EXPRESSION OF HERPES SIMPLEX VIRUS 1 GLYCOPROTEIN D IN PROKARYOTIC AND EUKARYOTIC CELLS

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Summary. – Recombinant plasmids encoding either the full-length glycoprotein D (FLgD) or truncated gDs were constructed. The recombinant plasmids were expressed in *Escherichia coli* and BHK-21 cells. The strongest expression was obtained with the recombinant plasmid encoding a truncated gD which corresponded to the gD ectodomain. The cells transformed with this plasmid showed good exponential growth ensuring satisfactory yields of the expressed polypeptide in the form of the fusion protein. The fusion protein was biotinylated and efficiently purified. The shortest truncated gD, which contained the main continuous antigenic locus VII binding neutralization antibody and additional continuous antibody binding epitopes, still reacted with specific antibody as proven by immunoblot analysis. In addition, a shuttle vector for expression of FLgD in mammalian cells was constructed. This vector-transfected BHK-21 cells expressed gD for 40 days during 9 consecutive passages. The expression of gD began on day 2 and culminated at day 9 post transfection (p.t.).

Key words: BHK-21 cells; Escherichia coli; expression; fusion protein; glycoprotein D; Herpes simplex virus 1; recombinant plasmid; shuttle vector

Introduction

gD is one of major components of Human herpesvirus 1 (HSV-1) and Human herpesvirus 2 (HSV-2) virion envelopes. It is an efficient tool of virulence and as such, it represents a prominent target for virus neutralizing antibody (Noble *et al*., 1983; Fuller and Spear, 1987; Highlander *et al*., 1987). The gD ORF (US6) spans from nt 138,415 to nt 139,601 of the HSV-1 strain 17 DNA (McGeoch *et al*., 1988; McGeoch *et al*., 1991). The 1185 bp long US6 ORF specifies 394 amino acids (aa) (Watson *et al*., 1982; McGeoch *et al*., 1991). FLgD contains 25 aa long signal sequence which is cleaved off during processing (Matthews *et al*., 1983). The estimated M_r of the non-glycosylated gD ranges from 49 to 52 K (Inglis and Newton, 1982), while the final glycosylated molecule has a M_r of about 59 K (Eisenberg *et al*., 1979). The ectodomain of gD has three Asn-X-Ser/Thr N-glycosylation sites (Sodora *et al*., 1991) and 6 cysteins which form 3 disulphidic bonds important for formation of discontinuous antigenic epitopes (Long *et al*., 1992).

Two continuous epitopes within the ectodomain have been defined by Eisenberg *et al*. (1985) and termed the locus VII (aa 36–44, i.e. aa 11–19 after removing the signal sequence) and locus II (aa 294–304, i.e. aa 268–287); an additional continuous epitope (locus XI, aa 309–326, i.e. aa 284–301) has been found still within the ectodomain close to the transmembrane (TM) sequence (Isola *et al*., 1989). The last continuous epitope recognized under denaturing conditions

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Abbreviations: aa = amino acid; ABC = avidin biotin complex; DMEM = Dulbecco's Modified Eagle's Medium; FLgD = full length gD; gB = glycoprotein B; gD = glycoprotein D; HCMV = Human cytomegalovirus; HSV-1 = Herpes simplex virus1; HSV-2 = Herpes simplex virus 2; HVEM = herpes virus entry mediator; IPTG = isopropyl ß-D-thiogalactopyranoside; ORF = open reading frame; MAb(s) = monoclonal antibody(ies); NK = natural killer; NP-40 = Nonidet P-40; PBS = phosphate-buffered saline; p.i. = post infection; PRP-1 = poliovirus receptor-related protein 1; PRP-2 = poliovirus receptor-related protein 2; p.t. = post transfection; Px = peroxidase; TM = transmembrane

(locus V, aa 365–381, i.e. 340–356) has been found within the cytoplasmic endodomain (Cohen *et al.*, 1988; Fig. 1). A long polypeptide stretch (from aa 149 to aa 258, i.e. from aa 124 to aa 233) between antigenic loci VII and II contains several overlapping discontinuous antigenic domains (Ia, Ib, III, IV and VI) which have been defined by means of various monoclonal antibodies (MAbs) using a "native" gel system (Cohen *et al.*, 1986). These domains, possibly associated with virus adsorption and neutralization, are critical for correct folding and processing of gD (Wilcox *et al.*, 1988; Muggeridge *et al.*, 1990).

Taken together, the most important virus neutralization sites are the gD loci VII (aa 36–44, i.e. aa 11–19) and Ib (aa 149–165, i.e. aa 124–140). According to Nicola and coworkers (1996) they comprise the functional regions I and II, both important for membrane fusion and virus penetration. The functional site III (aa 221/222–246/254, i.e. aa 250/251–271/279) was claimed to react with a herpes virus entry mediator (HVEM) such as HveA (tumor necrosis factor receptor) on the surface of susceptible cells (Montgomery *et al.*, 1996). In addition, gD binds to immunoglobulin superfamily related nectins (HveC, renamed as poliovirus receptor related protein 1 (PRP-1)), and possibly also to poliovirus receptor-related protein 2 (PRP-2); (former HveB) (Krummenacher *et al.*, 1998). The protein receptors interact also with the locus VII and an adjacent peptide sequence of aa 27–43 (i.e. aa 52–68), which is still a part of the functional site I (Whitbeck *et al.*, 1997). The importance of functional sites I and III has been confirmed by deletion experiments in which the truncated gD lacking the carboxy-terminus from aa 234 or aa 240 did not react with any HVEM receptor (reviewed by Rajčáni and Vojvodová, 1998; Whitbeck *et al.*, 1999).

Immunization with gD protects mice against lethal virus challenge (Long *et al.*, 1984). This effect can be also achieved with a truncated form of gD lacking 93 carboxy-terminal aa (Lasky *et al.*, 1984). In addition to the neutralizing antibody-binding epitopes, the protective effect of gD is related to at least 4 immunodominant regions (aa 49–82, i.e.73–107; aa 146–179, i.e. 171–204; aa 228–257, i.e. 253–282 and aa 287–317, i.e. 312–342), recognized by the receptors of T cells (BenMohamed *et al.*, 2003). It is generally accepted that both T/CD8 and T/CD4 cells contribute to the clearance of infectious HSV-2 producing cells in various tissues such as genital mucosa (Milligan *et al.*, 1998) and the sensory ganglia and cornea (Ghiasi *et al.*, 1999, 2000). The mediator function of helper T/CD4 cells is especially important during primary processing of HSV-1 antigens (Jennings *et al.*, 1991). Furthermore, gD activates the non-specific NK cells (Inoue *et al.*, 1990).

Experimental recombinant vaccines containing gD alone or in combination with glycoprotein B (gB) elicited significant protective and/or immunotherapeutic effects (reviewed by Stanberry, 2000; Vandepaliere, 2000). To prepare HSV-1 gD as vaccination antigen, different expression systems including bacteria, insect, mammalian and yeast cells have been used (Watson *et al.,* 1982; Steinberg *et al.*, 1986; Ghiasi *et al.*, 1991; van Kooij *et al.*, 2002; Cohen *et al.*, 1988).

Previous attempts to express gD-1 in bacterial cells have shown that HSV-1 FLgD, which included the signal sequence and the TM region was toxic for host cells (Steinberg *et al.*, 1986). Watson *et al.* (1982) shortened gD-1 by removing the first 52 aa from amino-terminus containing signal sequence. Various truncated polypeptides were formed due to premature termination or reinitiation events in *E. coli*. However, HSV-2 gD was not toxic when expressed in the same cells. This could be due to differences in TM and cytoplasmic anchor region of HSV-1 gD and HSV-2 gD (Steiberg *et al.*, 1986).

In this study we describe expression of HSV-1 gD of different length as biotinylated fusion proteins in *E. coli* and BHK-21 cells.

Materials and Methods

Virus and cells. HSV-1 strain HSZP (Rajčáni *et al.*, 1996, 1999) originated from our laboratory collection of viruses. HSV-1 strain 17 was obtained from the MRC Virology Unit, Institute of Virology, Glasgow, Scotland. Both viruses were propagated in Vero cells. Vero as well as the BHK-21 cells were propagated in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) of fetal calf serum (FCS), 25 U/ml penicillin, and 5 µg/ml streptomycin.

HSV-1 DNA for PCR was obtained from Vero cells infected with the strain HSZP or 17 as follows. Cell cultures were harvested and centrifuged at a low speed. The pelets were treated with Nonidet P-40 (NP-40) and a hypotonic Tris-HCl buffer (Rajčáni *et al.*, 1996). The clarified medium as well as the infected cell lysate were centrifuged on a 5–55% sucrose density gradient. The crude virus preparation was treated with proteinase K, extracted with phenol/chlorophorm and ethanol-precipitated (Košovský *et al.*, 2001).

PCR, agarose gel electrophoresis, purification and restriction analysis. gD fragments were amplified by PCR as follows. A 1x PCR reaction buffer (100 µl, Finnzymes) contained about 100 ng of HSV-1 DNA (strain HSZP or 17), 1.5 mmol/l Mg^{2+} (Finnzymes), 0.2 mmol/l dNTPs (Gibco), 100 pmoles of each gene-specific primer (Table 1), and 2 U of the high performance DynaZyme Ext DNA polymerase (Finnzymes). The PCR consisted of 35 cycles (denaturation at 95°C/1 min, annealing at 52°C/1 min, and elongation at 72°C/1 min prolonged by 3 secs at each cycle) and the PCR products were separated by electrophoresis in 0.8% agarose gel and purified by QIAquick PCR purification kit (Qiagen). Restriction analysis of the PCR products was performed by digestion with *Stu*I, *Pvu*II, *Eco*NI, *Ava*I, *Bst*XI and *Sty*I.

Construction of recombinant gD plasmids. The forward (gDF) and reverse (gDR) primers were designed to amplify the FLgD

Table 1. Primers for amplification of the gD gene

Name	Position (nt)	Oligonucleotide sequence
gDF	138,415–138,430	**5´-AAC**A*AGCTT*GTAT GGGGGGGGCTGC-3´*
gDR	139,601–139,581	**5´-CGC**CGGTAC*CTTTATT***CTA***GTAAAACAAGGGCTGGTC-3´*
gDF27	138,495–138,512	**5´-GGG** AAG CT**TATGCCTTGGTGGATGCC-3´*
gDR340	139,433–139,419	**5´-GGA** GGT AC**C***CTA**GTTGTTCGGGGTGGC-3´*

The clamp sequence is in bold; the restriction site for polylinker insertion is underlined; the gene-specific sequence is in italics (the translation initiation and stop codons, respectively, are in bold italics); the sites of the PinPoint Xa-1 plasmid cut by cloning are marked by asterisks.

Table 2. Recombinant plasmids, amplified gD DNA fragments, inserts, and encoded proteins

No.	Recombinant plasmid	Amplified fragment	Insert	Encoded protein	
					Length without fusion part (aa)
1	pPPXagD/HSZP	gD1211/HSZP	gD1199/HSZP	FLgD/HSZP	394
2	pPPXagD/17	gD1211/17	gD1199/17	FLgD/17	394
3	pPPXagD701/HSZP	gD1211/HSZP	gD701/HSZP	gD234/HSZP	234
4	pPPXagDdelTM/HSZP	gD955/HSZP	gD947/HSZP	gD313/HSZP[a]	313
5	pcDNA3.1-gD[b]	gD1211/HSZP	gD1199/HSZP	gD[b]	394[b]

For the abbreviations see their list at the front page.

[a]Surface ectodomain.

[b]Expressed as a non-fusion glycoprotein in BHK-21 cells.

USG gene sequence (1185 bp). The primers had a clamp sequence at 5´-end and the appropriate restriction site. The gDF primer contained a *Hind*III restriction site and the HSV-1 specific sequence started just 2 bp upstream of the gD translation initiation codon (Table 1). The inserted AGCTT sequence allowed an in frame translation of the fusion protein which was extended with two amino acids (Ala and Cys) inserted upstream of the Met codon. The gDR was designed to contain a *Kpn*I site. Both primers in question were used in amplification of gD1211/HSZP and gD1211/17. The amplified gD1211/HSZP or gD1211/17 fragments contained besides the complete gD sequence also 26 additional nucleotides, 6 of which belonged to the 5'-end clamp, 12 to the restriction sites and 6 to the polyA motif. The following DNA fragments were employed in construction of recombinant plasmids (Table 2): (i) the gD1211/HSZP fragment encoding the HSZP strain FLgD (FLgD/HSZP), (ii) the gD1211/17 fragment encoding the strain 17 FLgD (FLgD/17), (iii) the gD701/HSZP (prepared by *Stu*I digestion of gD1211/HSZP) encoding the truncated HSZP strain gD234 (gD234/HSZP), and (iv) the gD955/HSZP fragment (amplified by primers gD27F and gD340R) encoding the HSZP strain gD313 surface ectodomain (gD313/HSZP) (Fig. 1).

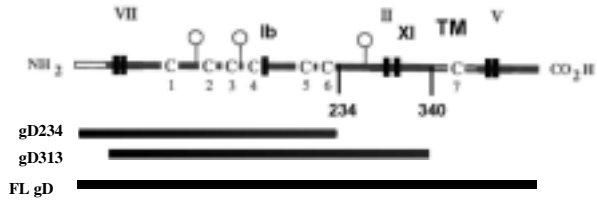

Fig. 1

gD polypeptides expressed through the PPXa-1 plasmid

FLgD consisted of 394 aa, while the truncated gD polypeptides gD234 and gD313 consisted of 234 aa and 313 aa, respectively. The position of 4 continuous epitopes as defined by Eisenberg *et al.* (1985) and Cohen *Æô ÂÍë* (1988) is shown (locus VII = aa 36–44, locus II = aa 297–312, locus XI = aa 309–326, and locus V = aa 365–381). gD234 contained the loci VII and Ib, only. gD313 (the entire ectodomain) lacked the cytoplasmic endodomain, the TM domain and the signal sequence. The position of cysteins is numbered. TM, i.e. the hydrophobic TM (anchor) region contained the C7 site, which formed no bisulfidic bridge. The signal peptide at the N-terminus is marked as an empty box. The N-glycosylation sites are labeled by empty circles.

The fragments under (i), (ii) and (iv) were digested with *Hind*III and *Kpn*I and inserted into the polylinker site of the PinPoint Xa-1 (pPPXa-1, 3331 bp) expression vector (Promega) (Košovský *et al.*, 2001). The following constructs were prepared: (i) pPPXagD/HSZP, (ii) pPPXagD/17, and (iv) pPPXagDdelTM/HSZP. (iii) The plasmid pPPXagD701/HSZP was made by insertion of the gD701/HSZP (obtained by digestion of gD1211/HSZP fragment with *Hin-d*ÌÌÌ and *Stu*I) into the pPPXa-1 plasmid. The construction of recombinant plasmids was verified by restriction analysis. The plasmid constructs as well as the original pPPXa-1 plasmid were used to transform competent *E. coli* JM109 cells. (v) The pcDNA3.1 (5.4 kbp) plasmid (Invitrogen) was double digested with *Hind*III and *Kpn*I. The gD1211/HSZP fragment (1189 bp) double digested in similar manner was inserted into the abovementioned plasmid to obtain the pcDNA3.1-gD construct. The recombinant plasmid was used in transfection of BHK-21 cells.

Expression of fusion proteins in E. coli and growth curve measurement. FLgD/HSZP, FLgD/17, gD234/HSZP (a truncated gD), and gD313/HSZP (the surface ectodomain of gD) were expressed in *E. coli* cells as biotinylated fusion proteins. The transformed

cells were grown in LB medium containing 2% glucose, 2 μmol/l biotin and 100 μg/ml ampicillin overnight at 37°C. Their growth was monitored at 3 hr intervals for 24 hrs by measuring A_{550} and by standard dilution method and by counting of viable colonies. To prepare larger stocks of fusion proteins, 1000 ml volumes of LB medium were inoculated with selected clones and incubated until the A_{550} reached 1.0–1.2. The expression was induced at A_{550} of 1.0–1.2 with 100 μmol/l IPTG and after 4 hr incubation at 37°C the cells were harvested by centrifugation at 2,600 x g for 12 mins at 4°C and stored at -70°C until use.

Purification of fusion proteins. The biotinylated fusion proteins were isolated using the monomeric avidin-conjugated resin (SoftLink™ Soft Release Avidin Resin, Promega). *E. coli* cells were suspended in 50 mmol/l Tris-HCl pH 7.5 containing 50 mmol/l NaCl, 5% glycerol, and 1 mmol/l phenylmethylsulfonylfluoride (PMSF), and were lysed in the lysis buffer (1 mg/ml lysozyme), 0.1% Triton X-100, and pancreatic DNase I (5 U/ml) (Košovský *et al*., 2001). Then the crude lysate was centrifuged at 10,000 x g for 10 mins and the supernatant was gently stirred with the SoftLink SoftRelease Avidin Resin. The bound proteins were eluted under non-denaturing conditions in the presence of 5 mmol/l biotin and concentrated with Sephadex G-200. Finally, the proteins were dialyzed against PBS pH 7.2 containing 10% glycerol. After determining the protein concentration the product was stored at -20°C.

Identification of the expressed fusion protein was done by SDS PAGE and Coomassie Blue staining as well as by Western blot analysis. In the latter procedure, the electrophoresed samples were transferred onto a Hybond-P membrane and stained either for biotinylated proteins or for gD antigen with an anti-gD MAb, which was kindly provided by Prof. B. Clements, Institute of Virology, Glasgow, UK. The blot strip was soaked in 5% non-fat milk in PBS pH 7.2 for 60 mins and was treated with an ABC mixture (an avidin-biotin complex) (Vectastain ABC kit, Vector Laboratories, USA) according to the manufacturer's instructions. After threefold washing in PBS containing 0.01% Tween 20 (T-PBS) the strips were stained with 0.1% DAB (diaminobenzidine tetrachloride) in the presence of 0.02% H_20_2. In immunostaining the blot strips, previously soaked in 5% non-fat milk, were treated with a mouse anti-gD MAb (diluted 1:2,000 in PBS containing 5% non-fat milk) for 60 mins, washed 3 times in T-PBS and treated with a peroxidase-conjugated swine anti-mouse immunoglobulin (Sw-AMo Ig/Px, Sevapharma, Czech Republic) diluted 1:1,000 for 60 mins. After repeated washing, the strips were stained with DAB solution.

Transfection of BHK-21 cells. The pcDNA3.1-gD/clone-3 plasmid was isolated using the QIAprep Spin Miniprep (Qiagen) Kit. The cells were grown for 24 hrs in 6-well microplates (Nunc) seeded with 4 x 10^5 cells in 2 ml. A transfection mixture was prepared by mixing the fusion reagent FuGENE6 (Roche) with the plasmid DNA in a ratio of 6 (μg) to 1 (μg). After 30 mins of incubation at room temperature, the transfection mixture was applied onto the cells grown in serum-free DMEM. After 24 hrs the medium was changed for a fresh one containing 5% FCS and 10 μg/ml G418. During further passaging the concentration of G418 was continuously raised from 25 μg/ml on day 2 to 250 μg/ml on day 20. On days 2 (passage 1), 9 (passage 2) and 30 (passage 5) cell samples were taken and grown separately on cover slips for immunofluorescence assay.

Immunofluorescence assay. The transfected BHK-21 cells and appropriate control cells grown on cover slips in Petri dishes were harvested, washed in PBS, air dried, fixed in acetone for 10 mins, and stored at -20°C until use. The cells were stained with an anti-gD MAb diluted 1:40 for 40 mins. After a 3-fold washing in PBS the cells were stained with an anti-mouse IgG conjugate (Sw-AMo/FITC, Sevapharma, Czech Republic) diluted 1:80 for 30 mins, washed and mounted into Tris-HCl pH 8.0-glycerol (1:10). The stained cells were viewed and photographed using an E400 Nikon fluorescence microscope.

Results

Expression of FLgD in E. coli

Prior to expression experiments the growth curves of transformed bacterial cells were determined. The growth of the cells transformed with pPPXagD/HSZP (Fig. 3A) or pPPXagD/17 as determined by A_{550} was delayed as compared with pPPXa-1-transformed cells.

The number of viable cells has been counted by dilution and seeding on agar plates with ampicillin at intervals from 3 to 24 hrs**.** At the onset of exponential growth, this number increased 10-fold at 6 hrs and 100-fold at 12 hrs in both cultures. Later on, the concentration of the pPPXagD/HSZP-transformed cells continued to increase very slowly, i.e. it doubled at 16 hrs and again at 24 hrs. No further exponential increase was noted as the peak concentration stopped at 8 x 10^3 cells/ml. In contrast, the number of mock-transformed cells containing the original pPPXa-1 plasmid increased again tenfold from 16 hrs reaching the peak concentration of about 5 x 10^5cells/ml at 20 hrs. Thus, the peak concentration of the pPPXagD/HSZP-transformed cells was about 6–7 times lower than that of pPPXa-1-transformed cells.

The slower growth of the pPPXagD/HSZP-transformed cells as compared to that of the pPPXa-1-transformed cells led to a relatively lower total protein yield (data not shown). The 49–52 K FLgD (Lee *et al*., 1982; Matthews *et al*., 1983) together with the 14.5 K biotinylated peptide (constituent of the fusion protein) should have a calculated M_r of 63.5–66.5 K. However, we found only a value of 45 K (Fig. 4, lane 3). Thus FLgD did not exceed the size 30–31 K, as already observed by Watson *et al*. (1982). The purified FLgD showing a M_r of about 27 K (Fig. 4, lanes 2, 4 and 5) thus contained probably only one third of the calculated gD. In the pPPXagD/17-transformed cells this polypeptide was present already before purification.

Summing up these results, we found that (i) the cells transformed with the pPPXagD/HSZP or pPPXagD/17 plasmid grew more slowly than the control plasmid (pPPXa-1) -transformed cells; (ii) an incomplete fusion gD was expressed in transformed cells.

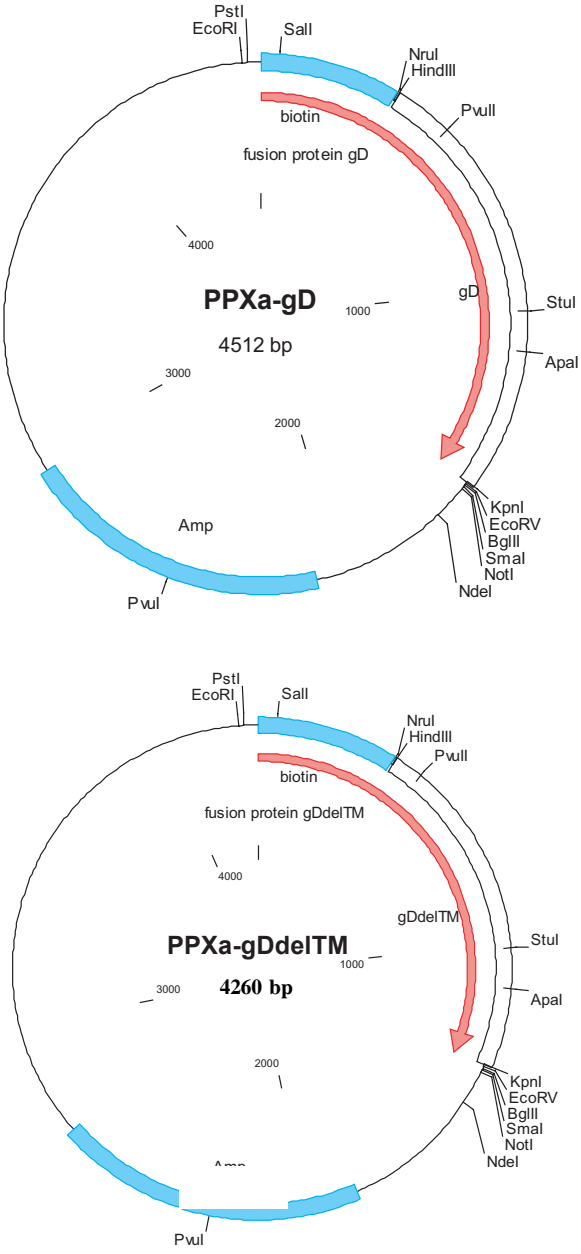

Fig. 2

The plasmid constructs pPPXagD and pPPXagDdelTM

The plasmid construct pPPXagD coded for a fusion protein containing the biotinylated peptide encoded by the pPPXa-1 vector and the FLgD protein encoded by the gD1199 insert. The pPPXagDdelTM plasmid had the gD/947 insert (Table 2), which, in addition to the biotinylated peptide, encoded the gD surface domain (gD313) lacking TM and signal sequences.

Expression of truncated gD in E. coli

In fact, the pPPXagD/HSZP- or pPPXagD/17-transformed *E. coli* cells (Fig. 2) expressed a truncated gD but not FLgD.

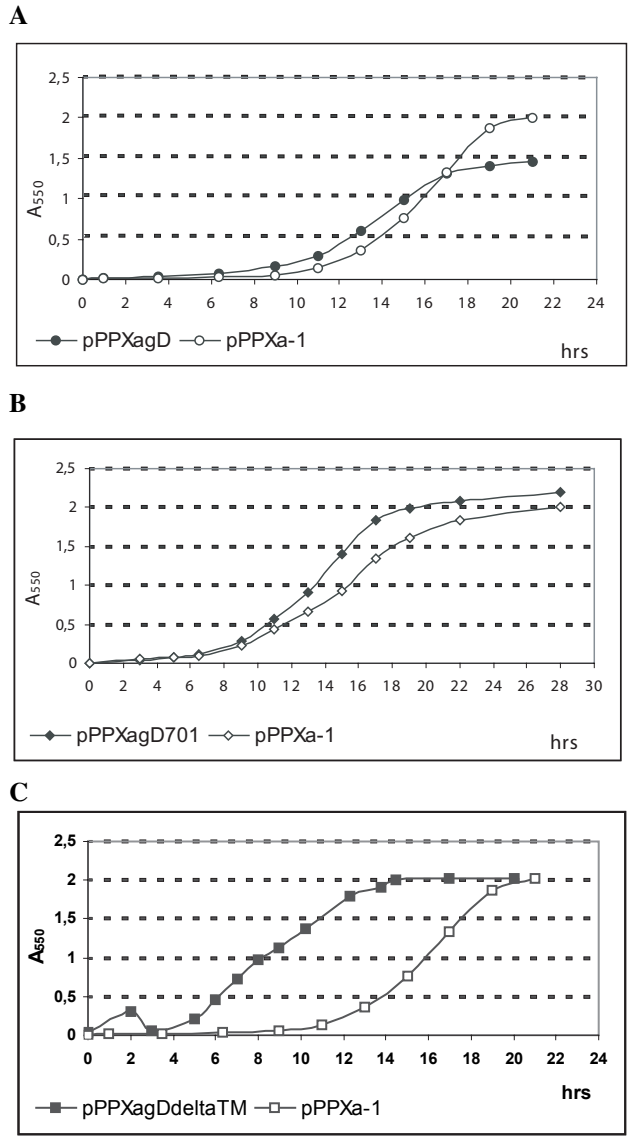

Fig. 3

Growth of *E. coli* cells transformed with the plasmids pPPXagD/HSZP (A), pPPXagD701/HSZP (B) and pPPXagDdelTM/HSZP (C)

Therefore, we prepared plasmid constructs encoding fusion proteins containing two different truncated gDs (Table 2, Fig. 1). *Stu*I was used to cleave the original gD1211/HSZP PCR product. After *Hind*III and *Stu*I digestions the 701 bp digested fragment (gD701/HSZP was inserted into the pPPXa-1 plasmid to form the pPPXagD701/HSZP plasmid. The latter encoded a 234 aa long gD (without a fusion part), which from 232 aa belonged to the truncated gD and 2 aa were artificial, specified by the codons positioned in the front of the gD initiation codon. The viability of *E. coli* cells transformed with the pPPXagD701/HSZP plasmid was relatively good, as judged from absorbance values which

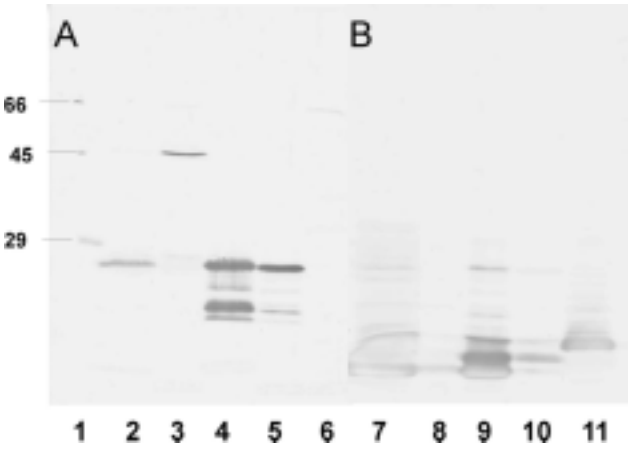

Fig. 4

Immunoblot analysis of expression of FLgD in *E. coli* cells

Blots of FLgD/HSZP expressed in pPPXagD/HSZP-transformed *E. coli* cells stained with the anti-gD serum (A) and with the ABC technique (B). Coomassie Blue-stained marker polypeptides (lane 1, M_r in K values). The purified fusion protein (lanes 2 and 7); the pPPXagD/HSZP-transformed cells (lanes 3 and 8); the pPPXagD/17-transformed cells (lanes 4 and 9); the purified FLgD/17 fusion protein (lanes 5 and 10); the mock-transformed *E. coli* cells (lanes 6 and 11). Note that the size of the FLgD/HSZP fusion protein in transformed cells did not correspond to the calculated M_r; it was present in amounts non-detectable by ABC staining. During purification, the fusion protein underwent additional cleavage. Extensive cleavage of the FLgD fusion protein occurred also in pPPXagD/17-transformed cells.

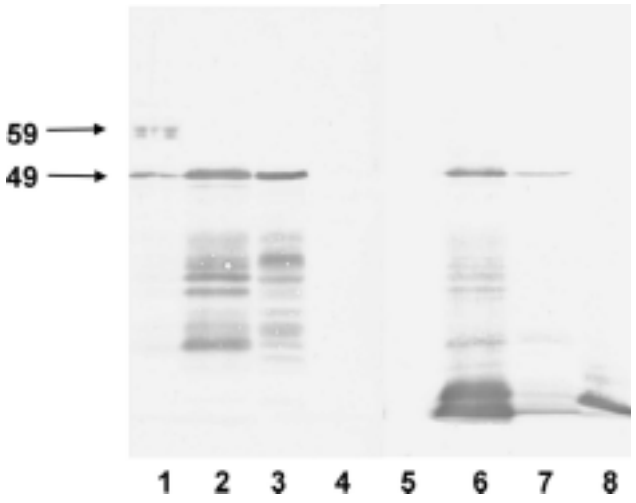

Fig. 5

Immunoblot analysis of expression of a truncated gD (gD234/HSZP) in *E. coli* cells

Blots of the gD234/HSZP fusion protein expressed in pPPXagD701/HSZP-transformed *E. coli* cells were stained with the anti-gD serum (A) and with the ABC technique (B). Coomassie Blue-stained marker polypeptides (lane M, M_r in K values). The strain HSZP virions purified by sucrose density gradient (lanes 1 and 5) (note the glycosylated gD bands at 57–59 K in lane 1); the cells transformed with pPPXagD701/HSZP (lanes 2 and 6); the purified fusion protein containing the truncated protein gD234/HSZP (lanes 3 and 7); the mock-transformed *E. coli* cells (lanes 4 and 8).

were close to those of pPPXa-1-transformed cells (Fig. 3B). The pPPXagD/HSZP-encoded fusion protein might have a calculated M_r of 46 K. The band corresponding to this protein (Fig. 5A, lane 2) reacted with an anti-gD MAb as well as with a Px-labeled avidin reagent (Fig. 5B, lane 6). A similar band was found in the purified proteins released from the avidin resin (Fig. 5B, lanes 3 and 7). It was of about the same size as the non-glycosylated FLgD of 49 K on Fig. 5A, lane 1, loaded with purified HSV-1. This lane displayed also a wider band corresponding to the glycosylated gD of 56–59 K (Eisenberg *et al.*, 1979; Zwaagstra *et al.*, 1988). No fusion protein was seen in the lanes 4 and 8 loaded with the pPPXa-1-transformed cells.

The growth curve of the pPPXagD701/HSZP-transformed *E. coli* cells clearly showed that the respective plasmid would be of advantage for preparation the recombinant gD. Removal of a part of the gD encoding the C-terminus of gD (cytoplasmic region) and the TM domain by *Stu*I digestion is a better solution than removal of the signal sequence (see Watson *et al.*, 1982), since the TM region is apparently responsible for the toxicity for *E. coli*.

However, gD234/HSZP seemed unsatisfactory, since it did not contain all the antigenic epitopes present in the gD ectodomain (Fig. 1). Therefore, we prepared a truncated gD DNA fragment (gD955/HSZP) encoding the total gD ectodomain but lacking the TM anchore sequence, the cytoplasmic domain and the signal sequence. The gDF27 and gDR340 primers were designed to start at the codon 27 and to end at the codon 340 of FLgD (Table 1). The total viral sequence had 939 bp specifying 313 aa. The gD955 fragment, as prepared on the HSV-1 strain HSZP DNA, was double-digested and inserted into pPPXa-1 plasmid to yield the pPPXagDdelTM/HSZP plasmid construct. The digested insert had altogether 947 bp and encoded one intervening Ala to enable the in frame reading of the fusion protein. The expressed gD313/HSZP protein was found non-toxic for producer cells (Fig. 3C) and could be relatively easily purified from the cell lysate. Two proteins of 56–59 K were found in a purified preparation stained either with Px-labeled avidin or an anti-gD antibody (Fig. 6, lanes 4 and 7). These proteins were of the same size as those detected in the pPPXagDdelTM/HSZP-transformed cells (Fig. 6, lanes 3 and 6). The lysates of control pPPXa-1-transformed cells did not show any positive bands (Fig. 6, lanes 2 and 5). Summing 14.5 K as the estimated M_r of the biotinylated part of the fusion protein and 41.5–44.5 K as the M_r of gD313/HSZP gave 56–59 K as the M_r for the fusion protein. This calculation was based on the assumption that the M_r of

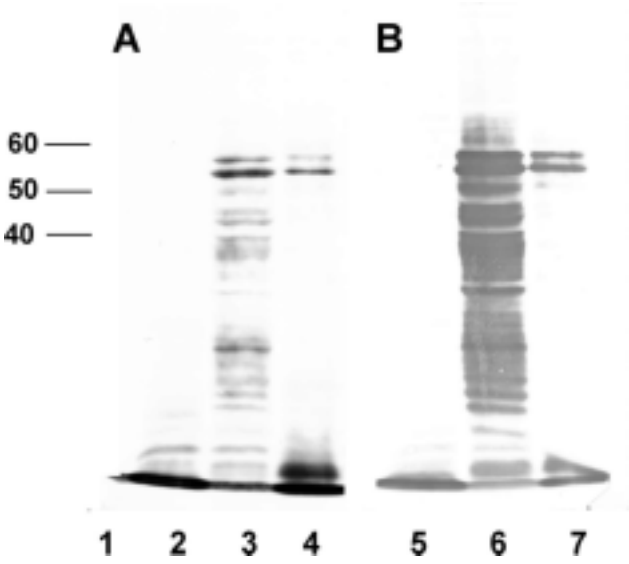

Fig. 6

Immunoblot analysis of expression of a truncated gD (gD313/HSZP) in . *coli* cells

Blots of the gD313/HSZP fusion protein expressed in pPPXagDdelTM/HSZP-transformed *E. coli* cells stained with the ABC technique (A) and with the anti-gD serum (B). Positions of marker polypeptides (lane 1, M_r in K values, bands not shown). The mock transformed *E. coli* cells (lanes 2 and 5); the cells transformed with the pPPXagDdelTM/ HSZP plasmid (lanes 3 and 6); the purified gD313/HSZP fusion protein (lanes 4 and 7). The gD313/HSZP fusion protein had an estimated M_r of 54–57 K.

gD313/HSZP was about 80% of the non-glycosylated FLgD (49–52 K; Lee *et al*., 1982; Matthews *et al*., 1983).

Expression of gD in mammalian BHK-21 cells

We made also an attempt to express gD in mammalian cells. The fragment gD1211/HSZP was double-digested with *Hind*III and *Kpn*I and inserted into the shuttle vector pcDNA3.1 to produce the pcDNA3.1-gD plasmid construct. The pcDNA3.1-gD clone 3 was purified by using of the Qiaprep Spin Miniprep Kit (Qiagen) and used for transfection of BHK-21 cells. We obtained a gD-producer cell line (BHK-gD) expressing gD under the control of immediate early Human cytomegalovirus (HCMV) promoter. During cell culture passaging in the presence of increasing G418 concentration, the ratio of gD producing cells to gD non-producing cells increased. A relatively low proportion of gD-positive cells were seen on day 2 p.t. (Fig. 7A). The number of gD-producer cells increased in the presence of 50 μg G418/ml added on day 5 p.t. On day 9 p.t. nearly each cell expressed gD (Fig. 7B, passage 2, G418 concentration of 100 μg/ml). The mock-transfected control cells remained negative throughout the experiment (Fig. 7C).

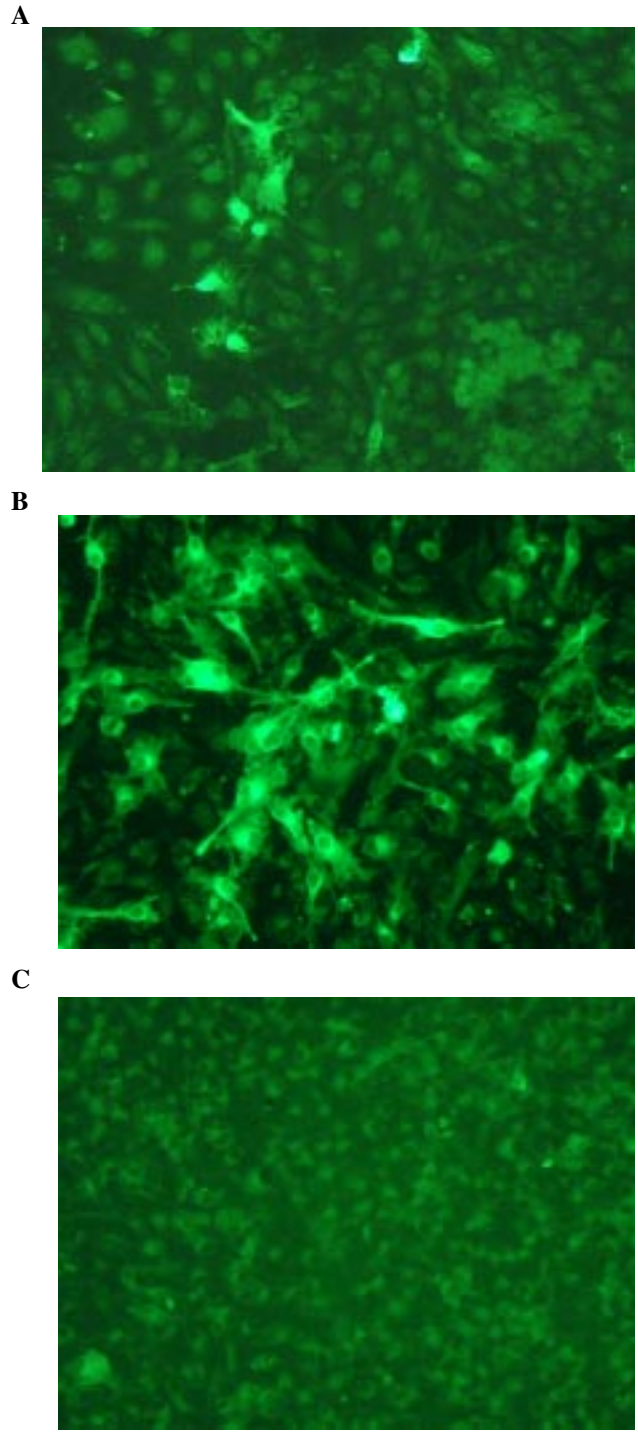

Fig. 7

Immunofluorescence of gD in gD-producer cell line BHK-21-gD

A. Immunofluorescence of gD in BHK-gD cells on day 2 p.t.
B. Overwhelming positivity of gD observed on day 9 p.t. at passage 2.
C. Control cells showing no fluorescence. Cells stained with the anti-gD MAb and with the SwAMo/FITC conjugate, respectively. Magnification 480x.

In gD-producer cells the virus antigen was seen mainly in the perinuclear area of the cytoplasm showing a ring-shaped appearance at the nuclear membrane and leaving the central part of the nucleus free of antigen. The cells continued expressing gD during 31 days p.t. The cells were frozen at the passage 9.

Discussion

Deletion of gD from the HSV-1 genome is lethal for the virus. However, in cells expressing gD a recombinant virus in which the gD/gI sequences were replaced with β-galactosidase sequences was able to replicate. A defective virus lacking the gD and gI genes was unable to initiate the synthesis of immediate early virus proteins suggesting that gD is essential for virus penetration (Ligas and Johnson, 1988). gD is not only an important tool of HSV-1 pathogenicity as a mediator of virus penetration into a host cell and into nerve endings (Izumi and Stevens, 1990) but also as a potent immunogen.

The bacterial host may not tolerate expression of certain stretches of eukaryotic proteins. These proteins include very often hydrophobic amino acid sequences such as a signal peptide or a TM region of a membrane–bound protein (Brosuis, 1984; Remaut *et al*., 1983). The presence of a signal sequence was conditionally lethal for *E. coli* as observed at expression of the vesicular stomatitis virus glycoprotein E gene (Rose and Shafferman, 1981). Watson *et al*. (1982) expressed 342 C-terminal codons of HSV-1 gD in frame with 24 codons of N-terminal bacteriophage l *cro* sequence; the sequence contained an initiation ATG codon placed under the control of the *lac* promoter. The authors were able to produce a protein of predicted M_r 46 K reacting with MAb against gD. However, they obtained comparable amounts of incomplete polypeptides of 36–38 K, also reacting with the MAb against gD. This can be seen also in our experiments described in this paper, in which the cells transformed with a plasmid containing the FLgD gene (pPPXagD/HSZP) expressed the incomplete 45 K fusion protein. In addition to expression of the incomplete fusion protein, we also observed inhibition of bacterial growth. Interestingly, HSV-2 gD expressed in *E. coli* cells did not inhibit their growth. Following induction of expression with IPTG the HSV-2 FLgD could be isolated, which, in contrast to HSV-1 gD, did not inhibit the growth of *E. coli* (Steinberg *et al*., 1986). We wanted to know if there is any difference between FLgD/17 and FLgD/HSZP expressed in *E. coli*. We found out that both were similarly toxic. Therefore we decided to remove the TM coding sequence from nt 701 of the gD1211/HSZP PCR product. The expression of this truncated protein (gD234/HSZP) did not inhibit the bacterial growth. The product in question could be easily isolated by means of the Soft Link Avidin Resin.

Anti-gD MAbs which neutralize the virus by inhibiting HSV-1 penetration (Highlander *et al.*, 1987) do not interfere with virion adsorption and *vice versa* (Fuller and Spear, 1985, 1987). The antigenic domain VII of HSV-1 gD was found to represent the site with which the majority of neutralizing antibodies react, since the peptide containing aa 8–23 (i.e. aa 33–48) was the most powerful immunogen (Cohen *et al*., 1984). According to Weijer and coworkers (1988), the antibodies to the peptides containing aa 1 to 30 (i.e. aa 26–55) neutralized the infectivity. The antibodies to the peptide containing aa 267–281 (i.e. aa 292–306) reacted strongly in Western blot analysis enhancing the neutralizing activity of the sera against the former peptides. Strynadka and coworkers (1988) have found virus neutralization activity in the sera reacting with the N-terminal domain VII (gD aa 2–21, i.e. aa 27–46), with the domain II (aa 267–297, i.e. aa 293–321) and with the domain adjacent to the TM region (aa 314–325, i.e. aa 338–347). In contrast, Geerlings and coworkers (1990) have claimed that only the antisera against the locus VII peptides but not against others neutralize HSV-1 *in vitro*. gD is responsible for binding HSV-1 to HveA (TNFaR) and HveC (nectin 1 also called poliovirus receptor 1) (Cocci *et al.,* 2001; Menotti *et al.,* 2002). Whitbeck and coworkers (2001) have shown that in the interaction with HveC there is possibly involved aa 222–254 (i.e. aa 246–279) of gD. This receptor binding site is near to or overlaps the antigenic domain II. The epitopes from the domains VII and II are present in the fusion protein gD313/HSZP, the virus-specific portion of which spans from aa 27 to aa 340.

Since the fusion protein gD234/HSZP lacked the aa corresponding to the antigenic domain II, we prepared another fusion protein (gD313/HSZP) containing 313 aa of the gD ectodomain without the signal sequence. With this truncated protein we achieved the best expression and we did not notice any inhibition of bacterial growth. Our preliminary data showed that this fusion protein (gD313/HSZP) prepared in *E. coli* protected mice against viral challenge with the pathogenic SC16 strain of HSV-1 (data not shown).

The vector constructs encoding FLgD or a TM-deleted FLgD of HSV-2 have been used to vaccinate mice and guinea pigs with promising protective results (Strasser *et al*., 2000). The protective immunity to HSV-1 generated by DNA vaccination did not strictly correlate with specific antibody levels in contrast to the immunity following natural infections (Nass *et al.*, 2001). Higgins and coworkers (2000) have shown that the plasmids expressing the TM-bound HSV-2 FLgD induce predominantly the TH1-type immune response, while the plasmid expressing gD induces predominantly a TH2-type response. Systemic immunization with a DNA vaccine encoding HSV-1 gD reduced the severity of ocular disease in mice challenged with corneal infection since the virus levels in the regional

trigeminal ganglion were 100 times lower than those in sham-immunized animals (Frye *et al.*, 2002). In a murine model the topical use of the gD-1/IL-2 DNA vaccine totally prevented the development of herpes keratitis (Nakao *et al.,* 1993; Inoue *et al*., 2002).

The aim of this work was to prepare a suitable gD antigen from the HSV-1 HSZP strain which had been earlier used for preparation of a subunit vaccine (Rajčáni *et al.,* 1995). The HSZP strain was chosen with regard to its low pathogenicity (Rajčáni *et al.*, 1996, 1999) but unchanged immunogenicity. The gD313/HSZP proteins as well as the plasmid pcDNA3.1-gD are the present candidates for immunization experiments aimed at comparison (i) of their protective effects in the same animal model and (ii) of their efficacy as a recombinant subunit vaccine versus a DNA vaccine.

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