Identification of the equine herpesvirus type 1 glycoprotein 17/18 as a homologue of herpes simplex virus glycoprotein D

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The DNA sequence of the equine herpesvirus type 1 (EHV-1) gD gene homologue has been determined for the strain Ab1 and compared with previously published sequences. A portion of the gene has been located to a region of the genome which also encodes homologues of the herpes simplex virus type 1 genes for gE and gI and is known to encode an epitope of the virion protein gp17/18. Analysis of the EHV-1 strain Kentucky A (KyA) by DNA hybridization showed the presence of a gD gene homologue and established the absence of genes for gI and gE. Western blot analysis, however, showed that KyA virus particles contain gp17/18, thus indicating that this protein is encoded by the gD gene homologue. The KyA gp17/18 was found to be smaller than that detected in other strains and this is accounted for by a frameshift mutation in the KyA sequence relative to Ab1. The mutation in the KyA strain results in an altered C-terminal sequence and could explain the apparent structural differences suggested by the activities with monoclonal antibodies (MAbs). We have also expressed part of the Ab1 gD gene as a fusion protein with glutathione S-transferase in Escherichia coli and shown that this reacts with the MAb 5H6 originally used to map gp17/18. These experiments establish that gp17/18 is encoded by the gD gene homologue.

Introduction

The glycoproteins of herpesviruses are potentially important in inducing cellular and humoral immunity in the host. For this reason much effort has been made to identify the glycoprotein genes and their products in several herpesviruses including herpes simplex virus type 1 (HSV-1) (Frink et al., 1983; Gompels & Minson, 1986; Highlander et al., 1988), pseudorabies virus (PRV) (Robbins et al., 1985) and varicella-zoster virus (VZV) (Keller et al., 1986; McGeech & Davison, 1986). The glycoproteins of equine herpesvirus type 1 (EHV-1) are less well characterized than those of some of the other herpesviruses, although some of their genes have been located (Allen & Yeargan, 1987) and sequenced (Allen & Coogle, 1988; Whalley et al., 1989; Audonnet et al., 1990, Elton et al., 1991b). Initially only one glycoprotein (gp17/18) was mapped to the short unique (Us) region of EHV-1 DNA (Allen & Yeargan, 1987). This is in contrast to other alphaherpesviruses analysed, some of which have several glycoprotein genes in Us. HSV-1 has at least four glycoprotein genes in this region, coding for gG, gD, gI and gE (McGeoch et al., 1985; Frame et al., 1986; Longnecker et al., 1987); PRV also has four genes, coding for gX, gp50, gp63 and gI (Rea et al., 1985; Petrovskis et al., 1986a, b) and VZV has two, encoding gpLV and gpI (Davison, 1983). DNA sequence analysis of EHV-1 has since confirmed the presence of three potential glycoprotein genes in the region to which gp17/18 was mapped (Audonnet et al., 1990; Elton et al., 1991a, b). Similar analysis of the Kentucky A (KyA) strain of EHV-1 has shown that no genes are located between the gD gene and the homologue of HSV-1 US9 (Flowers et al., 1991), a region in which Audonnet et al. (1990) and Elton et al. (1991a, b) have located genes homologous to those of HSV-1 gI and gE. This suggests that either a gene rearrangement has occurred or that the gE and gI gene homologues have been deleted in the KyA strain. It has been shown previously that gE and gI of HSV-1 are non-essential for virus growth in tissue culture (Longnecker et al., 1987); the extent of similarity between these glycoproteins and their counterparts in EHV-1 (Elton et al., 1991a) would suggest that they may have similar functions.

The aims of this study were to analyse the genome of KyA to confirm the presence or absence of genes for gE and gI and to compare the structure of the Us region with that of other strains of EHV-1. The nucleotide sequence of the entire gD gene of strain Ab1 was
determined as the previously published sequences (Audonnet et al., 1990; Flowers et al., 1991) differ in the predicted amino acid sequence at the C terminus. These experiments, in conjunction with Western blot analysis and prokaryotic gene expression, were designed to establish which of the three candidate genes in EHV-1 Us codes for gp17/18.

**Methods**

**Cells and media.** RK13 cells were grown as monolayers in autoclavable Dulbecco’s modified Eagle’s medium containing 10% calf serum.

**Viruses.** EHV-1 strains Ab1 and Ab4, both low passage isolates, were kindly provided by Dr J. Mumford, Animal Health Trust, Newmarket, U.K. KyA, a high passage laboratory strain, was kindly supplied by Dr Dr A. Davison, Institute of Virology, Glasgow, U.K. All strains were grown in RK13 cells and virus stocks were prepared as previously described (Meredith et al., 1989). DNA was extracted from cell-associated virus using the method of Robbins et al. (1988).

**Southern blotting and DNA hybridization.** Virus DNA was digested with restriction endonucleases as recommended by the supplier (Gibco-BRL). Approximately 1 μg samples of DNA were loaded onto 0.8% agarose gels, subjected to electrophoresis at 8 V per cm in E buffer (0.04 M-Tris base, 0.002 M-EDTA, adjusted to pH 7.9 with glacial acetic acid) and then stained in ethidium bromide. DNA fragments were transferred to a GeneScreen hybridization membrane (New England Nuclear) using vacuum blotting (Hybaid). The blots were air-dried, baked at 80 °C and hybridized with radioactively labelled DNA probes (Richby et al., 1977) using the membrane manufacturer’s recommended procedures.

**Western blotting.** Virus proteins were separated on 10% acrylamide gels (Laemmli, 1970) cross-linked with N,N′-diallyltartardiamine. Samples were prepared by boiling purified virus for 3 min in a loading buffer containing 0.125 M-Tris-HCl pH 6.8, 2% SDS, 0.1% bromophenol blue and 10% glycerol, either with or without the addition of DTT to 5 mM. Prokaryotic fusion proteins were prepared by centrifugation of 1.5 ml of induced or uninduced culture for 1 min in an Eppendorf centrifuge, washing the pellet in PBS and then resuspending the pellet in 300 μl PBS. An aliquot was mixed with loading buffer containing DTT as described above. Proteins were transferred from acrylamide gels to nitrocellulose as described by Towbin et al. (1979). Ascitic fluid containing the monoclonal antibody (MAb) SH6, (0-04 μg/ml) to gp17/18 gene, was diluted 1/500 in PBS prior to use. Binding was detected using sheep anti-mouse IgG–peroxidase conjugate (Dako) diluted to 1/1000. The peroxidase substrate was 0.6 mg/ml chloronaphthol, 0.01% H2O2 in PBS.

**DNA sequencing.** Recombinant plasmids, containing EHV-1 DNA cloned into pUC18, were constructed using standard techniques (Maniatis et al., 1982). Overlapping deletions were prepared using unidirectional digestion with exonuclease III (Henikoff, 1984), recircularized, then used to transform *Escherichia coli* strain TG1. Plasmid DNA was then sequenced using T7 DNA polymerase according to the manufacturer (Pharmacia). Nucleotide and derived amino acid sequences were analysed using DNASIS and PROSIS software packages (Hitachi).

**Results**

**Comparison of gD sequences from Ab1 and KyA**

The presence in EHV-1 of a gene homologous to HSV-1 gD was confirmed by sequence analysis of the DNA upstream of the gI and gE genes (Audonnet et al., 1990; Elton et al., 1991b). The complete nucleotide sequence of the EHV-1 strain Ab1 gD homologue was obtained from both strands of the DNA by sequencing a 1602 bp Smal to HindIII region from the Us region of the genome (GenBank accession number M60946). This sequence was almost identical to that obtained by Audonnet et al. (1990) for the Kentucky D (KyD) strain of EHV-1, although there were a number of differences in the region upstream of the predicted coding sequence of gD. However, comparison of the Ab1 sequence with that published for KyA by Flowers et al. (1991) identified a frameshift at nucleotide 1798 in the KyA sequence, which was due to a loss of two A residues. The derived amino acid sequences for Ab1 and KyD were identical, with the exception of a transition at position 195 from glutamine in Ab1 to arginine in KyD, and the features have been described for KyD by Audonnet et al. (1990). The amino acid sequence for the KyA gD homologue was ten amino acids shorter than that of Ab1 and the terminal 13 amino acids were different, as shown in Fig 1. The difference between the C termini of these polypeptide sequences resulted in a reduction in the predicted Mr of the primary translation product from 45.2K for Ab1 or KyD to 44.0K for KyA. From these data it can be predicted that the gD homologue of the KyA strain would be 1.2K smaller than that of the other two strains. The frameshift also resulted in the appearance of an extra cysteine residue which is predicted to fall in the cytoplasmic portion of the glycoprotein.

**Analysis of the glycoprotein genes in the Us region of the KyA strain of EHV-1**

A comparison of the published restriction analysis data for the KyA strain of EHV-1 with that of other strains for which data are available shows that KyA has a shorter Us region (Whalley et al., 1981; Henry et al., 1981; Elton et al., 1991a). Fig. 2 shows a comparison of the arrangement of the Us regions of strains Ab1 (Elton et al., 1991a, b) and KyA (Flowers et al., 1991). The data suggest that KyA may have lost the genes for gE and gI. To investigate this possibility, Southern blot analysis was carried out on KyA genomic DNA digested separately with the enzymes BamHI and EcoRI and compared with DNA from the EHV-1 strains Ab1 and Ab4, the latter of which contains a deletion of about 250 bp in Us (Fig. 3).
Glycoprotein D of EHV-1

The digested DNA was blotted onto a nylon membrane and probed sequentially with 32P-labelled EHV-1 DNA probes comprising total Ab1 DNA, gE coding sequence, gI coding sequence or gD coding sequence.

Fig. 3(a) shows that the KyA DNA hybridizes to the total Ab1 probe. Comparison of the KyA profiles in Fig. 3(a) (lanes 1 and 5) with the other strains (lanes 2 to 4 and 6 to 8) shows that the KyA strain differs extensively from the others. This is particularly evident with the EcoRI profiles (lanes 5 to 8). Fig. 3(b) clearly shows that whereas Ab1 and Ab4 contain DNA which hybridizes with the EHV-1 gE probe, the KyA DNA shows no hybridization at all, indicating that this gene is not present in this strain. As expected from the map location of the gE and gI gene homologues (Elton et al., 1991a), both probes hybridize to the BamHI D and EcoRI B and C fragments, as defined by the restriction maps of Whalley et al. (1981). The Southern blot was then probed with a fragment of DNA encoding the complete gD sequence (Fig. 3(d)). This blot shows that all the strains contain DNA to which the gD probe hybridizes, as expected from DNA sequence analysis. The BamHI fragment to which the gD probe hybridizes in KyA is, however, considerably smaller than that found in the other strains. This confirms the smaller size of the BamHI fragment in the centre of the KyA U9 region from the EHV-1 restriction map of Henry et al. (1981), compared to that of Whalley et al. (1981). Analysis of the EcoRI fragments (lanes 5 to 8) shows that in KyA, Ab1 and plaque-purified Ab4 the gD probe hybridized to three fragments, all of which were smaller in KyA. In Ab1 these bands correspond to EcoRI B, C and K. The additional smaller fragment seen in Fig. 3(d), compared to (b) and (c), hybridizes to the gD probe because this gene spans the EcoRI site within the BamHI D fragment. Lane 8 of Fig. 3(d) shows that the non-plaque-purified isolate of Ab4 contains a mixed population of molecules in approximately equal proportions, as the gD probe hybridizes to a doublet band corresponding to the single EcoRI K fragment of Ab1. This is the result of a deletion of approximately 250 bp within the BamHI D fragment. The upper band of the doublet is the same size as the EcoRI K fragment in Ab1, and the single band in the
plaque-purified Ab4 preparation is the same size as the lower band of the doublet, indicating that this isolate contains only DNA with the deletion in Us.

Confirmation of the identity of the product of the gD gene homologue

It has already been shown that the gene for gp17/18 maps to a 4.7 kb BamHI to EcoRI fragment (Allen & Yeargan, 1987) in which we, and others, have located the genes for gE and gI homologues (Audonnet et al., 1990; Elton et al., 1991 a, b). We have also shown that this fragment of DNA contains a significant portion of the gD gene homologue. To identify the gene coding for gp17/18 we have used Western blot analysis of purified virus from EHV-1 strains KyA, Abl and Ab4 and a MAb, 5H6, originally used to map the gene for gp17/18 (Allen & Yeargan, 1987). Fig. 4 shows the reaction of the antibody on purified virus proteins under both reducing and non-reducing conditions. In lanes 1 to 3, which contain samples treated by boiling without DTT, bands are clearly present in all three lanes showing that all three strains contain gp17/18. In Ab1 and Ab4 (lanes 1 and 2) 5H6 reacts with a band of 50K; with KyA there is a weak reaction with a similar band, but a much stronger band of 102K is also present. In lanes 4 to 6, containing reduced samples, 5H6 reacts with a species of 54K in Ab1 and Ab4 and a slightly smaller protein of 53K in KyA.

Fusion protein expression

Part of the gD gene of EHV-1 (nucleotides 1071 to 1602) was subcloned into the prokaryotic expression vector pGEX1 (Smith & Johnson, 1988). Ligation of DNA fragments, in frame, into this vector allows synthesis of foreign proteins fused to the C terminus of glutathione S-transferase. Expression is isopropyl β-D-thiogalactopyranoside (IPTG)-inducible, controlled by the lacZ promoter. Recombinant plasmids were constructed which had the gD sequence in either orientation; the fragment was inserted into the EcoRI site of pGEX1 and corresponded to the 3' end of the gene located within the 4.7 kb BamHI–EcoRI fragment to which gp17/18 had been mapped (Allen & Yeargan, 1987).
Western blot analysis of bacterial lysates from induced and non-induced cultures of *E. coli* harbouring the recombinant plasmids was performed (Fig. 5), using the anti-gp17/18 MAb 5H6. Fig. 5 shows that this antibody reacted with a polypeptide of approximately 45K in cultures containing pDE405/2 and pDE405/4 (two independently isolated plasmids which gave identical restriction enzyme profiles, indicating that the gD fragment was in the correct orientation for expression), but gave no reaction with pDE406 (a plasmid containing the gD fragment in the reverse orientation for expression). Expression of the fusion protein was significantly enhanced by the IPTG induction. The predicted M, of the glutathione S-transferase–gD fusion protein is 44K which is in agreement with the observed data. Together with the hybridization data, this provides conclusive evidence that gp17/18 is a homologue of HSV-1 gD.

**Discussion**

The gp17/18 gene was originally mapped to the Us region of the EHV-1 genome by Allen & Yeargan (1987). We, and others, have sequenced the DNA from this region (Audonnet *et al.*, 1990; Elton *et al.*, 1991a, b) and have located complete open reading frames for gI and gE gene homologues and part of an open reading frame potentially coding for a gD gene homologue. We have completed the sequence of the remaining portion of the putative EHV-1 gD gene for the strain Abl1 and shown that it shares significant identity with the gD of HSV-1. The derived amino acid sequence of the gD homologue was almost identical to that of KyD (Audonnet *et al.*, 1990) but differed significantly from the C terminus of KyA gD, suggesting that the latter may be atypical of EHV-1 strains. Analysis of the strain KyA using Southern blot analysis and DNA hybridization has confirmed that this strain has a smaller BamHI fragment in the centre of Us and lacks the genes for the EHV-1 gI and gE homologues. Hybridization studies have shown that the majority of natural isolates of this virus contain a fragment whose size is indistinguishable from that of the BamHI D fragment of Abl1 (data not shown), implying that they probably do contain genes for gI and gE. The result for KyA indicates that these genes are not essential for virus growth in tissue culture, which is known to be the case in HSV-1 (Longnecker *et al.*, 1987). This may corroborate the suggestion that these glycoproteins have a role in viral evasion of host immune response via Fc-receptor complex formation (Adler *et al.*, 1978; Bell *et al.*, 1990; Hanke *et al.*, 1990). As the KyA strain has been passaged extensively in tissue culture (Perdue *et al.*, 1974) it is possible that these genes have been lost through a deletion mutation arising in cell culture. This is consistent with these glycoproteins having a role only in natural infection and there being no selective pressure to maintain these genes in cell culture.

Analysis of the structural proteins of KyA using MAb 5H6 has confirmed the presence of gp17/18. This provides strong evidence that this glycoprotein is a product of the gD gene homologue, as this is the only glycoprotein gene present in KyA in the region to which gp17/18 was mapped. The difference in electrophoretic mobility of gp17/18 in KyA and Abl can be explained by the fact that the Abl strain, as with the KyD strain (Audonnet *et al.*, 1990), has a gp17/18 that is 10 amino acid residues longer than that found in the KyA isolate. The difference in both length and the amino acid sequence at the C termini could account for the difference in M, of 1-2K that was seen on the Western blots. In the absence of reducing agent KyA virus particles contained a protein with an apparent M, of 102K, a size consistent with the formation of a gD dimer. The C terminus of the KyA gD contains an additional cysteine residue, compared to Abl, which may play a part in the apparent dimerization seen on Western blots of purified KyA. The complex was disrupted only by the addition of DTT and not by boiling, as both non-reduced and reduced samples were heated prior to electrophoresis, consistent with the involvement of disulphide bridges. This may reflect the form in which the KyA gD homologue is found in mature virus particles or may result from the method used to prepare samples for electrophoresis. This is currently under further study.

The expression of the C-terminal portion of the gD gene in *E. coli* followed by the reaction of the product with MAb 5H6 confirmed that gp17/18 is encoded by the gD gene homologue. Other studies carried out on gp17/18 have shown that this glycoprotein shares similar characteristics with gD of HSV-1, implying that in addition to sequence homology these glycoproteins may also have a similar function. We have previously shown that anti-gp17/18 MAbs can induce passive immunity to EHV-1 in a small animal model (Stokes *et al.*, 1991), indicating that this glycoprotein carries epitopes important for recognition by the host immune system. We have also shown that antibodies to gp17/18 neutralize virus infectivity and inhibit virus penetration (Whittaker *et al.*, 1992). The C-terminal portion of HSV-1 gD may be important in the induction of a virus-neutralizing antibody response as three peptides from this region were successfully used to raise neutralizing antisera (Strynadka *et al.*, 1988). It is possible that the EHV-1 gD fusion protein described here may induce a neutralizing response when inoculated into a small animal model, because this protein contains the sequences equivalent to the HSV-1 peptides used by Strynadka *et al.* (1988). Studies are now in progress to evaluate the potential of
the EHV-1 gD fusion protein and other recombinant forms of this glycoprotein to stimulate a host immune response.

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