissociation was documented by the decrease in MAb 53S reactivity when HSV virions absorbed to J cells expressing nectin1 plus αvβ8 integrin. The extent of gL dissociation is lower with αvβ8 integrin as compared to αvβ6 integrin due to a lower expression of αvβ8 integrin. WB analysis of culture media harvested from HSV virion-cell mixtures. Only in J cells expressing nectin1 plus integrin αvβ8 gL was released in the culture media of HSV virion J cells mixture. Reference lysates of wild type HSV glycoproteins are shown on the left.

## 3.1.15 gL dissociation detected by means of immunofluoresce

To provide direct evidence of gL dissociation, fluorescent HSV virions (K26GFP) were employed to infect J cells expressing combinations of αvβ6 integrin and nectin1. The release of gL was monitored by fluorescence microscope. In particular, K26GFP HSV virions encase GFP fused with VP26, which is a viral protein contributing to HSV capsid formation; hence, K26GFP HSV virions emit

fluorescence. Fluorescence microscopy revealed that, upon 30' absorption, HSV virions lost a part of their reactivity to MAb 53S, or to a MAb directed against gL, only when encountered J cells expressing nectin1 together with integrin αvβ6, once again implying gL dissociation to happen. In particular, K26GFP HSV virions and J cells mixtures were fixed and incubated with MAb 52S, MAb 53S and MAb VIII-62 to gL; the latter two showed decreased secondary Ab reactivity only if J cells expressed nectin1 and integrin αvβ6. In contrast, no change in MAbs reactivity was shown if host J cells expressed only nectin1 or when MAb 52S was used for immunofluorescence (figure 17).

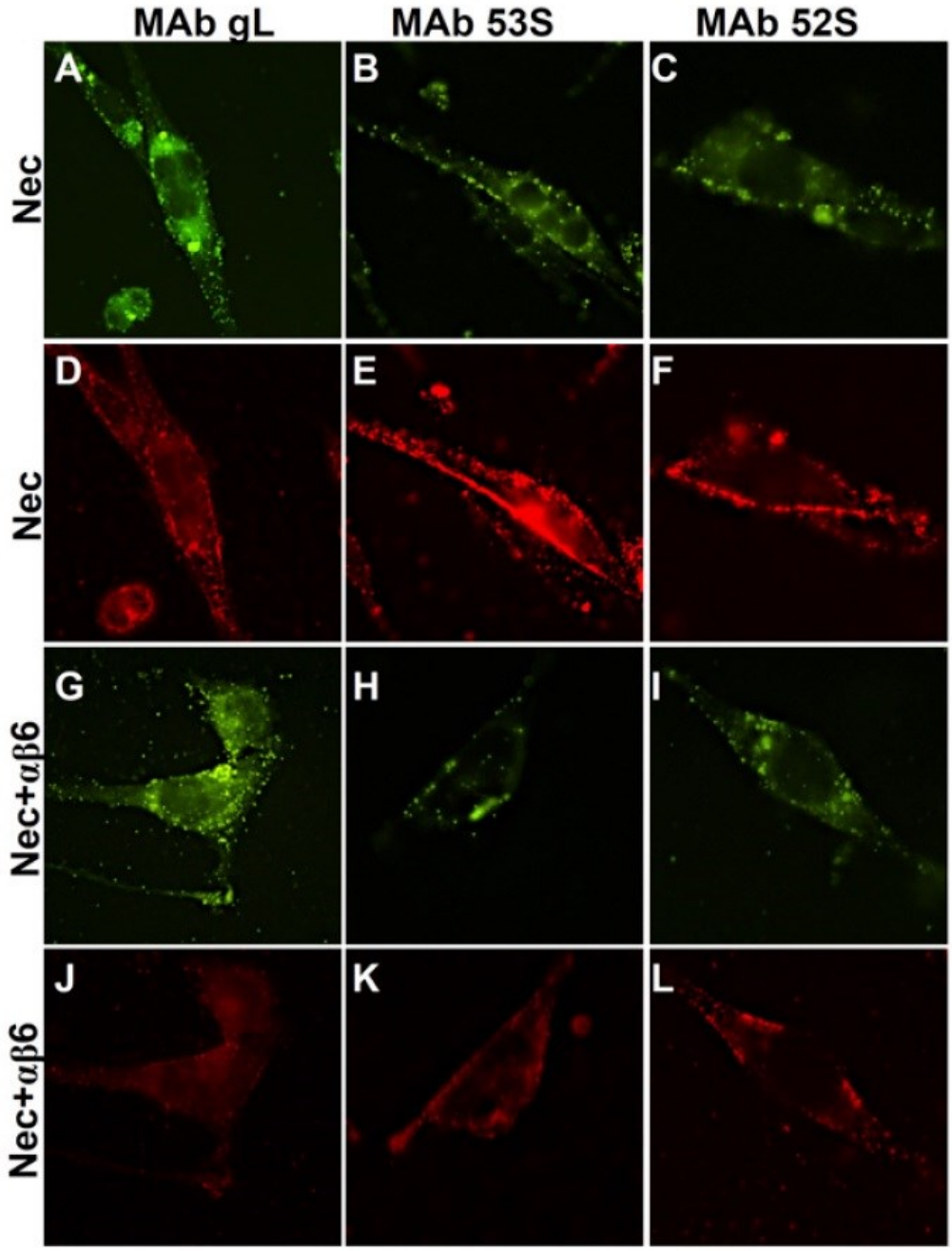



**Figure 17.** Immunofluorescence microscopical analysis of K26GFP HSV virions absorbed to J cells expressing nectin1 or nectin1 plus αvβ6 integrin. MAb 52S to gH (which monitors the whole amount of gH, both complexed or not with gL), MAb 53S to gH (which recognizes a gL dependent gH epitope and thus its decrease correlates with gL dissociation from gH/gL) and Mab VIII-62 to gL were used to screen J cell populations. Representative images are shown. GFP green channel, proportional to the amount of absorbed HSV virions, was used to normalize the output from the red channel, relative to the MAb selected. It is noticeable the lower red fluorescence in J,K panel (VII-62 and 53S, corresponding to nectin1 plus αvβ6 integrin expressing J cells, with gL dissociation) in respect to D,E (VIII-62 and 53S, corresponding to nectin1 only expressing J cells). 52S reactivity is supposed not to decrease, even if gL is released.

Fluorescence data acquired were elaborated so as to provide an indirect quantification of gL dissociation. 30-40 cells per case were scored both for GFP and secondary MAb red fluorescence intensities; mean ratio between red and GFP fluorescence was calculated to get normalization for every case scored. Reactivity of either the MAb to gL or MAb 53S decreased in HSV virions absorbed to J cells expressing both nectin1 and integrin αvβ6 to 50% of HSV virions absorbed to J cells expressing only nectin1, whereas reactivity of MAb 52S did not change (figure 18).

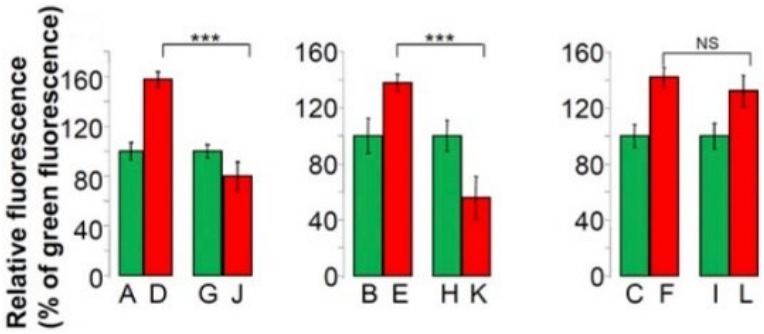
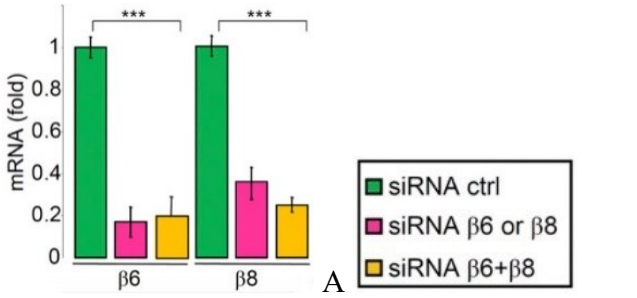



**Figure 18.** Quantification of the dataset exemplified in figure 17 obtained by immunofluorescence microscopical analysis. K26GFP HSV virions absorbed to J cells expressing nectin1 or nectin1 plus αvβ6 integrin. A statistical sample of 30-40 J cell was screened to investigate mean reactivity to MAb 52S to gH, MAb 53S to gH (which recognizes the gL dependent gH epitope) and MAb VIII-62 to gL. GFP green channel, proportional to the amount of absorbed HSV virions, was used as a normalizer (100%). 52S reactivity had not changed in the population considered. Mean gL MAb reactivity (as of panel D) decreased (as of panel J) and mean 53S Mab reactivity (as of panel E) decreased (as of panel K), both documenting gL dissociation from HSV virion gH/gL.

### 3.1.16 Silencing of β6 and β8 integrin subunit in SW480 human cell line prevented gL dissociation upon HSV absorption

Colon carcinoma cell line SW480 expressed a high level of integrin αvβ6 and αvβ8 and thus represented a suitable human epithelial cell model to extend results obtained in J cells.[176] HSV virion gL dissociation during HSV entry was detected again by means of MAb 52S and MAb 53S reactivity, while combinations of integrins were provided by selective subunits depletion with siRNAs against β6 and β8 integrin subunit. Knocking down specific integrin subunits in SW480 should prevent gL dissociation, as seen when HSV virions encountered J cells bearing only nectin1. Integrins depletion in

silenced cells was monitored by qRTPCR and by assaying reactivity to MAb 2077 to αvβ6 integrin and MAb 37E1 to αvβ8 integrin with a flow cytofluorimeter. β6 and β8 integrin subunits were both silenced at about 60-80% and the same extent of silencing was achieved when both β6 and β8 integrin subunits were silenced together. Control siRNA exerted no effect (figure 19, panel A). As detected by means of MAb 2077 to αvβ6 integrin, αvβ6 integrin expression was reduced to about 15% when β6 integrin subunit was silenced in SW480 cells, while silencing of both β6 and β8 integrin subunits lead to a decrease of about 20% in MAb 2077 reactivity to αvβ6 integrin (figure 19, panel B and C). Both the depletion of β8 integrin or the double depletion (β6 and β8) affected MAb 37E1 reactivity to αvβ8 integrin to about 30% (figure 19, panel B and C).



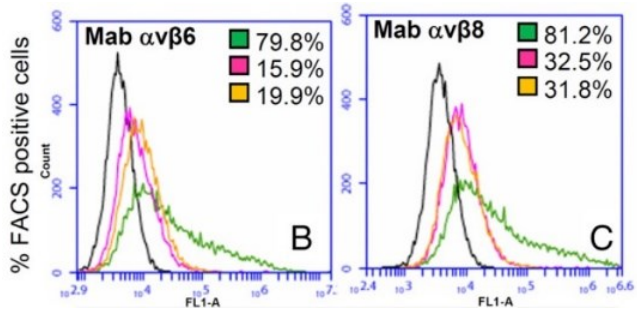
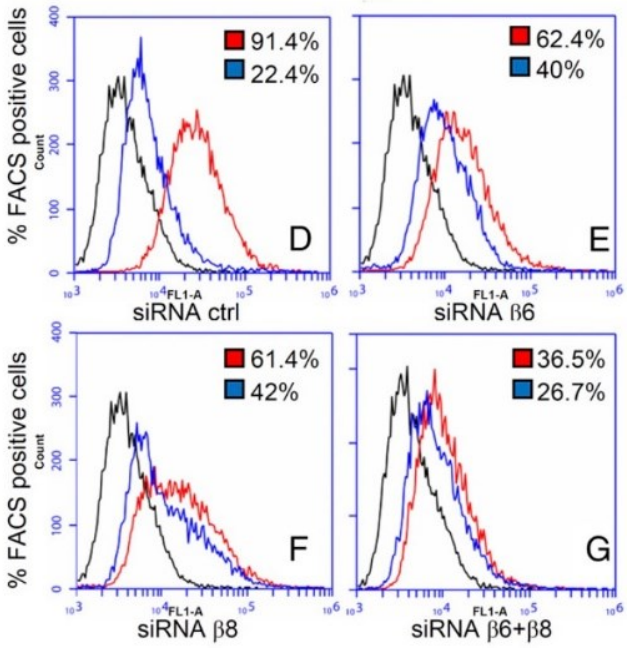




**Figure 19.** qRTPCR quantification of β6 and β8 integrin subunit expression and flow cytofluorimeter analysis of αvβ6 and αvβ8 integrin expression after β6 and β8 integrin subunits silencing with siRNA in SW480 human cell line. (A) Integrin reactivity was monitored after silencing with MAb 2077 to αvβ6 and MAb 37E1 to αvβ8 integrin. Integrin depletion reached 60-80% degree for either β6 or β8 integrin subunits, both for singly silenced subunit or doubly silenced subunit. Control siRNA exerted no effect. (B,C) αvβ6 integrins expression was reduced by about 15% and 20% respectively when β6 integrin subunit was singly or doubly (together with β8) silenced in SW480 cells, as detected by MAb 2077 reactivity decrease to αvβ6 integrin. Both β8 single integrin subunit or double silencing (both β6 and β8 integrin subunit) affected MAb 37E1 reactivity to αvβ8 integrin by about 30%.

Silencing of integrin subunits in SW480 cells reduced both the decrease in MAb 53S reactivity and the detection of gL in the media after HSV virion absorption. In particular, the reactivity to MAb 52S was different among control silenced cells (91%), the cells silenced for one or the other of the two integrin β subunits (61%) and the cells silenced for both β6 and β8 integrin subunits. As integrin αvβ6 and αvβ8 are involved in HSV virion tethering as receptors, depletion of one of them would have directly decreased HSV virion attachment and thus the total amount of HSV absorbed.[176] Simultaneous depletion of both β6 and β8 integrin subunits decreased HSV virion absorption, as estimated by MAb 52S reactivity compared to the single silencing. In fact, β6 and β8 integrin subunits are interchangeable, but both contribute to HSV virion tethering, a process which depends also on αv integrin subunit.[176] On the other hand, MAb 53S reactivity of absorbed HSV virions increased from control cells (22%) to cells silenced for one of the two integrin subunits (40%); but apparently decreased in doubly silenced cells (26%), probably because the absence of the two integrins reduced the level of HSV virion absorbed on cell membrane (figure 20). Nevertheless, when MAb 53S reactivities were normalized onto MAb 52S ones, it emerged that corrected reactivities for MAb 53S increased from 20% (control cells) to 73% (double silenced cells), implying that silencing integrin β6 and β8 subunits effectively prevented the dissociation of gL also in human SW480 (figure 20).



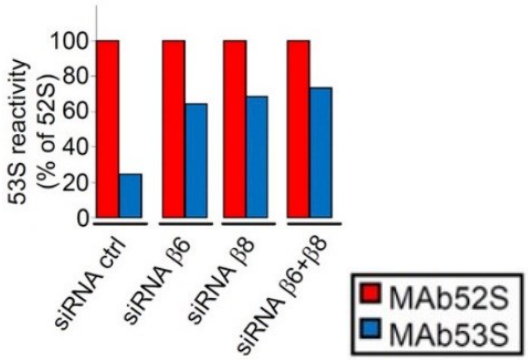



**Figure 20.** Flow cytofluorimetry analysis and corrected quantification of HSV virions absorbed to SW480 human cell line silenced for either β6 or β8 integrin subunits or both. Mab 52S recognizes an epitope in gH, while 53S epitope in gH is gL dependent. HSV virion reactivity to MAb 52S and 53S was measured after absorption to immobilized SW480 human cell line silenced with siRNA for either β6 or β8 integrin subunits or both (figure 19). In the cells that received control siRNA, gL was dissociated as documented by the decrease in MAb 53S reactivity. In double silenced SW480 cells, the cytofluorimeter analysis result of MAb 53S reactivity apparently argues for gL dissociation (26,7%, panel G). After normalization for MAb 52S reactivity (as integrin subunits are important for HSV virion tethering, and their deprivation might have influenced total HSV virion binding) also double integrin subunit silencing exerted inhibition effect on gL dissociation (detected as the increase in MAb 53S reactivity to 73%).

As described above, the decrease of MAb 53S reactivity during absorption of HSV virions reflected gL release in the media from HSV virion. MAb 53S reactivity was low in control cells, and gL is detected in the medium, whereas it increased in cells silenced for one of the two integrins or for both integrin β-subunits (where no gL was detected in the media) (figure 21).

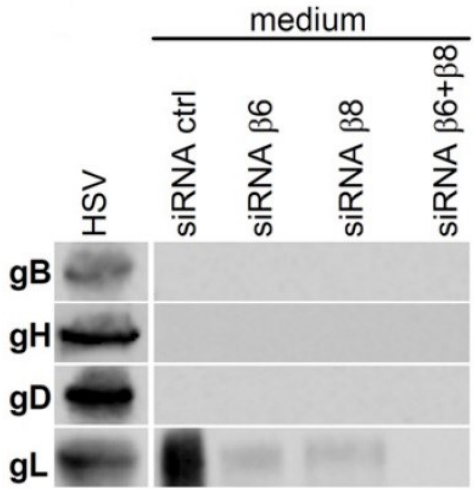


**Figure 21.** Western Blot analysis of culture media harvested from mixtures of absorbed HSV virion to immobilized SW480 human cell line silenced with siRNA for either β6 or β8 integrin subunits or both. In cells that received control siRNA, gL was released and detected in the culture media. The extent of gL release is lower when only either β6 or β8 integrin subunit is silenced, as the other integrin β subunit might promote a residual extent of gL release. In double silenced cells, no gL release was detected. Reference lysates of HSV virion glycoproteins are shown on the left.

### 3.1.17 $gH^{-/-}$ HSV virion infectivity is rescued to a higher degree when soluble $gH_t/gL$ is added to J cells expressing nectin1 and integrin αvβ6, as compared to J cells expressing nectin1 alone

The purpose of subsequent experiments was providing a further line of evidence that gH underwent a conformational change during virus entry. The rationale was as follows. If the interaction of gH/gL with integrins promotes a conformational change to gH/gL, critical for the cascade of glycoproteins activation, then virion bound gH/gL should be replaced with a soluble form of the heterodimer, $gH_t/gL$.[354,357] It was reported that a soluble form of gD ($gD_t$) allowed infection of susceptible cells by $gD^{-/-}$ HSV virion. Moreover, $gD_t$ rescue of $gD^{-/-}$ HSV virions implied that soluble $gD_t$ had changed its conformation to allow infection of susceptible cells.[219] Soluble form of HSV gH/gL allowed fusion of cell transfected with plasmid for nectin1 and gB, when gD was either encoded by a plasmid or administered as a soluble protein. J cells expressing nectin1 plus or minus αvβ6 integrin were exposed to $gH^{-/-}$ HSV virions in presence or absence of $gH_t/gL$ plus or minus $gD_t$ or control BSA. $gH^{-/-}$ HSV virion infectivity rescue was monitored by detecting β-galactosidase activity, proper of this HSV mutant. $gH^{-/-}$ HSV virions were rescued by $gH_t/gL$, and even better by combination of $gH_t/gL$ with $gD_t$, while no rescue was detected when BSA was added (figure 22). Endogenous hamster integrin contribution was lower than that exerted by over-expression of human αvβ6 integrin, as documented by the higher $gH^{-/-}$ HSV virion rescue upon binding J cells expressing both nectin1 and αvβ6 integrin. The results argue in favor of a conformational change to gH/gL which drives infectivity rescue of $gH^{-/-}$ HSV virions, leading to entry in J cells. $gH^{-/-}$ HSV virion rescue was dependent on J cells expression of the gH/gL receptor αvβ6 integrin, as the extent of $gH^{-/-}$ rescue increased when J cells expressed nectin1 plus integrin αvβ6 in respect to nectin1 alone (figure 22).

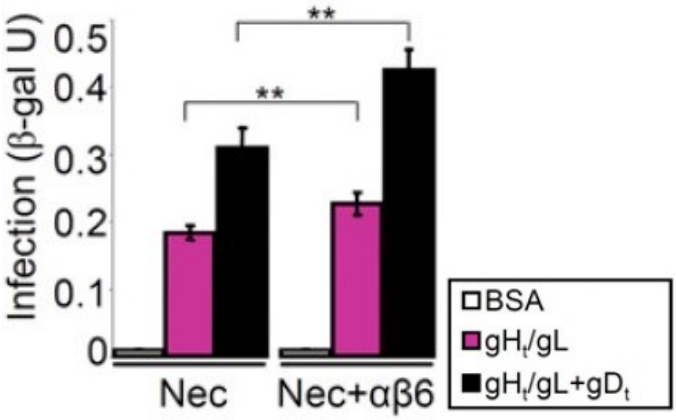



**Figure 22.** Quantification of infectivity rescue of HSV virions deprived of gH/gL upon addition of soluble form of gH/gL or gD and gH/gL. $gH^{-/-}$ HSV virion infection was monitored by β-galactosidase activity proper of this HSV mutant. J cells expressed nectin1 plus or minus integrin αvβ6. The extent of $gH^{-/-}$ HSV virions rescue was higher when both $gH_t$/gL and $gD_t$ were present, both in nectin1 only or nectin1 plus integrin αvβ6 expressing J cells. The extent of $gH^{-/-}$ HSV virions rescue was higher when soluble glycoproteins were added to assay infectivity on J cells expressing nectin1 plus αvβ6 integrin, as compared to J cells expressing nectin1 alone.

## 3.1.18 Infectivity of $gH^{-/-}$ HSV virion is not rescued by soluble $gH_t$/gL in SW480 human cell line depleted of integrin β6 or β8 or both by silencing.

$gH^{-/-}$ HSV virion infectivity rescue with soluble $gH_t$/gL (plus soluble $gD_t$) was assayed also in SW480 human cell line, after the silencing of human β6 and/or β8 integrin subunits. $gH_t$/gL plus soluble $gD_t$ rescued infectivity of $gH^{-/-}$ HSV virion only when control siRNA was used; when one or both integrin β subunits were silenced, no rescue was observed (figure 23).

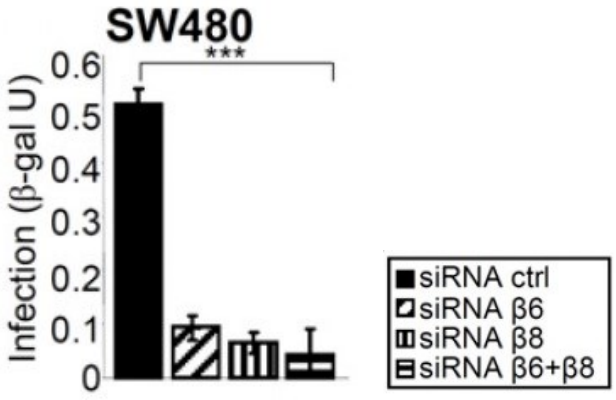



**Figure 23.** Quantification of infectivity rescue of $gH^{-/-}$ HSV virion upon addition of soluble form of gD and gH/gL. $gH^{-/-}$ HSV virions infectivity rescue was monitored by β-galactosidase activity proper of this HSV mutant. SW480 human cell line was control silenced or silenced for one or both integrin β6 and β8 subunits. $gH^{-/-}$ HSV virion rescue takes place only if control siRNA SW480 cells are used to test $gH^{-/-}$ HSV virion infectivity rescue with soluble $gH_t$/gL and $gD_t$.

Media from this experiment were collected and analyzed by WB. gL was present in the media of $gH^{-/-}$ HSV virions absorbed on cells transfected with control siRNA (figure 24). In this sample, the total amount of gL is the sum of the gL contained in the soluble $gH_t$/gL used for the rescue and the gL dissociated from gH. When SW480 cells were silenced for one of the integrin β subunits investigated, the total amount of gL in the media of virion cell mixtures appeared less and corresponded to the input of soluble $gH_t$/gL used; thus, no gL dissociation was detected (figure 24). Hence, the rescue of $gH^{-/-}$ HSV virion infectivity by soluble $gH_t$/gL relied on the presence of αvβ6 and αvβ8 integrins, leading to gL dissociation.

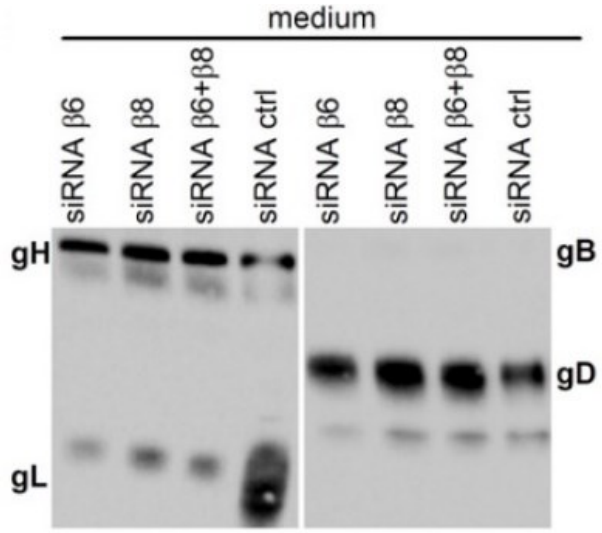



**Figure 24.** Western Blot analysis of culture media harvested from mixtures of absorbed $gH^{-/-}$ HSV virion to immobilized SW480 human cell line silenced with siRNA for either β6 or β8 integrin subunits or both. After soluble forms of gD and gH/gL were added, gL was detected in higher amount in the culture media of cells that received control siRNA, as compared to that of cells silenced with siRNA for either β6 or β8 integrin subunits or both. Soluble $gD_t$ elettrophoretic mobility is shown on the right.

## 3.1.19 Neutralizing MAb LP11 to gH prevents gL dissociation upon HSV virion binding to J cells expressing nectin1 plus αvβ6 integrin

gL dissociation and its release in the medium took place only in the typical conditions that lead to HSV infection of susceptible cells; in particular, only when gD, gH/gL, gB, nectin1 and integrin αvβ6 or αvβ8 are present together. As demonstrated, the absence of the entry receptor or of the host components led to conditions where no gL dissociation had been detected. Accordingly, MAbs to gH/gL which prevented gL dissociation without being neutralizing, could not be found. In fact, gL dissociation may represent an essential step for HSV entry. The different properties of known MAbs to gH/gL were exploited to hinder gL dissociation from gH/gL during HSV entry. In effect, it remained possible that some neutralizing MAbs to gH/gL exert their negative effect on HSV entry impeding gL dissociation from HSV gH/gL and thus inhibiting gH/gL activation. MAb LP11 to HSV gH epitope resides on the opposite surface in respect to the one recognized by MAb 52S and both these MAbs are neutralizing for HSV. MAb 53S recognizes a gL dependent epitope on gH and it is not neutralizing.[193,265,267] HSV virions were incubated with control Ig or the specified MAbs and then used

to infect J cells expressing nectin1 plus or minus integrin αvβ6; after 30', media was analyzed by WB. Pre-incubation of HSV virion with control Ig, MAb 52S or MAb 53S allowed the dissociation of gL from gH/gL and its release in the media when J cells expressed nectin1 together with αvβ6 integrin (figure 25). Contrariwise, pre-incubation with MAb LP11 prevented gL dissociation from gH/gL and its release in the media when J cells expressed nectin1 together with αvβ6 integrin. Therefore, neutralizing MAb LP11 could exert its strong HSV entry blockade effect by blocking gL to gH, finally joining HSV entry to gL dissociation and eventually disclosing a negative regulation function of gL on gH, which is αvβ6 or αvβ8 integrin dependent.

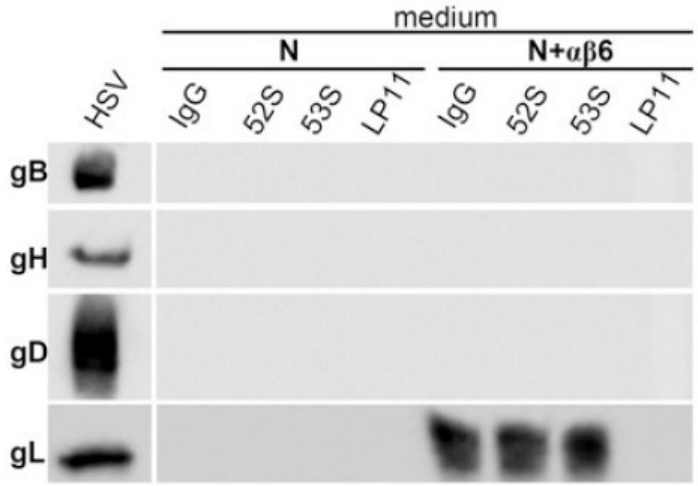



**Figure 25.** Western Blot analysis of culture media harvested from mixtures of absorbed pretreated HSV virion to immobilized J cell expressing nectin1 plus or minus αvβ6 integrin. When HSV virions were preincubated with control Ig, MAb 52S or MAb 53S and were used to infect J cells expressing nectin1 together with αvβ6 integrin, gL dissociation from gH/gL and its release in the media was allowed. If J cells expressing nectin1 together with αvβ6 integrin were infected with HSV virion preincubated with MAb LP11, gL dissociation from gH/gL and its release in the media were blocked. Elettrophoretic mobility of HSV virion essential glycoprotein is shown on the left.

# 3.2 HSV DISCUSSION

### 3.2.1 HSV entry summary

Entry of Herpesviruses is a highly controlled process.[164,165,222,372] gH/gL and gB are conserved across herpesviruses. Available gH/gL stuctures showed that gH/gL does not resemble other fusion glycoproteins, arguing at a regulator role gH/gL.[178,188,241] gL binds gH in its H1 N-terminal domain and gH/gL heterodimerization function is conserved in the majority of herpesviruses. Tropism of HSV is dictated mainly by gD, while gB structure features revealed that gB is the HSV fusogen.[175,279] HSV fusion begins with the encounter of gD with host receptors: nectin1 or HVEM. Upon binding, gD changes its conformation, displaying its profusion domain. gH/gL gathers fusion promoting signals from its own receptors, αvβ6 and αvβ8 integrins, and from activated gD, in order to trigger gB to execute fusion.[176] αvβ6 and αvβ8 integrins are HSV receptors for gH, but they do not allow HSV entry when present alone; integrins need one of the HSV gD receptors to exert their role. The stepwise activation model for HSV entry suggests that host nectin1 or HVEM and virion gD, gH/gL and gB are sequentially recluted to gain fusion of virion and host membrane. In contrast, in the HSV resting membrane, different preassembled complexes of glycoproteins are already interacting with each other at the equilibrium, without reaching the gB activation end point.[179] In fact, preassembled complexes of HSV virions glycoproteins were detected, i.e., gD interacting with gB, arguing against the sequential engagement proper of the stepwise activation model.[179,181] Hence, HSV glycoproteins might contact each other even when not triggered by their receptors, but the binding of glycoproteins to receptors promotes the conformational changes that favor the fusion process.[179]

### 3.2.2 αvβ6 and αvβ8 integrins

αvβ6 and αvβ8 integrins enable HSV virion entry, as depletion of these integrins with MAb or siRNA against αvβ6 and αvβ8 integrins reduced HSV infection.[176] Integrin antibodies might mimick ligand and induce integrin to proceed in endocytic pathway with consequent disappearance from the membrane. Antibodies against integrins may also act to stabilize an integrin conformation, or to mask the gH/gL docking site used to deliver triggering signal to the HSV entry machinery. αvβ6 and αvβ8 integrins promote an HSV entry pathway in host cells, which is dependent on endocytosis. Apart from

this entry mechanism, HSV could use other ways to infect cells: cells in which HSV entry takes place at plasma membrane or neutral compartment were described.[170,171,174,176] Through surface plasmon resonance, it was demonstrated that αvβ6 integrin interacts at high affinity with soluble $gH_t/gL$.[176] This argues for a direct binding between the ectodomain of gH/gL and host integrin heterodimers ectodomains, with a potential increase in absorption efficiency.[176] Apart from an enhancement in absorption, the increase in infection seen in presence of integrins might imply a further mechanism: integrins could improve HSV entry. In particular, αvβ6 or αvβ8 integrins might promote gH/gL transition to its active form, which prompts gB to execute fusion. Interaction between integrins and gH/gL might affect the interactions among HSV entry glycoproteins. Moreover, as αvβ6 and αvβ8 integrins are HSV receptors, it is plausible that they could trigger conformational modification to the glycoproteins they bind, namely gH/gL, during HSV entry.

### 3.2.3 HSV gL dissociation

αvβ6 or αvβ8 integrin interaction with gH/gL promoted the dissociation of gL from gH/gL and its release in culture media, but only when nectin1, gD and gB were also present. gL dissociates during HSV virion absorption or entry. gL dissociation is specifically inhibited by neutralizing MAb LP11 to gH, but not by neutralizing MAb 52S to gH, implying that a conformational modification takes place to gH when HSV enters cells expressing αvβ6 or αvβ8 integrins. gL dissociation might allow gH to reach its active conformation, which is necessary to trigger gB to fuse. It is possible that gL might act as an inhibitor that must be dissociated from gH to promote HSV entry and fusion.

gL dissociation upon gH/gL binding to αvβ6 or αvβ8 integrin was assayed by means of cotransfection, coculture and during HSV virion absorption and entry. In cotransfection, combinations of molecular interactors are expressed on the same cellular surface, while in coculture, a distinction is made between target cells, expressing combinations of receptors, and donor cells, expressing combinations of viral glycoproteins, in order to respectively mimick host cell membrane and virion envelope . In cotransfections, all actors are espressed *in cis*. One limit of this approach is that interactions eventually detected might take placein the secretory pathway and not on cell membrane, leading to false positive interactions in virus entry investigations. Also, the *in cis* approach is the least likely representation of the HSV entry phenomenon. A co-culture assay was established to bypass the limit of co-transfection system. The same assay conduced *in trans* is a better representation of the interactions between viral

envelope and cell membranes, as it follows the correct *in vivo* topology. In absence of nectin1, gD or gB no gL dissociation was detected upon gH/gL interaction with αvβ6 integrin, while gL dissociation was detected when endocytosis was hindered by BFLA, implying that endocytosis is not required for gL dissociation.

Co-seeding is a very clear and easy system to mimick molecular interactions among virus glycoproteins and their cellular receptors, but the better way to study HSV entry mechanism is to use directly HSV virions. Also during HSV entry the requirements for gL dissociation mediated by αvβ6 or αvβ8 integrin are the same as for HSV infection, namely that nectin1, gD, gH/gL and gB should be present together. In particular, gL dissociation required not only all the essential HSV virion glycoproteins, but also the host cellular receptors nectin1 and αvβ6 or αvβ8 integrins.

### 3.2.4 Molecular mechanism of HSV gL dissociation

The mechanism which dissociates gL from the gH/gL heterodimer depends on a conformational modification that ensues in gH/gL upon appropriate condition, and it is hampered by MAb LP11. MAb LP11 to gH might hamper a structural rearrangement of gH N-terminus, thus blocking gL in its original position, finally inhibiting the possibility for gH to activate gB for fusion. RGD integrin interactin motif resides at position 176-178 of gH; gL binds gH in positions 259-323 and LP11 contacts residues 86, 168, 300, 315-317 and 325 in gH as detected by MAb Resistance Mutants.[188,192,239] Instead of a direct competition of gL with αvβ6 integrin for a common binding site on gH surface, gL dissociation should depend on a gH/gL conformational change induced by integrin binding to gH/gL. It is possible that binding of MAb LP11 to residue 168 imposes steric hindrance to gH, thus preventing αvβ6 integrin binding to gH RGD (176-178) domain. To confirm this hypothesis, figure 8 shows that no gL dissociation was detected upon gH RGD to ADA mutation. Hence, gL dissociation from gH/gL depends on gH RGD domain, the same domain mediating HSV entry by αvβ6 integrin, while gH/gL binding to integrin αvβ8 does not depend on gH RGD.[176] Figure 9 demonstrates that a detectable amount of gH/gL heterodimer is pulled down by αvβ6 integrin, even when $gH_{ADA}$ is used instead of wild type HSV gH, implicating that integrin binding site on gH/gL extends over the gH RGD domain.

gL dissociation ensues during gH/gL activation by host receptor αvβ6 or αvβ8 integrin; gH conformational modification triggered by αvβ6 or αvβ8 integrin could be ultimately part of HSV entry mechanism. In particular, MAb LP11 to gH N-terminus (FR1) both neutralizes HSV and hampered gL dissociation, while other neutralizing MAb to gH (52S, which instead bind H2-H3 domain of gH, FR2)

or non-neutralizing MAb to gH (53S), do not, indicating that LP11 exerts a kind of specific inhibition to gH/gL conformational change and to HSV entry. Thus, the LP11 neutralizing effect on HSV might be exerted by preventing gL dissociation.

gH conformational modification takes place during HSV virion entry and might represent a metastable state of the glycoprotein induced during αvβ6 integrin dependent HSV infection. In fact, $gH^{-/-}$ HSV virions were rescued in their infectivity by adding a soluble form of gH/gL plus soluble gD, in an αvβ6 integrin dependent fashion. gL might act as a negative regulator of gH that, once dissociated, enables αvβ6 integrin dependent HSV entry, preventing premature exhaustion of gH fusogenic potential by introducing an additional rate limiting step control. Cross-linking the soluble $gH_t$/gL chemically with formaldehyde or engineering intermolecular disulphide bonds to costrain the heterodimer might prevent only the subordinate process of gL dissociation from gH/gL without exerting neutralization capabilities, namely without inhibiting the upper process of HSV entry. However, if integrin dependent HSV entry depends on gL dissociation it might not be possible to separate these events.

An established example of protein activation through inhibitor dissociation is the cytokine activator Nuclear factor κB (NFκB) which, upon triggering by cytosolic signals, undergoes a conformational change that dissociates its IκBα inhibitor subunit.[373] However, gL would represent more than an inhibitor for gH. Providing that gH did not resemble any known structure, its folding might require more than the host protein folding machinery (exemplified by chaperones), and gL is properly thought to accomplish this role. In fact, HSV gH needs gL both to gain proper folding, i.e. all of its standard epitopes, and to be correctly processed during secretion pathway.[231,235] Yet, gL is not a chaperone for gH because chaperones catalyze the folding of newly-synthetized proteins but do not remain tethered or cofold with the protein they facilitate, as gL does. It is possible that gL serves to prevent gH activation until the HSV multipartite apparatus is ready to encounter its receptors; hence, its dissociation would establish a no return point towards gB activation upon correct triggering by host receptors.

Thus, integrin interaction would play a similar role of the other HSV receptors, i.e. to induce conformational modifications to the entry glycoproteins. Once these modifications start, they should trigger an inter-molecular signaling cascade that breaks the existing equilibrium among preassembled glycoprotein complexes and culminates in fusion of HSV envelope with host membrane. These striking results emerged from J cells coexpressing on membrane surface arrangements of HSV glycoproteins, integrins and cognate HSV receptors. Conformational modifications triggered by αvβ6 integrin consisted in gH/gL heterodimer disassebly. This latter modification might allow synchronization of membrane fusion, a process exerted by HSV entry glycoprotein apparatus and host receptors, with

endocytosis, which is contributed by integrins. gL release during HSV entry was not inhibited by BFLA and was prevented by R8 PAb to gD or by the absence of gB, i.e. conditions that hamper HSV infection. Hence, gL dissociation might represent a necessary step for gH to gain its active conformation, with gL possibly working as an inhibitor of gH.

### 3.2.5 Refinement of HSV entry model

The first step in HSV entry is the gD interaction with one of its receptors, nectin1 or HVEM, called triggering step.

The second step involves gH/gL, which collects an activation signal from receptor bound gD and directly reacts to αvβ6 and αvβ8 integrins; this step is called activation step. αvβ6 or αvβ8 integrin-dependent HSV entry proceed through the activation step only if both signals are present. The outcome of the second step is gH/gL activation by gL dissociation (inhibitor release). This step might break the existing equilibrium among preassembled glycoprotein complexes delivering a different signal to gB, which is then allowed to mediate fusion between viral and host membranes. During HSV entry process, gH/gL still function as an upregolator of gB fusogenic properties. The heterodimer is self-inhibited by gL. The role gH/gL in HSV entry is thus to integrate signals from multiple sources and to deliver the fusogenic signal to gB. Since αvβ6 integrin, once bound to HSV gH/gL, promotes virion endocytosis by its signaling C-tail (β6 subunit), gL dissociation might provide correct timing necessary for fusion in the endosome compartment.

The third step is fusion and, in most herpesviruses, it is a gB driven process. In the case of HSV, gB might integrate signals from its receptor PILRα or other candidate gB receptors (figure 26).

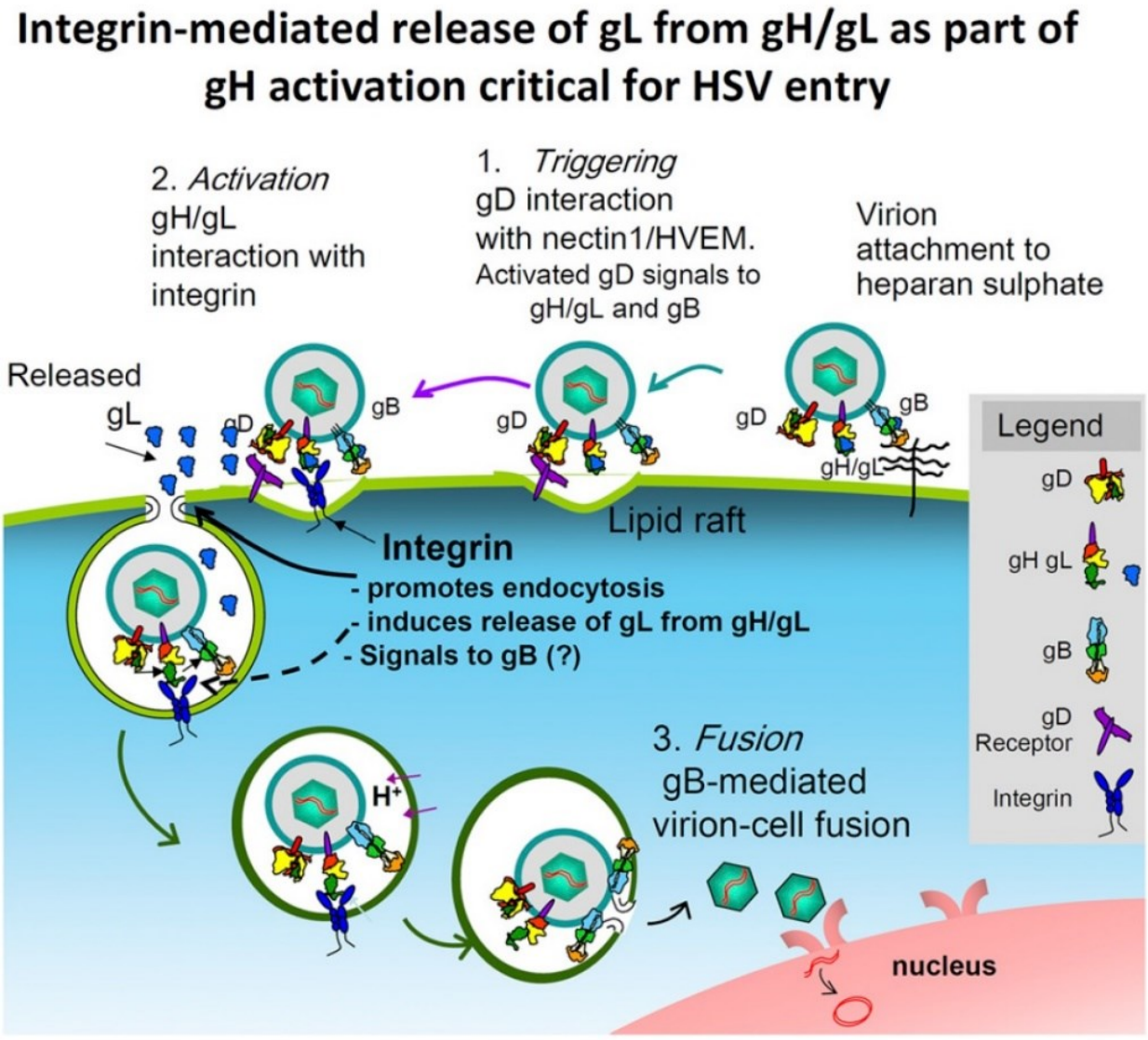



**Figure 26.** Schematic of αvβ6 integrin dependent HSV entry. Sequential steps that result in αvβ6 integrin dependent HSV entry proceed through the following events: Triggering (gD and host receptors), Activation (gH and αvβ6 integrin) and Fusion (gB). See full text for details. Adapted from ref. 374.

# 4.1 VZV RESULTS

## 4.1.1 Integrins mediate VZV fusion, especially when coexpressed with MAG

To investigate whether integrins of the αv family have a role in VZV fusion, the well-established luciferase-based assay was performed. In particular, donor cells mimic VZV virion and express combinations of VZV entry glycoproteins: gH/gL and gB plus or minus gE/gI. Target cells mimic host cell membranes, expressing combinations of integrins of the αv family plus or minus the VZV established receptor Myelin Associated Glycoprotein (MAG).[289]

The two COS cell populations were coseeded for 24 h and relative units of luciferase were measured.

Figure 27 shows that:

1. The highest level of cell-cell fusion was observed when all of the available actors, viral glycoproteins and cellular receptors, were contemporarily present. In particular, the highest level of fusion resulted from coculture of donor cells expressing combination of gH/gL, gB and gE/gI while target cells expressed MAG plus integrin αv and one of the β3, β5, β6 or β8 integrin subunits. This situation reflects what happens during VZV entry process.
2. A lower but still consistent extent of fusion was detected when gH/gL and gB were expressed on donor cells and co-seeded with target cells expressing MAG together with one of the αv integrin heterodimers investigated. This condition may represent the minimum set required for cell-cell fusion, where viral glycoproteins and their cellular counterpart are present.
3. When cells expressing gH/gL, gB and gE/gI were co-cultured with cells expressing integrins alone, the fusion level was comparable, or slightly higher, than that obtained when donor cells expressed gH/gL and gB and target cells expressed only MAG; a situation reported to induce the highest fusion level by others.[289] When donor cells expressed the five VZV glycoproteins and target cells expressed MAG, the level of fusion was higher than that observed when only integrins were expressed on target cells. This result suggests a primary role for MAG, when all VZV glycoproteins are present.
4. When cells expressing MAG alone or integrins alone were assayed with donor cells expressing only the three VZV glycoproteins, a very low level of fusion was detected. In particular there was no increase in fusion level as compared to fusion of donor cells with cells expressing

integrins or cells which expressed no receptors. The fusion level, was doubled when MAG was present.

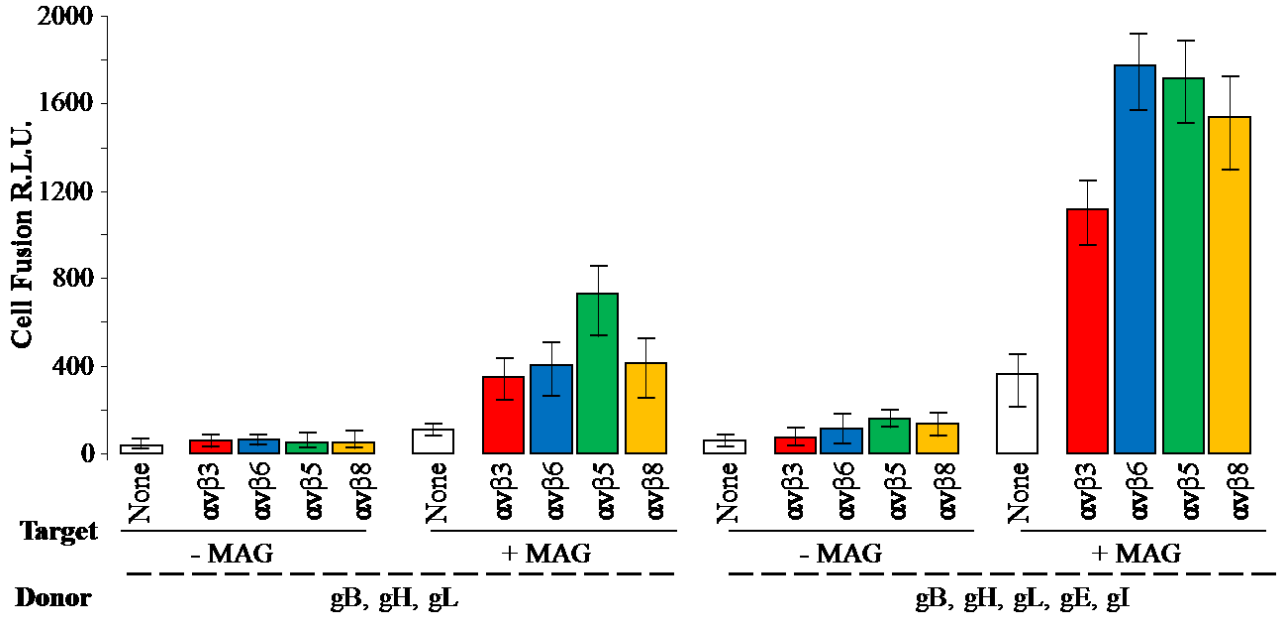



**Figure 27.** Luciferase assay. See full text for detailed results. Cell-to-cell fusion between donor COS cells expressing VZV gH and gL plus gB or VZV gH and gL plus the trio of gB, gE and gI, and target COS cells expressing MAG alone, or MAG plus αvβ- integrin heterodimers or integrins alone, or no receptors. Fusion was quantified by means of a T7 promoter-driven reporter luciferase gene transfected in target cells, and expressed as relative luciferase units (R.L.U.). Each point represents the average of triplicates. Bars show SD. Each value, proper of the specific combination tested, was normalized onto HLB MAG combination, the condition where VZV fusion was reported to reach its maximum extent.[289]

## 4.1.2 VZV pull-down assay

To investigate if VZV gH/gL and gB physically interact with integrins of the αv family, immunoprecipitation assay and western blot were performed in a coculture system, so as to mimick VZV Virion interaction with host membrane. In particular, 293T donor cells were tranfected with amounts of VZV gH/gL and gB that ensured similar expression. Target 293T cells were transfected

with STREP tagged integrin αv ($\alpha v_{STREP}$) alone, $\alpha v_{STREP}\beta 3$, $\alpha v_{STREP}\beta 6$ or control STREP tagged HSV gD ($gD_{STREP}$). VZV glycoproteins transfection was delayed by 12 h relative to integrins transfection, so as to prevent costitutive exhaustion of glycoprotein fusogenic potential before co-seeding. 16 h after integrins transfection target and donor cells were cocultured for 6 h or 10 h.

Figure 28 shows that $\alpha v_{STREP}$ integrin subunit pulled down increasing amounts of VZV gH in a time dependent manner, only when β3 subunit was present. In particular, $\alpha v_{STREP}\beta 3$ integrin interacts with VZV gH already at 6 h after co-seeding (lane 2). The amount of pulled down gH increased at 10 h (lane 6). $\alpha v_{STREP}$ integrin alone (lanes 4 and 8), or $\alpha v_{STREP}\beta 6$ (lanes 3 and 7), were capable to pull down modest amounts of gH. $\alpha v_{STREP}$ integrin over-expressed alone might have formed integrin heterodimer with a 293T resident human β3 integrin subunit, thus αvβ6 integrin might have been not involved in VZV gH interaction. There was no increase in gH retention by $\alpha v_{STREP}$ after β6 integrin over-expression, when compared to the amount pulled down by integrin $\alpha v_{STREP}$ alone (compare lanes 3 and 7 with lanes 4 and 8). The uncleaved form of VZV gB was pulled down aspecifically, as it was detected also when only HSV $gD_{STREP}$ was expressed. VZV gH was pulled down alone. In all tested conditions gL was absent, while gL was detected in the total cell lysates. gH was never pulled down by HSV $gD_{STREP}$, providing evidence for specificity (lanes 1 and 5). These observations are in accordance with recent findings that VZV enter human cells via integrins of the αv family.[290] Presently, the pull-down results are not in contrast with fusion assays findings, as the role of gE/gI heterodimer in pull-down assay has not been investigated yet.

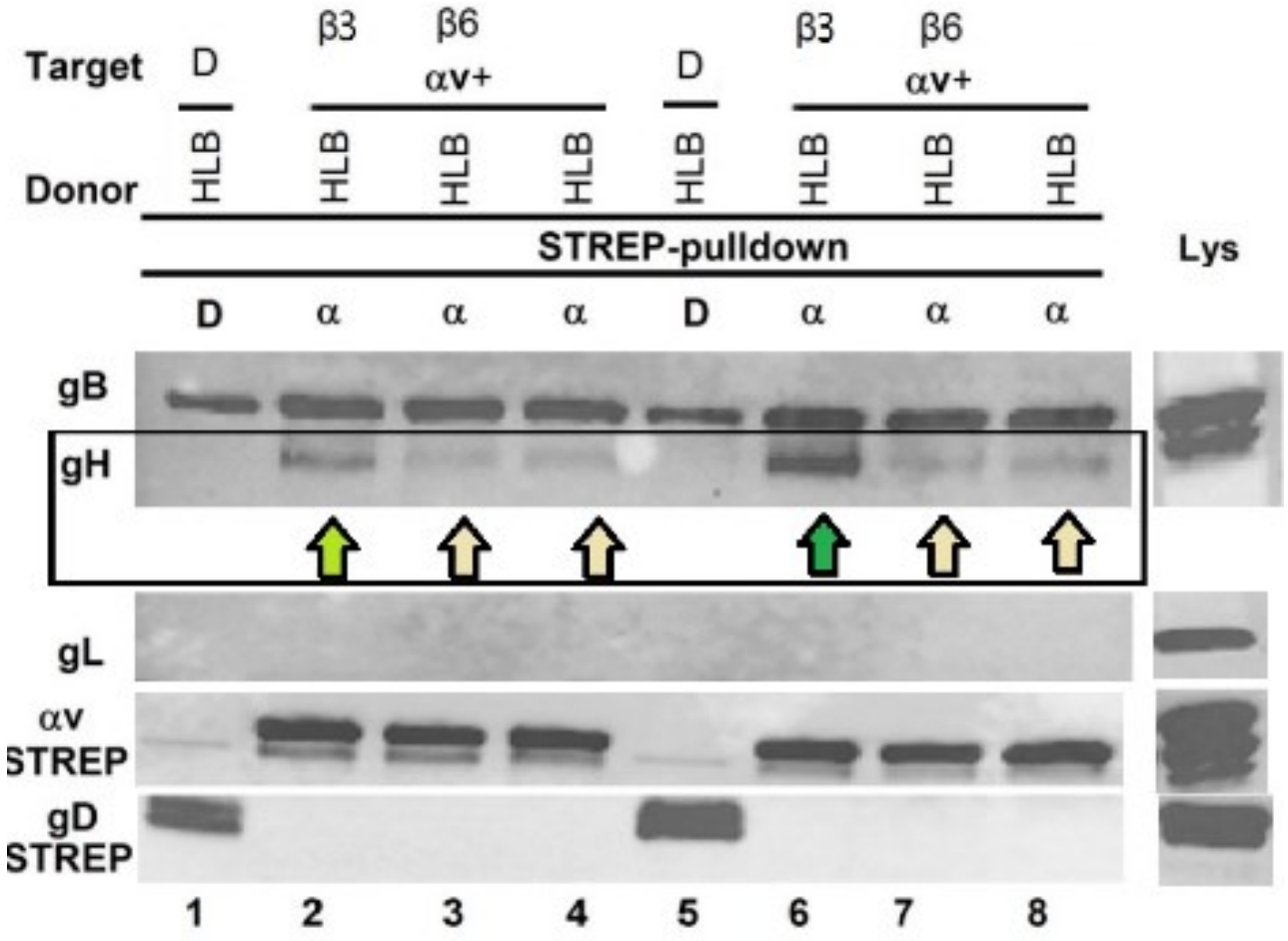



**Figure 28.** Western Blot analysis of VZV glycoprotein complexes assembled *in trans* on STREP tagged αv integrin. Tagged VZV gH, gL and gB were detected with MAbs raised against FLAG tag. $\alpha v_{STREP}$ and $gD_{STREP}$ were detected by specific antibody against STREP tag epitopes. Target 293T cells were transfected with $\alpha v_{STREP}$ alone, lanes 4 and 8, $\alpha v_{STREP}\beta 3$, lanes 2 and 6 or $\alpha v_{STREP}\beta 6$ lanes 3 and 7 or HSV $gD_{STREP}$ as negative control, lanes 1 and 5. 8 h later donor cells were transfected with FLAG tagged VZV gB, and gH/gL. 16 h later donor and target cells were coseeded. 6 h (lanes 1to 4) or 10 h (lanes 5 to 8) later cells were harvested for lysis and STREP pull-down. Green arrows indicate that VZV gH was pulled down by integrin $\alpha v_{STREP}\beta 3$ at high amount at 10 h post coculture (lane 6) and in lower amount at 6 h post coculture (lane 2). The yellow arrow indicates that VZV gH was pulled down by integrin $\alpha v_{STREP}\beta 6$ or $\alpha v_{STREP}$ at low level both at 6 h and 10 h post coculture (lanes 3, 4 and lanes 7, 8 respectively). HSV $gD_{STREP}$ pulled down no VZV gH FLAG. No VZV gL was detected, while uncleaved gB seems to be pulled down aspecifically. Elettophoretic mobility of reference proteins expressed in 293T cells is shown to the right.

# 4.2 VZV DISCUSSION

Most human herpesviruses use integrins as components of their entry machinery.[176,337,290,358,359] It was reported that HSV gH/gL interacts with αvβ6 and αvβ8 integrins.[176] HSV-1 gH/gL binds αvβ6 integrin, but not αvβ8 integrin, through its RGD domain. This interaction promotes HSV entry and modified HSV entry pathway. Epstein-Barr Virus (EBV) uses αvβ5, αvβ6 and αvβ8 integrins as receptors for epithelial cells. These integrins interact with EBV gH/gL at amino acid position 188KGD190. In Kaposi's sarcoma-associated herpesvirus (KSHV), it is gB that interacts with an αv integrin. This interaction is able to induce virus entry through an endocidic pathway. In addition, HCMV interacts with integrins in a very complex manner. It seems that integrin binding depends on the origin of the virus, such that clinical isolates bind different integrins as compared to laboratory strains. Presently, it remains elusive which viral glycoprotein, between gH/gL heterodimer and gB, is involved. However, it is clear that herperviruses have evolved to use integrins as cellular receptors. It is evident that integrin binding capacity resides in the conserved viral glycoproteins gH/gL or gB.

It was recently reported that, as for VZV, there is an involvement of αv integrins during virus infection, but it remains unclear which β subunit and expecially which viral glycoprotein is involved in integrin binding.[290] Here, for the first time, a physical interation between the VZV entry glycoproteins and integrins of the αv family was reported. In particular, it seems that $\alpha v_{STREP}\beta 3$ integrin interacts specifically with VZV gH, this result is in accordance with the recent finding that VZV enters human cells via integrin of the αv family.[290] αvβ3 integrin did not interact with gL. Integrins are also involved in VZV fusion, as expression of integrins together with MAG induces fusion mediated by gH/gL and gB, especially when also gE and gI are expressed, a condition mimicking the VZV infection. VZV gE and gI were considered for a long time essential in VZV entry, due to the lack of a tropism factor, as HSV-1 gD.

The entry machinery of herpesviruses accomodates various functions. During viral species evolution, the entry functions were unevenly distributed among glycoproteins. Thus, it is conceivable that gH/gL includes the tropism factor function and directly interacts with integrins in a RGD or KGD independent way, as these domains are not present in gH/gL. However, three KGD are present in VZV gE and gI. It is possible that VZV use ancillary proteins to increase or better define tropism determined by gH/gL, i.e. via gE/gI KGD mediated integrin interaction (a condition that has similarities with HCMV pentameric complex[375]). To this purpose, the interaction among gE/gI with integrins remains to be

elucidated, especially because well established examples of *in vivo* infection models revealed that part of gE/gI ectodomains are involved in VZV infection. It is known that MAG is a receptor for VZV gB, and that MAG over-expression allows VZV entry. MAG carries an RGD integrin binding domain and interacts with both VZV gB and gE.[289,309-311] It is conceivable that through its RGD domain MAG could relocalize integrins in specific sites of the cell membrane, favoring gH/gL interaction and VZV entry, as already reported for nectin1 in HSV-1 model.[353]

# 6 MATERIALS and METHOD

Cells, viruses, soluble proteins and antibodies

J cells were derived from baby hamster kidney (BHK-TK$^-$). J cells and do not express HSV entry receptors. J cells were grown in Dulbecco modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), as previously described.[204] SW480 human cell line was derived from colon carcinoma and was grown in Lebovitz L-15 10% FBS. 293T and COS cells were grown in DMEM 5% FBS.

Wild type HSV strain F was previously described.[204,376] K26GFP, an HSV recombinant, expressed a green fluorescent protein (GFP) in frame with VP26 capsid protein, was previously described.[376] The gB deletion mutant ΔgBKΔT was grown and titrated in complementing cells and previously described.[377] In the gH deletion mutant ΔgH SCgHZ, gH was substituted with LacZ gene and virions were grown F6 complementing cells, as previously described.[357] R8102 is an HSV recombinant which carries a LacZ reporter gene controlled by the α27 promoter.[204]

Soluble gD$\Delta_{290t\text{-}299t}$ was from Cohen G. H. and Eisenberg R.[378] $gH_t/gL$, a soluble form of gH/gL containing a One-Strep-Tag epitope for affinity purification, was previously described.[354]

R8 polyclonal antibody (PAb) to gD, MAb CK6 to Nectin1 and H12 monoclonal antibody (MAb) to gH were from Cohen G. H. and Eisenberg R. MAb VIII-62 to gL was from Spear P. G. Western Blot (WB) positive MAb H1817 to gB and WB positive MAb H170 to gD were from Goodwin Institute. PAb to gH/gL heterodimer, obtained against soluble $gH_t/gL$, was previously described.[354] Neutralizing MAb 52S to gH, recognizes a conformational epitope in gH; both not neutralizing MAb 53S to gH and neutralizing MAb LP11 to gH recognize conformational epitopes in gH, which are gL-dependent.[193,265] MAb 2077 to αvβ6 integrin heterodimer was from Chemicon. Antibody to Strep-Tag, conjugated with horseradish peroxidase was from IBA, Solutions for Life Sciences. MAb R1.302 to Nectin1 was from Lopez M.[204] MAb to FLAG epitope was from Sigma-Aldrich.

Plasmids

HSV gH, gL and gB genes were cloned in MTS vector; gD gene was cloned in pcDNA3.1. All genes were expressed under cytomegalovirus promoter, as previously described.[379] Plasmids encoding for Nectin1 and for epithelial growth factor receptor 2 Δ (EGFR2NΔ, which codified for a non-signaling EGF receptor termed Erb2Δ32), were previously described.[354,380] $gH_{ADA}$ carries the following substitutions in the RGD domain, gH176RGD178 to gH176ADA178, gHADA was previously described.[354] Plasmid encoding for $\alpha v_{STREP}$ tagged integrin was previously described.[338] Plasmid encoding for β8 was from Nishimura S. L.[381] Plasmids encoding for αv, β3, β5, β6 were from Blystone S.[355] Plasmid pCAGT7 containing the T7 RNA polymerase gene and plasmid pT7EMCVLuc expressing the firefly luciferase under the control of the T7 promoter were described.[382]
Plasmids encoding for FLAG tagged VZV gH, gL and gB, and plasmids encoding for VZV gE, gI and for Myelin Associated Glycoprotein (MAG) were from Suenaga T.

Constructs

To generate $gD_{STREP,}$ the $gD_N$ plasmid was digested with BglII-HindIII restriction enzymes to eliminate GFP N-terminus.[187] The following annealing oligonucleotides was used to amplify the Strep Tag epitope: 5′-GGA AGA TCT CTG GCT GGA GCC ACC CGC AGT TCG AGA AAG GTG GAG GTT CCG GAT CGG GAG GTG GAT CG-3′ and 5′-CCC AAG CTT CCC GGA TCC TCA TTT TTC GAA CTG CGG GTG GCT CCA CGA TCC ACC TCC CGA TCC GGA ACC T- 3′. The PCR product was digested with the same enzymes used before and ligated in frame with gD gene.

To generate $Nectin1_{STREP}$ encoding plasmid, was used a similar strategy applied for generation of plasmid encoding for $gD_{STREP.}$ In particular, $Nectin1_{STREP}$ was generated thorough digestion with BglII-XhoI restriction enzymes of $Nect_C$ plasmid in a way to eliminate GFP C-terminus.[187] The following annealing oligonucleotides were used to amplify Strep Tag epitope: 5′- GGA AGA TCT AGC GGA GGT GGA CAT CAT CAC CAT CA CAT AGC GGA GGT GGA AGC GCT TGG AGC CAC CCG CAG TTC GAG AAA GGT GGA GGT TCC GAG GGT- 3′ and 5′- CCG CTC GAG TCA TTT TTC GAA CTG CGG GTG GCT CCA CGA TCC ACC TCC CGA TCC ACC TCC GGA ACC TCC ACC

TTT CTC GAA CTG CGG GTG GCT CCA AGC G-3′. The fragment obtained from PCR was digested with the same enzymes and cloned in frame with Nectin1.

To generate β6N1 chimera, pCF18HNK was digested with BamHI-HpaI restriction enzymes in a way to eliminate only the ectodomain of Nectin1.[171] pCF18HNK encoded for full length Nectin1 but had three silent mutations for the insertion of the HNK (HpaI, NheI and KpnI) sites before the transmembrane domain of Nectin1.[171] The ectodomain of β6-integrin was PCR amplified from β6-integrin plasmid with the following oligonucleotides 5′- GAA CTG GGA TCC ATG GGG ATT GAA CTG CTT TGC C- 3′ and 5′- CAT GGG GTT AAC TGG AGG CTT CGG ACA ATC- 3′. The PCR product was digested with the same enzymes and ligated in frame to Nectin1 TMR and C-tail.

Effect of pharmacological inhibitors and role of β6 integrin C-tail in virus entry

J cells transfection was carried out with Lipofectamine 2000, as previously described.[345] Each time that J cells were transfected with Nectin1 plus integrins cells received a threefold dose of integrin αvβ6 plasmid in respect to Nectin1, while J cells expressing only Nectin1 received threefold dose of Erb2Δ32 control plasmid. 48 h after transfection, J cells expressing Nectin1 or Nectin1 plus a combination of integrin αVβ6 or β6N1, were pre-incubated for 1 h at 37 °C with bafilomycin A (BFLA) or wortmannin (WM), all from Sigma Aldrich. Cells were infected with R8012 in presence of inhibitors at 3 MOI (Multiplicity of infection = 3 Plaque Forming Unit (PFU) / 1 host cell) for 1.5 h at 37°C. Viral inoculum was removed and cells were left in culture media containing inhibitors for other 8 h. Alternatively, 48 h after transfection, J cells expressing Nectin1 or Nectin1 plus a combination of integrin αVβ6 or β6N, were infected with R8012 at increasing MOI. R8102 inoculum was removed and cells were left in culture media for other 18 h. Quantitation of infection was carried out with O-nitrophenyl–β-galactopyranoside (ONPG), a colorimetric substrate that allow measure of β-galactosidase activity proportional to R8102 infection, and read at 405 nm.

Strep-Tag Pull downs

In *cis* pull down experiments, J cells were co-transfected with appropriate combination of plasmids encoding HSV glycoprotein gD ($gD_{STREP}$ or wild type), gH ($gH_{ADA}$ or wild type), gL and gB. The same J cell population, was transfected with Nectin1 ($Nectin1_{STREP}$ or wild type), Nectin1 plus integrins $\alpha v_{STREP}$, β6 (β6N1 chimera or wild type) and β8 or $\alpha_{STREP}$ β6 integrin alone in appropriated combinations. In pull down *trans* experiments, donor J cells were transfected with a combination of HSV glycoproteins, target J cells were transfected with a combination of receptors. 24 h after transfection the two populations were co seeded for other 24 h. Cells were harvested and cell lysates subjected to Strep-Tag pulldown, as previously described.[181] Expression plasmid for Erb2Δ32, was added when required, to ensure equal amount of total transfected DNA. Proteins collected by means of Strep-Tactin resin were separated by Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS/PAGE) and analyzed by WB with appropriate antibodies using a ChemiDoc XRS+ and Image Lab Software (Biorad).

For VZV pull down experiments target 293T cells were transfected by means of Lipofectamine 2000 with $\alpha V_{STREP}$ plus β3 or β6 integrins (150ng /24 well) or with HSV $gD_{STREP}$ alone (50ng / 24 well) as negative control. 8 hours later 293T donor cells were transfected with VZV $gB_{FLAG}$ (150ng/24well) plus $gH_{FLAG}$ (100ng/24well) and $gL_{FLAG}$ (75ng/24well). The day after donor and target cells were co-seeded in equal amount in DMEM 5%FBS and 6 h or 10 h later cells were harvested. Lysates were pulled down by means of Strep-Tag as already described for HSV.

Silencing and qRTPCR

Integrins were silenced in SW480 human cell line by means of tranfection with Dharmafect One Target plus siRNAβ6-integrin, or siRNAβ8-integrin, or both (Dharmacon, ON-TARGET plus), as previously described.[345] siRNA to E.coli-polA_0054 (IBA, Solutions for Life Sciences ) was used as control. Extent of silencing was assayed by qRT-PCR by means of TaqMan gene expression assay (Applied Biosystems), as previously described.[176]

Immunofluorescence assay, flow cytometry and culture media analysis

HSV virions were preincubated or not with PAb R8 to gD for 1 h at 37°C. These virions, or ΔgBKΔT virions, were absorbed to J cells expressing a combination of integrin αVβ6 alone or Nectin1 alone or both Nectin1 and αvβ6 integrin for 1.5 h at 4°C at 1 MOI, or equivalent MOI for ΔgBKΔT. Then J cells where shifted at 37°C for 30 min. Alternatively, when BFLA effect was investigated, J cells expressing Nectin1 plus αvβ6 integrin were incubated with 30 nM BFLA from 1 h before infection and during infection. Cells were then fixed with 4% paraformaldehyde and reacted with MAb 52S or MAb 53S to gH, followed by anti- mouse secondary antibody conjugated with fluorescein isothiocyanate (FITC). Cytofluorimetric analyses was performed using an AccuriC6 (Becton Dickinson) cytofluorimeter. Control cells received only secondary antibody. In a similar manner SW480 human cells, transfected with siRNA against integrin β6 or β8 or both or mock transfected, were infected with HSV as described above. Cells were than staining with MAb 52S and MAb 53S to gH and analyzed by cytofluorimetric assay.

Culture media was harvested from virions-cells mixture and subjected to fivefold concentration, deprived of serum Ig by absorption to protein A Sepharose and then analyzed through SDS/PAGE and WB as already described.

For immunofluorescence assay (IFA) J cells were transfected with Nectin1 or Nectin1 plus αvβ6 integrin. After 24 h J cells were magnetically sorted for Nectin1 expression by means of MAb R1.302 to Nectin1 followed by anti-mouse IgG Micro Beads (Miltenyi Biotec). 48 h later, J cells were infected with K26GFP at 10 MOI for 0.5 h at 37°C and fixed with 4% paraformaldehyde. HSV glycoproteins were detected by MAbs 52S or 53S to gH, or by MAb VIII-62 to gL and stained with anti-mouse DyLight 549 secondary Ab (Jackson). Microphotographs were taken in a Nikon Eclipse Ni microscope and red and green fluorescence were analyzed with NIS Elements AR image analysis software. Red-to-green florescence ratio was determined for 30-40 J cells for each group. Briefly, membranes areas were circumscribed, pixels present in the green channel were analyzed and the green mean fluorescence intensity in each sample was made equal to 100. Same analysis was done for red fluorescence, but this was expressed as a percentage of green fluorescence in the same sample.

Rescue of $gH^{-/-}$ HSV virion by soluble $gH_t/gL$

$gH^{-/-}$ HSV virons were absorbed to J cells, expressing Nectin1 or Nectin1 plus αvβ6 integrins or to SW480 human cell line silenced for β6 or β8 integrins, or both, or mock silenced. 30 (equivalent) MOI of $gH^{-/-}$ virions were used. Virions were mixed together with BSA, used as negative control, or soluble $gH_t/gL$, or soluble $gH_t/gL$ plus soluble $gD_{\Delta 290t\text{-}299t}$ at 300 nM final concentration for each soluble protein in phosphate saline buffer (PBS).[354,378] After absorption for 2.5 h at 37°C the mixtures of $gH^{-/-}$ virons and soluble proteins were removed and J cells or SW480 cells were incubated in DMEM 1% FBS or L-15 1% FBS respectively. Rescue of $gH^{-/-}$ virons by soluble proteins was detected 24 h after infection analyzing β-galactosidase expression by means of ONPG quantification, as already described. When SW480 cells were used, the medium contained $gH^{-/-}$ virons plus soluble proteins were collected after cells absorption and subject to SDS PAGE and WB as already described.

VIRUS ELISA (VIR-ELISA)

$1 \times 10^8$ HSV virions per well were immobilized onto a 96 well plate by 18 h incubation using bicarbonate buffer pH 8, at 4 °C. J cells expressing Nectin1 or Nectin1 together with integrin αvβ6 were overlaid to wells containing immobilized virions for 1 h at 37 °C. Cell suspension was removed and HSV gH expressed on virion surface was reacted with MAbs 52S or 53S followed by anti-mouse peroxidase-conjugated secondary antibody. Quantification was done by reading the optical density of O-phenyilendiamine substrate at 490 nm .

VZV fusion assay

The luciferase-based cell-cell fusion assay was performed as described.[257,383]

Donor COS cells were transfected with the indicated glycoprotein mixtures: gB, gH and gL or gB, gH, gL gE and gI together with pCAGT7 plasmid. Targeted COS cells were transfected with pT7EMCVLuc plasmid in presence of integrins heterodimers plus or minus MAG or without receptors. 24 h after transfection donor and target cells were co seeded for other 24 h. Cell-cell fusion was

detected by means of a luciferase assay system (Promega) The total amount of transfected plasmid DNA was made equal by the addition of EGFR2Δ 2 plasmid. Each value represents the average of triplicate samples.

Statistical Analyses

When mean ± SD is presented, data included at least three independent experiments. Statistical significance was calculated by Student's *t* test and intervals were as follows: *P < 0.05, ** P < 0.01, ***P < 0.001.

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