CHARACTERIZATION OF GLYCOPROTEIN C OF HSZP STRAIN OF HERPES SIMPLEX VIRUS 1

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Summary. – Sequences of UL44 genes of strains HSZP, KOS and 17 of herpes simplex virus 1 (HSV-1) were determined and the amino acid sequences of corresponding glycoproteins (gC) were deduced. In comparison with the 17 strain, the HSZP strain showed specific changes in 3 nucleotides and in 2 amino acids (aa 139 and 147, both from Arg to Trp) in the antigenic locus LII. The change at aa 147 was situated within the GAG-binding epitope. In a similar comparison, KOS strain had changes in 3 nucleotides and 3 amino acids (aa 3, 14, and 300). The UL44 genes of HSZP and KOS strains were expressed in insect Sf-21 cells by means of the baculovirus (Bac-to-Bac™) expression system. As shown by immunoblot analysis, both the recombinant baculoviruses (B1-HSZP and B6-KOS) expressed a glycosylated gC, the M_r of which (116 K) was lower than that of gC synthesized in Vero cells (129 K) infected with strains HSZP or KOS. In addition, smaller gC-specific proteins (of apparent M_r of 50–58 K and 98 K) corresponding to a non-glycosylated precursor polypeptide and/or incomplete forms of the partially glycosylated gC were found. When Balb/c mice were immunized with Sf-21 cells expressing gC, the recombinant gC-HSZP represented a more efficient immunogen possibly due to its stronger expression in these cells. The corresponding gC-HSZP antiserum reacted in enzyme-linked immunosorbent assay (ELISA) equally well with HSZP and KOS virion antigens and neutralized HSZP strain at a low titer. Both gC-HSZP and gC-KOS antisera detected the homologous as well as the heterologous gC antigens in Vero cells regardless whether infected with strains HSZP, KOS or 17, revealing the presence of gC from 6 to 16 hrs post infection (p.i.) in the cytoplasm, on the nuclear membrane and at the cell surface.

Key words: HSV-1; HSZP strain; KOS strain; 17 strain; UL44 gene; glycoprotein C; mutations; immunogenicity; baculovirus expression system

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Abbreviations: BEM = Basal Eagle's Medium; gC-HSZP antiserum, gC-KOS antiserum = antisera against B1-HSZP and B6-KOS, respectively; B1-HSZP, B6-KOS = recombinant baculoviruses; CPE = cytopathic effect; GAG = glycosaminoglycan; gC = glycoprotein C; gC-HSZP, gC-KOS and gC-17 = gC of strains HSZP, KOS and 17, respectively; ELISA = enzyme-linked immunosorbent assay; FCS = fetal calf serum; HS = heparan sulfate; HSV-1 = herpes simplex virus 1; IF = immunofluorescence; MAb = monoclonal antibody; NP-40 = Nonidet P-40; UL44-HSZP, UL44-KOS, UL44-17 = UL44 genes of strains HSZP, KOS and 17, respectively; MOI = multiplicity of infection; PBS = phosphate-buffered saline; p.i. = post infection; PMSF = phe-nylmethylsulfonyl fluoride; RNase = ribonuclease; SDS = sodium dodecyl sulfate; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of SDS; VN = virus neutralization

Introduction

HSV specifies at least 11 glycoproteins designated B, C, D, E, G, H, I, J, K, L, and M, functional characterization (either as infected cell membrane and/or virion envelope components or both) of which has made considerable progress during the last two decades (Spear, 1993; Rajčáni and Vojvodová, 1998). As documented by several investigators (Campadelli-Fiume *et al.*, 1990; Lycke *et al.*, 1991), the initial phase of virion attachment to the surface of susceptible cells is mediated by gC. Though gC is dispensable for adsorption of virus to cells in culture, its absence decreases the virus infectivity about 10 times (Herold *et al.*, 1991). Attachment of HSV-1 virions to glycosaminoglycan (GAG) receptors on the cell surface is

mediated by gC and gB (Herold *et al.*, 1995). Furthermore, the interaction of gB with GAG becomes apparent only in the absence of gC. The most frequent GAG with which gC interacts is heparan sulfate (HS) and heparin interferes with this interaction (WuDunn and Spear, 1989). It was clearly demonstrated that the sulfate moiety content of HS is essential for this interaction (Herold *et al.*, 1995).

The mature gC molecule in HSV-1-infected mammalian cells is formed by addition of oligosaccharide moieties to the polypeptide using cellular glycosyltransferases (Lundström *et al.*, 1987). The predicted polypeptide encoded by the 1533 bp long UL44 gene (Frink *et al.*, 1983; McGeoch *et al.*, 1988) has a M_r of 55 K, while the determined M_r varied from 59 K to 69 K (Cohen *et al.*, 1980; Frink *et al.*, 1983). The HSV-1 gC polypeptide consists of 511 amino acids of which 25 comprise the signal sequence, 453 form the surface ectodomain, 23 constitute the hydrophobic transmembrane domain, and 10 represent a very short C-terminal cytoplasmic domain (Homa *et al.*, 1986). Due to the presence of 9 N-linked glycosylation sites (Kikuchi *et al.*, 1987) and several O-linked glycosylation sites, the mature gC has a M_r of 130 K and interacts with the *Helix pomatia* lectin by its terminal N-acetylglucosamine moieties (Svennerholm *et al.*, 1984). The two antigenic loci (LI and LII) as described by Marlin *et al.* (1985) were found either resistant (LI) or sensitive (LII) to periodate-sialidase treatment (Holland *et al.*, 1984; Sjöblom *et al.*, 1987). They consist of at least 15 epitopes located in two distinct clusters (Wu *et al.*, 1990). LII has a site essential for GAG binding, where Arg at positions 143, 145 and 147 together with Thr^{250} and Gly^{247} play an important role (Trybala *et al.*, 1994; Olofsson *et al.*, 1999). After deleting aa 33–123 the gC-mediated adsorption of HSV-1 to cell surface was reduced (Tal-Singer *et al.*, 1995).

During our previous efforts in preparing an experimental (candidate) subunit HSV-1 vaccine (Rajčáni *et al.*, 1988; Rajčáni *et al.*, 1995), we adapted to chick and quail embryo cells the HSZP strain originally isolated from a labial lesion in ZP (rabbit lung) cells (Szántó, 1960). This strain showed several unique properties, such as (1) giant cell formation due to a syn^3gB mutation (Rajčáni *et al.*, 1996), (2) an impaired early host shutoff due to several mutations within the *vhs* polypeptide encoded by the UL41 gene (Vojvodová *et al.*, 1997), (3) a limited pathogenicity, possibly due to several mutations in the gB surface domain IV (aa 59–77) shared with gB-KOS (Košovský *et al.*, 2000), and (4) a lower affinity to the *Helix pomatia* lectin (Raučina *et al.*, 1984), a property possibly related to modified glycosylation of gC.

To identify the possible contribution of gC to the unique properties of HSZP strain, we determined and compared the nucleotide and amino acid sequences of gC of strains HSZP, KOS and 17 of HSV-1 and compared the expression and the immunogenicity of gC-HSZP with the most frequently studied gC-KOS.

Materials and Methods

HSV-1 strains. The HSZP strain was used in its 150th passage. This virus strain, originally isolated in rabbit lung cells (ZP) from a patient with labial herpes (Szántó, 1960), was later on passaged in chick embryo and Vero cells. The KOS strain originated from the WHO Collaborating Center for Virus Reference Research, Houston, TX, USA, while the 17 strain was provided by Dr. V. Preston, Institute of Virology, MRC, University of Glasgow, Glasgow, UK.

Cells. Vero cells were maintained in Basal Eagle's Medium (BEM) containing 5% calf serum, glutamine and gentamicine (40 μg/ml). Quail embryo cells were obtained by trypsinization of 10–12 day-old embryonated eggs. Sf-21 cells from *Spodoptera frugiperda* were grown in Sf-900II medium (Gibco-BRL) supplemented with 5% of fetal calf serum (FCS) and gentamicine (40 μg/ml).

Isolation of viral DNA. Vero cells were infected with strains HSZP, KOS or 17 at a multiplicity of infection (MOI) of 0.1 PFU/cell. When showing cytopathic effect (CPE) at 18–20 hrs p.i., viral DNA was isolated according to Kintner *et al.* (1994).

PCR. The DNA fragments containing the UL44 gene were amplified from the DNAs of strains HSZP, KOS or 17. About 5–10 ng of viral DNA was denatured (95°C/10 mins) and added to a reaction mixture (total volume of 50 μl containing 1 mmol/l dNTPs (Perkin Elmer Cetus), 50 pmoles of each primer (either gC7*Xho*I and gC8*Xho*I or gC11 and gC8*Xho*I, Table 1) and 2.5 U of Taq DNA polymerase (Gibco-BRL) or Pfu DNA polymerase (Stratagene) in a reaction buffer containing 2.5 mmol/l Mg^{2+}. The fragments designated UL44-HSZP, UL44-KOS*a* and UL44-17 (prepared using the downstream primer gC7*Xho*I), and UL44-KOS*b* (prepared using the downstream primer gC11) were amplified in 30 cycles (95°C for 45 secs, 56°C for 45 secs, and 72°C for 2 mins) and purified by means of the Wizard™ PCR Preps DNA Purification Kit (Promega).

Cloning of UL44 genes. All the 4 fragments (UL44-HSZP, UL44-KOS*a*, UL44-KOS*b* and UL44-17) were cloned in the pGEM-T Easy™ vector (Promega). The plasmid DNA was isolated as described by Sambrook *et al.* (1990). Positive clones were checked by agarose electrophoresis after cleavage with *Eco*RI, *Pvu*II and *Sal*I.

DNA sequencing. UL44-HSZP, UL44-KOS*a*, and UL44-17 genes were sequenced as PCR products using the TAQuence Cycle Sequencing Kit (USB) and well as plasmid clones using the T7 Sequencing Kit (Pharmacia) (Table 1). Each fragment (clone) was sequenced in both directions. The sequence of the gC-HSZP was evaluated using the DNASIS program and was submitted to the GeneBank Database (Acc. No. AJ 133757) and the sequence of the gC-KOS was compared with that of gC-KOS retrieved from the GeneBank Database under Acc. No. J02216. The both gC sequences were compared with that of gC-17.

Analysis of secondary structure. The secondary structure of corresponding gC polypeptides was predicted according to Chou and Fasman (1978) and Garnier *et al.* (1978) using the PROSIS program with the probability of β-turns at $P_t = 125 \times 10^{-6}$.

Recloning of UL44 genes into the expression vector pFASTBAC1. The UL44 genes, originally cloned in the pGEM-T

Table 1. Primers used for amplification and sequencing of UL44 genes

Primer	Location (nt)	Sequences	Orientation
gC7*Xho*I	96227–96251	5'-*CAGCTCGAG*TGGTCCGTGTGGAGGTCGTTTTCA-3'	D
gC11	96291–96308	5'-GCG AGG GGG AGG CGT CGG G-3'	D
gC3	96506–96527	5'-CCA AAC CCC AAC AAT GTC ACA-3'	D
gC13	96798–96818	5'-TGG GTC CGT CCC CCC CAA TC-3'	D
gC5	96952–96973	5'-CCC TCC GTT GTA TTC TGT CAC-3'	D
gC4	97049–97028	5'-CCA GGC CAA GTA ATA CAT TCC-3'	U
gC15	97341–97359	5'-CCT TCA CCT GCC AGA TGA C-3'	D
gC9	97443–97464	5'-ATT TGG GGT CCG CAT TGT GGT-3'	D
gC6	97615–97594	5'-TAC GAA ATG GGC AGG GTG GAC-3'	U
gC10	97757–97740	5'-TTC CAA TCC CCA CCC ACT-3'	U
gC8*Xho*I	97952–97928	5'-*CGGCTCGAG*GACCTGAGGGAAGAGAGGGTGGCG-3'	U

The linker/insertion restriction sites are in italics.
D = downstream; U = upstream.

plasmid, were cleaved with *Xho*I (gC-HSZP and gC-KOS*a*) or *Not*I (gC-KOS*b*), respectively, and then recloned into the baculovirus expression vector pFASTBAC1 (Life Technologies, Inc.). The orientation of the fragments was ascertained by *Eco*RI cleavage.

Construction of recombinant baculoviruses. The Bac-to-Bac baculovirus expression system (Life Technologies, Inc.) (Luckow *et al.*, 1993) was used as recommended by the manufacturer. Briefly, the donor plasmids carrying each gC gene were transfected into the competent *E. coli* DH10 Bac cells. The first positive clones were selected in the presence of kanamycine (50 μg/ml), gentamicine (7 μg/ml), tetracycline (10 μg/ml), X-gal (300 μg/ml) and IPTG (40 μg/ml). The second selection was done in the presence of gentamicine, kanamycine, X-gal and IPTG only. The recombinant bacmid DNA (a shuttle vector containing a mini-F replicon, a kanamycin resistance marker, and a mini attTn7 target site for the Tn7 transposon inserted into the lacZα region) was isolated using standard methods (Sambrook *et al.*, 1990) and stored in TE buffer containing ribonuclease (RNase) A (20 μg/ml) at -20°C.

About 2 x 10^6 Sf-21cells were allowed to attach to the flask surface during 1–2 hrs and then were supplemented with TC-100 medium (Gibco-BRL) containing gentamicine but no serum. The transfection mixture contained 1 ml of Cellfectin Reagent™ (Life Technologies, Inc.), 10 μl of recombinant bacmid DNA and $CaCl_2$ in final concentration of 0.125 mol/l. After very gentle washing of the cells, the transfection mixture was added for 20 mins at room temperature and then the cells were fed with the TC-100 medium containing gentamicine and FCS. After overnight incubation at 27°C, the cells were refed with the same medium and further incubated for 4 days. The titers of recombinant virus stocks (B1-HSZP and B6-KOS) were determined in a standard way (Summers and Smith, 1987); they ranged from 1 x 10^7 to 5 x 10^7 PFU/ml.

Immunoblot analysis. Monolayers of SF-21 cells were infected with the recombinant baculoviruses (B1-HSZP and B6-KOS) at MOI of 2.5, 5 and 10 PFU/cell. By 72 hrs p.i., the cultures were harvested while the cells were palleted by centrifugation (800 x g for 10 mins at 4°C). The cells were then were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in 300 μl of the lysis buffer I (50 mmol/l Tris-HCl pH 7.5, 100 mmol/l NaCl, 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mmol/l phenylmethylsulfonylfluoride (PMSF)) on ice for 10–30 mins. The lysate was centrifuged for 10 mins at 14 000 x g to obtain a soluble fraction, while the pellet represented an insoluble fraction. The latter was dissolved in the sample buffer (50 mmol/l Tris-HCl pH 6.8, 8% SDS, 10% glycerol, 5% mercaptoethanol, and 0.05% Bromphenol Blue). The soluble fraction was diluted with the same buffer containing 2% SDS only. Both the insoluble and soluble fractions were then heated at 100°C for 2 mins and stored in aliquots at -70°C.

Monolayers of Vero and quail embryo cells were infected with strains HSZP or KOS at MOI of 10 PFU/cell. The infected Vero and quail cells were harvested 16 hrs and 20 hrs p.i., respectively. The cells were lysed with the lysis buffer II (50 mmol/l Tris-HCl pH 7.5, 1% NP-40, 100 mmol/l NaCl, and 1 mmol/l PMSF) on ice for 30 mins. The lysate was centrifuged for 10 mins at 14 000 x g. The soluble and insoluble fractions were treated as described above and subjected to immunoblot analysis. After electrophoresis in 12.5% polyacrylamide gels in the presence of SDS (SDS-PAGE), the gels were blotted onto nitrocellulose membranes (0.45 μm; Schleicher and Schuell). The blots were incubated in PBS containing 5% skim milk for 2 hrs, washed 3 times in PBS containing 0.1% NP-40 and then incubated with the anti-gC monoclonal antibody (MAb) T60 (purified IgG, Bystrická *et al.*, 1991) diluted 1:1000 in PBS containing 5% skim milk and 0.1% NP-40 for 1 hr. After washing, the blots were stained with peroxidase-labeled goat anti-mouse IgG (DAKO) diluted 1:1500 in PBS containing 5% skim milk and 0.1% NP-40 for 1 hr. After the final wash, the binding of antibodies was visualized with Luminol (ECL-Western Light, Amersham) by autoradiography (RTG-Rapid film, FOMA).

Immunofluorescence (IF) test. To detect the expression of gC-HSZP or gC-KOS, Sf-21 cells grown on cover slips were infected with the recombinant baculovirus B1-HSZP or B6-KOS at MOI of 10 PFU/cell. By days 3, 6, and 7 p.i., the cells were washed with PBS, dried, fixed in cold acetone, reacted with MAb T60 (diluted 1:50), washed again and stained with goat anti-mouse IgG/FITC (DAKO) diluted 1:50. The results were viewed under a fluorescence microscope (E400, Nikon).

Immunization of mice with gC-expressing Sf-21 cells. Sf-21 cells infected with the recombinant baculoviruses B1-HSZP or B6-KOS at MOI of 10 PFU/cell were harvested at 72 hrs p.i., pelleted, washed, and resuspended in PBS. Balb/c mice (6–8 week-old, 10 in each group) were immunized intraperitoneally with 3 doses of SF-21 cells (2×10^6) expressing gC-HSZP or gC-KOS or mock-immunized with non-infected (control) Sf-21 cells at days 0, 21 and 42. Another group of mice was immunized with semipurified (see below) formalin-inactivated KOS or HSZP strains (2×10^5 PFU, 3 doses). Sera of animals of the same groups were pooled and used in the IF test, immunoblot analysis, enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) test.

Indirect IF tests with the mouse gC-HSZP and gC-KOS antisera were performed on Vero cells infected with strains HSZP, KOS and 17 at MOI of 0.1 and 2 PFU/cell and harvested at 8, 12, and 16 hrs p.i. and 6, 8, 10, and 12 hrs p.i., respectively. At the given intervals, the infected cover slip cultures were removed, washed and fixed as described above. The cells were then stained with either of the 3 mouse antisera (gC-HSZP, gC-KOS, and control antisera) diluted 1:50 as well as with the MAb T60 (positive control) diluted 1:100. After washing, the cells were stained with the goat anti-mouse conjugate IgG/FITC (Sigma) diluted 1:100, washed and viewed as described above.

ELISA was performed with semipurified KOS and HSZP virion envelope antigens prepared as described (Rajčáni *et al.*, 1988). The antigens at a concentration of 10 μg/ml were adsorbed onto the immunoplate wells overnight at 37°C; the wells were then saturated with 1% BSA at room temperature. The following MAb or antisera were used (1) MAb T60 diluted from 1:100 to 1:6 400, (2) the mouse gC-HSZP antiserum diluted from 1:100 to 1:3 200, (3) the mouse gC-KOS antiserum diluted from 1:100 to 1:3 200, (4) a negative control mouse serum diluted from 1:100 to 1:3 200. The antiserum titer was determined as the ratio of its absorbance to that of the negative control serum at corresponding dilution (a ratio over 2.1 was regarded as positive). Each antiserum dilution was applied to 3 wells coated with each antigen according to standard procedures (Cambell, 1987) using peroxidase-labeled goat anti-mouse IgG (Sepharma, Prague, diluted 1:1000) and OPB for visualization of positive reaction.

VN test in the presence and absence of complement was made with each antiserum according to standard procedures. About 30 PFU of strains HSZP or KOS were incubated with increasing antiserum dilutions (from 1:5 to 1:80 or 1:1280, respectively) for 60 mins and then inoculated to Vero cell monolayers on 24-well microplates (Nunc). The results were read after 4 days of incubation at 37°C in 3% CO_2.

Results

Sequencing of the UL44 gene

When comparing the sequences of UL44-HSZP and UL44-KOS DNA fragments with that of UL44-17 (a reference sequence), a total of 17 nucleotide (nt) differences were found. Of these, only 5 nucleotide changes were specific for the HSZP strain, 9 were common for strains HSZP and KOS, and 3 were specific for the KOS strain (Table 2). The nucleotide sequence of UL44-17 determined by us was found identical with the published by Frink *et al.* (1983) (GeneBank Acc. No. D10879).

In the primary structure of the polypeptide gC-HSZP we found 2 amino acid differences (aa 139, 147) specific for HSZP. In addition, 4 nucleotide differences common for HSZP and KOS caused 3 amino acid differences (aa 23, 306, and 383). Three amino acid differences were specific for gC-KOS (aa 3, 14, and 300). The two gC-HSZP-specific amino acid differences in question (Arg to Trp at positions 139 and 147) were located in the antigenic locus LII (Fig. 1). From the 3 gC-KOS-specific amino acid differences one was localized in the polypeptide itself (aa 300), while the rest was found in the signal sequence (aa 3 and 14). None of these amino acid alterations changed the N-linked glycosylation site. The regions sequenced by us encompass 84 nt upstream of the initiation codon, i.e. about half of the promoter region. In the non-coding region of UL44-HSZP there were 2 nucleotide differences, namely at nt 96268 (27 nucleotides upstream of the initiation codon) and at nt 96284 (43 nt upstream of the initiation codon).

Expression of gC in the baculovirus system

In the beginning, both the UL44-HSZP and UL44-KOS*a* 1726 bp fragments were cloned by help of the *Xho*I site present in the both flanking primers into the plasmid pFASTBAC1 and were used for construction of either the gC-HSZP or the gC-KOS carrying recombinant baculoviruses. These fragments covered 84 nucleotides upstream of the initiation codon and 106 nucleotides downstream of the stop codon. Because the expression of gC-KOS was extremely low even after repeated trials, a new UL44-KOS*b* PCR fragment (1662 bp) was prepared by help of the downstream primer gC11 (Table 1). The expression of this shorter fragment in Sf-21 cells was clearly superior to that of UL44-KOS*a*. Two

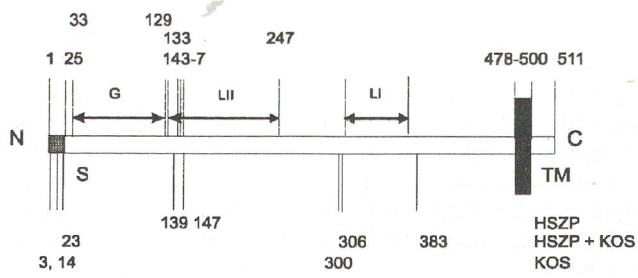

Fig. 1

Scheme of HSV-1 gC with amino acid differences specific for strains HSZP and KOS and common for strains HSZP and KOS in comparison with the 17 strain

LI, LII = antigenic loci; G = domain with cumulation of N-linked and O-linked glycosylation sites; S = signal sequence; TM = transmembrane domain.

Table 2. Differences in nucleotide and amino acid sequences of gC genes and polypeptides of strains HSZP, KOS and 17 of HSV-1

Nucleotide No.	Nucleotides in strains				Amino acid No.	Amino acids in strains			
	HSZP	KOS[a]	KOS[b]	17		HSZP	KOS[a]	KOS[b]	17
96268	**T**	C	C	C	–	–	–	–	–
96284	**G**	A	A	A	–	–	–	–	–
96319	G	**T**	G	G	3	Pro	**Leu**	Pro	Pro
96350	A	**G**	**G**	A	14	Ser	**Gly**	**Gly**	Ser
96377	**G**	**G**	**G**	T	23	**Ala**	**Ala**	**Ala**	Ser
96487	**A**	**A**	**A**	G	59	Glu	Glu	Glu	Glu
96725	**T**	C	C	C	139	**Trp**	Arg	Arg	Arg
96749	**T**	C	C	C	147	**Trp**	Arg	Arg	Arg
96904	**G**	**G**	**G**	A	198	Leu	Leu	Leu	Leu
97209	T	**A**	**A**	T	300	Val	**Asp**	**Asp**	Val
97227	**G**	**G**	**G**	A	306	**Arg**	**Arg**	**Arg**	His
97366	**C**	T	T	T	352	His	His	His	His
97456	**G**	**G**	**G**	C	382	Arg	Arg	Arg	Arg
97457	**C**	**C**	**C**	A	383	**His**	**His**	**His**	Ile
97458	**A**	**A**	**A**	T	383	**His**	**His**	**His**	Ile
97675	**G**	**G**	**G**	A	455	Glu	Glu	Glu	Glu
97850	**G**	**G**	**G**	A	–	–	–	–	–

[a]Sequence determined in this study.
[b]Sequence published by Frink *et al.* (1983); Acc. No. J02216.
The nucleotide and amino acids differing from those of the 17 strain are in bold. Notice: note the 2 nucleotide changes upstream to the UL44 initiation codon in the HSZP sequence.

producer baculoviruses (B1-HSZP and B6-KOS) were selected and used to infect Sf-21 cells.

Lysates of these cells examined by immunoblot analysis after staining with MAb T60 showed better results with B1-HSZP than with B6-KOS. The former revealed two distinct bands, one in the range of 116 K and another in the range of 59 K. Quite clearly, one band corresponded to the glycosylated form of gC, while another to the non-glycolysated (precursor) polypeptide. The mobility and apparent M_r of gC-HSZP and gC-KOS were similar, though the mobility of gC-HSZP seemed to be slightly higher. At MOI of 2.5 and 5 (Fig. 2) at least 2 precursor polypeptide bands could be recognized in the range of 50–58 K. Additional precursor polypeptides of about 68 K could be recognized in the lysates of Sf-21 cells infected at MOI of 10. Below the gC bands, partially glycosylated glycoprotein bands of 98–110 K were visible. Indirect IF test confirmed expression of both gC-HSZP and gC-KOS in a few foci of infected SF-21 cells at day 3 p.i. and a widespread production of the abovementioned antigens in the majority of infected cells (in the cytoplasm and on the cell surface) at days 6 (Fig. 3) and 7 p.i.

The both gC-HSZP and gC-KOS glycoproteins were compared when expressed Sf-21 (insect), quail embryo (avian) and Vero (mammalian) cells inoculated at MOI of 5 or 10. The results (Fig. 4) showed that M_r of either glycoprotein was slightly higher in Vero cells than in Sf-21 cells. The electrophoretic mobility of either gC synthesized in the avian cells (data not shown) was between that of gCs

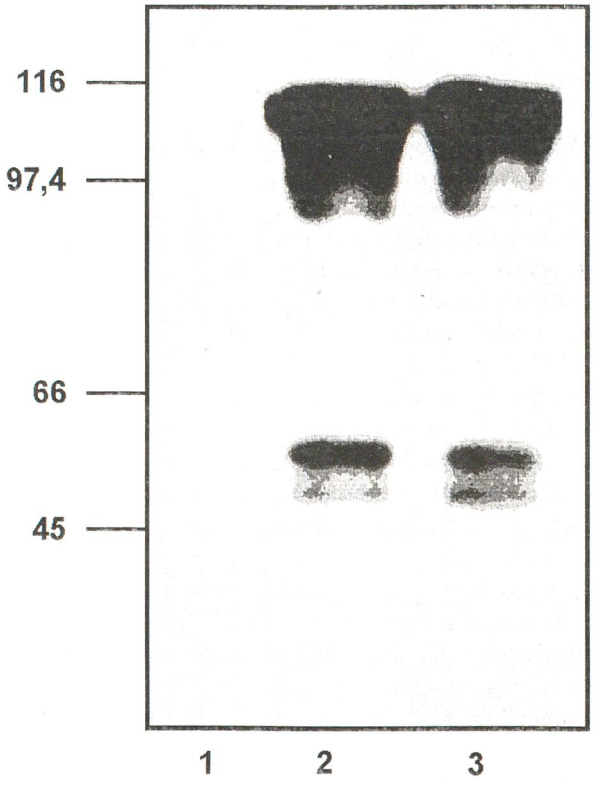

Fig. 2
Immunoblot analysis of expression of gC-HSZP and gC-KOS in Sf-21 cells
Control cells (lane 1), cells infected with B1-HSZP at MOI of 5 (lane 2), and cells infected with B6-KOS at MOI of 5 (lane 3).

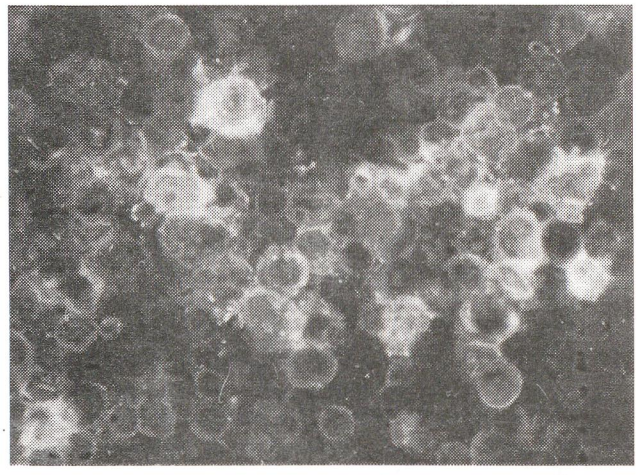

Fig. 3
Positive focal immunofluorescence of gC-HSZP in the cytoplasm of Sf-21 cells infected with B1-HSZP
Indirect IF test, 6 days p.i., MAb T60, magnification 200 x.

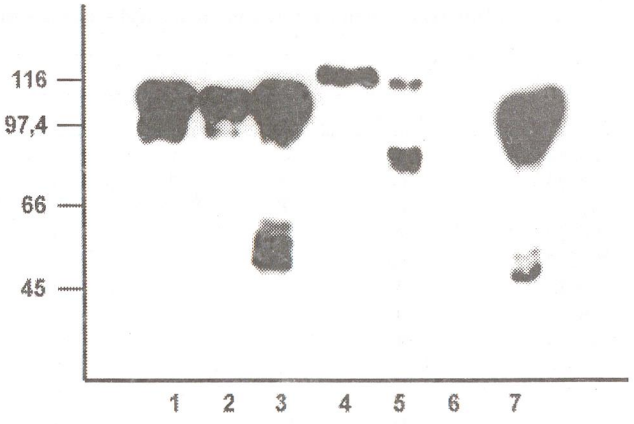

Fig. 4
Immunoblot analysis of expression of gC in Sf-21 and Vero cells
Sf-21 cells (lanes 1–3, 6 and 7). Vero cells (lanes 4 and 5). B1-HSZP at MOI of 10 (lane 1) and 5 (lane 2). B6-KOS at MOI of 10 (lane 3) and 5 (lane 7). HSZP (lane 4) and KOS (lane 5) at MOI of 5. Mock-infected Sf-21 cells (lane 6).

from Sf-21 cells and that of gCs from Vero cells, indicating that the M_r and the degree of glycosylation are dependend on the host cell system. Furthermore, the gC-KOS expressing Vero cells at given intervals still showed the presence of precursor polypeptides, which was not the case of Vero cells expressing gC-HSZP.

Properties of gC antisera and MAb T60 in ELISA and VN test

ELISA showed that the titer of MAb T60 against both semipurified virion antigens (HSZP and KOS) was 1 :1 600, the titer of the gC-HSZP antiserum against both antigens was 1:800, while the titer of the gC-KOS antiserum was only 1:200. Thus, the gC-HSZP antiserum had a higher titer with both (homologous as well as heterologous) antigens than the gC-KOS antiserum, which, in turn, reacted with both antigens at a lower titer (Table 3). The gC-HSZP and gC-KOS antisera were used in the VN test in the presence as well as in the absence of complement against relatively low amounts of each virus strain antigen. On the other hand positive control antisera prepared by immunization of mice with the inactivated purified HSZP and KOS strains neutralized both virus strains at titres of 1:40–1:80, respectively. At repeated testing, however, the gC-HSZP antiserum showed a low VN titer (1:10) against the HSZP but not against the KOS strain antigen.

Glycoprotein C in Vero cells

MAb T60 and the polyclonal gC-HSZP and gC-KOS antisera were used to localize gC in infected Vero cells by indirect IF test. Vero cells were infected at MOI of 1 (strains HSZP and KOS) and 0.1 (strains HSZP, KOS, and 17). MAb T60 and the gC-HSZP antiserum yielded similar results at dilutions of 1:100 and 1:50, respectively, regardless of the gC antigen used (Table 3). As expected, the intensity of IF staining was weaker with the gC-KOS antiserum. At higher MOI, traces of gC appeared in the cytoplasm of a few cells at 6 hrs p.i. At 8 hrs p.i., intensive granular IF could be seen in the cytoplasm, mainly in the vicinity of nuclei (data not shown). At 10–12 hrs p.i. the intensity of granular IF increased filling the whole cytoplasm, and showing occasional accumulation of larger antigen masses in the

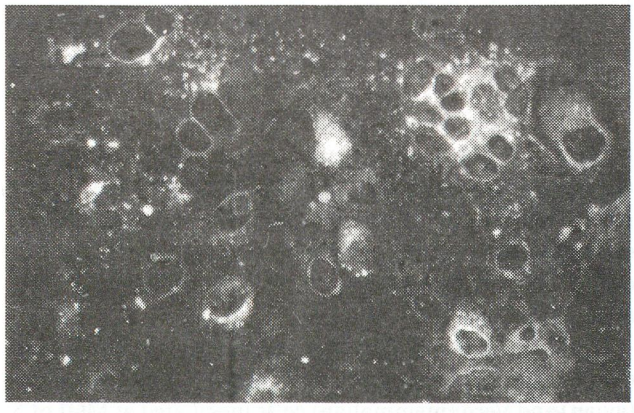

Fig. 5
Immunofluorescence of gC-HSZP in HSZP-infected Vero cells
Indirect IF test, 12 hrs p.i., the gC-HSZP antiserum, magnification 200 x. Predominantly cytoplasmic granules in the vicinity of nucleus and at the nuclear membrane. Typical giant cells.

Table 3. Reactivities of gC antisera and MAb T60 in ELISA and indirect IF test

MAb/antiserum	ELISA titers			IF test		
	KOS	HSZP	KOS 8 hrs	KOS 12 hrs	HSZP 8 hrs	HSZP 12 hrs
MAb	1:1600	1:1600	+	+++	+	+++
gC-HSZP antiserum	1:800	1:800	+	++	+	++
gC-KOS antiserum	1:200	1:200	+	++	+	++

Total virion envelope proteins were used as antigens. Vero cells were infected at MOI of 1. MAb T60 (diluted 1:100) and the antisera (diluted 1:50) were used as primary antibodies.
(+) = single (scattered) positive cells, (++) = many positive cells, (+++) = confluent positivity of the monolayer.

paranuclear area (Fig. 5). In many HSZP-infected or KOS-infected cells, nuclear membranes showed intensive gC antigen staining at 10–12 hrs p.i. Whereas the accumulation of gC at MOI of 1 reached peak at 10 hrs p.i., at MOI of 0.1 it culminated at 12 hrs p.i. In some cells, infected with either strain, fluorescent granules were occasionally found also over the nucleoplasm, mainly forming small foci located closer to the inner lamella of nuclear membrane. At 16 hrs p.i. at MOI of 0.1, faint fluorescence of gC was found at the cell surface membrane, but this was much less prominent than the ring-shaped staining of the nuclear membrane.

Discussion

Recombinant gC-HSZP and gC-KOS were prepared in the baculovirus expression system based on site-specific transposon-mediated insertion (Luckow *et al.*, 1993). Expression of respective recombinant baculovirus in Sf-21 cells was successful in the case of the UL44-HSZP fragment, which, unlike the UL44-KOS*a* fragment, had 2 nucleotide changes located upstream of the translation initiation codon by 27 bp (A to G, nt 96284) and 43 bp (C to T, nt 96268), respectively. The UL44-KOS*a* fragment sequence, which ended 84 bp upstream of the translation initiation codon at nt 96227 and was identical with the 17 strain sequence, could not be efficiently expressed unless it was shortened by a 64 bp region (in preparing the UL44-KOS*b* fragment). The 63 bp portion of the gC promoter (between nt –34 to +29 as related to the initiation site of transcription) containing the TATA homologue and a putative operative element sufficient to induce CAT activity (Shapira *et al.*, 1987) lies just upstream of the sequence amplified within the range of the UL44-KOS*a* fragment. According to the nucleotide numbering published by McGeoch *et al.* (1988) and used in our paper, the distance between the TATA homologue and the translation initiation codon in the UL44 promoter region takes 170 bp but not 158 bp as claimed by Shapira *et al.* (1987). This fact, however, had no influence on our observation that the remaining promoter region of 84 nt seemed to interfere with the transcription under the control of the Ppolh promoter in the pFASTBAC1 plasmid. This effect could be eliminated by use of the UL44-KOS*b* fragment prepared by removing nt 21–84 upstream of the translation initiation codon. Why the promoter region in question became functionally modified as result of gC-HSZP alternations within the transcribed DNA region upstream of the translation initiation codon should be further investigated.

The gC-KOS expressed in Sf-9 cells by Ghiasi *et al.* (1992c) was partially resistant to Endo-H and susceptible to tunicamycine inhibition, indicating that the N-linked glycosylation of the consensus Asn residues (Wenske and Courtney, 1983) must have taken place in Sf-21 cells. In these cells we also observed glycosylated, partially glycosylated as well as non-glycosylated or partially digested precursor forms of gC. Nevertheless, the M_r of mature gC-HSZP as well as gC-KOS was higher in Vero cells than in the Sf-21 cells. This difference could be caused by an incomplete N-linked glycosylation (no addition of acetylgalactosamine sugar residues to mannose) as well as by an unfinished O-linked glycosylation. In both cases a partial glycosylation could occur, e.g., due to the lack of N-galactosyltransferase as described for mouse neuroblastoma cells (Lundström *et al.*, 1987; Olofsson *et al.*, 1990; Ghosh-Choudhury *et al.*, 1994). Furthermore, gC-HSZP produced in mammalian cells showed a relatively low affinity to *Helix pomatia* lectin as described for strains KOS (Raučina *et al.*, 1984) and F of of HSV-1(Svennerholm *et al.*, 1984). Whether the latter difference might be associated with the lower content of N-acetylgalactosamine residues as related to sialic acid or N-acetylglucosamine within the O-linked sugar side chains of gC-HSZP (Lundström *et al.*, 1987) needs further elucidation.

In determining the amino acid differences in gC described here as KOS-specific and as KOS and HSZP-common (in comparison to the 17 strain), we confirmed the presence of two KOS-specific differences at aa 14 (Gly) and aa 300 (Asp). At aa 3 of gC-KOS we found Leu (codon CCT) but not Pro (codon CCG) described by others. However, G (not

T) at nt 96319 was found in both the HSZP and 17 strains. The rest of the UL44-KOS gene was in agreement with the corresponding database data.

The gC domain binding to cell surface GAG is situated within the immunodominant antigenic domain LII (Marlin *et al.*, 1985) which spreads from aa 129 to aa 247 (Wu *et al.*, 1990; Rux *et al.*, 1996). The predicted amino acid sequence of gC-HSZP showed 2 differences (aa 139 and 147) as compared to gC-17, changing Arg to Trp. Clearly, the predicted glycosylation site 148-Asn-X-Ser/Thr-150 (Elbein, 1987) was not affected by the Trp^{147} alteration indicating that the expected number of 9 consensus N-linked glycosylation sites in gC-HSZP was not altered. None of the four C3b binding sites localized within aa 124–366 (Hung *et al.*, 1992) was affected by the HSZP-specific changes. The HSZP-specific amino acid changes in question are located within the aa 137–151 motif xxRxxxRCRFR xxxR, from which at least one (Arg^{147}) represents a critical Arg responsible for the interaction with the cell surface HS (Trybala *et al.*, 1994). Their role in modification of HSZP attachment to cell surface should be seriously considered.

The average titer of the HSZP strain stock propagated in Vero cells for over 15 passages was 5-fold lower than that of KOS strain grown under the same conditions. Absence of gC in various gC-deficient HSV-1 mutants reduced the virus titer about 10-fold (Herold *et al.*, 1991). As recently reported, replacement of Thr^{150} by Ile, which destroyed the N-linked glycosylation site Asn^{148}, also reduced the infectivity titer of the corresponding mutant 5-fold, namely from $10^{7.5}$ to $10^{6.8}$ PFU/ml (Olofsson *et al.*, 1999). We can assume that the Arg^{147} alternation in gC-HSZP reduced the titer of HSZP versus KOS about 5-fold. In our previous work, in preparing the infected cell-derived glycoprotein (candidate vaccine) extracts (Rajčáni *et al.*, 1995), we observed that the PFU titer of the same HSZP strain stock as assayed in quail embryo cells versus Vero cells was at least 5 times higher. The change at aa 139 in the motif aa 137–151 (Trybala *et al.*, 1994) of gC-HSZP could be also connected with a stronger attachment of this strain to chick and/or quail embryo cells.

According to the prediction method of Garnier, the changes at aa 300 and 306 in gC-KOS abolished a short a-helix between aa 298–305, but this effect was not seen in gC-HSZP which had a change at aa 306 (Hist to Arg) but not aa 300 (Val to Asp), accepting 4 amino acids as the lowest possible limit for a-helix formation.

The secondary structure constructed according to the Chou-Fasman prediction revealed two regions of alteration. The short a-helix between aa 301–305 in gC-17 has moved into the region of aa 287–301 in gC-KOS, while in gC-HSZP it disappeared completely. This prediction showed secondary structure changes in gC-HSZP also in association with the alternations at aa 139 and 147. Namely, the short β-sheet became slightly extended ending aa at 147 and covering both changes. A similar slight extension of a β-sheet structure could be observed in association with aa 383. However, the changes at aa 306 and 383, common for gC-HSZP and gC-KOS were situated outside of the major antigenic domains.

Summing up, in comparison to gC-17, gC-HSZP contains 2 specific amino acid changes (Trp at positions 139 and 147), while gC-HSZP and gC-KOS contain 3 common amino acid changes from which one (aa 306) seems to influence the secondary structure. The possible effect of the changes to Arg at positions 139 and 147 on the secondary structure could be assessed according to the prediction of Chou and Fasman only.

The gC-HSZP antiserum as well as MAb T60, reported to react with an epitope on the antigenic locus LII by Bystrická *et al.* (1997), showed similar reactivity in ELISA, immunoblot analysis, indirect IF and VN tests. The ELISA titers of the antiserum using whole virus envelope proteins of strains HSZP and KOS as antigens were 1:800 and 1:1600, respectively. The titers of these antisera were the same regardless whether the KOS or HSZP antigen was used. The titer of the gC-KOS antiserum was lower than that of the gC-HSZP antiserum.

As expected (Bystrická *et al.*, 1997), MAb T60 did not neutralize the HSZP or KOS strain antigen in the VN test, when 30 PFU of either antigen was preincubated for 30 mins in the presence or absence of complement. Under these conditions, the mouse antisera against whole virions of strains HSZP and KOS titers of 1:40 and 1:80, respectively, but the gC-HSZP antiserum showed only an insignificant titer of 1:10 against the HSZP antigen only. This finding is consistent with earlier results of Eing *et al.* (1898) indicating that the non-essential glycoproteins such as gC and gE are not the main targets of VN antibodies as detected in a classical assay.

When used to detect gC-HSZP, gC-KOS and gC-17 antigens in infected Vero cells by the indirect IF test, each gC was found in the beginning of infection. Incorporation of gC into the nuclear and cytoplasmic membranes was observed. Possibly due to giant cell formation, the fluorescence of gC was more prominent in the nuclear than in the cellular membranes of HSZP-infected Vero cells. A similar pattern of fluorescence was observed in different mammalian cells expressing gC after transfection with a cloned subfragment carrying the UL44 gene derived from the *Hind*III fragment L (Ghosh-Choudhury *et al.*, 1990).

The results reported here confirm that there are some differences in the synthesis and processing of gC in mammalian, avian and insect cells infected with the same HSV-1 strain as well as in the same cells infected with various HSV-1 strains. Despite of expected minor differences in the glycosylation patterns gCs of strains HSZP and KOS expressed in the baculovirus system the gC-HSZP and gC-

KOS polyclonal antisera reacted similarly, indicating an overlapping antigenic reactivity. The recombinant gC-HSZP represented a stronger immunogen than the recombinant gC-KOS, possibly due to a stronger expression. The gC-HSZP and gC-KOS antisera cross-reacted with all the three gC antigens (HSZP, KOS and 17).

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