Expression from cloned DNA of biologically active glycoprotein C of herpes simplex virus type 1 in mammalian cells

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A DNA fragment of the herpes simplex virus type 1 genome encoding glycoprotein C (gC-1) has been cloned into different eukaryotic expression vectors for transient and stable expression of the glycoprotein in a number of cell lines. All of these expression vectors use a non-HSV promoter, such as the adenovirus major late promoter or murine leukaemia virus long terminal repeat promoter to express gC-1 in COS and CHO cells or 3T3 cells. The gC-1 protein synthesized was fully glycosylated with both N- and O-linked oligosaccharides. Synthesis of the mature 120K gC-1 glycoprotein involved partially glycosylated 100K and 105K proteins and the non-glycosylated 70K protein as intermediate molecules. Immunofluorescence studies showed that the expressed gC-1 was localized intracellularly in the nuclear envelope as well as on the cell surface. The expressed gC-1 was biologically active and could act as a receptor for the complement component C3b in the absence of other HSV proteins.

Introduction

Herpes simplex virus type 1 (HSV-1) buds from the inner membrane of the nucleus (Darlington & Moss, 1968) and codes for at least seven envelope glycoproteins (g); gB, gC, gD, gE, gG, gH and gI (Spear, 1984, 1987; Gompels & Minson, 1986; Richman et al., 1986; Longnecker & Roizman, 1987). gB, gC and gD are present on the infected cell surface (Glorioso et al., 1983) and act as the major antigenic determinants of HSV-1 (Cohen et al., 1972; Glorioso et al., 1984). In infected cells the gC of HSV-1 (gC-1) also functions as a receptor for the complement component C3b (Friedman et al., 1984, 1986). Recently gC-1 has been shown to be the immunodominant antigen recognized by HSV-1-specific cytotoxic T lymphocytes (Glorioso et al., 1985; Rosenthal et al., 1987).

The nucleotide sequence of the gC-1 gene and a deduced amino acid sequence of the glycoprotein have been published (Frink et al., 1983; Homa et al., 1986). In common with other transmembrane glycoproteins (Wickner & Lodish, 1985) gC-1 contains a 25 amino acid long, amino-terminal signal sequence and a 23 amino acid long, membrane anchoring sequence near the C terminus (Frink et al., 1983; Kikuchi et al., 1984). The amino acid sequence also shows the presence of at least seven potential N-glycosylation sites (Frink et al., 1983). However, gC-1 contains both N- and O-linked oligosaccharides (Olofsson et al., 1983; Spear, 1984; Campadelli-Fiume & Serafini-Cessi, 1984; Dall'Olio et al., 1985).

As a first step in the study of the role of various domains of gC-1 in the immunological response to HSV-1 infection, as well as to study the mechanism of targeting of proteins into the nuclear membrane, we have cloned the gC-1 gene into three eukaryotic expression vectors for transient and stable expression of the glycoprotein in different cell lines. Previous reports on expression of gC-1 in mammalian cells were limited to expression systems dependent on infection of the cells carrying the gC-1 gene with HSV-1 (Arsenakis et al., 1986; Rosenthal et al., 1987). Recently, gC-1 protein was transiently expressed in NIH 3T3 cells (Seidel-Dugan et al., 1988) and the expressed protein was shown to act as a C3b receptor. Here we show that gC-1 can be expressed in a transient or constitutive fashion in various mammalian cell lines in the absence of any other HSV-1 gene products. The gC-1 synthesized was fully glycosylated with both N- and O-linked oligosaccharides. Immunofluorescence studies showed that the expressed gC-1 protein was located both in the nuclear envelope and on the cell surface. In addition, gC-1 expressed from cloned genes could bind to the complement component C3b, showing that gC-1 can act as a C3b receptor in the absence of any other HSV-1 gene products.

Methods

A cloned 4 kb SalI–HindIII fragment of the HSV-1 genome containing the gC (pGC) coding sequence (Frink et al., 1983; Homa et al., 1986) was provided by Dr F. L. Homa, University of Michigan,
Ann Arbor, Mich., U.S.A. The expression vectors p91023 and pRK1-4 (Kaufman, 1985 and personal communication) were obtained from Dr R. Kaufman, Genetics Institute, Cambridge, Mass., U.S.A. The retrovirus vector pDOLMP10 (Korman et al., 1987) and $\psi$-2 cells (Mann et al., 1983) were obtained from Dr R. Mulligan, M.I.T. Antiserum to HSV-1 was from Dr M. Suh, Montreal Cancer Institute or Dako Laboratories. Monoclonal antibodies to HSV-1 gC and gB proteins (Showalter et al., 1981) were obtained from Dr M. Zweig of the National Cancer Institute, Frederick, Md., U.S.A.

Construction of gC expression plasmids; transient expression in COS cells. A 2.0 kb $Bgl$II- BamHI fragment from pGC was subcloned into the BamHI site of pUC18, which resulted in the insertion of a gC-1 coding sequence lacking the endogenous promoter (Frink et al., 1983). To generate Psrl sites at both ends of the gC-1 coding sequence we isolated a 1.5 kb EcoRI- HindIII fragment from one orientation of the above clone and a 460 bp EcoRI-PstI fragment from the other orientation of the above clone. Ligation of these fragments followed by cloning in PstI-HindIII sites in pUC18 generated pUCgCp4b, which contains the full coding sequence of gC-1 flanked by Psrl sites at both ends. The 2.0 kb Psrl fragment from pUCgCp4b was cloned in the unique Psrl cloning site of the vector p91023 (Kaufman, 1985).

Constitutive expression of gC-1 in stable cell line. A CHO cell line constitutively expressing gC-1 glycoprotein was obtained by using the vector pRK1-4 derived from the vector p91023 (Kaufman, 1985 and personal communication). This vector was constructed by introducing into the vector p91023 a mouse dihydrofolate reductase (dhfr) gene under the control of the simian virus 40 (SV40) promoter, in a separate cassette. When $dhfr^-$ mutants of CHO cells (Urlaub & Chasin, 1980) were transfected with this vector the second $dhfr$ gene provided a functional enzyme and transformed the cells to a $dhfr^+$ phenotype. The gC-1 coding sequence cloned in pUC18 was excised by complete digestion with Psrl, followed by partial digestion with EcoRI. The 2.0 kb DNA fragment containing the complete gC-1 coding sequence with a Psrl recognition sequence 30 bp upstream of the initiator ATG codon and an EcoRI recognition sequence present at the 3' end of the termination codon was cloned into pRK1-4 plasmid digested with Psrl and EcoRI enzymes. The resulting pRK-gC clone was used for generating CHO cell lines expressing gC-1 glycoprotein.

The retroviral vector, pDOLMP10, was used to produce 3T3 cell lines containing a stably integrated single copy of the gC-1 gene and constitutively expressing the glycoprotein. The vector pDOLMP10 was constructed (Korman et al., 1987) from the retroviral vector pZipNeo SV40 (Cepko et al., 1984). The gC-1 coding sequence contained in the 2.0 kb $Bgl$II- BamHI DNA fragment lacking the endogenous HSV promoter was cloned in the BamHI cloning site of pDOLMP10. The recombinant plasmid pDOL-gC was used to produce recombinant retrovirus containing the gC-1 gene (DOL-gC) by transfecting $\psi$-2 cells. 3T3 cells expressing the gC-1 gene were then generated by infecting them with the recombinant DOL-gC virus (Cepko et al., 1984; Korman et al., 1987).

Transfection of mammalian cells with plasmid DNA. $\psi$-2, COS-1 or $dhfr^-$ CHO (DUKX-22) cells were transfected with plasmid DNA using a modified calcium phosphate precipitation method (Graham & van der Eb, 1973). An approximately 60% confluent monolayer of cells in 60 mm plastic culture dishes was incubated for 4 h at 37 °C with a calcium phosphate precipitate of plasmid DNA prepared as follows. Ten μg of plasmid DNA was mixed with 25 μl of 2.5 M-CaCl$_2$ and the volume was made up to 250 μl with sterile water. This DNA mixture was added to 250 μl of 2 x HEPES-buffered saline (2 x HEBES; 42 mM-HEPES, 27 mM-NaCl, 10 mM-KCl, 1.4 mM-Mg$_2$SO$_4$, and 11 mM-dextrose, pH 7.05 to 7.1) with slow bubbling of air. The DNA-calcium phosphate precipitate was formed by keeping the mixture at room temperature for 30 to 40 min. The precipitate (500 μl) was then added to 5 ml culture medium in dishes containing the cells to be transfected and was incubated at 37 °C for 4 h. After the 4 h incubation period the medium containing the calcium phosphate-DNA precipitate was aspirated off the cell monolayer and the monolayer was treated with 15% glycerol in growth medium for 2 min at room temperature. The glycerol was removed by adding fresh medium to it and then washing the monolayer twice with fresh medium. Finally, the cells were fed with fresh medium and were incubated at 37 °C for required periods of time.

Establishment of cell lines from single colonies of mammalian cells. To establish permanently transformed cell lines the appropriate cells were transfected with the required plasmid DNA containing the selectable marker gene. To obtain a CHO cell line constitutively expressing gC, $dhfr^-$ CHO cells grown in a-DEG containing ribo- and deoxyribonucleotides were transfected with pRK-gC plasmids using the calcium phosphate precipitation method (Graham & van der Eb, 1973). At 40 h post-transfection the cells were trypsinized and were plated at a 1:5 dilution in a-DEG containing no additional ribo- or deoxyribonucleotides. The cells were maintained in this selection medium for 10 to 15 days and a number of $dhfr^+$ colonies were obtained from each set of transfections.

Recombinant retroviruses containing the gC-1 gene were obtained by transfecting $\psi$-2 cells with the recombinant pDOL-gC plasmid. Clones of $\psi$-2 cells resistant to geneticin (G418, 0.75 mg/ml; Gibco) were isolated and grown as recombinant virus producer cells. The virus produced was titrated in 3T3 cells. Typically, 10$^5$ to 10$^6$ c.f.u./ml of the retrovirus were produced from the producer $\psi$-2 cells. 3T3 cells were infected in the presence of 2 μg/ml polybrene with the recombinant retrovirus and cells resistant to 0.75 mg/ml of G418 were selected and cloned. The G418-resistant (G418$^+$) 3T3 cells were analysed for expression of the inserted glycoprotein genes.

Labeling of cells. To label proteins expressed in the transfected cells or in the established cell lines, cells were starved (for 1 h) of the component with which they would subsequently be labelled (i.e. methionine or sugar). At the end of the starvation period medium containing L-$[^{35}$S]methionine (50 μCi/ml, specific activity 1000 Ci/mmol), D-2-[3H]mannose (100 μCi/ml, specific activity 54 Ci/mmol), or D-[3H]galactose (100 μCi/ml, specific activity 54 Ci/mmol) was added and the cells were labelled for various time periods.

Immunoprecipitation and gel analysis of labelled proteins. Cells labelled with $[^{35}$S]methionine, $[^{3}$H]galactose or $[^{3}$H]mannose were harvested, after removal of the labelling medium at the end of the labelling period. The monolayer of cells was washed twice with ice-cold phosphate-buffered saline (PBS; 138 mM-NaCl, 2.7 mM-KCl, 8 mM-Na$_2$HPO$_4$, 1.46 mM-K$_2$HPO$_4$, pH 7.3) and then lysed with a solution containing 1% Nonidet P-40 (BDH), 0.4% sodium deoxycholate (Calbiochem), 100 units of aprotinin (Sigma) per ml, 66 mM-EDTA (BDH) and 10 mM-Tris- HCl (Bio-Rad) adjusted to pH 7.4 (Rose & Bergman, 1982). After lysis nuclei were removed by centrifuging at 10000 g for 3 min. The clear supernatant was then treated for 30 min at 0 °C with anti-HSV-1 antiserum or monoclonal antibodies to HSV-1 glycoproteins. Control immunoprecipitations were carried out using normal rabbit serum or ascites fluid. At the end of the incubation period the antigen–antibody complexes were mixed with 80 μl of 10% (w/v) Protein-A-Sepharose bead suspension (Pharmacia) for 2 to 4 h at 4 °C. This was followed by washing the beads four times with the above cell lysate buffer containing 0.5% SDS (BDH) and three times with Tris-buffered saline (50 mM-Tris-HCl, 150 mM-NaCl and 100 units/ml aprotinin, pH 7.2). The immunoprecipitated proteins were then extracted from the beads by boiling with 20 to 30 μl of 2 x sample buffer (0.125 M-Tris-HCl pH 6.8, 6% SDS, 20% glycerol, 0% 2-mercaptoethanol).

The immunoprecipitated proteins were analysed by SDS-PAGE (Laemmli, 1970) using discontinuous buffer systems and processed for
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COS cells were transfected with p9-gC plasmid, labelled with [35S]methionine and the intracellular proteins were immunoprecipitated with either polyclonal anti-HSV-1 antiserum or with the monoclonal antibody 19S, which recognizes HSV-1 gC (Showalter et al., 1981). As shown in Fig. 1(a), each of these antibodies recognized 120K and 100K to 105K proteins synthesized in COS cells transfected with p9-gC. The recognition of these proteins by specific monoclonal antibodies identifies them as the mature and precursor (pgC-1) forms of gC-1 (Campadelli-Fiume & Serafini-Cessi, 1984).

To show that gC-1 expressed in the transfected COS-1 cells was properly processed and glycosylated, the protein was labelled with [3H]mannose, [3H]galactose in the presence or absence of the glycosylation inhibitors tunicamycin and monensin.

Tunicamycin, a potent inhibitor of protein glycosylation, blocks the first step in the lipid-linked pathway of glycosylation in eukaryotes and allows the synthesis of proteins lacking N-linked oligosaccharides (Elbein, 1987). Monensin, on the other hand, blocks glycosylation and processing steps occurring in the Golgi complex (Tartakoff, 1983). Results shown in Fig. 1(b) show the pattern of the different forms of [35S]methionine-labelled gC-1 synthesized in the absence (lane 1) or in the presence (lanes 2 and 3) of tunicamycin and monensin. The 120K and 100K forms of gC-1 and pgC-1, respectively, were replaced by the 105K and 70K pgC-1 proteins in the presence of tunicamycin (Fig. 1b, lane 2). The sizes of pgC-1 synthesized in the transfected cells in the presence of tunicamycin agreed with the sizes of pgC-1 synthesized in HSV-1-infected cells treated with tunicamycin (Wenske & Courtney, 1983). The observed differences in the sizes of the gC-1 and pgC-1 proteins synthesized in the HSV-1-infected cells (130K and 105K in the absence and 92K and 75K in the presence of tunicamycin, respectively) with the sizes of gC-1 and pgC-1 proteins observed in the transfected cells (120K and 100K in the absence and 105K and 70K in the presence of tunicamycin, respectively), are probably due to the different gel systems used. In infected cells monensin has been reported to inhibit the processing of the high-mannose precursor forms of the HSV-1 glycoproteins to the fully mature product (Wenske et al., 1982; Johnson & Spear, 1982). In the presence of monensin the fully processed 120K pgC-1 protein synthesized in the transfected COS-1 cells was replaced by smaller size proteins of about 100K (Fig. 1b, lane 3). This 100K form of pgC-1 may be the high-mannose precursor form of gC-1 (pgC).

Labelling of the transfected COS cells with [3H]mannose showed that both the 120K and 100K forms of gC-1 and pgC-1 incorporated [3H]mannose (Fig. 1c, lane 4). As expected, in the presence of tunicamycin the gC-1 synthesized was not mannose-labelled (Fig. 1c, lane 5). The incorporation of [3H]mannose into the 100K protein in the presence of monensin (Fig. 1c, lane 6) further suggests that this protein is a high-mannose precursor of gC-1.

Labelling of cells with [3H]galactose showed both the 120K and 105K proteins to be labelled (Fig. 1c, lane 1). The incorporation of [3H]galactose into the 100K pgC-1 protein in the presence of tunicamycin suggested that it could represent pgC-1 protein containing only O-glycosylated oligosaccharides (Fig. 1c, lane 2). As
Fig. 1. (a) Expression of gC-1 in p9gC-transfected COS-1 cells. SDS–polyacrylamide gel analysis of immunoprecipitated [35S]methionine-labelled proteins from COS-1 cells transfected with p91023 vector (lanes 1 and 2) and p9gC-1 plasmid (lanes 3 to 6). Lanes 1, 3 and 4 and lanes 2, 5 and 6 were treated with anti-HSV-1 serum and anti-gC-1 19S monoclonal antibodies, respectively. (b and c) Glycosylation of gC-1 in p9gC-transfected COS-1 cells. SDS–polyacrylamide gel analysis of (b) [3S]methionine-labelled and (c) [3H]galactose- (lanes 1 to 3) and [3H]mannose- (lanes 4 to 6) labelled gC-1, expressed in p9gC-transfected COS-1 cells in the absence [lanes (b) 1, (c) 1 and 4] or in the presence of 3 μg/ml tunicamycin [lanes (b) 2, (c) 2 and 5] or 1 μM-monomesin [lanes (b) 3, (c) 3 and 6]. The proteins were immunoprecipitated with 19S monoclonal antibody.

As expected of O-glycosylated proteins, the presence of monensin blocked the incorporation of [3H]galactose in the gC-1 protein (Fig. 1c, lane 3). The apparent sizes of the mature, precursor and non-glycosylated forms of gC-1 present in the wild-type HSV-1-infected cells, as reported by various workers, are 115K to 130K, 86K to 105K and 70K to 75K, respectively (Frink et al., 1983; Wenske & Courtney, 1983; Wenske et al., 1982; Campadelli-Fiume & Serafini-Cessi, 1984; Holland et al., 1984). Therefore, the observed 120K, 100K to 105K and 70K proteins expressed in transfected COS-1 cells possibly correspond to mature gC-1, precursor pgC-1 and non-glycosylated pgC-1, respectively.

The precursor–product relationship of various forms of gC-1 synthesized in the transfected COS-1 cells in the presence and in the absence of tunicamycin was shown by a series of pulse–chase experiments. The results show that in the absence of tunicamycin the co-translationally glycosylated, high-mannose glycan-containing 100K pgC-1 was formed during the 20 min pulse period and was processed to the fully mature 120K gC-1 protein during the chase periods of 1 and 2 h. In fact, after a 2 h
chase only the mature gC-1 was observed. In the presence of tunicamycin the unglycosylated 70K pgC-1 was predominantly present during the pulse period. The disappearance of this protein was accompanied by the appearance of a pgC-1 protein of 105K during the 1 h and 2 h chase periods (data not shown).

The enzyme endo-H specifically hydrolyses the glycosidic linkage between the two proximal N-acetylglucosamine (GlcNAc) residues linked to the asparagine of the polypeptide containing the high-mannose core oligosaccharide (Tarentino et al., 1974). Thus, only the precursor form of glycoproteins containing high-mannose oligosaccharide show endo-H sensitivity, whereas the processed complex forms of N-linked oligosaccharide chains are resistant (Robbins et al., 1977). Therefore, the endo-H sensitivities of different forms of gC-1 synthesized in the transfected COS-1 cells in the presence and in the absence of these inhibitors were studied. In the absence of any inhibitor only pgC was found to be sensitive to endo-H digestion, giving rise to a 72K protein band (Fig. 2, lane 2). As expected, the 120K mature gC-1 was resistant to endo-H digestion, showing that it was the fully processed mature gC-1 glycoprotein. The pgC-1 synthesized in the presence of monensin was also completely digested by endo-H to the 72K form (Fig. 2, lanes 5 and 6).

However, the pgC-1 proteins synthesized in the presence of tunicamycin were found to be resistant to endo-H digestion (Fig. 2, lanes 3 and 4) and the 80K band present in these two lanes could represent an intermediate of the O-glycosylated pgC-1 protein. The other two protein bands represented the non-glycosylated and O-glycosylated forms of pgC-1 protein (70K and 105K, respectively) and they lacked the N-glycosidic bond required for endo-H sensitivity. Taken together these results showed that the 70K, 100K and 105K forms of pgC-1 synthesized by the transfected COS-1 cells represented the unglycosylated, high-mannose oligosaccharide-containing N-glycosylated and O-glycosylated intermediates of the gC-1 protein. The 120K protein represents the mature form of the gC-1, containing both N- and O-glycosylated residues. The difference between the 72K protein band arising from the endo H digestion of the high-mannose pgC and the unglycosylated 70K pgC-1 can be accounted for by the residual GlcNAc units still attached to the 72K form, as opposed to the totally unglycosylated 70K pgC-1.

Establishment of cell lines constitutively expressing gC-1

In order to avoid the various drawbacks of transient expression systems it was decided to establish cell lines constitutively expressing gC-1, so two different approaches were used. In the first approach a vector, pRK1-4 derived from p91023 (Kaufman, 1985), was used to transfer the gC-1 gene into CHO cells deficient in the dhfr gene product. The second dhfr gene present in pRK1-4 would provide the enzyme and thus transform the CHO cells into a dhfr+ phenotype. The gC-1 gene lacking the endogenous promoter was, therefore, cloned into the unique PstI-EcoRI sites of the vector. The ability of the pRK-gC plasmid to express the gC-1 gene properly was checked by transfecting COS cells. Expression of gC-1 protein was observed in COS cells transfected with pRK-gC in the absence and in the presence of tunicamycin (data not shown). To obtain stable expression of gC-1, dhfr-deficient CHO cells (DUKX-22) were transected with pRK-gC plasmid and the colonies of CHO cells were incubated in medium lacking glycine, hypoxanthine and thymidine (Urlaub & Chasin, 1980) to select the dhfr+ phenotype. A number of dhfr+ colonies were chosen and these were shown to express gC-1 protein.
Fig. 3. Indirect immunofluorescence staining of cells expressing gC-1 protein. Cells were fixed, reacted with rabbit anti-HSV-1 antisera and stained as described in Methods. The immunofluorescence of COS-1 cells transfected with p9-gC DNA (a, c and e) and p91023 DNA (g); CHO-gC cells (i and k) and 3T3-gC cells (n and o) are shown. (b, d, f, h, j, l, n and p) Corresponding phase contrast pictures. Short arrows indicate nuclear membrane staining. (a, c, g, i and m) Internal immunofluorescence observed with permeabilized cells; (e, k and o) surface staining of the cells. Bar marker in (a) represents 50 μm for (a) to (h); bar marker in (l) represents 100 μm.
In the second approach, the recombinant retrovirus vector pDOLMP10 was used to clone the gC-1 gene and transfer it into mouse cell lines. The pDOL-gC plasmid was used to transfect COS cells. As expected, the transfected cells expressed gC-1 proteins that were of a similar size to those produced in COS cells transfected with the p9-gC plasmid. The producer G418-resistant ψ-2 cells were obtained by transfection of ψ-2 cells with pDOL-gC and isolation of antibiotic-resistant cells. 3T3 cells expressing gC-1 protein and the selectable neomycin resistance gene that which confers G418 resistance were generated by infecting 3T3 cells with recombinant DOL-gC virus produced by the producer ψ-2 cells. The transformed 3T3 cell line could express gC-1 protein even after 20 passages.

**Intracellular localization of gC-1**

Since HSV is known to bud from the inner nuclear membrane it is expected that the viral glycoproteins, being the major viral envelope components, will be localized at the nuclear membrane. Earlier studies have shown that HSV glycoproteins are localized in the nuclear fraction of HSV-1-infected cells (Compton & Courtney, 1984). Immunofluorescence studies using monoclonal antibodies also showed that in HSV-1-infected cells gB, gC and gD are located on the nuclear envelope and cell surface (Koga et al., 1986). We have recently shown that gB-1 expressed from cloned DNA in COS cells are targeted to both the nuclear envelope and to the cell surface (Ali et al., 1987).

Indirect immunofluorescence studies showed that in transfected COS cells gC-1 protein was localized in the nuclear envelope (Fig. 3a and b). Diffused perinuclear staining corresponding to the endoplasmic reticulum and Golgi complex was also observed (Fig. 3c and d) and gC-1 was also detected at the cell surface (Fig. 3e and f). The dhfr+/ CHO cell lines and G418R 3T3 cell lines constitutively expressing gC-1 protein also showed the presence of glycoprotein in the nuclear envelope and in the cytoplasm (Fig. 3i, j, m and n). As expected, gC-1 protein was also detected on the surface of the CHO and 3T3 cells expressing this protein (Fig. 3k, l, o and p). Almost all of the CHO and 3T3 cells present in the field were stained with the anti-gC antibody, indicating that the majority of the cells in the CHO-gC and 3T3-gC cell lines are expressing gC-1 protein.

The distribution of gC-1 proteins in the intracellular membranes of COS cells expressing the glycoprotein was determined using a biochemical fractionation procedure (Puddington et al., 1985) that separates nuclei from organelles such as endoplasmic reticulum, Golgi complexes and plasma membranes. Analyses of the labelled proteins present in the nuclear and the post-nuclear membrane fractions showed that about 50% of the total gC-1 protein was associated with the nuclear fraction (data not shown).

**Complement component C3b receptor activity**

Binding of HSV-1-infected cells to erythrocytes coated with the complement component C3b to form rosettes was proved to be due to the specific receptor activity for C3b of gC-1 (Friedman et al., 1984, 1986). In all of these cases the C3b receptor activity of gC-1 was detected in the presence of other HSV-1 gene products. In an effort to ascertain whether the gC-1 expressed from cloned genes was biologically active we determined the C3b binding activities of cells expressing the gC-1 protein. However, in this assay we used human erythrocytes coated with C3b, rather than sheep erythrocytes as used by Friedman et al. (1984). Since treatment with neuraminidase was shown to stimulate the C3b binding activity (Smiley & Friedman, 1985) we tested the rosette forming ability of COS cells infected with HSV-1 before and after treatment with neuraminidase. In agreement with the previous findings we also observed that COS cells infected with HSV-1 formed rosettes with C3b-coated human erythrocytes better after the cells had been treated with neuraminidase (Fig. 4c and d). Control COS cells or COS cells infected with the MP mutant of HSV-1, which lacks gC-1 (Spear, 1976, 1984), did not form rosettes either before or after treatment with neuraminidase (Fig. 4a, b, g and h). Treatment of HSV-1-infected COS cells with anti-HSV-1 antibody prior to the rosette assay, in the absence or in the presence of neuraminidase, did not allow the formation of any rosettes (Fig. 5e and f). These results are in agreement with the previous reports using C3b-coated sheep erythrocytes (Friedman et al., 1984; Smiley & Friedman, 1985) and thus show that human erythrocytes can be used in the C3b receptor binding assay. Results presented in Fig. 5 further show that COS cells transfected with p9-gC and expressing the gC-1 protein formed rosettes only after treatment with neuraminidase (Fig. 4i and j). The two cell lines CHO-gC and 3T3-gC constitutively expressing gC-1 also formed rosettes in the presence of C3b-coated erythrocytes, but only after digestion with neuraminidase (Fig. 4i and n). These results show that gC-1 can bind to C3b in the absence of any other HSV-1 proteins. The sizes of rosettes formed with COS cells were larger than the CHO-gC and 3T3-gC cell lines. This was due to the high expression of proteins from genes cloned in the p91023 vector (Kaufman, 1985). This plasmid is highly amplified in COS cells due to the presence of the SV40 origin of replication. The strong adenovirus major late promoter used, as well as the presence of adenovirus VA gene products, further
increase the level of gene expression in this system. As in COS cells transfected with p91023 without inserts, CHO and 3T3 cell lines transformed with the vectors pRK1-4 and pDOLMP10, respectively, failed to bind C3b-coated erythrocytes either before or after digestion with neuraminidase (data not presented). Furthermore, incubation of the cells with anti-HSV-1 antibody resulted in the complete inhibition of rosette formation (data not shown). Similar results have recently been reported with NIH 3T3 cells transfected with plasmids containing gC-1 or gC-2 genes and the Rous sarcoma virus long terminal repeat (LTR) promoter (Seidel-Dugan et al., 1988).
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Fig. 5. C3b receptor activity of gC-1 protein is located on the extracytoplasmic domain. COS cells were transfected with p9-gCgB or p9-gBgC plasmids. After 40 h the cells were analysed for rosette formation with C3b-coated erythrocytes. (a and b); Cells transfected with p9-gCgB; (c and d) cells transfected with p9-gBgC. (a and c) and (b and d) show rosette formation before and after treatment with neuraminidase, respectively. Bar marker represents 50 μm.

The ability to determine C3b receptor activity of the gC-1 protein expressed from cloned DNA in the absence of any other HSV-1 proteins allowed determination of the structure–function relationship of gC-1 by mutagenesis. As a first step we constructed two hybrid glycoproteins. One, gCgB, contained the ectodomain of gC-1 fused to the transmembrane and cytoplasmic domain of gB-1. In the other hybrid protein, gBgC, the ectodomain of gC-1 was replaced with that of gB-1. Both of these hybrid genes were expressed in COS cells to produce the correct fusion proteins, which were transported to the nuclear and cell surface locations (unpublished). When COS cells expressing either gCgB or gBgC hybrid proteins were assayed for rosette formation only cells expressing the gCgB hybrid glycoprotein were able to form rosettes after digestion with neuraminidase (Fig. 5b). These results show that the C3b binding activity is located on the ectodomain of gC-1.

Discussion

The salient feature of this report is the constitutive expression of the HSV-1 gC gene in mammalian cells using non-HSV promoters. Previously the gC-1 gene could be expressed in an L cell line only after infection with HSV-1 or a gC− HSV-1 MP mutant (Arsenakis et al., 1986). Attempts to express the gC-1 gene by transactivation with an ICP4 gene expressed from a transformed cell line, Z4, were also unsuccessful (Persson et al., 1985). A cell line derived by transfecting the Z4 cell line with a plasmid containing a hybrid gC-1 gene coupled to the gD-1 promoter expressed barely detectable levels of gC-1. However, infection of these cells with the MP strain of HSV-1, which lacks gC-1, resulted in increased expression of the glycoprotein (Rosenthal et al., 1987). In contrast COS cells transfected with p9-gC expressed significant amounts of gC-1 glycoprotein. Thus the adenovirus major late promoter present in the p91023 vector was sufficient for transcription of the gC-1 gene and no HSV-1 function was necessary. The CHO-gC cell line derived from dhfr− CHO cells by using the pRK1-4 vector also constitutively expressed gC-1. Similar constitutive expression of the gC-1 gene in the 3T3-gC cell line was possible by using the murine leukaemia virus LTR as a promoter. However, the level of expression in both of these cell lines was lower than that obtained with transfected COS cells due to a large amplification of genes cloned in the p91023 vector in these cells (Kaufman, 1985). Transfected COS cells showed a similar level of gC-1 protein expression when compared with HSV-1-infected COS cells. However, in the case of transfected COS cells only about 5% of the cells expressed the gC-1 gene. The levels of gC-1 protein produced in CHO-gC-1 and 3T3-gC-1 cell lines were about 2 to 10% of the amount of gC-1 protein expressed in COS cells infected with HSV-1 (data not shown). The availability of such constitutive expression systems allows one to study the biological roles that gC-1 might play in the absence of other HSV-1 proteins. The gC-1 synthesized in these expression systems was processed, glycosylated, targeted and transported intracellularly in a fashion identical to that observed with HSV-1-infected cells. The steps and regulation involved in these events can now be studied in detail by using molecular and cellular biological approaches without infecting the cells with viruses.

The gC-1 protein has been shown to be non-essential for HSV-1 replication in cultured cells (Heine et al., 1974; Holland et al., 1984; Spear, 1984; Homa et al., 1986). However, gC-1 has been implicated in a number of biological activities, including antigenicity (Eberle & Courtney, 1980; Glorioso et al., 1984), cytotoxic T lymphocyte recognition (Eberle et al., 1981; Glorioso et al., 1985; Torseth et al., 1987; Rosenthal et al., 1987) and binding of complement component C3b (Friedman et al., 1984, 1986).

The gC-1 synthesized in both the transient and stable expression systems was antigenically active and recognized by polyclonal and monoclonal antibodies. Cells
expressing gC-1 also showed C3b binding activity and the receptor activity was dependent on the neuraminidase removal of gC-1 sialic acid. The expression systems studied in this report therefore produce biologically competent gC-1 molecules. As a first step in determining the structure–function relationship of the C3b receptor activity of gC-1 we decided to study the role of the different domains of gC-1 in C3b binding by using molecular genetic approaches. Studies with chimeric proteins in which the cytoplasmic and membrane anchor domains of gC-1 were replaced with those from gB-1, or the extra-cytoplasmic domain of gC-1 was switched with that of gB-1, showed that the extra-cytoplasmic domain of gC-1 contained C3b binding activity. The transmembrane and cytoplasmic domains of gC-1 showed no role in this binding process.

Following these experiments Friedman et al. (1989) reported high level expression of gC-1 using a glucocorticoid-inducible promoter. However, the induced gC-1 protein was highly cytotoxic so stable gC-1 expressing clones could not be established. In contrast, the CHO-gC-1 and 3T3-gC-1 cell lines reported here express the gC-1 protein constitutively. Several possible reasons could account for the CHO-gC-1 and 3T3-gC-1 cell lines behaving differently to the cell lines expressing gC-1 using an inducible promoter (Friedman et al., 1989). This may be due to the very high level of expression of gC-1 in the induced system, as compared to the relatively low level of constitutive expression of gC-1 protein by the CHO-gC-1 and 3T3-gC-1 cell lines. Alternatively, over-expression of gC-1 by glucocorticoid stimulations may have resulted in cytotoxicity, which led to cell death and loss of the cell lines expressing gC-1. In contrast, the CHO-gC-1 and 3T3-gC-1 cell lines express a very low level of gC-1, which is not cytotoxic and thus allowed selection of the cell lines. That overexpression of gC-1 caused cytotoxic effects was also observed by us and all attempts to amplify the co-expression of dhfr and gC-1 genes in the presence of methotrexate (Alt et al., 1978; Kaufman & Sharp, 1982) resulted in loss of the CHO-gC-1 cell lines. The presence of glucocorticoid could further increase the cytotoxicity of gC-1, as observed by Friedman et al. (1989). Also, gC-1 protein could be less toxic to CHO and 3T3 cells than to L cells.

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References


Expression of HSV-1 gC


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