THE ROLE OF HERPES SIMPLEX VIRUS GLYCOPROTEINS IN THE VIRUS REPLICATION CYCLE

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Summary. – At least nine of the eleven herpes simplex virus (HSV) glycoproteins so far known have been widely characterised as regards their role in the virus replication cycle. During early virus-to-cell adsorption („adsorption"), glycoprotein C (gC) interacts with the glycosoaminoglycan (GAG) heparan sulphate (HS), located on the cell membrane surface. This interaction is labile until other glycoproteins such as B and D (gB and gD) begin to participate in the entry process. gB also harbours a site for interaction with GAGs, while gD provides a stabile attachment to cellular receptors („receptors") such as the herpesvirus entry mediator (HVEM). Late adsorption is associated with a conformation change of gD occurring after the receptor binding, a step followed by interaction of gD with the gH/gL heterodimer (complex). Fusion domains of the gH/gL complex and gB enable the pH-independent virus-into-cell penetration („penetration"). The gE/gI complex and gM interact with the receptors at cell junctions in order to facilitate cell-to-cell spread of the virus along the basolateral surface of polarised cells and/or a similar intercellular spread in nonpolarised cells by avoiding virion release. gK, the only so far known HSV-coded glycoprotein which is not incorporated into virions, plays an essential role in the virus capsid envelopment at the nuclear membrane and in the virion transport to the cell surface. Unusually large polykaryocytes arise due to mutations in syn (syncytium) loci of the viral genome, which were mapped to UL53 (syn^1) and UL27 (syn^3) genes coding for gK and gB, respectively, while the genes UL20 and UL24 (both syn^5) code for nonglycosylated cell membrane-associated proteins („membrane proteins"). The products of nonmutated *syn* genes either downregulate the fusion of plasma membranes of infected cells („membrane fusion") or protect them from undesirable fusion events.

Key words: herpes simplex virus; glycoproteins; cell receptors; adsorption; penetration; egress; syncytium

Abbreviations: aa = amino acid; Ab = antibody; CHS = chondroitin sulphate; CPE = cytopathic effect; DS = dermatan sulphate; ER = endoplasmic reticulum; FFWO = fusion from without; GA = Golgi apparatus; GAG = glycosoaminoglycan; gB, gC, gD, gE, gH, gI, gK, gL and gM = glycoproteins B, C, D, E, H, I, K, L, M; gC1 = HSV-1 gC; gC2 = HSV-2 gC; gCt = truncated gC; HA = haemagglutinin; HS = heparan sulphate; HSV = herpes simplex virus; HVEM = herpesvirus entry mediator; ILNM = internal lamella of nuclear membrane; mar = MoAb-resistant; MoAb = monoclonal antibody; MOI = multiplicity of infection; m.u. = map unit; MPR = mannose-6-phosphate; NGF = nerve growth factor; NSF = N-ethylmaleimide-sensitive factor; PBS = phosphate-buffered saline; pgB = precursor of gB; p.i. = post infection; roe = rate of entry; SNAP = soluble NSF attachment protein; TM = transmembrane; TNF = tumour necrosis factor; ts = thermosensitive

Outline of the HSV replication cycle

Following adsorption, penetration results from fusion of the viral envelope with the cell plasma membrane („envelope-membrane fusion"), a process that proceeds at neutral pH. After entering the cytosol, the deenveloped virion is transported to nuclear pores, uncoated and its DNA is discharged into the nucleoplasm, where the transcription as well as replication of viral genes take place. The immediate early (α) transcripts are spliced and processed into α mRNAs which leave the nucleus and are translated on the ribosomes into α-polypeptides. Except for ICP47 these are transported back to the nucleus to activate the next wave of formation of early (β) and late (γ) transcripts. The β (mainly nonstructural) proteins and a portion of the γ (structural) pro-

teins are returned to the nucleus in order to participate in the synthesis of viral DNA and to supply material for the assembly of viral capsids. The synthesised viral DNA molecules are packaged into the capsids during a multistep maturation process. The capsids acquire their „immature" envelope at the inner lamella of nuclear membrane (ILNM). Precursor polypeptides of viral glycoproteins are synthesised in the rough endoplasmic reticulum (ER) and transported either to the nuclear or cytoplasmic membranes. A great majority of viral glycoproteins is glycosylated in the Golgi apparatus (GA). Maturation of envelope glycoproteins proceeds during the egress of virions from cells through a system of cytoplasmic vesicle-like structures. The whole replication process in permissive cells lasts 18 – 20 hrs. In addition to their release into the intercellular space, the virions can reach neighbor cells by membrane fusion, „a cell-to-cell spread" of the virus, without being discharged into the intercellular space and thus avoiding further adsorption and penetration.

HSV glycoproteins as tools facilitating virus replication

The entry of virions into susceptible cells proceeds in two phases: (1) adsorption (attachment and binding) of virion to cell surface and (2) an irreversible interaction between the virion envelope and cell membrane resulting in envelope-membrane fusion which enables virion penetration. Virions can loose their envelopes within endocytic vesicles, which are protrusions of the cell plasma membrane. Nevertheless, a direct fusion of virion envelope with cell membrane frequently occurs in the absence of internalisation. Some investigators (Wittels and Spear, 1991) regard the endocytosis as an aberrant form of virus entry leading, especially after the discharge of lysosome contents, to early degradation of the engulfed virion components, while others claim that gB and gD induce the envelope-membrane fusion also at low pH (Butcher *et al.*, 1990). The dynamics of the entry is determined by interactions of several viral envelope glycoproteins (Table 1).

Adsorption

HSV infects different cell types such as fibroblasts, squamous epithelium cells including keratinocytes, polarised cylindric epithelium cells, glial cells and nerve endings. *In vivo*, membrane receptor proteins located at the apical surface of polarised epithelium cells, differ from those present at the basal and lateral surfaces. *In vitro*, however, the fibroblasts and epithelium cells are usually not polarised. Thus, the virions can attach to membranes with varying composition of receptor proteins and interact with different receptor molecules. The first contact of the virions with the surface of susceptible cells is mediated by GAGs (former mucopolysaccharides) present at the cell surface (Spear, 1993; Herold *et al.*, 1996). The most frequent GAGs with which the HSV virions interact are HS, chondroitin sulphate (CHS) and dermatan sulphate (DS). Incubation of cells with substanc-

Table 1. HSV glycoproteins and their role in virus replication

Glycoprotein	Gene	Function
gB	UL27	Interacts with GAGs during adsorption. Participates in envelope-membrane fusion. Its ectodomain contains many neutralising epitopes. Its cytoplasmic domain regulates fusion The *syn* mutations are clustered at its C-terminal regions I and II
gC	UL44	Cooperates with other glycoproteins during early adsorption. Interacts with GAGs and complement (C3). Contains discontinuous epitopes which can be modified by O-glycosylation
gD	US6	Adsorbs to protein receptors such as HVEM. Participates in membrane fusion. Contains several neutralising epitopes. Participates in the cell-to-cell and transsynaptic spread of virus Functions as fusion mediator but does not contain *syn* mutations.
gE	US8	Reacts with the Fc receptor.
gI	US7	Forms complexes with gE. The gE/gI complex adsorbs to receptors at intercellular junctions mediating the cell-to-cell and transsynaptic spread of the virus.
gH	UL22	Forms a complex with gL. Initiates envelope-membrane fusion and stabilises adsorption. Contains several neutralising epitopes and fusion domains but not *syn* mutations.
gL	UL1	Forms a complex with gH. Some of its epitopes are present in the complex only.
gK	UL53	Is present in the nuclear membrane but not in virions Is involved in virion transport across the cytoplasm. Participates in the envelopment of particles at the nuclear membrane. Contains *syn* mutations.

es which block the attachment of HSV to cell surface such as heparin or polylysine interferes with the cellular receptor function (Lageland *et al.*, 1988). Enzymatic cleavage of HS but not of CHS or DS destroys the capability of cells to interact with HSV and decreases the number of plaques post infection (p.i.) (WuDunn and Spear, 1989).

The aminoglycoside neomycin interferes with the early binding of the virus to the HS receptor provided by gC and also with the internalisation of HSV virions following adsorption (Langeland *et al.*, 1987; Herold and Spear; 1994). Because the first contact between the virus envelope and HS is mediated by gC, the ability of truncated gC (gCt) to attach to cells has been studied (Tal-Singer *et al.*, 1995). The gC-deficient strains show at least 10 times lower plating efficiency as compared to the wild type virus, but still retain a relatively high proportion of infectivity (Herold *et al.*, 1991). Thefore, gC is not considered essential for HSV replication *in vitro*. Using KOS which are mutants gC-deficient due to frameshift mutations, the rest of infectivity mediated by gB was demonstrated to cause an early termination of the gC ectodomain synthesis. Drastically decreased virus binding was observed with a KOS double mutant, which, in addition, carried an inserted termination codon near to the N-terminus of gB (Herold *et al.*, 1994).

In the absence of HS on the surface of HS-deficient CHO or L cells, or provided that the degree of sulphatation of HS was lower, the efficiency of plating decreased by 3 – 4 log units (Spear, 1993; Gruenheid *et al.*, 1993). Because small amounts of HSV could still adsorb to the cells, these experiments showed that GAGs other than HS also interact with the viral envelope. The ability of HSV virions to attach to GAGs other than HS was attributed to gB rather than to gC. In L cells deficient in the synthesis of GAGs, free purified CHS of type A, B or C interfered in a dose-dependent manner with HSV adsorption (Banfield *et al.*, 1995a,b). The most marked effect was observed with CHS of type B or DS upmost resembling HS. The authors concluded that the inhibitory effect of added CHS was not only related to its anionic charge, but also to the structure of its carbon backbone. Lycke *et al.* (1991) studied the influence of chain length of a GAG saccharide on HSV adsorption and found that the shortest oligosaccharide capable of interaction should have consisted of at least 10 monosaccharide units. In principle, however, the negatively charged regions of the polysaccharide (i.e. carboxyl and N-sulphate groups) are the most important feature of HSV gC interactions which mediate the first contact of the virus with HS at cell surface (Herold *et al.*, 1995). The 6-O and the 2,3-O sulphates are of primary importance for HSV type 1 gC (gC1) but are not essential for HSV type 2 gC (gC2) binding (Herold *et al.*, 1996).

The polarised MDCK cells have at least two types of receptors. One type interacts with gC and is situated at the apical surface. The apical gC receptor is possibly identical with that which reacts with the complement component C1 (Roizman and Sears, 1996). However, HSV preferentially enters MDCK cells at the basolateral surface after attachment to the 275 K mannose-6-phosphate receptor (MPR) (Topp *et al.*, 1997). This second type receptor is located at the lateral and basal surfaces and does not react with gC (Sears *et al.*, 1991). The latter interaction is mediated by gD. In GAG-deficient L cells, in which the plating efficiency decreased to 0.5% of that of the wild type cells (Banfield *et al.*, 1994), the residual susceptibility to HSV was attributed to gD-associated adsorption. In swine ST and SK cells, which lack the non-HS protein receptor interacting with gD, no synthesis of immediate early polypeptides occurred indicating that, despite of the gC-mediated adsorption, the virus penetration did not take place (Perez *et al.*, 1995; Subramanian *et al.*, 1994, 1995).

Lee and Fuller (1993) found that UV-inactivated virions could saturate the cell surface and block the challenge with HSV-1 or pseudorabies virus. While the HSV virions lacking gB were able to prevent infection with the challenging virus, virions lacking gD were not. The authors concluded that HSV could attach to the non-GAG receptor(s) present in a limited amount by means of gD and possibly also gH. This conclusion is in accord with that of Forrester *et al.* (1992) who ascribed the major role in blocking the superinfection to gD and the minor role to gH. These reports confirmed the finding of Campadelli-Fiume *et al.* (1988a), who showed that gD expressed on the surface of gD-transfected cells rendered them resistant to HSV infection. As mentioned above, gD binds to either a 275 K or 46 K MPR (Brunetti *et al.*, 1995). Because HSV surprisingly produced plaques in MPR-deficient cells, it has been concluded that several cell receptor molecules must participate in the interactions of HSV gD at the cell surface to facilitate virion entry. Indeed, an another protein belonging to the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor family was found to interact with HSV gD and was designated HVEM. It seems that gD binds to a variety of non-HS receptors as demonstrated with antiidiotypic antibodies, which mimicked the reactive epitope of the 62 K receptor protein on the surface of Vero, HeLa, BHK, Hep-2 and other cells (Huang *et al.*, 1996).

Summing up, the adsorption of HSV has three phases. The phase 1 (early, attachment phase) is resistant to washing with phosphate-buffered saline (PBS) and can be prevented by adding heparin or citric acid. During this phase, gC interacts with HS. In gC-deficient mutants gB interacts with HS. The binding of gC to GAGs only concentrates the virions at cell surface. A more stable adsorption occurs during the phase 2, when gD interacts with the non-GAG protein receptor(s). This phase is resistant to heparin but not to acid pH. gD, possibly in cooperation with gB, initiates envelope-membrane fusion and penetration. In phase

3, the interaction of gD with the protein receptor(s) is stabilised by further complexing with the gH/gL heterodimer (Cai and Person, 1988a; Roop *et al.*, 1993).

Penetration

During penetration, the cell membrane structure is disrupted and the lipids and proteins of the virion envelope and cell plasma membrane are rearranged. After a short lag phase, about 80 – 90% of adsorbed virions penetrate into the cell within 20 mins. Virions cross the cell plasma membrane either directly from the cell surface or from endocytic vesicles, but the endocytosis is not a prerequisite for the penetration (Wittels and Spear, 1990; Shieh and Spear, 1994). In contrast to influenza virus haemagglutinin (HA) which needs acid pH to activate the fusion domain, the HSV entry proceeds at neutral pH and is reduced at acidic pH (Rosenthal *et al.*, 1989). Nevertheless, both gB and gD can induce envelope-membrane fusion also at low pH (Butcher *et al.*, 1990). Glycoproteins participating in penetration may aggregate before undergoing activation by conformational changes. As demonstrated with purified envelope glycoproteins, a cross-linking occurs between gC and gB, gC and gD, gB and gD, and between gD and the gH/gL heterodimer, but never between gB and the gH/gL complex (Handler *et al.*, 1996a,b). Penetration is initiated during the late phases (phases 2 and 3) of adsorption following a conformation change after binding of gD to the non-HS (protein) receptor and interaction with the gH/gL heterodimer. The virus entry is possibly related also to gB-gD complexing, but this has not been confirmed *in vivo*.

In studying the entry process of HSV, Fuller and Lee (1992) found that noninfectious virus-antibody complexes are formed as result of blocking the gD and gH neutralising domains. The virus preincubated with neutralising anti-gD antibody (Ab) was arrested prior to formation of a fusion bridge between the virus envelope and cell membrane. In contrast, the virus inactivated with anti-gH Ab could form a fusion bridge, which was followed neither by expansion nor by rearrangement of the envelope and tegument components. The initiation of a fusion bridge results from the conformational change of gD after its stabile binding to the non-HS receptor. In addition, the process requires a tegument protein coded by the UL25 gene (Addison *et al.*, 1984; Preston, 1990).

Egress of virions

While the capsid envelopment starts at ILNM, the enveloped capsids must cross the cytoplasm of infected cells. The completed capsids containing packaged DNA get attached to ILMN modified by preceding insertion of virus-coded glycoproteins, but empty capsids do not become enveloped. In addition, „full" capsids undergo modifications such as self-cleavage of the scaffold protein 22a, while empty capsids lack these maturation changes. The mature virions cross the ILNM within 50 –60 mins and accumulate in perinuclear cisterns (Rixon, 1993; Church and Wilson, 1997). According to one interpretation, the virions enveloped at ILNM are passing through a system of tubular channels (or vesicle-like structures) formed from ER or the outer lamella of the nuclear membrane. They are further transferred to the trans-Golgi cisterns and/or to Golgi-derived transport vesicles in order to reach the cytoplasmic surface membrane. If this is true, the envelope should contain a complete set of glycoproteins already at crossing ILNM, the only diference being their „immature" (core-glycosylated) saccharide composition. According to other authors, however, the initially enveloped virions when crossing the ILNM loose their envelope and enter the cytosol. These capsids then reenter the cisterns of GA or the vacuoles derived from them to acquire their final envelope (compare this hypothesis with the gK function described below). Browne *et al.* (1996a) used a recombinant virus in which the gH gene had been modified by replacing its original signal sequence. The envelope of newly formed recombinant virions then lacked gH due to its impaired transport. Though the infectivity titer decreased, the total number of virions produced was not lower than that in cells infected with the wild type virus. These data favour an assumption that capsids present in the cytosol complete their envelopment at membranes derived from GA or at the plasma membrane. Which of these interpretations is correct could not be settled from the available electron microscopic findings. The fact that monensin which inhibits a GA-related secretion also inhibits the transport of HSV virions (Stannard *et al.*, 1996) shows that the transport always requires some terminal glycosylation of the envelope proteins. In cells, in which the terminal glycosylation of the mannose residues is blocked, the virions accumulate in the cytoplasm and do not reach the extracellular space (Roizman and Sears, 1996). Many fresh HSV isolates form small plaques and give low infectivity yields at early passages in culture because of incomplete glycosylation of their envelope glycoproteins (Dick and Rosenthal, 1995). The pattern of glycosylation of the envelope glycoproteins points at their movement along the cellular secretion pathway. Precursors of gB, gC and gD bind to calnexin, an important internal cytoplasmic membrane protein, which controls the sequence and folding of the newly inserted polypeptides (Yamashita *et al.*,1996). The latter become glycosylated during their passage across ER and GA. However, the mechanism of insertion of the glycoproteins into ILNM is unclear. Interesting data were published by Stannard *et al.* (1996) who found that the gB and gD precursor polypeptides associated with mature capsids appeared in the nucleus, and then were incorporated in the envelope at the budding site of ILNM.

The envelopment of HSV particles was not found altered in mutants with deletions in gB, gD and gH (Cai *et al.*, 1988b; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Roop *et al.*, 1993). However, the egress of particles was impaired in the case of gD and gH mutations (Desai *et al.*, 1988, Campadelli-Fiume *et al.*, 1991). The tsQ26 mutant containing an amino acid substitution in gH (at position 244) entered cells efficiently but, at nonpermissive temperature, produced gH-deficient non-infectious virions. When infected cells were disrupted it was found that they contained infectious particles, which were not excreted. Further HSV genes related to the egress of virions are UL53 (gK gene) (Hutchinson and Johnson, 1995; Jayachandra *et al.*, 1997), UL20 (Baines *et al.*, 1991; Ward *et al.*, 1994), UL11 (Baines and Roizman, 1992) and IR_L1 (ICP 34.5gene) (Brown *et al.*, 1994). gK-deficient mutants do not become enveloped and accumulate within the nucleus. UL20-deficient mutants get enveloped but accumulate in perinuclear cisterns. Some cell lines complement the defect in the virion transport of gK-deficient mutants or that caused by the absence of the UL20 gene product (Baines *et al.*, 1991; Avitabile *et al.* 1994).

Cell-to-cell spread of HSV

HSV can spread from one to neighbor cells without being released to the environment from the maternal infected cell and avoiding repeated adsorption and penetration across the surface of surrounding uninfected cells. This kind of spread is effective in the stratified squamous epithelium but can occur also in cell culture. The cellular receptors for intercellular spread differ from those involved in the adsorption and penetration and interact with the gE/gI complex which participates in the cell-to-cell spread via cell junctions (Dingwell *et al.*, 1994, 1995; Balan *et al.*, 1994). Relatively small giant cells which randomly occur in HSV infection in the squamous epithelium *in vivo* should not be mistaken for the *syn* phenotype. The latter occurs in some HSV strains *in vitro* due to mutations in the *syn* genes which cause unregulated (uninhibited) membrane fusion and large giant cell formation (Table 2). The *syn* mutants frequently develop in the course of repeated passaging in cell culture, while small polykaryocytes occur in association with the cytopathic effect (CPE) caused by fresh isolates. Small polykaryocytes appear during the first 1 – 2 hrs p.i. in contrast to unregulated fusion which starts from 4 or 6 hrs p.i. (Read *et al.*, 1980). Unlike *syn* mutations, the intercellular spread and formation of small polykaryocytes is related to the action of naturally present envelope components such as the gE/gI complex. Glycoproteins participating in the adsorption and penetration (gB, gC, gD, gH and gL) are not identical with those (gE, gI and gM) which are predominantly involved into cell-to-cell spread (Davis-Pointner *et al.*, 1994).

Cell membrane fusion and polykaryocyte formation

The cell membrane fusion is a common event which mediates housekeeping functions in cells such as endocytosis, secretion and recycling of membrane components. The envelope-membrane fusion participates in the virion entry into and in the virion egress from host cells. This fusion is initiated and regulated by at least one fusion protein or by a complex of such proteins. A well known example is the HA of influenza virus. The fusion domain localised at the N-terminus of the HA2 subunit is a conserved sequence containing many hydrophobic amino acids. At neutral pH, the 3 fusion domains of each HA2 subunit are tightly tucked by hydrogen bonds to the fibrous stem about 3 nm away from the site where the trimer enters the viral envelope. In response to low pH (about 5), the tertiary folding of the whole trimer is altered to expose the buried fusion ectodomain and to dissociate its globular tip structure. The exposed fusion peptide renders the globular tip of the trimer hydrophobic and fosters its attachment to the target membrane before significant lipid mixing occurs. During a lag phase whose length depends on pH, temparature and density of the target membrane receptor, additional motions of the HA subunits take place in the layer of the viral envelope resulting in aggregation of several HA trimers and formation of a fusion pore.

Overcoming of the repulsive hydration force by the attractive hydrophobic force is the dominant mechanism how the hydrophobic domains provide fusion. The hydrophobic

Table 2. *syn* mutations in HSV DNA

Gene	Product	Locus (classical)	Reference
UL53	gK	syn^1	Ruyechan *et al.* (1979)
UL44	gC	syn^{2*}	Pogue-Geile *et al.* (1984), Draper *et al.* (1984)
UL27	gB	syn^3	Weise *et al.* (1987), Cai *et al.* (1988)
UL24	membrane-associated protein	syn^5	Tognon *et al.* (1990)
IR_L1	ICP 34.5	syn^6	Romanelli *et al.* (1991)

*Data not confirmed by other authors.

domain of the fusion peptide is usually an integral part of the envelope glycoprotein. It consists of a stretch of 16 (in the Semliki forest virus E1 glycoprotein) to 26 (in the simian vacuolating virus 5 fusion protein F1) hydrophobic amino acids (Ile, Phe, Val, Leu, Trp and Met). Viral fusion proteins could serve as a basis for understanding the function of more complicated cell fusion systems. The fusion of transport vesicles in the cytoplasm is a very complex process requiring several proteins. At least 3 basic components form the core of the „fusion machine" at the interface of the transport vesicle and acceptor cistern: N-ethylmaleinimide-sensitive factor (NSF) associates with several soluble NSF attachment proteins (SNAP) to a receptor at the acceptor membrane. The alpha-SNAP receptor has recently been identified as an integral membrane component (White, 1992).

According to Ruyechan *et al.* (1979), HSV strains inducing unusually large giant cells have the *syn* phenotype, while those causing cell rounding only have the *syn*$^+$ (non-*syn*) phenotype. Six syn loci were identified by classical recombination techniques, from which at least 2 were later on confirmed and localised by DNA sequencing (Table 2), namely syn^1 gK (UL53) and syn^3 gB (UL27). The syn^5 (UL24) locus has been determined indirectly. Another 2 loci (syn^2 and syn^6) are still questionable (see below), while syn^4 locus was not confirmed at all. In addition, however, the UL20 gene was found related to the syncytium formation.

The majority of *syn* mutations is located in gK. Because this glycoprotein is neither present in extracellular virions nor on the cell surface, the effect of gK mutations was attributed to impaired fusion regulation and to delayed action of such disregulation because of halted virion transport. The gK-related giant cell formation starts from the 6th hr p.i.; only about 10% of cells remain unaffacted at MOI of 0.4 –1.8 (Person *et al.*, 1976). In gB-related giant cell formation, the corresponding mutations located in the cytoplasmic domain of gB block its regulatory function for membrane fusion. When HEL cells were infected with either the syn gK mutant MP or the syn gB mutant tsB5, the transport and glycosylation of gB, gD and gH were impaired (possibly due to delayed virion trafficking to the GA) as compared to cells infected with the wild type HFEM virus-infected cells (Person *et al.*, 1982). No difference, however, was found in the accumulation of precursor polypeptides of the main viral glycoproteins in Vero cells infected with HSZP strain (Raučina *et al.*, 1985). Also the UL20 and UL24 gene products downregulate membrane fusion. Thus, their mutations then cause a *syn* phenotype. As far as it is known, other fusogenic glycoproteins as gD, gH and gL, in contrast to gB, do not possess any *syn* mutations.

Cyclosporin A inhibits polykaryocyte formation induced by gK and UL24-related mutations (McKenzie *et al.*, 1987). From inhibitors of the Na/K pump, melittin inhibits *syn* gK-related fusion, while ouabain inhibits fusion induced by *syn* gK as well as *syn* gB mutations (Baghian and Kousoulas, 1993). Heparin inhibits the *syn* gB-related fusion. The different effect of inhibitors on the *syn* gB- and *syn* gK-related fusions confirm their different mechanism of action (Seck *et al.*, 1994). Both *syn* gB and *syn* gK phenotypes can be inhibited in the range of pH of 6.7 – 7.0, the optimum pH being pH 7.5 (Baghian *et al.*, 1992).

HSV glycoproteins as tools for virus adsorption and envelope-membrane and membrane fusion

Glycoprotein B (gB)

The precursor polypeptide of this largest glycoprotein encoded by the UL27 gene has 904 aa.Twenty-nine or 30 of them constitute the signal sequence cleaved off from the N-terminus (Claesson-Welsh and Spear, 1987). The gB surface domain consists of 696 aa. There are 6 Asn, of which at least 5 can be N-glycosylated (Cai *et al.*, 1988a). In addition, the precursor gB (pgB) molecule has a 69 aa long transmembrane (TM) domain (aa 727–795) and a 109 aa long cytoplasmic (C) domain (Pellet *et al.*, 1985). The gB molecule is inserted in the membrane by means of 3 hydrophobic segments of the TM domain (Gilbert *et al.*, 1994). If the TM domain is deleted, the gB molecule cannot be inserted in the lipid bilayer of the membrane (Cai *et al.*, 1988b). gB molecules deleted in the TM and C domains are secreted from transfected cells (Ali, 1987). Desai *et al.* (1994) found that gB deleted in segments covering aa 43–711 cannot be incorporated into virions. After being inserted in the ER, gB forms homodimers by interaction of two regions at aa 98–282 and 596–711 (Highlander *et al.*, 1991; Quadri *et al.*, 1991). This means that the C domain is not essential for the dimer formation (Ali, 1990; Lin *et al.*, 1996). Neither does the C domain (related to fusion regulation as described above) participate in the heparin binding (Lin *et al.*, 1996). The site for interaction of gB with HS possibly resides in the N-terminus of the gB ectodomain possessing a cluster of basic aa (Herold *et al.*, 1994).

The epitopes of the surface domain which interact with neutralising monoclonal antibodies (MoAbs) fall into two groups, namely those which react also in a denatured state (continuous epitopes) and those which react in native state only (discontinous epitopes). The latter occur mainly in dimeric structures (Chapsal and Pereira, 1988), but they can also result from the conformation of the monomer (Pereira *et al.*, 1989). The antigenic epitopes and their partially overlapping clusters were characterised using the HSV strains KOS and F with similar but not identical results. The appearance of MoAb-resistant (*mar*) mutations confirmed the correct localisation of several neutralising epitopes. Marlin *et al.* (1986)

Table 3. Important sites and epitopes of HSV gB

HSV-1 strain	Site	Position (aa)	*mar* mutation (aa)	Function
KOS	I	381–441	473	Contains continuous epitopes. Antibody binding requires complement. Is involved in penetration.
	II	597–737	594	Antibody binding requires complement. Contains *roe* and *ts* mutations. Is involved in adsorption and indirectly also in penetration.
	III	283–380	305	No complement is needed for antibody binding. Is the main site of penetration. Contains *ts* mutations.
	IV	?–282	85	Reacts with MoAb B6 which blocks penetration.
F	D1	1–47		Contains continuous neutralising epitopes.
	D2	273–298		Is the main neutralising epitope. Corresponds to site III of KOS and overlaps with site IV of KOS.
	D3	380–457		Corresponds to site I of KOS.
	D5	600–690		Corresponds to site II of KOS. Contains discontinuous epitope(s). Is indirectly involved in penetration.

mar = MoAB-resistant; *roe* = rate of entry; *ts* = thermosensitive.

described five antigenic loci in KOS strain gB in comparison to the three neutralising regions of F strain gB (Kousoulas *et al.*, 1988; Pereira *et al.*, 1989; Quadri *et al.*, 1991; Navarro *et al.*, 1992). The neutralising epitopes of gB were characterised by a MoAb, which blocked mainly adsorption and/or penetration by preventing the virus entry.

Table 3 summarises the main antigenic epitopes of gB. From these, the neutralising epitopes are located either in the middle of the gB polypeptide or span towards the N-terminus (designated as sites D1 and D2 or I, III and IV). An additional site (D5 or II) which is indirectly involved in the penetration is located close to the TM domain, situated far away from the main neutralising epitope cluster D2 (Highlander *et al.*, 1988, 1989). All the MoAbs, especially anti-D2 MoAbs directed against the latter neutralising epitopes, reduced or inhibited plaque formation. Some of them could also interfere with the cell-to-cell spread. A group of anti-D1 and anti-D2 MoAbs, which blocked virus penetration in the presence of complement, did not inhibit plaque formation. Anti-D1 antibodies (Abs) inhibited plaque formation in the absence of complement, but neutralised the free virus in the presence of complement only. This points at the possibility that plaque formation may also result from the cell-to-cell spread in which gB coparticipates. MoAbs against KOS sites I and IV interfered with penetration, but the most efficient MoAbs in preventing penetration were directed against the KOS site III. This has been confirmed by substitution of aa 273 and 377 and/or by insertion at positions 313 and 437 (Bzik *et al.*, 1984; Cai *et al.*, 1988b). While anti-III Abs directly prevented the penetration, anti-II Abs impaired plaque formation. This suggested that the site II, which is the site of *ts* and/or *mar* mutations, is indirectly involved in penetration and possibly coparticipates in adsorption. The same function has been attributed to the D5 site of the F strain.

The *syn* gB phenotype is associated with mutations scattered over two sites of the C-terminal domain as the cell fusion is downregulated by this domain and its mutation causes unregulated fusion. The cytoplasmic site I of gB is located in the vicinity of the TM domain between aa 796 and 817. The cytoplasmic site II encompasses aa 848–877 (Gage *et al.*, 1993). Mutations at aa 817 and 855 cause polykaryocyte formation but do not affect the ability of the mutant to penetrate into cells (Goodman and Engel, 1991; Engel *et al.*, 1993). Cai *et al.* (1988b) ascribed the ability to induce giant cells to aa 816/817 (site I). Extensive giant cell formation, however, was found with mutants lacking the last 28 aa, i.e. when the truncation was made downstream of aa 876 (Baghian *et al.*, 1993). The C-terminal domain has two α helices, which can be related to the fusion regulation. If the C-terminus is shortened by more than 49 or 64 aa, both helices become distorted. In the latter case, the virus becomes noninfectious. Site II *syn* gB mutations often participate in the fusion from without (FFWO) which does not require translation *de novo* (Walev *et al.*, 1994). This kind of fusion is extremely quick (may occur 60 mins p.i.) and widespread, especially in the case of high MOI. FFWO is not inhibited at 40 – 60 mmol/l concentrations of cytochalasin A, which usually inihibit polykaryocyte formation (Walev *et al.*, 1991a). Typical HSV strains inducing FFWO are ANGpath (Lingen *et al.*, 1995a) and HSZP (Rajčáni *et al.*, 1996), in which mutations were found at aa 854 and 857, respectively. It has been postulated that the cytoplasmic gB domain acts during FFWO in accord with the rate of entry (roe) ectodomain, especially at aa 553 (Saharkhiz-Langroodi and Holland, 1997). A possible relationship of the site II syn gB to the penetration region I of gD (see below) should be also taken into account.The *syn* gB mutations at aa 817 and 854–857 (or 855–858; the different numbering is caused by the varying length of the signal

sequence cleaved off in different strains) (Rajčáni *et al.*, 1996) increased the rigidity, hydrophobicity and positive charge of the cytoplasmic domain. It seems that Arg 857/8 is crucial for its correct functioning. The *syn* tsB5 mutation at aa 857/8 of gB induced large polykaryocytes even in the case of double infection with the non-syn KOS strain (Bond *et al.*, 1982). Mutation at aa 851 of KOS gB, i.e. in the close vicinity of site II, reduced the intensity of penetration of the wild type non-syn KOS strain but did not cause polykaryocyte formation (Gage *et al.*, 1993). Because the *roe* mutations were found at the ectodomain aa 553 (Bzik *et al.*, 1984; DeLuca *et al.* 1982) and 594 (Highlander *et al.*, 1989), close to the site II (D5), the ectodomain possibly acts in accord with the cytoplasmic domain.

Glycoprotein C (gC)

The 1,536 bp UL44 gene specifies the 511 aa long precursor of gC (pgC) (Frink *et al.*, 1983). Using a set of MoAbs against KOS gC, three epitopes were assigned to the antigenic site I and six epitopes were found clustered in the site II composed of 3 distinct subsites (Marlin *et al.*, 1985). According to Homa *et al.* (1986), gC1 contains a 25 aa signal sequence at the N-terminus, a long surface domain (453 aa), a 23 aa TM anchoring domain (aa 478–500) and a short C-terminal cytoplasmic domain. Deletion of the latter domain, even if preserving the TM domain, allows secretion of the whole gC (Holland *et al.*, 1984). The HSV type 2 gC (gC2) was previously designated gF, because of the considerably different molecular mass and distinct antigenic specificity as compared to gC1. Sequencing of gC1 and gF genes showed their wide homology. On the other hand, a deletion of 31 aa in gC2 near to the N-terminus has been found (Dowbenko and Lasky, *et al.*, 1984; Swain *et al.*, 1985).

Whereas the predicted M_r of pgC was 55 K, SDS-PAGE yielded a value of 120 – 130 K indicating its heavy glycosylation (Kikuchi *et al.*, 1987). Sequential removal of peripheral monosaccharides (galactose and sialic acid) from the gC molecule showed that saccharides do not directly participate in the antigenic reaction but rather force the polypeptide into a conformation which is essential for the expression of the site II epitopes (aa 129–247, Sjöblom *et al.*, 1987). The site I antigens are expressed also in the absence of peripheral saccharides. The high affinity of gC to lectins was assigned to the multiple O-linked saccharides (Lundstrom *et al.*, 1987a) whose biosynthesis depends of the host cell enzymes (Lundstrom *et al.*, 1987b). In infected cells, gC can function as a receptor for the C3b component of complement (Friedman *et al.*, 1984; Ghosh-Choudry *et al.*, 1990). The role of gC in the initiation of HSV adsorption is desribed above. The truncated gC (gCt) lacking aa 33–123 adsorbed less efficiently to susceptible cells indicating the function of the N-terminal ectodomain. Of the first 100 aa in this particular region, 16 showed alkaline dissociation and may represent the HS binding site. The GAG binding site of gC has been localised to the first 120 aa (aa 33–123) from the N-terminus (Tal-Singer *et al.*, 1995). However, MoAbs recognising the epitopes of antigenic domain II (aa 129–247) also inhibited the virus attachment (Trybala *et al.*, 1994). The corresponding *mar* mutations mapped to aa 143, 150 and 247.

The relationship of gC to syncytium formation became a matter of confusion since several *syn* mutants turned out gC-deficient or at least possessing gCt. E.g., the MP strain variant (syn gK) MP10311 revealed the *syn* phenotype and was gC-deficient in subconfluent Vero, Hep-2 and MDBK cells (Bartoletti *et al.*, 1985). In general, the *syn* phenotype could be more effectively expressed especially in Hep-2 cells if the mutant virus had a truncated or defective gC (Manservigi *et al.*, 1977; Bond *et al.*, 1982; Tognon *et al.*, 1984; Goodman and Engel, 1991; Pertel and Spear, 1996). The exact role of gCt in the potentiation of the gK and/or gB *syn* mutations has not been cleared yet.

Glycoprotein D (gD)

The 1182 bp long US6 gene specifies a 394 aa precursor of gD (pgD, 52 K), which after splitting off the 25 aa long signal peptide has 369 aa with 3 glycosylation sites (McGeoch *et al.*, 1985; Watson *et al.*, 1982). The N-linked oligomannosyl core moiety is further glycosylated and sialylated so that the mature gD has a M_r of 59 K (Eisenberg *et al.*, 1979). The last 32 aa from the C-terminus (aa 338–369) form the cytoplasmic domain (Minson *et al.*, 1986). The most MoAbs react with gD1 as well as gD2 proteins, a finding confirmed by 80% DNA sequence homology (Lasky and Dowbenko, 1984). The ectodomain has 6 Cys at positions 66, 106, 118, 127, 189 and 202 forming disulphide bonds for the configuration of discontinuous epitopes Ia, Ib, III, IV and VI (Long *et al.*, 1992). The pgD polypeptide contains 3 Asn-X-Ser/Thr sites for addition of N-linked saccharides (N-CHO) (Sodora *et al.*, 1991a). The gD mutant without N-CHO but with all the three Ser/Thr substituted by Ala (the so called mutant AAA) was structurally altered but still able to complement the infectivity of a gD-deficient mutant. The mutant QAA gD with the first Ser/Thr substituted by Gln showed the same antigenic conformation as the wild type gD synthesised in the presence of tunicamycine. This result pointed at the importance of the first N-CHO glycosylation site at aa 94–96 for the formation and preservation the gD polypeptide conformation.

A majority of HSV neutralising Abs precipitate gD, while a minority react with the gB and gH envelope components (Eing *et al.*, 1989). The most efficient gD-reactive neutralising Abs which decrease virus infectivity and inhibit plaque

Table 4. Important sites, regions and epitopes of HSV gD

Site[a]	Position (aa)	*mar* mutation (aa)	Function
VII	11–19	16	Is a type-common continuous neutralisation epitope which reacts with MoAb LP14.
II	268–287	273	Is a type-common continuous epitope which reacts with MoAb DL6. Complement is not required for antibody binding
V	340–356		Is a continuous type-specific epitope.
XI	284–301		Is a continuous type-specific epitope.
Ia	?–233	216	Is a discontinuous neutralisation epitope which reacts with MoAb LP2.
Ib	124–140	129	Is a discontinuous epitope which reacts with MoAbs AP12, D2, AP12 and 4S. Complement is not required for antibody binding.
		132	
		140	
III	all three	180	Is a discontinuous epitope.
IV	aa ?–183		Is a discontinuous epitope.
VI			Is a discontinuous epitope involved in adsorption.
Region[b]			
I	25–43	25–27	Contains a site for direct penetration. Reacts with MoAb AP7.
II	126–161	125–6	Includes the site Ib and is related to fusion.
		140	
III	221–246		Binds to HVEM receptor.
IV	277–310		Mediates virus entry and reacts with AP7.

[a]Data according to Eisenberg *et al.* (1985). [b]Data according to Chiang *et al.* (1994).
For the legend see Table 3.

formation can directly interfere with the virus penetration without preventing the virus attachment. In contrast, anti-gD MoAbs neutralising the virus at higher concentrations only, hamper the virus interactions with the cell receptors and interfere with giant cell formation as demonstrated in the case of the HFEM *syn* mutant (Fuller and Spear, 1985, 1987). One of the most important continuous antigenic sites is located within the first 23 aa from the N-terminus (Dietzschold *et al.*, 1984). This epitope (site VII) can be neutralised with MoAb LP14 (Eisenberg *et al.*, 1985; Minson *et* al., 1986; Bosch *et al.*, 1987). Another important site, located between aa 27–43 and adjacent to the site VII forms a part of the MoAb AP7 binding epitope. The latter continuous epitope was associated with *mar* mutations at aa 25–27 (Chiang *et al.*, 1994; Dean *et al.* 1994). MoAb AP7 neutralised the virus in the presence of complement and MoAb LP14, and in addition to neutralisation, also inhibited the envelope-membrane fusion. A further continuous epitope (epitope II) was localised at aa 268–287 (Isola *et al.*, 1989). Continuous antigenic epitopes were also found near the TM domain at aa 284–301 (site XI), aa 340–356 (site V) and aa 358–369 in the cytoplasmic domain (Table 4).

While antibodies in groups II, V, VII and XI recognize denatured gD, another set of MoAbs reacts at least with four additional discontinuous epitopes referred to as sites I (aa 233–259), III, IV and VI (?–182). The site I has been found related to membrane fusion and the site VI to irreversible adsorption (Cohen *et al.*, 1986). Furthermore, the group I type-common MoAbs were divided into subgroups Ia and Ib with different *mar* mutations preventing neutralisation (Muggeridge *et al.*, 1990). The MoAbs Ia-related mutations were localised to aa 216, while the MoAbs Ib-related mutations to aa 132 and 140.

Using insertion-deletion mutants, Nicola *et al.* (1996) defined functional regions I (aa 27–33), II (aa 126–161, includes the site Ib), III (aa 225–246) and IV (aa 277–310). Surprisingly, the region IV, located close to the TM domain, was found to contain at least two AP7-positive epitopes (one of them between aa 290–300) and to be important for the gD-mediated virus entry. The regions I and IV may constitute a single functional domain after getting close to each other in the folded native gD molecule (Chiang *et al.*, 1994).

Based on the complement requirement, Highlander *et al.* (1987) selected nine anti-gD MoAbs neutralising the wild type KOS strain. The group I, II, IX and X MoAbs neutralised the virus in the absence of complement. At least sites I and II related to these MoAbs were identical with the Eisenberg's classification (Eisenberg *et al.*, 1985) (Table 4), but sites IX and X were newly defined. In the MoAb groups in question, the neutralisation in no case interfered with the virion attachment suggesting that the corresponding MoAb prevented the virion penetration. This was in accord with the statement of Minson *et al.* (1986) that an Ab against the site Ia (*mar* mutation at aa 216) neutralised SC16 strain but did not prevent polykarocyte formation by the HFEM *syn* mutant. In contrast, an Ab against the site Ib (*mar* mutation at aa 129) inhibited the HFEM *syn* mutant-mediated fusion but did not neutralise the virus. Furthermore, a *mar* mutation at aa 16 (site VII) provided evasion from anti-syn protection of the corresponding group VII MoAbs. Both the

anti-VII and anti-Ib MoAbs had a prominent neutralising activity. MoAbs against gD blocking late HSV adsorption reacted with the sites VII and Ib constituted by the first 182 aa from the N-terminus (Long *et al.*, 1992; Nicola *et al.*, 1996). These sites encompass the main neutralising domains I and II (Fuller and Spear, 1985, 1987). The HVEM receptor (Warner *et al.*, 1995; Montgomery *et al.*, 1996) interacts with site VII because mutations at aa 25 or 27 abolish the ability of the virus to penetrate into cell (Montgomery *et al.*, 1996; Whitback *et al.*, 1997). Additional data suggest that also aa 230 is directly involved. Truncated recombinant gD (gDt) lacking the first 306 aa cannot interact with HVEM. Purified recombinants of gD1 and truncated gD (gD 306t lacking the TM and cytoplasmic domain) still interact with the a truncated recombinant HVEM (HVEM200t) forming complexes which mimic the direct receptor for gD binding. As described above (see *Adsorption*), the anti-idiotype gD MoAbs which mimic gD react with a 62 K protein located on the surface of many cells (Huang *et al.*, 1996). Furthermore, the M-6-P receptor (MPR) was found important for gD-mediated virion-cell interactions, and at least, two forms of MPR were identified (46 K and 275 K).

Cells expressing gD are resistant to HSV challenge (Campadelli-Fiume *et al.*, 1988). This phenomenon occurs due to saturation of the corresponding protein receptors. The developing resistance is dose-dependent (Johnson *et al.*, 1990). However, the phenomenon is not solely related to adsorption. The intereference-resistant strains and mutants (see below) do not interact with the HVEM receptor only, but also with an alternative minor receptor(s) though at lower affinity (Brandimarti *et al.*, 1994). The gD molecules expressed in transfected cells may directly interfere with the envelope-membrane fusion. Because incubation of transfected cells with MoAb AP7 reversed the interfering activity of gD expressed on their surface, the functional region I has been implicated in direct penetration (Campadelli-Fiume *et al.*, 1990). The corresponding *mar* mutation of strain F maped to aa 25 by selecting a mutant (with Pro instead of Leu at this position) which still remained infectious for the gD-transfected cells. The AP7-gD region I is directly involved in virus penetration and functions immediately after attachment to the cell receptor.

Spontaneous KOS mutants rid1 and rid2 were isolated on the basis of their ability to grow in Hep-2 cells expressing gD (Dean *et al.*, 1994). These mutants had either Pro (rid1) or Gly (rid2) instead of Glu at position 27 (the region I), a change which increased their ability to infect the resistant CHO cells. Another interesting mutation concerns the region II, namely its site Ib (aa 140) as demonstrated on Hep-2 cells expressing wild type gD. These cells were less sensitive to challenge with KOS strain than with strains 17, ANG and F (Dean *et al.*, 1995). The differences were attributed to aa substitutions at positions 27 and/or 25. The ANG strain with both aa substitutions was most resistant to the gD-mediated interference. In strain F, additional mutations at aa 185 and 140 were found as relatively important. If purified truncated forms of gD (gDts) were adsorbed to Vero cells to prevent plaque formation by HSV, the purified gDt expressing the N-terminal ectodomain consisting of the first 301 aa residues had a protective effect analogous to that of the wild type gD. A deletion of aa 290–299 (site XI, region IV) enhanced the interference in a paradoxic manner as compared to the wild type gD. As expected, a gDt with a deletion in the region I did not block the infectivity and gDts with deletions in the regions II and III had only a minimal blocking effect (Nicola *et al.*, 1996, 1997). A deletion in the region IV increased the resistance to challenge with the KOS mutants rid1 and rid2 stressing the specific role of the AP7 reaction site. Taken together, it is likely that different regions of gD are involved in various successive steps of the virus entry, i.e. in the late (stabile) phase of adsorption, envelope-membrane fusion and penetration. Some strains (ANG, ANGpath and HSZP) able to penetrate in Vero cells at 4°C also revealed mutations in the N-terminal region of gD at aa 25 and 27 (Lingen *et al.*, 1995b). The functional role of gD-mediated interference may reside in keeping the virions away from reentering the cytoplasm of infected cells when crossing the system of cytoplasma membrane network. It could also protect the infected cells from reinfection with already released virions present in the intercellular space. A *in vitro* infection with gD-deficient HSV mutants showed limited formation of extremely small plaques because the virus was unable to spread to neighbour cells. Alternatively, the transsynaptic spread of a recombinant gD (in which gD of ANGpath was replaced by KOS gD) beyond the first neuron was highly reduced *in vivo* (Rajčáni *et al.*, 1994). In another experiments, the pathogenic SC16 strain was altered by replacing its C-terminal (syn^3 gB) region with that from the nonpathogenic ANG strain, and/or by deleting its gD gene (Davis-Poynter *et al.*, 1994). The results showed that the latter recombinant did not spread to adjacent cells confirming the essential role of gD in penetration.

Glycoprotein G (gG)

gG, a product of the US4 gene (Ackerman *et al.*, 1986), is incorporated into the nuclear and cytoplasmic membranes of the cell (Frame *et al.*, 1986; Sullivan and Smith, 1987). It is essential neither for the virus entry into nor for the virus release from infected non-polarised cells. The gG-deficient virions, however, are arrested at an so far unclear postadsorbtion phase of virus entry (Roizman and Sears, 1996). A SC16 recombinant with ANG gD showed normal plaque count and morphology as compared to the wild type strain (Balan *et al.*, 1994b). However, in animal experiments, the

yield of the recombinant was lower. The *syn* phenotype of the recombinant was not altered (Davis-Poynter *et al.*, 1994).

Glycoproteins E and I (gE and gI)

While the US8 gene codes for gE (Lee *et al.*, 1982), the neighbor US7 gene does so for gI (Longnecker *et al.*, 1987). They both form a single membrane-anchored complex showing higher affinity to the Fc portion of IgG than each of them alone. Cells transformed with the US8 gene did not bind IgG significantly (Johnson and Feenstra, 1987). The gE/gI complex is quickly formed during the synthesis and transport of both glycoproteins before their incorporation into the virion envelope (Para *et al.*, 1982; Sullivan and Smith, 1988). The gE/gI-deficient mutants of strains F and 17 formed small plaques in human fibroblasts and/or epithelium cells. At high MOI, the yields of these mutants did not differ from those of the wild type strains. After repeated passages at low MOI, the yields of the gE/gI-deficient mutants decreased as compared to those of wild type strains. Under the latter conditions, the yields of the mutants were lower in the presence of neutralising Abs than in their absence. This was attributed to a limited cell-to-cell spread which was not influenced by the presence of polyclonal HSV-neutralising Abs (Dingwell *et al.*, 1994). SC16 gE/gI-deficient mutants formed plaques of usual size in BHK-21 cells indicating that the gE/gI complex did not influence the plaque size (Balan *et al.*, 1994b). Nevertheless, in the culture of trigeminal ganglion neurons, the intercellular spread of the gE/gI mutants was reduced by 50%. Here again, the virus growth after high MOI remained unchanged. These results of Dingwell *et al.* (1995) indicate that the gE/gI heterodimer might be highly effective in the neuron-to-neuron or transsynaptic spread. Though the gE/gI-deficient mutants were less efficient in crossing the cytoplasmic membrane of neighbour cells or in attachment to cell junctions, the latter function seems dominating from the pathogenetic point of view, because the ability of the gE/gI-deficient mutants to cross synapses or enter the nerve endings was considerably reduced. *In vivo,* the gE/gI mutants showed a limited spread along the neural pathway possibly due to their impaired ability to adsorb to synaptic receptors (Rajčáni *et al.*, 1990; Kúdelová *et al.*, 1991). Even when the gE/gI complex is not indespensable for infection of cells *in vitro* (Neidhard *et al.*, 1987), it may be very important for viral spread *in vivo*.

In the body, the gE/gI complex binds to the IgG Fc fragment providing evasion from the specific Ab action, but its main function resides in assisting the cell-to-cell spread which implies fusion of the membranes between neighbour cells. Again, the role of the gE/gI complex in the cell-to-cell spread of HSV, namely in crossing basolateral membranes of polarised cells, might be especially important *in vivo* (Roizman and Sears, 1996). Because the gE/gI-deficient mutants penetrated into cells at a normal rate, the fusion of membranes of infected and uninfected cells differs from the envelope-membrane fusion. Nevertheless, the *syn* phenotype of the gK mutant MP could be neutralised by an anti-gE serum (Chatterjee *et al.*, 1989). The artificially created fusogenic capacity of an SC16/ANG recombinant, in which the cytoplasmic domain syn^3 of SC16 gB was replaced with that from ANG strain, was lost or highly reduced when the recombinant was, in addition, deleted in the gE/gI genes (Balan *et al.*, 1994b; Davis-Pointer *et al.*, 1994). In contrast, an ANG mutant deleted in gE/gI retained its *syn* phenotype, though the polykaryocytes formed were smaller than those caused by the wild type ANG strain (Neidhard *et al.*, 1987). This might be explained by a cooperation of the gE/gI complex with gD, namely its region I, which is mutated at aa 25 and 27 in the ANG mutant but not in the SC16/ANG recombinant.

Glycoproteins H and L (gH and gL)

The UL22 gene codes for gH, a virion envelope component essential for virus penetration into cell. In addition, gH participates in the egress of virions from the infected cells and in their cell-to-cell spread. The infected cell membranes contain a heterodimer of gH with gL, the product of gene UL1 (Hutchinson *et al.*, 1992). When the UL22 gene was expressed in transfected cells in the absence of UL1, the resulting polypeptide was neither correctly processed nor folded. Because it could not be transported to the cytoplasmic membrane it remained retained in the ER where it underwent self-aggregation. The gH monomer did not react with the specific MoAbs LP11 and 52S, which specifically recognised the epitopes on the heterodimer (Forrester *et al.*, 1991; Foa-Tomasi *et al.*, 1991; Robets *et al.*, 1991). Alternatively, cells infected with a gL-deficient virus did not produce virions containing the gH/gL complex. When cells were infected with a gH-deficient virus, gL was neither correctly processed nor incorporated into the cell plasma membrane (Hutchinson *et al.*, 1992; Roop *et al.*, 1993). The secreted form of gL found in the culture medium under such conditions had M_r of 30 K (Dubin *et al.*, 1995). When both genes UL22 and UL1 were coexpressed in mammalian cells, the antigenic conformation of the formed gH/gL complex was identical with that found in virus-infected cells, and both the MoAbs 52S and LP11 were able to immunoprecipitate the complex. It appears that gL is not an integral membrane glycoprotein, but becomes tightly associated with the cell membrane through an interaction with gH (Dubin *et al.*, 1995). The mutual interaction of both glycoproteins is mediated by the N-terminal region of the first 69 aa of gL after cleaving off the 25 aa long signal sequence (Roop *et al.*, 1993), while gH reacts through its central region.

Hybrid forms of gH containing the N-terminal surface domain of gH of various length and the C-terminal domain of gC, and *vice versa,* were obtained. Hybrid proteins carrying the 325 N-terminal aa of gH upstream of mature gC polypeptide were efficiently transported. In the absence of gL, the central part of the external gH domain (aa 325–430) inhibited the cell surface trafficking of the gHt/gC hybrid (Schnabel *et al.*, 1995).

Virions lacking the gH/gL complex could not enter cells though they could attach to the cell surface. The adsorption of gH-deficient virus to Vero cells blocked the superinection with wild type virus. These observations indicated the role of gH in envelope-membrane fusion but not in the virion attachment and receptor binding. Forrester *et al.* (1992) prepared a gH-deficient mutant of SC16 strain which together with a gH-deficient *ts* mutant of KOS designated Q26 (Desai *et al.*, 1988) were used for infection of Vero cells transfected with the gH gene (F6 cells). Though both the KOS and Q26 mutants had non-*syn* phenotype, they produced syncytia confirmed the possible role of overexpressed gH in membrane fusion.

Mutants with insertions at aa 691–692, 709 and 791 adjacent to the TM domain considerably reduced the ability of gH to complement gH-deficient mutant in infectivity and cell fusion assays. Especially the former two insertions disrupting the hydrophobic stretch of 108 aa upstream of the TM domain were manifested by the loss of *syn* phenotype. Not only the region located upstream of the TM domain is important for membrane fusion. A similar function may be attributed to a short C-terminal internal domain of gH (SVP motif, aa 891–892). Deletion of the SVP motif or mutation of Val at 831 abrogated the *syn* phenotype (Wilson *et al.*, 1994). The fact that the SVP motif contributed to virus entry (Browne *et al.* 1996) but did not represent a *syn* locus confirmed its active role in membrane fusion.

Characterisation of the potentially functional domains in gH relied largely on the analysis of mutants conferring resistance to the neutralising MoAbs LP11 and 52S. The neutralisation by MoAb LP11 was as efficient as by MoAb AP7 reacting with the gD region I when tested in BHK cells. Insertion mutants at aa 201, 300, 313, 316/17 and 325/6 had an impaired reactivity with MoAb LP11. The majority of these insertions were located in the epitope III. This epitope is a part of a discontinuous, conformation-dependent region in the surface domain of gH, which is 243 aa long and can be stabilised by gL. The region in question is regarded as essential for gH function. It is composed of at least 3 epitopes (sites I, II and III) defined by *mar* mutations at aa 86 (site I), 168 (site II) and 329 (site III), respectively (Gompels and Minson, 1986). The mutations in epitopes I and II rendered the HFEM strain escape mutants resistant to neutralisation. A further conformation-dependent epitope (IVa, IVb) reacting with the neutralising MoAb 52S was localised in the region specified by *mar* mutations at aa 536–537 (Gompels *et al.*, 1991). A RDG motif in the gH polypeptide external domain (aa 176–178) which is important for the entry of picornaviruses and adenoviruses, and which is also present in gH, was not found related to the HSV entry (Galdiero *et al.*, 1997).

Polyclonal Abs as well as MoAbs were used to detect the antigenic epitopes in gL. The Ab-reactive regions in gL are located between aa 34 and 224, but they are mainly clustered within a stretch of 55 aa at positions168–224. A MoAb against this region could differentiate KOS strain from strains SC16 and HFEM (Novotný *et al.*, 1996). Anti-KOS gL MoAbs reacting with the C-terminal region of gL inhibited the membrane fusion by the *syn* gK mutant of KOS strain (KOS 804) but did not inhibit polykaryocyte formation by the *syn* gB mutant of HFEM. It may be concluded that the anti-gL MoAbs inhibited the membrane fusion caused by virus strains expressing this determinant, but did not inhibit the virus infectivity. The role of gL in the cell fusion differs from its role in the virus entry. The low inhibitory effect of the anti-gL MoAbs on the virion entry distinguished the mechanism of gL action from those of gD and gB.

Glycoprotein K (gK)

The hydrophobic precursor polypeptide of gK encoded by the UL53gene is 338 aa long and comprises four TM domains (Debroy *et al.*, 1985; Pertel and Spear, 1996). It contains only two Asn at positions 48 and 58 which become glycosylated by N-linked mannose (Ramaswamy and Holand, 1992; Hutchinson *et al.*, 1992). Anti-gK MoAbs detect gK in the nuclear membrane and membranes of ER. Since a gK-deficient mutant with the UL53 gene substituted by the *lac*Z gene did not yield infectious progeny in cell culture, gKwas found essential for HSV replication *in vitro* (MacLean *et al.*, 1991). Vero cells infected with an UL53 deletion-insertion mutant of F strain expressed a fusion protein containing the 112 aa residues from the N-terminus of pgK (Hutchinson *et al.*, 1995). In comparison to cells infected with the wild type virus, the cells infected with the mutant contained a higher number of naked particles and many aberrant virions in cytoplasm. The aberrant virions were less infectious and did not reach the extracellular space. Many capsids remained retained in the nucleus so that the perinuclear cisterns contained very few virions. These findings confirm the essential role of gK in the envelopment and release of virus particles. Because gK is present mainly in the cell nuclear membrane and membranes of ER but not in the virion envelope, it has been postulated that gK could be important for the capsid envelopment at ILNM. Furthermore, functional analysis of gK supported the hypothesis that virions move from the perinuclear cisterns along a cytoplasmic membrane network to trans-Golgi vesicles

and that they are not uncoated and reenveloped before reaching the cell surface. If so, gK could interfere with undesired fusion occurring between the virion envelope and the cytoplasmic membranes. In the absence of gK, an unregulated fusion occurs during the virion transport across cytoplasm. As a result, naked capsid-like particles accumulate in the cytoplasm and the virion release becomes hampered.

Jayachandra *et al.* (1997) used an UL53 deletion mutant of KOS strain and the abovementioned UL53 mutant of F strain to infect Vero and BHK cells. They found that the gK-deficient KOS mutant in contrast to the gK-deficient F mutant did not fuse 143 TK$^-$ cells. This difference could be explained by expression of the N-terminal portion of gK in the latter mutant. The former mutant yielded virus progeny which could not reach the cell surface. Many naked capsids accumulated in the nucleus and similar particles were seen in the cytoplasmic vesicles. Even when virions were formed by budding at ILNM in actively growing cells, they were not translocated to the extracellular space. Thus, though the envelopment of particles could be in part compensated in growing cells, the egress of virions still remained seriously impaired.

In the case of *syn* gK mutations, the halted virion transport caused disregulation of fusion events associated with the virion release. Read *et al.* (1980) observed larger plaques with *syn* mutants than with the wild type virus and giant cells having over 100 nuclei. The *syn*^{1,2} mutants mapping to 0.735 – 0.740 m.u. (Bond and Person, 1984; Poge-Geile *et al.*, 1984) were later characterised as *syn* gK mutants. Fusion induced by *syn* gK mutants was supressed in stably tranformed cells expressing gK to high levels. Infection of these cells with *syn* gK mutants but not with *syn* gB mutants interfered with the syncytium formation in a dose-dependent manner, i.e. depending on the amount of gK expressed before infection. This observation was explained not by a classical gene complementation but by a functional repair of fusion in the presence of the non-mutated gK expressed before infection (Hutchinson *et al.*, 1993). As already mentioned, mellitin (a component of honey bee venom which acts as an inhibitor of the Na/K pump) inhibited the giant cell formation caused by *syn* gK mutants but not *syn* gB mutants (Baghian and Kousoulas, 1993). Another inhibitor of the Na-K pump, ouabaine, inhibited the polykaryocyte formation mediated by both the *syn* mutants. Taking into account the signal sequence of pgK, crucial *syn* gK mutations were found at aa 40 (Ala replaced by Val or Thr) and 99 (Dolter *et al.*, 1994). The latter mutation created a new N-glycosylation site which might alter the conformation of the whole polypeptide. Additional mutations found at aa 33, 85 and 121 pointed at the importance of the N-terminal region of gK. At least two important *syn* mutations (aa 304 and 310) were found closer to the C-terminus. None of these mutations could be detetcted in gK of the syn gB strains HSZP and ANGpath (Kúdelová *et al.*, 1998).

Glycoprotein M (gM)

The product of the UL10 gene is a hydrophobic glycosylated polypeptide with 8 predicted TM domains (Bainess and Roizman, 1993). gM becomes a part of the virion envelope and is present in the cytoplasmic membrane of infected cells (MacLean *et al.*, 1993). gM-deficient mutants grew to 10 – 20 times lower titers in Vero or BHK cells forming slightly smaller plaques (MacLean *et al.*, 1991, 1993). Davis-Poynter *et al.* (1994) following the replication of a SC16/ANG recombinant that contained ANG gB but was deleted in the UL10 gene found that gM (similarly to the gE/gI complex) might be involved in the cell-to-cell spread. gM acts possibly at the virion penetration across intercellular junctions.

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opposite river bank. WN virus was previously isolated from *Aedes cantans* mosquitoes collected at Malacky, West Slovakia, in June 1972 *(3)*. The ecosystem at Lanžhot (floodplain forest and meadows) is remarkably similar to that at Malacky (including the mosquito and bird fauna), and the air distance between the two localities is only about 25 km. WN virus was also isolated from migratory birds of several species in South and East Slovakia *(4,5)*. Other European countries where the virus was detected involve Portugal, Spain, South France, Hungary, Romania, Moldavia, Ukraine, Belarus and South Russia *(6)*.

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