Stable constitutive expression of glycoprotein B (gpUL55) of human cytomegalovirus in permissive astrocytoma cells

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Permanent cell lines showing homogeneous constitutive expression of glycoprotein B (gpUL55; gB) of human cytomegalovirus (HCMV) were selected, in the presence of geneticin, from human astrocytoma cells (U373) after transfection with recombinant pRC/CMV-gB carrying the complete coding sequence for HCMV gB and for aminoglycoside phosphotransferase. The biosynthesis and processing including specific proteolytic cleavage, formation of disulphide-linked oligomers as well as transport of recombinant gB in three of four established transformed cell lines essentially resembled that found in infected parental U373 except for eventual degradation after 2 h of gB synthesis. Analysis of the fourth transformant expressing uncleaved gB suggested that proteolytic cleavage is not required for normal intracellular transport. The stable transformants retained permissiveness for productive superinfection with HCMV. The application of cell lines transformed with mutagenized HCMV gB for the rescue of genetically engineered HCMV mutants is discussed.

Introduction

Construction of mutant viruses by use of the genetic methods available has become a powerful tool for the functional analysis of viral gene products. Using directed mutagenesis of specific viral genes by insertion of marker genes or deletion of defined coding gene segments, considerable progress has been achieved even with complex agents such as herpesviruses, e.g. herpes simplex virus (HSV) and pseudorabies virus (Ligas & Johnson, 1988; Mettenleiter et al., 1990; Peeters et al., 1992; Spaete & Mocarski, 1987). In the various systems viral gene products were recognized that were indispensable for growth under experimental conditions (Longnecker & Roizman, 1987; Rauh & Mettenleiter, 1991). For growth of the respective virus mutants the deleted product had to be provided in trans by the cell culture used for propagation, i.e. the cells were transformed for expression of the respective viral protein. By this approach phenotypic complementation of viable progeny virions was achieved for biological characterization (Cai et al., 1987, 1988; Rauh & Mettenleiter, 1991).

In the case of human cytomegalovirus (HCMV) so far only mutants deficient in genes dispensable for growth in cell culture have been described (Kollert-Jøns et al., 1991) or constructed (Jones et al., 1991; Jones & Muzithras, 1992) mainly because permissive transfected cells for complementation have not been available. A major limitation in the HCMV system has been the species- as well as cell-type-specificity of this agent (Rapp, 1983). The cell culture system generally used for productive infection of HCMV is human fibroblasts (Rapp, 1983) which we have found to exhibit a restricted potential for stable transformation (unpublished observation). Recently, permissiveness for HCMV has been shown for human astrocytoma cells (U373; Kari et al., 1992). Here we describe the stable transformation of U373 with the complete gene for glycoprotein B (gB) of HCMV. It is shown that HCMV gB is essentially correctly processed and transported in transformed cell lines which also maintained permissiveness for HCMV.

Methods

Cell cultures, virus propagation and virus titration. Monolayers of human foreskin fibroblasts (HFF) and of human astrocytoma cells (U373; ATCC HTB17, U-373MG; kindly provided by Dr G. Jahn, Erlangen, Germany) were cultured in Eagle’s MEM supplemented with 10% fetal calf serum, vitamins, non-essential amino acids, glutamine, penicillin at 0.5 units/ml, and gentamicin at 60 µg/ml. For propagation of strain AD169 of HCMV confluent monolayers of HFF cells (1.5 × 10^7) were infected with an m.o.i. of approximately 0.01 and serum concentration was lowered to 2%, for experimental infection an m.o.i. of about 3 for HFF and 5 for U373 cells was used. To determine infectious units, coverslip cultures were infected with the virus suspensions prior to examination at 48 h post-infection for early antigen (EA) production by indirect immunofluorescence using a commercial monoclonal antibody (MAb) (DuPont; Radsak et al., 1990). For quantification EA-positive nuclei were counted; the amount of virus inducing EA production in a single cell was defined as 1 infectious unit (IU).

Construction of plasmids, transfection conditions and establishment of stable transformants of U373. The strategy for subcloning of the
DNA sequencing. To exclude cloning artefacts, sequencing of the gB gene in the recombinant constructs pUC-gB and pRC/CMV-gB was performed after alkaline denaturation by a modification of the dideoxynucleotide chain termination method of Sanger et al. (1977). In order to resolve GC-rich regions with possible intrastand secondary structures, the dGTP of the commercial sequencing kit used (Deaza dGTP, Pharmacia), was substituted by its analogues, c7dGTP or c7dITP (Spaete et al., 1988). The appropriate oligonucleotide primers were synthesized at the Institut für Molekularbiologie, Marburg, Germany. For separation the samples were run on 6% polyacrylamide-urea gels in Tris-borate-EDTA buffer (Kollert-Jöns et al., 1991).

Northern blotting. Total cellular RNA was extracted according to the method of Chomezynski & Sacchi (1987). Briefly, 1.5 x 10^7 infected U373 or transformed U373 derivatives were lysed in 4 ml of lysis buffer (4 m-guanidinium thiocyanate, 25 mM-sodium citrate pH 7.0, 0.5% w/v sodium N-laurylsarcosinate, 0.1 M-2-mercaptoethanol). After addition of 0.5 ml 2 M-sodium acetate pH 4.0, RNA was extracted by vigorous shaking with 5.0 ml equilibrated phenol and 1.0 ml chloroform/isooamy alcohol (24:1) for 10 s. After incubation on ice for 15 min the emulsion was centrifuged in the Beckman SW41 rotor of a Beckman L8-M ultracentrifuge for 15 min at 10000 r.p.m and 4°C. The aqueous phase was collected and the RNA was precipitated with 50 ml 2-propanol at 4°C overnight. In order to protect the RNA from degradation by contaminating RNases, RNase inhibitor (Boehringer) was added to the aqueous phase at a concentration of 5 units/μg according to the instructions of the supplier.

Formaldehyde–agarose gel electrophoresis and Northern blotting were done according to Sambrook et al. (1989). Ethidium bromide staining of parallel gels was used to monitor RNA degradation during extraction. Hybridization was performed at 42°C using pUC-gB radiolabelled with a commercial random primer labelling kit (BRL Life Technologies) as the detection probe. The conditions used were identical to those described previously (Kollert-Jöns et al., 1991) except that the hybridization buffer contained 5% formamide and the washing temperature was 55°C.

Immunofluorescence. Visualization of gB expression was carried out by indirect immunofluorescence with MAb 27-156 (Spaete et al., 1988) or glycoprotein H (gH)-specific MAb 14-4b (Bogner et al., 1992) and a secondary fluorescence-labelled rabbit anti-mouse IgG (Dakopatts) or glycoprotein H (gH)-specific MAb 14-4b (Bogner et al., 1992) and a secondary fluorescence-labelled rabbit anti-mouse IgG (Dakopatts) or anti-gB MAb 27-156 (Spaete et al., 1988). The appropriate oligonucleotide primers were synthesized at the Institut für Molekularbiologie, Marburg, Germany. For separation the samples were run on 6% polyacrylamide-urea gels in Tris-borate-EDTA buffer (Kollert-Jöns et al., 1991) except that the hybridization buffer contained 5% formamide and the washing temperature was 55°C.

Radiolabelling, immunoPrecipitation and Immunoblottting. For radiolabelling prior to immunoprecipitation, the complete culture medium was replaced by labelling medium consisting of MEM lacking methionine plus [35S]methionine (50 to 100 μCi/ml; specific activity >1000 Ci/mmol; Amersham Buchler) (see Results). Monolayers (1.5 x 10^6 to 5 x 10^6 cells) were harvested by scraping, centrifuged and extracts prepared by dissolving the cell pellets in 20 mM-Tris-HCl pH 9, 0.3 M-NaCl, 10% glycerol, 1 mM-CaCl2, 0.5 mM-MgCl2, 2 mM-EDTA, 0.5% NP40, 0.5 mM-PMSF, 100 units Trasylol/ml (Radsak et
Constitutive HCMV gB expression in U373

al., 1990; 0.5 ml/5 x 10⁶ cells). For immunoprecipitation (Radsak et al., 1990), samples of cell extracts of comparable protein content were pre-cleared by incubation with Protein A-Sepharose CL4B beads (Pierce) prior to incubation overnight at RT with MAb 27-156 or 14-4b. Immunocomplexes were adsorbed for 1.5 h RT onto Protein A-Sepharose CL4B beads coated with rabbit anti-mouse IgG (Dakopatts). Following seven cycles of washing the beads with PBS plus 0.1% NP40, 0.1% SDS and one washing cycle with distilled water the precipitates were subjected to SDS-PAGE (Laemmli, 1970) and consecutive fixation and fluorography (Bonner & Laskey, 1974) of the dried slab gels. For immunoblotting extracts prepared as described above were separated using SDs-PAGE under reducing or non-reducing conditions, transferred to nitrocellulose sheets and probed with gH-specific polyclonal antibody pAb gH (Bogner et al., 1992) and successively with HCMV pp65-specific MAb 28-19 (kindly provided by W. Britt) or with convalescent or hyperimmune serum, as described previously (Bogner et al., 1992).

Endoglycosidase digestion. Digestion with endoglycosidase H (endo H) and N-glucosidase F (PNGase F or pF) (Bogner et al., 1992; Radsak et al., 1990) was performed on immunoprecipitates of gB after one additional washing cycle with 100 mM-sodium phosphate pH 6, 0.05% SDS, 0.5% octylglycoside, 2 mM-EDTA, 0.5 mM-PMSF (glycosidase buffer) by incubation overnight at 37°C in 80 μl of glycosidase buffer plus 0.1 M-2-mercaptoethanol. The instructions of the manufacturer (Boehringer) were followed for the enzyme concentrations.

Results

Establishment of stable HCMV gB transformants derived from human astrocytoma cells

The complete coding sequence of the HCMV gB gene was initially subcloned from AD169-derived cosmid pCM1029 containing genomic HindIII fragments D and F (Fig. 1; Fleckenstein et al., 1982), into pUC18 and examined for its authenticity by sequencing (Sanger et al., 1977). The gB gene was subsequently transferred into the eukaryotic expression vector pRC/CMV which carries the aminoglycoside phosphotransferase gene for selection of transformants by the use of geneticin (Fig. 1; Chen & Okayama, 1987). The correct insertion of the gB gene for the expression of gB under the control of the vector-specific CMV IE promoter/enhancer was examined by restriction analysis and sequencing (see Methods). The recombinant pRC/CMV-gB was used for transfection of U373 by the calcium phosphate method. In a representative transfection assay, between 30 and 50 geneticin-resistant clones were recovered after about 3 weeks. Expression of gB was identified with gB-specific MAb 27-156 (Spaete et al., 1988; Radsak et al., 1990) on an average of 50 to 80% of the cells. By subcloning single cells of the gB-positive clones, four clones showing homogeneous gB expression (U373-gB1 to -4) were obtained (Fig. 2a). By using the pUC-gB construct as the probe, specific transcripts of about 3.5 kb (Spaete et al., 1988) were identified in Northern blots of RNA from gB-expressing cultures and that of AD169-infected cells (Fig. 3, lanes 1 and 3 to 6) whereas no specific signal was obtained for RNA from control U373 cultures (Fig. 3, lane 2).

Comparison of the expression of gB in transformed and infected cultures

In HCMV-infected permissive cells biosynthesis of gB includes cotranslational modification of the polypeptide backbone (100K) by N-glycosylation (150K) and subsequent processing by proteolytic cleavage into the N-terminal product of 90K to 110K and the C-terminal product of 58K (Britt & Auger, 1986; Britt & Vugler, 1989). In order to examine the biosynthesis of the transfected gB gene product U373-gB cultures were...
Fig. 4. Biosynthesis of HCMV gB in transformed cell lines. Cultures (1.5 x 10⁶ cells) of transformed cell lines U373-gB1 and -4 (c and d), AD169-infected HFF (a) and of AD169-infected U373 (b) were pulsed-labelled at 72 h and 96 h p.i., respectively, with [³⁵S]methionine (100 μCi/ml) for 2 h (lanes 1) and chased with unlabelled methionine for 2 h (lanes 2), 4 h (lanes 3) or 6 h (lanes 4). Extracts were prepared as described in Methods prior to immunoprecipitation with gB-specific MAb 27-156, separation of the precipitates by SDS-PAGE under reducing conditions and fluorography. Immunoprecipitations obtained from infected cultures with MAb 27-156 frequently resulted in coprecipitation of an unidentified 65K polypeptide (c) as observed previously. The representative fluorograms for transformed cell lines (c, d) show results obtained with U373-gB1 and -4. The positions of gB precursor (p), the N-terminal (gB 'N') and C-terminal (gB 'C') cleavage products, and putative degradation products (d) are indicated on the right. The positions of marker proteins are indicated on the left: α₂-macroglobulin (170K), β-galactosidase (116K), fructose-6-phosphate kinase (85K), BSA (68K), glutamate dehydrogenase (55K) and aldolase (39K).

pulse-labelled with [³⁵S]methionine and subjected to various consecutive chase intervals with unlabelled methionine prior to preparation of cellular extracts, immunoprecipitation with MAb 27-156 and analysis of the precipitates by SDS-PAGE (Fig. 4c, d). U373 cultures and HFF infected with HCMV strain AD169 and radiolabelled 96 h and 72 h p.i., respectively, were analysed by the same procedure to serve as controls. In the infected cultures only the uncleaved gB precursor of 150K was precipitated after the pulse (Fig. 4a, b). The radiolabel in the cleavage products of 90K to 110K and 58K was clearly present after 2 h of chase in HFF; in U373 cleavage appeared to be delayed and significant amounts of cleavage products were observed only after 6 h of chase. From immunoprecipitates of three of the four transformed cell lines, on the other hand, gB precursor as well as cleavage products were recovered as early as after the pulse interval (Fig. 4c). During the chase, radiolabel in the position of the expected gB-specific products was continuously lost; after 4 h of chase significant amounts of radiolabel were observed in additional precipitates that exhibited unusual Mr's of approximately 68K and 35K (Fig. 4, indicated on the right-hand side by d). This observation suggested that in these transformed cell lines gB was probably partially degraded during the chase period. In contrast, analysis of the immunoprecipitates of U373-gB4 revealed uncleaved gB precursor after both pulse and chase, indicating resistance to specific proteolytic cleavage of the recombinant gB polypeptide in this particular cell line. In addition, no obvious immunoprecipitable degradation products were recovered. In immunoblots with extracts from the transformed cell lines, recombinant gB was also recognized by HCMV-positive convalescent or hyperimmune serum (not shown).

Processing by glycosylation of gB in transformed cultures

To examine whether modification of the gB gene product of transformed cells by glycosylation was comparable to that of gB in infected cells, gB-specific immuno-
Fig. 5. Glycosidase sensitivity of HCMV gB in transformed cell lines. Cultures (1.5 x 10^6 cells) of transformed cell lines U373-gB1 (a), U373-gB4 (b) and of the AD169-infected U373 parental cell line (c) were pulse-labelled for 2 h and extracts were prepared as described in the legend of Fig. 4. Immunoprecipitates obtained with gB-specific MAb 27-156 were subjected to endo H (lanes 2) or pF (lanes 3) digestion or incubated in digestion buffer without enzyme (lanes 1) prior to separation by SDS-PAGE under reducing conditions and fluorography. The positions of gB precursor (p) and cleavage product of 58K (gB 58) are indicated on the right along with small arrows showing the reduced Mr of the glycosidase digestion products. The positions of marker proteins are shown on the left: myosin (200K), phosphorylase b (97K), BSA (69K) and ovalbumin (46K).

Fig. 6. Formation of disulphide-linked complexes of HCMV gB (gcI) in transformed cell lines. Immunoprecipitates obtained with gB-specific MAb 27-156 were prepared from radiolabelled extracts of transformed cell lines U373-gB1 to -4 (lanes 1 to 4) or AD169-infected parental cell line U373 (lane A) as described in the legends of Fig. 4 and 5 and subjected to SDS-PAGE under non-reducing conditions and fluorography. The arrows on the right indicate the positions of glycoprotein complex I (gcI) and gB precursor; the positions of marker proteins myosin (200K) and phosphorylase b (97K) are shown on the left.
Fig. 8. Production of early nuclear antigen following superinfection with HCMV strain AD169. Coverslip cultures of AD169-infected parental U373 (panel a) and of transformed cells (b, c) were subjected to acetone fixation at 24 h p.i. prior to indirect immunofluorescence with a commercial MAb recognizing HCMV-induced early nuclear antigen as described in Methods. The epifluorescence equipment of a Zeiss microscope (Axiophot) was used for photography. The micrographs in (b) and (c) show representative results obtained for U373-gB1 and -4.

Fig. 9. Production of late viral antigens in transformed cell lines following superinfection with HCMV strain AD169. Parallel cultures (5 x 10⁶ cells) of AD169-infected transformed cell lines (columns gB1 and gB4) and the parental cell line (U373) were harvested at 9 days p.i. and extract samples were subjected to immunoblotting with successive staining for HCMV gH and pp65 as described in Methods using 4-chloro-1-naphthol and N-3,3'-diaminobenzidine, respectively, to obtain different colours for the two viral proteins. Positions for gH (86K) and pp65 (65K) and for marker proteins phosphorylase b (97K) and BSA (68K) are indicated.

the other hand, only a gB precursor of about 145K was identified under these conditions (Fig. 6, lane 1) indicating that oligomers were not formed in this particular transformed culture. Furthermore, in all of the transformed cell lines including U373-gB4 unimpaired transport of the gB gene products to the outer cell membrane was observed as examined by surface immunofluorescence with MAb 27-156 (Fig. 7a).

Fig. 10. Production of viral progeny in transformed cell lines following superinfection with HCMV strain AD169. Parallel cultures (1 x 10⁶ cells) of AD169-infected transformed cell lines (columns gB1 and gB4) and the parental cell line (U373) were harvested at 2 (■) or 9 (□) days p.i. by scraping into the culture medium (4 ml). To liberate intracellular virus the cell medium suspension was subjected to three cycles of freezing and thawing prior to determination of the amount of infectious viral progeny as described in Methods. The columns for transformed cells show representative results obtained for U373-gB1 and -4.

Multiplication of HCMV in transformed cultures

Permissiveness of the complementing transformed cell lines is required for the use of these systems in future rescue studies of viral mutants. Parallel cultures of U373-gB cells were therefore infected with HCMV strain AD169 (m.o.i. approximately 5); then at various intervals p.i. cultures were examined by immunoreactions for production of viral antigens other than gB. In addition, titres of progeny virus were determined. As a control viral multiplication was examined in the parental U373 cell line by the same procedures. Both parental cells (Fig. 8a) and transformed cells (Fig. 8b, c) exhibited comparable permissiveness for HCMV as concluded from results from immunofluorescence for early nuclear antigen 24 h p.i. and from immunoblotting for the late viral antigens, gH and lower matrix protein pp65, 9 days p.i. (Fig. 9) as well as from the titres of infectious viral progeny produced (Fig. 10). Compared with HCMV multiplication in human fibroblasts, however, the production of infectious virus was delayed and virus yield was reduced by at least one order of magnitude (not shown).

Discussion

In recent studies the expression of the complete or truncated HCMV gB gene has been analysed in the vaccinia virus system (Cranage et al., 1986; Britt et al.,
Constitutive HCMV gB expression in U373

1990) or in stable transformants of Chinese hamster ovary cells (Spaete et al., 1988, 1990), respectively. In both instances it was demonstrated that the expressed viral gene product was processed correctly in the absence of other HCMV-specific products in cells that are non-permissive for HCMV. Our experiments extend these observations to permissive astrocytoma clones which were recovered after stable transformation with the complete gB gene. The transformed cell lines were competent in the constitutive expression of gB while showing unimpaired proliferation potential. This indicates that the viral product lacks cytotoxicity, unlike HSV gH (Gompels & Minson, 1989). The gB polypeptide in these cultures appeared to be stable for about 2 h, thereafter degradation was observed resulting in immunoprecipitable products of decreased Mr. Furthermore, normal proteolytic cleavage of the gB precursor was not observed in one out of four clones. This could be due either to altered cellular protease activity (Spaete et al., 1990) in this particular cell clone or to a mutation of the cleavage site (Spaete et al., 1990) in the transfected gene. Superinfection of U373-gB4 with the AD169 strain, however, resulted in normal cleavage of gB (unpublished observation); this observation strongly suggests the latter alternative. Sequencing of the cleavage site of the transfected gB gene should verify this interpretation. Given the unimpaired exposure of gB at the cell surface of this artefactual transformant it can be concluded that proteolytic cleavage of gB is not a prerequisite for its natural transport. This view is in agreement with data reported for bovine herpesvirus (Blewett & Misra, 1991). In the context of transport of gB to the cell surface it is noteworthy that in transformed cell lines an increasing number of multinucleated cells was found upon prolonged culturing (unpublished observation), an observation which may imply a function for gB in membrane fusion.

With respect to modification by glycosylation of membrane glycoproteins, cellular transport generally includes trimming of mannose-rich intermediates during rough endoplasmic reticulum–cis-Golgi transit followed by addition of terminal sugars to form complex carbohydrate side-chains and, in the case of HCMV gB, proteolytic cleavage during passage through the medial and/or trans-Golgi compartments (Britt & Vugler, 1989). These sequential processes imply that HCMV gB shows complete endo H sensitivity for the majority of the uncleaved precursor molecules and endo H resistance for a significant portion of the mature product(s) as was observed here for gB in transformed cell lines U373-gB1 to -3 as well as in infected U373. It is intriguing that most of the uncleaved gB precursor of U373-gB4 retained mannose-rich carbohydrate chains, i.e. endo H sensitivity, in spite of apparent transport through the distal Golgi and trans-Golgi network compartments for presentation at the cell surface. It can be concluded from the only small additional reduction of Mr after pF digestion that few complex carbohydrate side-chains are present in the uncleaved molecule, as is also seen in infected cells.

Regarding cell lines U373-gB1 to -3 our data are in agreement with previous observations in other systems that the correct processing of the gB polypeptide obviously does not depend on the presence of additional viral products and that thus all regulatory elements are contained in the gB gene sequence itself (Cranage et al., 1986; Spaete et al., 1988). Establishment of recombinant U373 derivatives transformed with specifically mutagenized gB genes therefore provides a relevant alternative system for the determination of amino acid motifs that regulate the maturation of HCMV gB. This experimental approach requires technical effort comparable to that in other systems but offers obvious advantages over the use of the vaccinia virus system for example.

It has been demonstrated for HSV that stably transformed cell lines are indispensable as an experimental tool for the precise functional characterization of the envelope glycoproteins in viral infectivity (Campadelli-Fiune et al., 1988a, b, 1990). For the rescue of genetically engineered HCMV mutants deficient in essential structural components, the cultures used for phenotypic complementation must provide the potential to support HCMV growth. Compared with HSV this aspect represented a major experimental obstacle in the case of the species- and cell type-specific HCMV. Previous reports showed that multiplication of HCMV in the parental U373 cell line used for our experiments was essentially equivalent to that obtained in fibroblast cultures; release of infectious virus was, however, somewhat restricted (Kari et al., 1992). We verified the permissiveness of U373 to HCMV and the titres of progeny virus obtained appeared to be reduced by approximately one order of magnitude as compared with those obtained in fibroblasts (unpublished observation). The permissiveness of the recombinant clones derived from the parental cell line after transformation was essentially unaltered. Relative resistance to viral superinfection, as reported for cells transformed for glycoprotein expression in the HSV system (Johnson & Spear, 1989; Johnson et al., 1990), was not observed.

The use of such permissive U373-derived cultures transformed by mutagenized HCMV gB genes in rescue studies with gB-negative HCMV should yield phenotypically complemented mutant virus for identification and characterization of gB domains that are relevant for its putative function in viral infectivity (Rasmussen et al., 1988). For instance, whereas in the influenza virus system proteolytic cleavage of the haemagglutinin is an essential event for viral penetration (Garten et al., 1981), the
significance of proteolytic cleavage of gB for HCMV maturation and biological activity remains unknown. The system described here should provide a key tool to elucidate these open questions.

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References


Constitutive HCMV gB expression in U373


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