Characterization of glycoprotein B of the gammaherpesvirus equine herpesvirus-2

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Twenty-two monoclonal antibodies (MAbs) were generated to the gammaherpesvirus equine herpesvirus-2 (EHV-2). Using Western blot analysis, eight MAbs recognized an Escherichia coli glutathione S-transferase (GST)–glycoprotein B (gB) fusion protein and, using overlapping GST–gB fusion proteins, a neutralization epitope was mapped to amino acids 29–74. One of the gB-specific MAbs was used to characterize the glycosylation and kinetics of synthesis of EHV-2 gB. EHV-2 gB is synthesized as a 97 kDa polypeptide that is co-translationally modified to a 130 kDa high-mannose precursor that forms a 260 kDa dimer shortly after synthesis. Each 130 kDa precursor is endoproteolytically cleaved to disulphide-linked subunits of 75 and 58 kDa prior to further processing to complex oligosaccharide-containing subunits of 89 and 65/62 kDa. The 89 and 65/62 kDa subunits of EHV-2 gB contain 39 and 17 kDa of N-linked oligosaccharides, respectively, and do not contain any O-linked oligosaccharides. Western blot analysis of purified EHV-2 virions established that gB exists as a 320 kDa dimer in the virion envelope.

Introduction

Equine herpesvirus 2 (EHV-2) is a slow-growing, highly cell-associated virus that has been linked with respiratory disease, conjunctivitis and general malaise in the horse (Blakeslee et al., 1975; Jolly et al., 1986; Palfi et al., 1978; Studdert, 1971). EHV-2 was originally classified as a betaherpesvirus but has recently been classified based on partial DNA sequence analysis as a member of the Gammaherpesvirinae (Telford et al., 1993). Subsequently, the complete sequence of the EHV-2 genome was determined and this placed EHV-2 in the Rhadinovirus genus on the basis of closer overall genetic similarity to herpesvirus saimiri (HVS) than to Epstein–Barr virus (EBV) (Telford et al., 1995). Additionally, EHV-2 establishes latent infection in B lymphocytes, a characteristic of some members of the Gammaherpesvirinae (Drummer et al., 1996).

The presence of the gene for glycoprotein B (gB) is highly conserved among members of the family Herpesviridae and protein homologues have been identified for all the herpesviruses. Among the subfamilies of herpesviruses, gB has been shown to be an abundant envelope glycoprotein of the alpha and betaherpesviruses and a major target for the immune response (Britt & Mach, 1996; Pereira, 1994). By comparison, the gB homologues of the gamma-1 herpesvirus EBV (gp110) and the unclassified murine herpesvirus 68 (MHV-68) are retained in the infected cell, contain only high-mannose oligosaccharides, and are not cleaved into two subunits (Gong & Kieff, 1990; Herrold et al., 1996; Stewart et al., 1994). Sequencing of the genomes of EBV, HVS, MHV-68, Kaposi’s sarcoma herpesvirus and bovine herpesvirus-4 (BHV-4) has identified gB homologues (Albrecht et al., 1992; Baer et al., 1984; Lomonte et al., 1997; Stewart et al., 1994; Telford et al., 1995). However, of the gamma-2 herpesviruses, only BHV-4 gB has been characterized in detail (Lomonte et al., 1997). BHV-4 gB is found as an envelope glycoprotein and undergoes proteolytic cleavage by a cellular protease to produce a disulphide-linked protein with subunits of 128 and 58 kDa and an apparent overall molecular mass of 210 kDa. The 128 kDa subunit of BHV-4 contains both N-linked and O-linked oligosaccharides while the 58 kDa subunit apparently contains only N-linked oligosaccharides.

The nucleotide sequence of EHV-2 gB has been determined (Telford et al., 1995) but its physical characteristics have only been partly investigated (Agius et al., 1994). Using rabbit antisera made to an Escherichia coli-derived glutathione S-transferase (GST) fusion protein, Agius et al. (1994) determined

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that EHV-2 gB was 64 kDa in size but no other subunits of gB were identified. In this paper, we further characterize EHV-2 gB using a panel of MAbs and show that EHV-2 gB consists of two disulphide-linked subunits that exist as a homodimer in the viral envelope and that the extreme N terminus of the protein contains linear neutralizing epitopes.

Methods

■ Virus and cell culture. EHV-2 strain 86/67 (EHV-2.86/67) was propagated in equine foetal kidney (EFK) monolayer cell cultures. Infected cells were maintained in minimum essential medium (MEM; Gibco BRL) containing Earle’s salts, l-glutamine and non-essential amino acids, and supplemented with 1% foetal bovine serum (FBS), 50 µg/ml ampicillin, 0.13 M NaHCO₃ and 0.015 M HEPES (Sigma) at pH 7.5.

■ Antibodies. Twenty-two MAbs were produced against EHV-2.86/67 using the method of Kohler & Milstein (1975). Six- to eight-week-old specific pathogen-free BALB/c mice were immunized with 200 µg purified EHV-2.86/67. Mice were inoculated three times, one dose in Freund’s complete adjuvant followed by two injections 3 weeks apart in incomplete Freund’s adjuvant. Four days prior to fusion, 100 µl live EHV-2 in 100 µl PBS was administered via the dorsal tail vein. Hybridomas were screened by ELISA against whole EHV-2.86/67-coated microtitre plates. Cells from positive wells were cloned twice by limiting dilution and ascites fluid was produced in Pristane (Sigma)-treated BALB/c mice.

Antiserum to EHV-2.86/67 was raised following infection of an equine foetus (Gleeson & Studdert, 1977). After birth, the infected foal was deprived of colostrum and artificially reared in an environment free from other equine pathogens. The serum neutralizing antibody titre reached a peak at 28 days after birth of the foal, and this serum was used to detect EHV-2.86/67 proteins in Western blotting.

■ Infection, radiolabelling and virus purification. These were performed as described previously (Crabb & Studdert, 1990) except that 10 µCi [³⁵S]methionine/cysteine TRAN³⁵S-LABEL (ICN) was added at 48 h post infection (p.i.) and infected cell pellets were lysed at 168 h p.i. in buffer containing 0.1% SDS, 1% NP40 and 1% deoxycholate in Tris-buffered saline (0.05 M Tris–HCl pH 7.3, 0.15 M NaCl).

■ Pulse–chase analysis. EFK monolayer cell cultures were infected with EHV-2.86/67 or were uninfected (mock). At 48 h p.i., cells were washed and incubated with methionine/cysteine-deficient DMEM for 30 min prior to the addition of 200 µCi [³⁵S]methionine/cysteine TRAN³⁵S-LABEL (ICN) to individual flasks. EFK cell cultures were incubated for 15 min prior to washing with PBS and incubated for various times with MEM containing ten times the normal concentration of methionine. At 15 min intervals thereafter, cells were subsequently scraped into the medium for a total of 210 min and radiolabelled antigen was prepared and analysed as described for radioimmunoprecipitation (RIP).

■ Carbohydrate analysis of EHV-2 gB. EFK monolayer cell cultures were infected with EHV-2.86/67 at an m.o.i. of 10. After adsorption for 48 h at 37 °C, media was removed and replaced with methionine/cysteine-deficient DMEM containing either 2 µg/ml tunicamycin (Boehringer) or 1 mM 1-deoxymannojirimycin (1-DMJ; Sigma), added in medium containing 2% FBS. After a further 30 min incubation, 10 µCi [³⁵S]methionine/cysteine TRAN³⁵S-LABEL was added to individual flasks. Incubation was continued until cytopathic effect (CPE) greater than 90% was observed. Radiolabelled antigen was prepared as described above.

Deglycosylation reactions were performed on radiolabelled, immunoprecipitated samples. Briefly, following RIP, protein G beads (Pharmacia) were washed in PBS and resuspended in 50 µl deglycosylation buffer [100 mM sodium acetate pH 5.5, 0.01% SDS, 0.1% n-octyl-β-D-glucopyranoside (β-DOG; Sigma) and 0.5 mM PMSF (Boehringer)]. For endoglycosidase H (EndoH) digestion, 1 M 2-mercaptoethanol (2-ME) was added to the buffer. The protein G beads were then heated to 100 °C for 5 min and chilled on ice prior to the addition of either EndoH (10 µl; Boehringer) for 10 h at 37 °C or neuraminidase (10 µl; Sigma) for 1 h at 37 °C, or neuraminidase (10 µl) for 1 h at 37 °C followed by O-glycosidase (1:5; Boehringer) for 4 h. Samples were then analysed by SDS–PAGE and subjected to autoradiography as described above.

■ Virus neutralization. Virus titration and serum neutralization assays were performed in sterile 96-well flat-bottomed polystyrene plates (Nunc). EHV-2.86/67 was obtained from cell culture supernatants and half-log serial dilutions were made in MEM supplemented with 2.5% FBS, 50 µg/ml ampicillin and 0.013 M NaHCO₃ (incubation medium). In each well, 50 µl diluted virus was added to 50 µl incubation medium; EFK cells (approximately 2.4 x 10⁵ in 50 µl) were then added to each well. Plates were incubated in an atmosphere of 5% CO₂ at 37 °C for 7 days, examined for CPE and the 50% tissue culture infectious dose (TCID₅₀) per ml was calculated.

For neutralization assays, MAbs were tested by incubating 50 µl incubation medium containing 100 TCID₅₀ of EHV-2.86/67 with serial twofold dilutions of antibody in a volume of 100 µl for 1 h at 37 °C. EFK cells (2.4 x 10⁵) in 50 µl incubation medium were then added to the wells and the plates incubated as described above. The neutralization titre of each MAb was defined as the reciprocal of the highest dilution of MAb giving a 90% reduction in CPE at 6 days p.i.

■ SDS–PAGE and Western blotting. Proteins from EHV-2.86/67-infected EFK cell cultures or purified virions were solubilized by boiling for 3 min in buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS and 10% glycerol with the addition of 1 M 2-ME (reducing). Following SDS–PAGE on 5–15% gradient gels, the proteins were transferred to PVDF membranes (Millipore) for Western blotting and blocked in 10 mg/ml bovine serum albumin (BSA) containing 5% skim milk powder; gels were cut into strips and probed with MAb diluted in PBS containing 5 mg/ml BSA and 0.05% Tween 20. MAb binding was detected after incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulin (1:1000; Dako) followed by staining with 0.4 mg/ml diaminobenzide (Sigma) in 0.1% H₂O₂. Alternatively, for some experiments a chemiluminescent detection system (ECL; Amersham) was used with exposure on autoradiographic film (Biomas MS; Kodak). For radio-labelling experiments, polycrylamide gels were dried under vacuum prior to autoradiography on Kodak Biomax MS film.

■ RIP. Preparation of ³⁵S-labelled viral antigens was carried out as described previously (Crabb et al., 1991). To saturate protein G beads with MAb, 10 µl packed 50% protein G Sepharose beads was incubated with 50 µl of a 1/50 dilution of ascitic fluid in RIP buffer (0.05 M Tris–HCl, pH 8.0, containing 1 mM EDTA, 0.15 M NaCl, 0.25% w/v BSA and 1% v/v Triton X-100) for 1 h at 4 °C with shaking. After three washes in RIP buffer, 200 µl radiolabelled EHV-2 protein was added and incubated for a further 1 h at 4 °C with shaking. Immunoprecipitated proteins were washed three times in RIP buffer, resuspended in sample buffer and analysed by SDS–PAGE and autoradiography.

■ ELISA. ELISAs were performed as described previously (Crabb et al., 1991) except that 96-well plates were coated with EHV-2.86/67 virions at a concentration of 5 µg/ml and added to plates in a total volume of
100 µl and horseradish peroxidase-conjugated affinity-purified rabbit anti-mouse antibody (Dako) diluted 1:1000 was used.

**Immunooaffinity chromatography.** EHV-2.86/67-infected EFK cells were solubilized in a buffer containing 50 mM Tris–HCl 0.15 M NaCl, 10 mM EDTA, 0.3 mM PMSF, 4 µM leupeptin and 1 % Triton X-100. EHV-2.86/67 gB was purified from the solubilized fluid extract by immunooaffinity chromatography using columns with the purified MAb 15C7 coupled to CNBr-activated Sepharose 4B (Sigma) according to the manufacturer's directions. Bound antigen was eluted from the column with 50 mM diethylamine, pH 11.5, containing 0.05% Triton X-100. Fractions containing EHV-2 gB, identified by a dot-blot immunobinding assay, were pooled, dialysed against PBS containing 0.05% Triton X-100, and concentrated in a Centricon 50 centrifugal concentrator (Amicon). Antigen was further concentrated by precipitation in 6% trichloroacetic acid followed by washing in 80% ether/20% ethanol three times prior to suspension in SDS–PAGE sample buffer and SDS–PAGE.

**N-terminal sequencing of gB.** Purified gB was placed in sample buffer containing 1 M 2-ME, incubated at 100 °C for 2 min as described for Western blotting (above) and transferred to PVDF membrane (Bio-Rad) using a buffer containing 10 mM CAPS (cyclohexylaminopropane sulfonic acid) and 20% methanol, pH 11. Protein bands were detected by staining with 0.05% Coomassie blue for 15 min and destained in 10% acetic acid and 45% methanol. The Coomassie blue-stained glycoprotein bands were cut out of the membrane and subjected to N-terminal sequencing by Edman degradation on an ABI amino acid analyser (Applied Biosystems).

**Construction of recombinant GST–gB plasmids.** Standard DNA cloning techniques were used in the construction of gB expression clones (Sambrook et al., 1989). The regions of EHV-2 gB used for the construction of GST–gB plasmids were amplified using PCR from viral DNA. EHV-2.86/67 DNA was isolated by digestion of purified virions with 0.1 mg/ml proteinase K at 37 °C for 2 h in buffer containing 0.2% SDS, 10 mM Tris–HCl, pH 8.0, and 10 mM EDTA, followed by phenol–chloroform extraction. The extracted DNA was used as the template for PCR. Plasmid gBL, encoding the large subunit of gB, expressed the region of EHV-2 gB between amino acids 27 and 425 whereas plasmid gBS, encoding the small subunit of gB, expressed the region between amino acids 454 and 692. For construction of plasmid gBL, DNA was amplified by PCR using the oligonucleotide primers 5' TCGGATCCTCAGGTCCTCGCTCAGGA 3' and 5' TCGGATCCCGCTCCCGCCCTCAGGA 3', which contained BamHI restriction sites (underlined). For plasmid gBS, oligonucleotide primers 5' TCGGATCCTCAGGTCCTCGCTCAGGA 3' and 5' TCGGATCCCGCTCCCGCCCTCAGGA 3' (BamHI restriction sites underlined) were used. For construction of plasmids gBL and gBS, PCR products were digested with BamHI and cloned into pUC18 then directionally cloned as an EcoRI–SamI fragment into the bacterial expression vector pGEX-4T (Pharmacia). For plasmid gBL, which encodes a truncated large subunit of gB, DNA expressing the region between amino acids 12 and 74 of EHV-2 gB was amplified with the oligonucleotide primers 5' CTGGATCCAGGGCCCTCAGGAGG 3' and 5' GGTCCCGCTCAGGAGG 3' by PCR and cloned into the vector pGEMT (Promega) followed by directional cloning as an EcoRI–SamI fragment into pGEX-4T.

**Synthesis of bacterial fusion proteins.** E. coli JM109 cells were transformed with plasmids gBL, gBS and gBL. The cells were grown to a density of 0.4 (A600) and production of the fusion protein was initiated by the addition of 1 mM IPTG (Boehringer); the cultures were incubated with shaking for 3 h at 37 °C. The fusion proteins were purified using Glutathione Sepharose beads (Pharmacia) and eluted using 50 mM glutathione according to the protocol of Smith & Johnson (1988). In some experiments, 10 mM PMSF (Sigma) was included during the purification procedure to prevent proteolysis of the fusion protein occurring.

**Results**

**gB-specific MAbs recognize an 89 kDa protein in Western blots of purified virions**

Western blot analysis of the twenty-two MAbs produced to EHV-2.86/67 demonstrated that eight MAbs recognized an 89 kDa protein under reducing conditions (Fig. 1a). Under non-reducing conditions, these eight MAbs identified a single protein band of approximately 320 kDa with five MAbs recognizing an additional protein of 160 kDa. A RIP assay was performed on EHV-2.86/67-infected cells to determine if the 320 kDa band seen in non-reducing Western blots was formed by a complex of viral proteins. All eight MAbs precipitated a complex of four major proteins of 130, 89, 65 and 62 kDa from EHV-2.86/67-infected EFK cells, but did not precipitate proteins from mock-infected cells. All eight MAbs identified 130 and 89 kDa proteins in Western blots of proteins from EHV-2-infected EFK cells but only revealed the 89 kDa protein in purified virions. A representative MAb 4F10 is shown in Fig. 1(c, d).

**Identification of the signal cleavage site of gB**

gB is the most abundant envelope protein of most herpesviruses and commonly exists as a disulphide-linked heterodimer (Pereira, 1994). As all of the MAbs directed against the 89 kDa protein recognized a higher molecular mass, disulphide-linked protein complex, we investigated the possibility that the MAbs recognized EHV-2 gB. The product of the EHV-2.86/67 gB gene is 874 amino acids long with a predicted molecular mass of 98 kDa. It shows the typical features of a type I integral membrane glycoprotein with a possible signal peptide of approximately 30 amino acids at the N terminus and a second hydrophobic region between amino acids 682 and 760 most likely representing a transmembrane region similar to other herpesvirus gB homologues.

To determine the signal cleavage site of EHV-2 gB, N-terminal sequencing was performed (Fig. 2). The signal cleavage site was determined by alignment of the N-terminal amino acid sequence with the predicted amino acid sequence derived from translation of the nucleotide sequence of EHV-2.86/67 gB. The signal cleavage site was located between Val29 and Arg30. These findings confirmed that the 89 kDa protein represents the N-terminal part of EHV-2 gB.

**Antibody 4F10 recognizes an epitope located at the N terminus of EHV-2 gB**

The eight MAbs that recognized the 89 kDa protein in reducing Western blots of EHV-2.86/67 were tested by Western blotting with the GST fusion proteins of gBL and gBS.
Fig. 1. (a and b) Western blot analysis of EHV-2 gB using a panel of MAb s generated to EHV-2.86/67. EHV-2.86/67 virions were separated by SDS–PAGE under reducing (a) or non-reducing conditions (b) on 5–15% continuous gels. Eq Poly, EHV-2 monospecific equine polyclonal antiserum. The positions of the molecular mass standards (in kDa) are indicated on the left and approximate molecular masses of the viral bands detected are on the right. (c) Radioimmunoprecipitation of [35S]methionine-
Two additional isotype-matched control MAbs identifying other EHV-2.86/67 MAbs were used as controls in the Western blot experiments. Seven of the eight MAbs identifying the epitope of these MAbs were located in the N-terminal region of EHV-2 gB (Fig. 3a). None of the MAbs reacted with GST or the gBS fusion protein in Western blotting (data not shown). The predicted molecular mass of the gB fusion protein was 77 kDa. However, MAb 15C7 reacted weakly with the full-length fusion protein and the other seven MAbs reacted with a protein product of molecular mass 37 kDa in Western blots and not the full-length fusion protein. The lower molecular mass proteins presumably represent proteolytic products of the full-length fusion protein. To prevent proteolysis of the GST–gB fusion protein, PMSF was included during the purification procedure and this resulted in the production of a 70 kDa fusion protein and smaller amounts of the 77 kDa full-length fusion protein. However, in contrast to the findings observed with the first Western blot, only MAb 15C7 reacted with the 70 kDa gB fusion protein produced (Fig. 3b). Production of the full-length GST–gB fusion protein was confirmed by Western blotting using antiserum specific to GST and EHV-2.86/67-specific equine polyclonal antiserum.

These findings suggest that, following Western transfer, the full-length recombinant GST–gB fusion protein formed a conformational structure that prevented binding of seven of the MAbs. This secondary conformational structure apparently was not formed by the proteolytic products. As only gB amino acid sequences attached to the N-terminal GST tag are purified on Glutathione Sepharose, an assumption was made that the epitope of the MAbs was located in the N-terminal 10 kDa of the gB molecule immediately C-terminal to the 27 kDa GST protein. A truncated form of gB (gBLt) containing amino acids 12–74 was constructed and the purified fusion protein was subjected to SDS–PAGE and transferred for Western blotting. MAb 4F10 and six other MAbs that reacted with the shortened 40 kDa gBL fusion protein recognized epitopes located within the gBLt fusion protein. Two MAbs identifying other EHV-2.86/67 proteins did not react with the gBLt fusion protein confirming the specificity of the seven MAbs reacting with the gBLt fusion protein (Fig. 3c). MAb 15C7 recognized the 89 kDa EHV-2 protein in reducing Western blots and reacted with the 70 kDa gBL fusion protein, but did not react with the 37 kDa gBL fusion protein or the gBLt fusion protein. These findings suggest that the epitope of MAb 15C7 was located C-terminal to the epitopes recognized by the seven other gB-specific MAbs.

Analysis of EHV-2 gB in the presence of glycosylation inhibitors

To investigate the post-translational processing of EHV-2 gB, RIP analysis of radiolabelled proteomes from EHV-2-infected cells propagated in the presence of 1-DMJ or tunicamycin was performed (Fig. 4). The antibiotic tunicamycin prevents the addition of high-mannose N-linked oligosaccharides in the endoplasmic reticulum (ER) while 1-DMJ is an inhibitor of N-mannosidase I, which prevents the conversion of high-mannose intermediate forms to complex carbohydrate forms (Campadelli-Fiume & Serafini-Cessi, 1982). In the presence of tunicamycin, a 97 kDa protein was immunoprecipitated; this corresponds with the predicted size of the gB polypeptide without the addition of oligosaccharides. In the presence of 1-DMJ, proteins of molecular mass 130, 75, 62 and 59 kDa were immunoprecipitated. The 130 kDa protein was unchanged in the presence of 1-DMJ, suggesting that, in EHV-2-infected cells, the 130 kDa protein is a high-mannose, uncleaved precursor form of gB.

Analysis of EHV-2 gB using a panel of deglycosylation enzymes

Although most herpesvirus gB have similar sized polypeptide backbones, considerable variation in the molecular mass of the fully processed gB is reported (Pereira, 1994). Variation in the molecular mass of the gB homologues of different herpesviruses is predominantly dependent on the type and presence of O-linked and N-linked oligosaccharides. Analysis of the amino acid sequence of EHV-2 gB predicts the presence of 14 potential sites for N-linked glycosylation. To determine the type and amount of glycosylation present on EHV-2 gB, a panel of deglycosylation enzymes was used. Treatment with the enzyme α-glycosidase F, which removes N-linked oligosaccharides, reduced the molecular mass of the radioimmunoprecipitated 130, 89, 65 and 62 kDa proteins to 97, 63, 50, 49 and 47 kDa. However, the appearance of the
Fig. 3. Western blot analysis of E. coli-expressed GST–gB fusion proteins using EHV-2.86/67-specific MAbs and an EHV-2 monospecific equine polyclonal antiserum (Eq poly). Purified GST–gB fusion proteins were separated by SDS–PAGE on (a) 5–15% continuous and (b, c) 12.5% gels and probed with eight MAbs that recognized an 89 kDa EHV-2 protein in Western blot of EHV-2 virions. Two MAbs that did not recognize the 89 kDa protein in Western blot were used as a control (MAb con). Lane anti GST contains antiserum specific to GST. (a) Western blot analysis of the GST–gBL fusion protein expressing amino acids 27–425 of EHV-2 gB. (b) Western blot analysis of the GST–gBL fusion protein purified with the inclusion of PMSF. (c) Western blot analysis of the GST–gBLt fusion protein expressing amino acids 12–74 of EHV-2 gB. For (b) and (c), a Coomassie blue stained strip is shown to the right of the Western blot strips. Mobilities of the molecular mass standards (in kDa) are indicated on the left and approximate molecular masses of protein bands detected are on the right.

63 kDa protein suggested that it was the product of incomplete deglycosylation of the 89 kDa protein. Treatment with neuraminidase followed by O-glycosidase was used to assess the amount of O-linked oligosaccharide. Treatment with neuraminidase alone, which removes terminal sialic acid residues, reduced the 89, 65 and 62 kDa proteins by a small amount to 80, 63 and 60 kDa forms, respectively (Fig. 5). Further digestion with O-glycosidase did not increase the mobility of the neuraminidase-treated proteins and suggests that EHV-2 gB contains no O-linked oligosaccharides. As a control, EHV-1 gp300, a protein known to contain O-linked oligosaccharides, was reduced in molecular mass using this protocol, thus verifying the activity of the enzymes (data not shown) (Wellington et al., 1996). The addition of neuraminidase to digests containing N-glycosidase F appeared to facilitate removal of N-linked oligosaccharides by N-glycosidase F. Treatment with neuraminidase, O-glycosidase and N-glycosidase F reduced the molecular masses of the 130, 89, 65 and 62 kDa forms to 97, 50, 49 and 47 kDa, respectively (Fig. 5). These species appeared to represent the completely deglycosylated forms of the radioimmunoprecipitated proteins. The 63 kDa protein seen following treatment with N-glycosidase F alone was absent when neuraminidase and O-glycosidase were included in the digestion. However, a broad smear was observed between the 47 and 63 kDa bands which represented some residual, partly deglycosylated gB species.
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Fig. 4. Analysis of the glycosylation of gB using inhibitors of glycosylation. RIP was performed with MAb 4F10 and the precipitated proteins separated by SDS–PAGE under reducing conditions on 5–15% continuous gels. Lane: Nil, EHV-2-infected untreated cell proteins; 1-DMJ, 1-DMJ-treated infected cell proteins; Tun, tunicamycin-treated infected cell proteins; and Mock, mock-infected cell proteins. Mobilities of the molecular mass standards (in kDa) are indicated on the left and approximate molecular masses of the viral bands detected are on the right.

Kinetics of post-translational processing of gB

Many gB homologues of herpesviruses are proteolytically cleaved generating disulphide-linked products (Pereira, 1994). In the presence of 1-DMJ, the 130 kDa band remained unchanged suggesting that it is a high-mannose precursor form of EHV-2 gB in infected cells. The presence of three additional proteins of molecular mass 89, 65 and 62 kDa in RIP experiments suggested that the 130 kDa precursor form of gB may be endoproteolytically cleaved to form the 89, 65 and 62 kDa proteins. In the presence of 1-DMJ, the 89 kDa protein was reduced to 75 kDa and the 65 and 62 kDa proteins reduced to 62 and 59 kDa, respectively, suggesting that they contain complex carbohydrate. Furthermore, infected cells grown in the presence of tunicamycin accumulated a protein with a molecular mass of 97 kDa, which corresponds to the predicted molecular mass of the non-glycosylated gB polypeptide. To further characterize the post-translational processing of gB, a pulse–chase experiment was conducted.

Under reducing conditions, the 130 kDa high-mannose precursor form of gB is present throughout the pulse–chase period (Fig. 6a). After 15 min chase, a 75 kDa protein was detected and this species increased in molecular mass to 89 kDa during the period from 30 to 210 min. Also after 15 min chase, a 65 kDa protein was present and this species increased in intensity during the chase period.

A gradual loss of EndoH sensitivity of the immunoprecipitated 89 and 65 kDa proteins was apparent throughout the chase period (Fig. 6b). During the 15 to 180 min chase period, multiple proteins between 47 and 89 kDa were detected which indicated variability in the number of N-linked high-mannose oligosaccharides that had been modified to complex oligosaccharides during this time. EndoH reduced the 130 kDa protein to a 97 kDa protein throughout the 210 min chase, again confirming that it is a high-mannose precursor form of gB. The 97 kDa form seen following EndoH treatment compares closely with the size of the immunoprecipitated protein labelled in the presence of tunicamycin and also with the predicted molecular mass of 95 kDa for the gB polypeptide following removal of the signal sequence.

Pulse–chase analysis under non-reducing conditions showed that EHV-2 gB dimerizes to a protein of approximate molecular mass of 300 kDa as early as 15 min chase (Fig. 6c).
Fig. 6. For legend see facing page.
Ability of MAb 4F10 to neutralize EHV-2.86/67

Previous studies conducted in herpes simplex virus type 1 and human cytomegalovirus (HCMV) using potent neutralizing MAbs demonstrated the presence of linear neutralization epitopes located at the N terminus of gB (Basgöz et al., 1992; Kousoulas et al., 1988). As seven of the eight MAbs that recognized the 89 kDa subunit of gB had been shown to recognize an epitope between amino acids 12 and 74 of EHV-2 gB, we undertook serum neutralization tests to determine if the gB MAbs were neutralizing. The results of serum neutralization tests demonstrated complement-independent neutralization of EHV-2.86/67 by MAbs 17A7, 17D8, 4F10, 3D10, 19B1, 19F10 and 3B1 to a titre of between 1 in 28 000 to 1 in 56 000.

Discussion

The presence of gB as a major envelope glycoprotein is well-documented for the alpha and betaherpesvirus subfamilies (Britt & Mach, 1996; Pereira, 1994). Using Western blot analysis, we have detected gB in purified virion preparations and this suggests that gB is a resident virion envelope protein of EHV-2. Western blot analysis using equine polyclonal serum demonstrated that EHV-2 gB is a target for the immune response in horses infected with EHV-2. Using seven MAbs, we have identified a linear neutralization epitope located between amino acids 29 and 74 of EHV-2 gB. Polyclonal equine EHV-2.86/67-specific antiserum recognized the same region in the N terminus of EHV-2 gB suggesting that the N terminus region of gB is immunogenic during natural infection.

The results of N-terminal sequencing and comparison with the predicted sequence of EHV-2.86/67 showed the 89 kDa subunit to be the N-terminal portion of gB (Fig. 7a). The results of the pulse–chase experiment suggested that the EHV-2 gB first appears as a 130 kDa monomer prior to cleavage to 75 and 58 kDa high-mannose-containing subunits and conversion of the subunits to the 89 and 65 kDa complex carbohydrate-containing forms (Fig. 7b). EndoH treatment confirmed that both the N-terminal and C-terminal subunits of gB contained some high-mannose carbohydrate until 180 min chase. Under non-reducing conditions, EHV-2 gB exists as a homodimer of two 160 kDa proteins, with an approximate molecular mass of 320 kDa. The formation of a higher molecular mass form of gB was apparently not caused by boiling samples prior to SDS–PAGE as the results shown under non-reducing were identical regardless of whether the samples were boiled or not.

In the presence of 1-DMJ, EHV-2 gB was cleaved suggesting that modification of gB from a high-mannose form to a complex carbohydrate form is not required for endoproteolytic cleavage to occur as has been suggested for HCMV gB and pseudorabies virus gB (Britt & Vugler, 1989; Whealey et al., 1990). Tunicamycin prevented glycosylation and cleavage of the 130 kDa gB monomer into the two subunits suggesting that passage into the Golgi and N-linked glycosylation is required for endoproteolytic cleavage to occur.

The consensus sequence at the cleavage site of gB homologues that are endoproteolytically cleaved has been reported to be the basic tetrapeptide Arg-Xaa-(Arg/Lys)-Arg-Ser (Wellington et al., 1996) or Arg-(Thr/Ser/Arg)-(Lys/Arg)-Arg (Spaete et al., 1990). EHV-2.86/67 has a predicted endoproteolytic cleavage motif (Arg-Arg-Arg-Arg) at amino acids 431–435. The two polypeptides created by endoproteolytic cleavage and corresponding to the N-terminal and C-terminal subunits have a predicted molecular mass of 47 and 49 kDa. These figures compared closely with the results seen following removal of carbohydrate moieties with neuraminidase, O-glycosidase and N-glycosidase F digestions suggesting that EHV-2 gB may be cleaved at amino acids 431–435. BHV-4 gB is similarly cleaved and contains an Arg-Gln-Lys-Arg cleavage motif at amino acids 461–465 (Lomonte et al., 1997).

Analysis of the pulse–chase experiment suggests that the mature form of EHV-2 gB consists of 89 and 65 kDa disulphide-linked subunits. In a previous study using rabbit antiserum raised to an EHV-2 gB fusion protein, EHV-2 gB was identified as a 64 kDa protein (Agius et al., 1994). Retrospectively, EHV-2 gB fusion protein produced by Agius et al. (1994) spanned amino acids located in the predicted N-terminal and C-terminal subunits of EHV-2 gB. This information was unavailable in 1994 as the complete nucleotide sequence of EHV-2 gB was not determined until 1995. Also, this serum should have had specificity for the N-terminal and C-terminal subunits. It seems likely that the 64 kDa protein identified by Agius et al. (1994) is the same protein as the 65 kDa C-terminal subunit identified in our RIP experiments using MAbs. However, we have been unable to repeat this work using the rabbit serum, possibly due to low levels of EHV-2 gB antibody found in this serum.

We are unable to explain the presence of a 62 kDa protein in RIP analysis of EHV-2 gB (Fig. 1c). The 62 kDa protein appeared to be present in lower molarity than the 65 kDa protein and is apparently a glycoprotein as deglycosylation...
Fig. 7. (a) Predicted proteolytic cleavage sites of EHV-2 gB. A signal sequence cleavage site (closed arrow) and two predicted endoproteolytic cleavage sites (open arrows) are shown. A hydrophobic transmembrane region (labelled TM) and 14 predicted N-linked glycosylation sites (circles) are shown. (b) Proposed model of EHV-2 gB synthesis and processing.

with N-glycosidase F reduced the molecular mass by the same amount as the 65 kDa protein suggesting they contain identical amounts of N-linked carbohydrate. The protein is apparently not a high-mannose precursor of the C-terminal subunit as the molecular mass was reduced by 3 kDa in 1-DMJ-treated, immunoprecipitated samples when compared with untreated samples. It is possible that the 62 kDa protein may represent a variably glycosylated form of the 65 kDa protein. There is some precedence for this as rhesus cytomegalovirus gB has been identified as having variably glycosylated 55 and 52 kDa C-terminal subunits (Kropff & Mach, 1997). Another possible explanation may be that the 62 kDa protein is an alternatively cleaved form of the small subunit of gB. EHV-1 gB has been shown to have an additional proteolytic cleavage site 28 amino acids C-terminal of the expected cleavage site (Wellington et al., 1996). Following complete deglycosylation of gB, 50 and 47 kDa subunits were clearly visible. Additionally, a 49 kDa subunit was visible at lower molarity, possibly representing an alternatively cleaved, deglycosylated C-terminal subunit. However, we were unable to perform N-terminal sequence analysis of the 62 and 65 kDa proteins to prove this was the case.

Among the gamma-2 herpesviruses, BHV-4 gB has been determined to have 40 kDa of O-linked oligosaccharide and approximately 40 kDa of N-linked oligosaccharide (Lomonte et al., 1997). By comparison, the N-terminal large subunit of EHV-2 gB contains 39 kDa of N-linked carbohydrate and the C-terminal subunit contains 17 kDa of N-linked carbohydrate with no evidence for O-linked glycosylation. The significance of O-linked carbohydrate in the function of gB is unknown although several herpesvirus gBs contain O-linked oligosaccharides (Britt & Vugler, 1989).

This study has demonstrated that the gamma-2 herpesvirus EHV-2 contains gB as an integral virion membrane glycoprotein and is similar to BHV-4 and members of the alpha and betaherpesviruses in this respect. By comparison, EBV and MHV-68 retain gB as an uncleaved high-mannose form in the ER. EBV is not known to establish primary infection in epithelial cells and, although MHV-68 is able to replicate in vitro in epithelial cells, antibodies to MHV-68 gB are not neutralizing, suggesting that MHV-68 gB is not involved in infection of epithelial cells (Stewart et al., 1994). These findings suggest that the ER retention of gB may be a characteristic restricted to some members of the gammaherpesvirus subfamily and suggests that the gamma-2 herpesviruses EHV-2 and BHV-4 may be more similar in regard to pathogenesis of epithelial infection to the members of alpha and beta-herpesvirus subfamilies. Both EBV and MHV-68 establish latent infection in B lymphocytes and EBV has evolved a unique glycoprotein gp350 for attachment to B cells (Kieff, 1996). Although EHV-2 has been demonstrated to be latent in B lymphocytes (Drummer et al., 1996) and BHV-4 has been suggested to be latent in mononuclear cells (Egyed et al., 1996), little is known about the function of gB during gamma-2 herpesvirus infection of lymphocytes.

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