Excretion of Non-infectious Virus Particles Lacking Glycoprotein H by a Temperature-sensitive Mutant of Herpes Simplex Virus Type 1: Evidence that gH Is Essential for Virion Infectivity

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SUMMARY

A temperature-sensitive mutant of herpes simplex virus type 1, tsQ26, was shown to contain an amino acid substitution in glycoprotein H (gH). The mutant entered cells efficiently at the non-permissive temperature and replicated to give nearly normal yields of intracellular infectivity. The intracellular virions contained, predominantly, an immature form of gH and no gH was found on the surface of infected cells. Excreted virions were devoid of gH and were not infectious. Virions excreted at the permissive temperature were infectious and contained gH and no loss of gH resulted from incubation of these virions at the non-permissive temperature. The temperature-sensitive phenotype apparently results from the loss of gH from virions during their transport to the cell surface, and since loss of gH is accompanied by loss of infectivity we conclude that gH is an essential component of the infectious virion.

INTRODUCTION

Glycoprotein H (gH) of herpes simplex virus type 1 (HSV-1) is a protein of apparent molecular weight approximately 110000 that has been identified in HSV-1-infected cells and in purified virions using monoclonal antibodies (Showalter et al., 1981; Buckmaster et al., 1984; Richman et al., 1986). The gene for this protein has been mapped, using intertypic recombinants, to the immediate left of the thymidine kinase gene in the prototype orientation of the virus genome (Buckmaster et al., 1984; Gompels & Minson, 1986), and sequencing studies in this region of the genome have revealed an open reading frame that would code for an amino acid sequence of the correct size with the properties of a transmembrane glycoprotein (Gompels & Minson, 1986; McGeoch & Davison, 1986). Although we know nothing of the functions of gH, two lines of evidence suggest that it plays a central role in the virus replication cycle. Firstly, coding sequences homologous to those of HSV-1 gH have been identified in varicella-zoster virus, Epstein–Barr virus (McGeoch & Davison, 1986), human cytomegalovirus (B. Barrell, personal communication) and herpesvirus saimiri (U. Gompels & R. W. Honess, personal communication) and it seems likely that gH, like gB, is ubiquitous among herpesviruses. Secondly, monoclonal antibodies to gH exert pronounced biological effects in vitro; like some gD antibodies they strongly neutralize virus infectivity and inhibit cell fusion by syncytial virus strains (Noble et al., 1983). In addition monoclonal antibodies to HSV-1 gH, unlike most other neutralizing antibodies, inhibit the transfer of virus from infected to uninfected cells during plaque formation by syncytial or non-syncytial virus strains (Buckmaster et al., 1984; Gompels & Minson, 1986) and similar observations have recently been made using antibodies against the varicella-zoster virus homologue of gH (Keller et al., 1987). The properties of antibodies to gH imply roles for gH in virus entry, and in virus release, or in the formation of intercellular junctions between infected and uninfected cells.
normal pattern of protein synthesis was observed at the non-permissive temperature. Marker rescue experiments located the genetic lesion of tsQ26 in an EcoRI to PvuII fragment corresponding to map units 0.301 to 0.304 (Weller et al., 1983). As noted by McGeoch & Davison (1986) this fragment lies within the coding sequence of gH. In this paper we show that tsQ26 contains an amino acid substitution in gH and we investigate the phenotype of the mutant. We find that gH is not expressed on the surface of cells infected by tsQ26, and that virus particles excreted by such cells contain no detectable gH and are not infectious. However, normal levels of infectious virus accumulate within tsQ26-infected cells and these virus particles contain an immature form of gH.

METHODS

Cells and viruses. BHK-21 and HEp-2 cells were grown in Glasgow-modified Eagle's medium supplemented with 10% calf serum and 10% tryptose phosphate broth (ETC). Stocks of KOS and tsQ26 were prepared in BHK cells using an m.o.i. of 0:1 at 37 °C and 33 °C respectively and assayed in BHK cells. All stocks of tsQ26 used in the experiments described gave relative plating efficiencies of less than 5 x 10^-5 at 39 °C relative to the titre at 34 °C. Cultures were maintained at 39 °C by submerging them in a water-bath, and manipulation of these cultures was done in a 37 °C warm room using solutions pre-warmed to 39 °C. Cultures at 34 °C were kept in an incubator and manipulated at room temperature.

Antibodies. VP16 (Vmw65) and glycoprotein D were detected with monoclonal antibodies LP1 and LP14 respectively (McLean et al., 1982; Minson et al., 1986). Antibody LP11 is a murine monoclonal antibody against HSV-1 gH (Buckmaster et al., 1984). Since this antibody will not react with denatured gH (i.e. does not react in Western blots), a polyclonal rabbit antibody was prepared against an Escherichia coli fusion protein containing gH sequences. This was achieved as follows. The EcoRI to SalI fragment from within the gH coding sequence of HSV-1 strain HFEM was cloned into the EcoRI and SalI sites of the trpE vector pHAT11 (Spindler et al., 1984) and the resulting plasmid was used to transform E. coli HB101. Induction of the trp operon by tryptophan starvation and addition of 2-indole-acrylic acid (Spindler et al., 1984) resulted in the synthesis of a protein of approx. M, 80000, a size consistent with the fusion of the trpE gene product to amino acids 270 to 690 of HSV-1 gH. The protein was purified by gel electrophoresis and rabbits were immunized with approximately 300 µg of the protein with Freund's complete adjuvant by intramuscular injection. Subsequent immunizations were done with the same amount of protein and incomplete adjuvant after 3 and 10 weeks. Animals were bled 10 days after the final immunization. The resulting sera reacted in Western blots with a polypeptide of approx. M, 110000 from HSV-1-infected BHK cells, but gave no reaction with extracts of HSV-2-infected cells or uninfected cells. Serum from a single rabbit was used in all the experiments described here and is designated anti-trpE-gH.

Biochemical methods. Nucleotide sequencing was performed by the dideoxy chain termination method using sequences cloned into phage M13mp8 (Sanger et al., 1977; Messing & Vieira, 1982), and with the modifications to the methods described by Bankier & Barrell (1983).

Antigens were detected after acrylamide gel electrophoresis by Western blotting. Following electrophoretic transfer to nitrocellulose, the membrane was blocked for at least 2 h in a solution containing 5% skim milk powder, 1% bovine foetal calf serum (FCS) and 2 mM-KI in phosphate-buffered saline (PBS) (blocking buffer). Antiserum was then added to a final dilution of 1/200 and allowed to react overnight at 4 °C with gentle shaking. The membrane was then washed in three changes of 10 mM-sodium phosphate pH 7.5 containing 0.5% sodium deoxycholate, 0.5% NP40, 1% FCS and 0.1 M-NaCl (wash buffer) for 20 min. The membrane was then soaked in blocking buffer for 30 min before addition of 125I-labelled Protein A to a final concentration of 0.05 µCi/ml. After shaking for 1 h at room temperature the membrane was washed three times for 20 min in wash buffer and subjected to autoradiography.

DNA sequences were detected after agarose electrophoresis as described by Southern (1975) using nick-translated probes (Rigby et al., 1977).

Intracellular cytoplasmic virus was purified from infected BHK cells as follows. A cytoplasmic fraction was prepared by Dounce homogenization followed by removal of nuclei by centrifugation at 2000 g. The supernatant was layered on a 10 ml cushion of 36% sucrose in 10 mM-Tris–HCl pH 8.0 and centrifuged at 25000 r.p.m. in a Beckman SW27 rotor for 2 h. The pellet was resuspended in PBS containing 5% FCS and centrifuged through a 10 to 40% potassium tartrate gradient in PBS plus 5% FCS for 1 h at 25000 r.p.m. in an SW27 rotor. The resulting visible virus band was harvested and pelleted by centrifugation at 200000 g for 1 h.

Radiolabelling of infected cells and immune precipitation of antigen was performed as described in Richman et al. (1986).
**HSV-1 gH is essential for virion infectivity**

**RESULTS**

**TsQ26 contains an amino acid substitution in gH**

The lesion in tsQ26 has been mapped by marker rescue in an EcoRI to PvuII fragment within map units 0.301 to 0.304 (Weller et al., 1983). To confirm the existence of a mutation in gH of tsQ26 the BglII m restriction fragments (map units 0.282 to 0.312) from DNA of tsQ26 and its parent KOS were cloned into the BglII site of pKC7 and the recombinant plasmids were digested with EcoRI and PvuII. The resulting 495 bp fragments, from within the gH coding sequence, were end-repaired using *E. coli* DNA polymerase I Klenow fragment and cloned into the Sinai site of M13mp8. Inserts in both orientations were then sequenced and the resulting KOS sequence compared with that of tsQ26. The sequences, given in Fig. 1, showed the presence of a single base pair substitution in tsQ26. The change would result in the substitution of Trp 244 (TGT) by Cys (TGG), thus confirming the presence of an alteration in gH in mutant tsQ26.

**TsQ26 enters cells normally at the non-permissive temperature**

Since monoclonal antibodies to gH strongly neutralize virus infectivity it is probable that gH is involved in virus entry. To discover whether tsQ26 was defective in entry functions, BHK cells were infected at an m.o.i. of 5 with KOS or tsQ26 at 34 °C or 39 °C. At various times after infection the inoculum was removed, non-penetrated particles were inactivated by treatment of...
Fig. 2. Adsorption and penetration by tsQ26. Monolayers of approximately $10^7$ BHK cells were inoculated with $5 \times 10^7$ p.f.u. HSV-1 KOS or tsQ26 in 2 ml ETC, and incubated at 34 °C or 39 °C. Monolayers and inoculum were pre-warmed to the incubation temperature before addition of inoculum. After various times the inoculum was removed and the monolayers were washed for 2 min with 2 ml 0.1 M-glycine-HCl pH 3.0. The monolayers were then washed twice with ETC, harvested with trypsin and assayed for infectious centres by plaque assay on BHK cells. KOS at 39 °C (■); tsQ26 at 39 °C (□); KOS at 34 °C (●); tsQ26 at 34 °C (○). An additional monolayer was inoculated with KOS but maintained at 4 °C to permit adsorption but not penetration (●).

Fig. 3. Replication and secretion of tsQ26 under one-step growth conditions. Monolayers of approximately $5 \times 10^6$ HEp-2 cells were infected with KOS or tsQ26 at an m.o.i. of 5. After 1 h the inoculum was removed and residual virus inactivated by treating the monolayers for 2 min with 0-1 M-glycine-HCl pH 3.0, before addition of fresh medium. At various times after infection the medium was removed and replaced with fresh pre-warmed medium. After a further 15 min the medium was removed, centrifuged for 5 min at 2000 g to remove infected cells and assayed for released virus (b). At the same time the monolayers were harvested, and the cells were disrupted and assayed for intracellular infectivity (a). ●, ■: KOS-infected cells at 34 or 39 °C respectively. ○, □: tsQ26 at 34 or 39 °C respectively.
the monolayer with 0.1 M-glycine-HCl pH 3.0 for 2 min, and the number of infectious centres was assayed after trypsinization. The results in Fig. 2 show that the number of infectious centres was independent of virus and temperature. When adsorption was allowed to proceed at 4 °C, such that penetration did not occur, the number of infectious centres was reduced by a factor of 10³, demonstrating that virus that had failed to penetrate was effectively inactivated under the conditions used.

Infectious tsQ26 virions accumulate at the non-permissive temperature but are not excreted

Chu et al. (1979) classified tsQ26 as a ‘late’ mutant because it exhibited a normal pattern of protein synthesis at the non-permissive temperature. We obtained similar results but also noted that despite its inability to form plaques at 39 °C, tsQ26 gave high yields of infectivity following high multiplicity infections at this temperature. We therefore examined the ability of tsQ26-infected cells to excrete virus into the culture medium. HEp-2 cells were used for these experiments since these cells excreted wild-type virus more efficiently than BHK cells. HEp-2 cells were infected at an m.o.i. of 5 with KOS or tsQ26 at 34 °C or 39 °C and at various times after infection the medium was removed and replaced with fresh, pre-warmed medium. After 15 min this medium was removed, transferred to ice and assayed for released virus, while at the same time the cells were harvested, disrupted by freezing and thawing and assayed for intracellular virus. The results in Fig. 3 (a and b) show that tsQ26 is similar to wild-type virus in its ability to accumulate within infected cells, but that infectious virus is not released into the culture medium at 39 °C. The very low levels of tsQ26 infectivity found in the culture medium at 39 °C probably represent ‘inoculum background’ in this experiment because there was no significant increase in the rate of virus release between 6 and 12 h after infection, but levels of intracellular virus increased by at least 100-fold during the same period. When the culture supernatant was removed from tsQ26-infected cells cultured at 34 °C and incubated at 39 °C for 15 min no loss of infectivity resulted (data not shown). Failure of tsQ26-infected cells at 39 °C to release infectivity into the medium is not therefore due to inactivation after release. The intracellular infectivity that accumulated in tsQ26-infected cells at 37 °C was of mutant phenotype, having the same relative plaquing efficiency at 34 °C and 39 °C as the input inoculum. We conclude that tsQ26 exhibits normal intracellular growth at the non-permissive temperature, but is defective in excretion of infectious virus.

Excreted tsQ26 virions are not infectious and lack gH

To find whether tsQ26 virions were excreted in a non-infectious form at 39 °C, 10⁸ HEp-2 cells were infected at 39 °C with KOS or tsQ26 at an m.o.i. of 5 and the medium was replaced after 8 h. Virus that was excreted between 8 and 12 h post-infection was then harvested, and to avoid problems that might result from degradation of particles during prolonged incubation at 39 °C, the medium was harvested and replaced at 30 min intervals over the 4 h harvest period. The pooled harvests were centrifuged at low speed to remove any cells and cell debris, and virus particles were then pelleted by centrifugation at 25000 r.p.m. in an SW27 rotor for 1 h. The resulting (invisible) pellets were resuspended and divided into two equal parts. DNA was extracted from one part and subjected to restriction enzyme digestion and Southern blotting, while the second part was subjected to polyacrylamide gel electrophoresis and Western blotting. The results of these experiments are given in Fig. 4 (a and b). On the basis of viral DNA content, tsQ26-infected cells excreted at least as many, and probably more, virus particles at 39 °C than did KOS-infected cells. Similarly, monoclonal antibodies LP14 and LP1 detected approximately equivalent amounts of gD and Vmw65 in high speed pellets from medium harvested from tsQ26- or KOS-infected cells. In contrast no gH was detected in tsQ26 pellets. We were unable to obtain adequate yields of extracellular virus from cultures infected with KOS or tsQ26 to measure particle numbers by electron microscopic methods, but the results strongly imply that at 39 °C tsQ26-infected cells excrete non-infectious virions that lack gH. The results shown in Fig. 4 (a, b) could be explained if extracellular gH was rapidly broken down at 39 °C such that the molecule was degraded after the excretion of otherwise normal virions. This possibility was eliminated by harvesting medium from cells infected with tsQ26 at 34 °C and incubating the medium at 39 °C.
Fig. 4. Properties of released virions. HEp-2 cells (10⁸) were infected with tsQ26 (lanes 1) or KOS (lanes 2) at an m.o.i. of 5 and incubated at 39 °C for 8 h. Released virus was harvested over the subsequent 4 h period by removal and replacement of medium at 30 min intervals. The supernatant medium was centrifuged at 2000 g to remove cells or cell debris and the pooled harvest was centrifuged at 150000 g for 1 h to pellet virus particles. The pellets were resuspended in 10 mM-Tris-HCl, 1 mM-EDTA pH 8.0. (a) Samples of the resuspended pellets were electrophoresed in 10% SDS-polyacrylamide gels, the products transferred to nitrocellulose and reacted with antibodies to detect the presence of gH, gD and VP16 (Vmw65). (b) Further samples were treated with 0.5% SDS and 1 mg/ml Pronase for 15 min and extracted with phenol in the presence of 100 µg calf thymus DNA. DNA was precipitated from the aqueous phase with ethanol after addition of NaCl to 0.1 M. The resulting DNA preparations were digested with BgII, electrophoresed in 1% agarose and transferred to nitrocellulose. The relative virus DNA content was then assessed by hybridization with a nick-translated BgII m probe.

for 30 or 60 min. The virus particles were then pelleted, subjected to SDS-PAGE and Western blotting, and analysed for gH content. As shown in Fig. 5, tsQ26 virions excreted at 34 °C contain gH and no detectable loss results from subsequent incubation at 39 °C.

TsQ26-infected cells are deficient in mature gH

Although cells infected with tsQ26 at 39 °C excrete non-infectious virions without gH the infectious virions that accumulate within cells under the same conditions (Fig. 3) clearly contain gH because their infectivity is neutralized by antibody LP11, which is specific for gH (data not shown). In order to examine the form of gH in tsQ26-infected cells and in intracellular virions, 10⁸ HEp-2 cells were infected at an m.o.i. of 5 with KOS or tsQ26 and after 12 h at 39 °C the cytoplasmic virus was purified. Extracts of infected cells and purified virus were subjected to electrophoresis, transferred to nitrocellulose and reacted with anti-trpE-gH antibody. The result is shown in Fig. 6(a). The antibody detects two protein species of approx. M₆, 110000 which correspond to the two species immunoprecipitated by antibody LP11 (Buckmaster et al., 1984),
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Fig. 5. Stability of gH in secreted tsQ26 virions. HEp-2 cells (10⁶) were infected at an m.o.i. of 5 with KOS (lanes 1, 3, 5) or tsQ26 (lanes 2, 4, 6) and the medium harvested after 12 h. Aliquots of the harvested medium were incubated at 39 °C for 0 (lanes 1, 2), 30 (lanes 3, 4) or 60 (lanes 5, 6) min and pellets containing extracellular virus were then prepared and compared for their gH and VP16 content as described in the legend to Fig. 4.

and pulse-chase experiments (Fig. 6b) indicate that the higher mobility form is a precursor of the lower mobility form. Only the latter (mature gH) is found in extracellular KOS virions (Fig. 4). The results given in Fig. 6 show that tsQ26-infected cells at 39 °C contain greatly reduced amounts of mature gH and that the gH found in intracellular KOS virions is mostly of the mature form while gH in tsQ26 virions is almost exclusively of the precursor form. The fact that intracellular tsQ26 virions containing immature gH are infectious is consistent with reports that glycoprotein maturation is not essential for infectivity (Campadelli-Fiume et al., 1982; Johnson & Spear, 1982). We were unable to examine directly the synthesis and processing of gH in tsQ26-infected cells due to the inefficiency of immunoprecipitation by antibody LP11. Several attempts were made to immunoprecipitate gH from tsQ26-infected cells after pulse-labelling, but no signal was detected. Similar experiments with KOS-infected cells were successful but the signal was very weak (Fig. 6d). Despite this failure the results shown in Fig. 6 suggest a defect in the synthesis, or in the stability, of the mature form of tsQ26 gH at the non-permissive temperature. This conclusion is consistent with the results of immunofluorescence experiments: LP11 entirely failed to react with the surface of cells infected with tsQ26 at 39 °C, while KOS-infected cells gave strong surface fluorescence (data not shown).

DISCUSSION

The phenotype of tsQ26 is unusual in that, despite its inability to form plaques at the non-permissive temperature, the virus enters cells efficiently and nearly normal yields of intracellular infectious virus accumulate after high multiplicity infection. TsQ26-infected cells, and the resulting intracellular virions, contain predominantly an immature form of gH. Excreted tsQ26
Fig. 6. Analysis of gH in intracellular virus. HEp-2 cells (10⁶) were infected with KOS (lanes 1) or 
tsQ26 (lanes 2) at an m.o.i. of 5. After 12 h the cells were harvested and a cytoplasmic fraction was 
purified from the cytoplasmic fraction as described in Methods. Samples of the cytoplasmic fraction (a) or purified virions (b and c) were electrophoresed in 7.5% SDS-acrylamide gels, transferred to nitrocellulose and reacted with anti-trpE-gH serum to detect gH and a 
related polypeptide (pgH). To establish the precursor-product relationship of the species detected in 
(a), (b) and (c), a pulse–chase experiment was performed (d). HEp-2 cells (10⁶) were infected with KOS 
at an m.o.i of 5 and after 6 h the medium was replaced with methionine-free medium containing 50 μCi 
[³⁵S]methionine (Amersham). After 30 min the cells were harvested (pulse); lane 3 or the medium was 
replaced with medium containing methionine and incubated for a further 4 h before the cells were 
harvested (chase; lane 4). Antibody LP11 was used to prepare immune precipitates from cell lysates as 
described in Richman et al. (1986).

Virions, on the other hand, are devoid of gH and are non-infectious, and no gH can be detected 
on the surface of infected cells. However, the fully processed form of tsQ26 gH is not intrinsically 
unstable at the non-permissive temperature because 39 °C incubation of tsQ26 virions excreted 
at 34 °C results in no loss of gH. An unambiguous explanation of these observations requires an 
examination of the kinetics of synthesis, processing and turnover of gH in tsQ26-infected cells, 
but this has proved impossible with the reagents presently available. Nevertheless our 
observations are most simply interpreted by supposing that gH is synthesized normally in tsQ26-
infected cells at 39 °C and is incorporated into virions. The subsequent maturation of the virion 
glycoproteins during the transfer of the virions from the perinuclear space via the Golgi 
apparatus to the cell surface involves subcellular compartments in which gH of tsQ26 is unstable 
and is degraded at 39 °C. A similar fate presumably befalls the molecule during its transfer from 
the endoplasmic reticulum to the cell surface. This reasoning would account for the deficiency of 
the mature form of gH in tsQ26-infected cells and the absence of all forms of gH on the cell 
surface or in the excreted particles. An analogous phenomenon has been proposed to account for 
the behaviour of a ts mutant of vesicular stomatitis virus that synthesizes virus glycoprotein at 
the non-permissive temperature, but which excretes bald particles that lack the globular domain 
of the glycoprotein (Metsikko & Simons, 1986). It is worth noting that the amino acid 
substitution in gH of tsQ26 would result in a free sulphhydrly group that is likely to be susceptible 
to oxidizing environments.
An alternative explanation for the lack of gH on excreted tsQ26 virions would be that egress of HSV involves a process of membrane exchange such that extracellular virions have acquired an envelope derived from plasma membrane, which in tsQ26-infected cells at 39 °C lacks gH. On the basis of electron microscopic studies it has been suggested that enveloped herpesviruses in the perinuclear space are released into the cytoplasm as nucleocapsids as a result of fusion of the virion envelope with the outer lamella of the nuclear envelope, and that egress results from subsequent re-envelopment at cytoplasmic membranes (Siminoff & Menefee, 1966). However, the weight of biochemical and electron microscopic evidence favours a single envelopment process followed by egress via a process of reverse endocytosis (reviewed by Spear, 1984), and on this basis the phenotype of tsQ26 is more readily explained by degradation of gH during transport of the enveloped particle to the cell surface.

Whatever the mechanism involved, the loss of gH in excreted tsQ26 virions is accompanied by loss of infectivity, implying that gH is an essential virion component in vitro, a conclusion consistent with the efficient neutralizing activity of antibodies to gH. It is now apparent that many of the defined HSV-1 glycoproteins are not essential for growth in vitro. Mutations in gC do not affect viability (Heine et al., 1974; Holland et al., 1984), and more recent evidence shows that gE, gG and gI are also dispensable in vitro (Longnecker & Roizman, 1986, 1987; Longnecker et al., 1987; Weber et al., 1987). To date only gB, which is highly conserved in the herpesviruses, has been shown unambiguously to be essential for infectivity (Little et al., 1981; Cai et al., 1987). The data presented here show that gH, which is also conserved in the herpesvirus group, is also an essential component of the infectious virion.

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