The processing, transport and heterologous expression of Epstein–Barr virus gp110

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Epstein–Barr virus (EBV) glycoprotein gp110 has substantial structural and sequence homology with herpes simplex virus (HSV) gB and gBs of other alpha- and betaherpesviruses but unlike HSV gB localizes differently in infected cells and is absent from virions. To facilitate the analysis of EBV gp110, antisera were raised to fragments of gp110 expressed in a bacterial system. They recognized a protein of the predicted size in recombinant bacterial lysates, in lymphoblastoid cells and in recombinant vaccinia virus–gp110 infected cells. gp110 from all sources possessed a high-mannose type of N-glycosylation implying that gp110 has not passed through the Golgi. Immunofluorescence and immuno-electron microscopy confirmed this conclusion and demonstrated that, in contrast to HSV gB, the majority of immunoreactive gp110 was present at the nuclear membrane or endoplasmic reticulum (ER) but not at the cell membrane. Unexpectedly, a truncated version of gp110 lacking the hydrophobic C-terminal region, despite forming dimers analogous to HSV dimers, was transported in a similar manner to full-length gp110. Two chimeric proteins constructed by replacing the N- and C-terminal domains of gp110 with corresponding regions of gp340/220 were also transported to the nuclear membrane/ER. These data suggest that unlike HSV gB both the N- and C-terminal portions of EBV gp110 contain independent signals sufficient to direct the molecule to the ER/nuclear membrane. Specific transport of gammaherpesvirus gB homologues to the nuclear membrane, from where herpesviruses bud, suggests that they may be involved in the egress of virus from the nucleus.

Introduction

The Epstein–Barr virus (EBV) genome encodes at least seven glycoproteins. Among these gp110, the herpesvirus gB homologue (Gong et al., 1987), is the most conserved within the herpesvirus family and can be used to estimate evolutionary relationships between herpesvirus family members (McGeoch et al., 1995). Glycoprotein homologues of EBV gp110 have been identified in each of the three herpesvirus subfamilies (McGeoch et al., 1995) and are typified by herpes simplex virus (HSV-1) gB (DeLuca et al., 1982) from the α-subfamily; human cytomegalovirus (HCMV) gp55–116 (Cranage et al., 1986) from the β-subfamily; and equine herpesvirus type 2 gBs (Telford et al., 1995), murine herpesvirus 68 (MHV-68) gB (Stewart et al., 1994) and EBV gp110 (Gong et al., 1987; Gong & Kieff, 1990) from the γ-subfamily. All of the gB homologues characterized thus far are predicted to contain a large ecto-domain containing potential N-glycosylation sites, a hydrophobic transmembrane region close to the C terminus and a short cytosolic domain. EBV gp110 (open reading frame BALF4 of EBV) (Baer et al., 1984) is predicted to be 857 amino acids (aa) long with a 24 aa signal sequence, a transmembrane region of 46 aa (aa 710–756), nine potential N-linked glycosylation sites and a cytoplasmic tail of approximately 100 aa. Sequence and structural homology between gB glycoproteins reflects their functional similarities and it has been demonstrated that gBs of closely related viruses can functionally substitute for each other (Mettenleiter & Spear, 1994, and references therein).

gBs analysed from alpha- and betaherpesviruses were...
found to be at least partially expressed on the cell surface and included into the viral envelopes in their fully glycosylated forms. These glycoproteins seem to follow the classic exocytotic pathway involving folding and initial N-linked glycosylation in the endoplasmic reticulum (ER), transport to the Golgi followed by Golgi-specific modifications to carbohydrate residues (reviewed by Doms et al., 1993); finally, a significant proportion of the fully mature gB is transported to the cell surface. There is some debate as to where the viral particle acquires gB but it is probably in the trans-Golgi network where virus particles are assembled (see Discussion). Recent studies on HSV-1 gB demonstrate, however, that at least a fraction of gB molecules ‘escape’ this classic pathway and are targeted to the nuclear envelope in high-mannose glycosylated forms (Ali et al., 1987; Torrisi et al., 1992; Gilbert et al., 1994).

The only gammaherpesvirus gBs studied in detail so far are EBV gp110 (Gong et al., 1987; Gong & Kieff, 1990) and MHV-68 gB (Stewart et al., 1994). Both seem to possess an exclusively high-mannose type of glycosylation and unlike other gBs appear to be absent from the viral particle. A few reports based on immunofluorescence suggest that EBV gp110 is expressed on the surface of the lymphoid cell line B95-8 and consequently might be included into the viral particle during EBV envelopment at the plasma membrane, the site at which proteins are normally incorporated into the particle (Emini et al., 1987; Chan et al., 1989). Also, EBV gp110 expressed in recombinant vaccinia virus-infected cells can serve as a target for antibody-dependent cell-mediated cytoxicity (Jilg et al., 1994). This supports the hypothesis that at least some gp110 is expressed at the cell surface. On the other hand, lack of cell surface localization of EBV gp110 was clearly demonstrated using biochemical studies, immunofluorescence and immuno-electron microscopy (Gong et al., 1987; Gong & Kieff, 1990) where gp110 was detected only in the nuclear envelope and in cytoplasmic vesicles.

To extend and clarify the data concerning EBV gp110 we have investigated the properties of gp110 expressed by several EBV strains and by recombinant vaccinia virus. Preliminary studies with a mutant gp110 and several chimaeras expressed by vaccinia virus have been undertaken in an initial attempt to define molecular motifs responsible for transport and processing of EBV gp110. We hoped to exclude a number of trivial explanations for the observations and to analyse expression of gp110 in epithelial cells, as nasopharyngeal epithelial cells have been postulated as a site of productive EBV replication.

Methods

**Virus, cells and media.** Vaccinia virus Western Reserve (WR) strain (ATCC VR119) and recombinants based on the strain were propagated by standard methods (Mackett, 1995). Lymphoid cell lines Akata (Taikada, 1984), B95-8 (European Collection of Animal Cell Cultures (ECACC) 85011419), C2-BL16 (gift of J. Stewart), Ramos (ECACC 91030710) (Klein et al., 1975) and Raji (ECACC 85011429) (Pulvertaft, 1964) were maintained in RPMI 1640 (Gibco BRL) containing 10% foetal calf serum (FCS) (Sera-labs). RK13 (ECACC 88062427), HeLa (ECACC 85060701) and HeLa TK-143 human osteosarcoma derived cell line (ECACC 88022409) were maintained in MEM (Gibco BRL) containing 10% FCS. MRC-5 (ECACC 84101801) was maintained in Dulbecco’s MEM (Gibco BRL) supplemented with non-essential amino acids and 10% FCS.

MEM containing 5% FCS, 25 mg/l mycophenolic acid (MPA) (Sigma), 250 mg/l xanthine (Sigma), 15 mg/l hypoxanthine (Sigma) was used for the selection of vaccinia recombinants expressing the *E. coli* guanine phosphoribosyltransferase (*gpt*) gene.

**DNA cloning.** All cloning techniques were as described by Sambrook et al. (1989). In all cases where read-through was required to produce fusion proteins in either prokaryotic or eukaryotic expression systems the junctions of the constructs concerned were sequenced to ensure authenticity.

**Cloning of gp110.** BamHI A (Arrand et al., 1981) from the B95-8 strain of virus was cleaved with Xbal and NrdI and overhangs filled in with Klenow DNA polymerase and then religated. The resulting plasmid p500 was used as a source of the EcoRI (159853)–SalI (157753) fragment containing the gp110 gene for cloning into pUC13 cleaved with EcoRI and SalI. A 201 bp *MvlI* fragment (159124–159325) isolated from the resulting plasmid p502 was filled in with Klenow DNA polymerase and cloned into *HincII* cleaved pUC13. This gave a plasmid designated p505 containing the 5′ end of the gp110 gene. p502 was partially cleaved with Xhol, overhangs filled in with Klenow DNA polymerase and BamHI linkers inserted. A plasmid containing the *BamHI* linker at the 3′ end of the gp110 gene was identified and designated p504. The 3′ end of the gp110 gene was isolated by cleavage of p504 with *MluI* and *BamHI*. The 5′ end of the gene was isolated as a *BamHI–MluI* fragment from p505 and together with the 3′ end of the gene ligated into pGSP20 (Mackett et al., 1984). The resultant plasmid p514 was used to generate recombinant vaccinia virus (vgp110/7.5) (see below) and the *BamHI* fragment containing the gp110 gene was cloned into the *BamHI* site of pUC13 resulting in the plasmid p780.

**Cloning for expression in *E. coli.*** All fragments were blunt ended and isolated from p502 and cloned into the pUC119 site of pSET151 (Invitrogen) or the XmnI site of pMAL-c2 (New England Biolabs) and propagated in *E. coli* DH5α. For expression studies the constructs were transferred to *E. coli* BL21, BL21 LysS or BL21 LysE. The blunt ended fragments cloned into pSET151 were *DraI–EcoRV* (155856), *DraI* (159065)–*DraI* (157487), *PvuII* (155989)–*PvuII* (157607), *SmaI* (157987)–*SmaI* (157013) and *SmaI* (157874)–*SmaI* (157987), resulting in the plasmids pD2, pD9, pP9 and pS15. The blunt ended fragment *SmaI* (157013)–*PvuII* (155989) was cloned into the *PvuII*–XmnI site resulting in plasmid pD12. Numbers refer to the co-ordinates of the restriction sites indicated in the EBV genome (Baer et al., 1984). pD9 contains aa 79–605 of gp110, pS15 contains aa 192–445 of gp110 and pP12 contains the C-terminal 88 aa of gp110. Antibodies raised against proteins expressed in *E. coli* by these plasmids were given the same designation as the plasmids. For example the antisera most frequently used, anti-S15, was raised against protein expressed by pS15.

**Cloning for transfer to vaccinia virus.** p780 was used as the source of the gp110 containing *BamHI* fragment for cloning into the vaccinia transfer vector p-gpt-ATA-18-2 (Stunnenberg et al., 1988). Plasmids with the gp110 gene in both orientations were isolated and designated pggp110 for the sense orientation and pggp110/rev for the antisense orientation with respect to the vaccinia promoters. pggp110 was cleaved withBgIII plus SalI and a stop codon inserted using self
Table 1. Vaccinia virus recombinants used

<table>
<thead>
<tr>
<th>Vaccinia transfer vector</th>
<th>Virus designation</th>
<th>Genes</th>
<th>Promoter used</th>
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<td>vgp340</td>
<td>gp340</td>
<td>11K</td>
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<tr>
<td>p514</td>
<td>vgp110/7.5</td>
<td>gp110</td>
<td>7.5K</td>
</tr>
<tr>
<td>pgp110</td>
<td>vgp110</td>
<td>gp110</td>
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<tr>
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<td>vgp110/rev</td>
<td>Antisense gp110</td>
<td>11K</td>
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<td>Truncated gp110</td>
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<tr>
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<tr>
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<td>730 aa gp340 fused to 286 aa C terminus containing the putative membrane anchor of gp110</td>
<td>11K</td>
</tr>
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Recombinant vaccinia generation. Vaccinia virus EBV-gB recombinants were generated by standard procedures (Mackett, 1995) using either MPA selection for recombinants co-expressing E. coli gpt and the gene of interest (i.e. with transfer vectors pgp110, pgp110A, pgp110/rev, pgp110/340, pgp340/110 and pgp340) or with BUDR in HTK-143 cells where insertion into the TK gene was appropriate (p514). A recombinant vaccinia expressing gp110 from the 7.5K early late promoter was generated from p514. Recombinant vaccinia viruses were designated as indicated in Table 1.

Expression and purification of recombinant proteins expressed in the E. coli pRSET system. The conditions used for the T7 based pRSET-vector system (Invitrogen) were essentially as described (Studier & Moffatt, 1988; Studier, 1991). In all cases the resulting gp110–His fusion proteins were insoluble making them difficult to purify on nickel affinity columns. Consequently, all products were purified by excision from polyacrylamide gels followed by electroelution.

pMAL system. The conditions used for expression of gp110 fusion proteins in the pMAL system were essentially those described by the manufacturers (New England Biolabs).

Antisera. gp110 fusion proteins expressed and purified from E. coli were used to immunize rabbits in conjunction with Hunter’s Titermax adjuvant (CytRX, Norcross, Ga., USA). A set of serum samples from individuals who were vaccinia virus negative and EBV positive was also used for immunoprecipitation of gp110 from recombinant vaccinia infected cells. Where appropriate non-specific binding was prevented by adsorption to acetone powder extracts of lymphoblastoid cells (Harlow & Lane, 1988).

Immunofluorescence analysis. Cells were grown directly on slides or spun down from suspension, infected with vaccinia recombinants at a multiplicity of 5 p.f.u. per cell or mock infected and fixed with either acetone at −20 °C or a freshly dissolved solution of 2% paraformaldehyde before being stained. The staining procedures are as described previously (Mackett, 1995). Primary antibody concentrations were determined empirically.

Radioimmunoprecipitation and immunoblotting. EBV positive lymphoblastoid cell lines were induced for late EBV gene expression as described previously (Takada, 1984; Stewart & Rooney, 1992). Radiolabelling of cells and immunoprecipitation was done as described previously (Stewart & Rooney, 1992). Briefly, cells were washed several times with methionine free medium followed by incubation for 10 h in 95% methionine-free medium containing 0.5 mM [35S]methionine/ml. Cells were washed twice with cold PBS and lysed at 4 °C for 10 min in 1% NP40 lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF and 10 µg aprotinin/ml. Insoluble material was removed by centrifugation at 4 °C for 15 min at 10000 g. Cell lysates were precleared overnight with normal rabbit serum adsorbed to protein A–Sepharose. Precleared lysates were incubated with affinity purified rabbit serum for 4–8 h at 4 °C and the antigen–antibody complexes collected by incubation with an equal volume of a 50% slurry of protein A–Sepharose at 4 °C for 1 h and precipitated by centrifugation at 15 000 g for 5 min in a microcentrifuge. The immunoprecipitates were washed four times with 1% NP40 lysis buffer, twice with 0.5 M LiCl–0.1 M Tris (pH 7.4) and twice with 1% NP40 lysis buffer before immunoprecipitated proteins were removed from the immune complex by boiling in SDS sample buffer. Samples were run on 7.5% polyacrylamide gels, fixed, dried and autoradiographed.

In the case of vaccinia recombinants, 5 × 10⁴ cells were infected at 20 p.f.u. per cell and labelled for the times indicated in the Figure legends at 12 h post-infection with 20 µCi/ml [35S]methionine in medium containing 10% of the normal levels of methionine.
Western transfers were carried out in cold 0.25 M Tris–0.19 M glycine (pH 8.8), 20% methanol and 0.1% SDS in a Bio-Rad minigel system at 175 mA for 1 h. Nitrocellulose membranes were then blocked overnight at 4°C with TBS (20 mM Tris–HCl; 150 mM NaCl; pH 8.2)–5% dried milk powder and probed with the appropriate antisera. Membranes were then washed, incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (DAKO) and developed using 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (Sigma).

**Carbohydrate analysis.** Endoglycosidase H (endoHI), O-glycanase, N-glycanase and sialidase (neuraminidase) were used according to the manufacturer’s (Boehringer Mannheim) instructions. Glycosylation of proteins in cells infected with 10 p.f.u. per cell of recombinant vaccinia virus was blocked by the addition of 20 μg/ml tunicamycin at 4 h post-infection for 3 h before addition of 20 μCi [35S]methionine in medium containing 10% of the normal levels of methionine and 20 μg/ml tunicamycin. Immunoprecipitation was then carried out as described above.

**Immunoelectron microscopy.** Vaccinia recombinant RK13 infected cells were pelleted and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in PBS, pH 7.4, at 20°C. After brief rinsing in buffer and then distilled water, specimens were dehydrated sequentially in ethanol at low temperature as follows: 30% for 30 min at 0°C then 50, 70, 95, 100 for 1 h each at −20°C. Specimens were then embedded in Lowicryl HM20 resin (Agar Scientific) at −30°C for 14 h in conical capsules. Embeddings were polymerized with diffuse UV for 48 h at −30°C.

Sections (60 nm thick) were mounted on 300 mesh gold grids and rinsed for 1 h in 10% normal goat serum in PBS, then incubated for 2 h in either pre-immune rabbit serum or rabbit anti-gp110 diluted 1:5 in PBS. After rinsing with buffer, specimens were incubated with goat anti-rabbit IgG conjugated to 5 nm colloidal gold (Amersham) diluted 1:25 in TBS. After further buffer rinsing specimens were dipped in distilled water and dried. Grids were subsequently stained with uranyl acetate and lead citrate and examined at 60 or 80 kV in an EM400 electron microscope.

**Results**

To facilitate the analysis of EBV gp110 we expressed a number of fragments of the gp110 gene in *E. coli* (see Methods). Interestingly, the clones that spanned the hydrophobic anchor sequence of gp110 – pS9, pD2 and pP10 – all grew poorly and could not be used. However pS15, pD9 and pP12 were all expressed at high levels and protein isolated from gel slices was used to raise antisera (referred to as anti-S15, anti-D9 or anti-P12 antisera respectively). pD9 contains aa 79–605 of gp110, pS15 contains aa 192–445 of gp110 and pP12 contains the C-terminal 88 aa of gp110.

**gp110 in lymphoid cell lines**

Since all previous studies of EBV gp110 have used the traditional laboratory EBV strains adapted over many passes in culture, for example, the mononucleosis derived marmoset cell line B95-8, and since different strains of EBV vary in their activity on B-cells and epithelial cells (Li et al., 1992) we decided to analyse gp110 from other more recently derived EBV strains of both A and B types carried in human cell lines. This allowed us to examine the possibility that the reported features of EBV gp110 (lack of complex carbohydrates and lack of cell surface transport) are a consequence of adaptation during propagation in culture. Human cell lines Akata (EBV type A) and C2-BL16 (EBV type B) along with B95-8 were induced to enter the EBV replicative cycle and gp110 expression was initially analysed by immunofluorescence. Following induction, between 5 and 15% of cells in all the EBV-propagating lines examined could be labelled with anti-gp110 antibodies corresponding to either the N-terminal (S15 and D9) or C-terminal (P12) part of the molecule. No reactivity was observed with pre-immune sera on either induced or uninduced cells and none of the sera used reacted with Raji (EBV genome positive, virus defective) and Ramos (EBV negative) cells used as controls. Dual immunofluorescence showed that all cells labelled with anti-gp110 sera also expressed the late viral protein gp340/220 as detected with the monoclonal antibody 72A1 (data not shown).

The proteins synthesized during the late phase of the EBV replicative cycle were metabolically labelled and used for immunoprecipitation with anti-gp110 sera in an attempt to establish the molecular mass and glycosylation status of gp110 expressed by various cell lines (Fig. 1). The major proteins immunoprecipitated with anti-S15 serum from Akata (Fig. 1a), C2-BL16 (Fig. 1b) and B95-8 (Fig. 1c) all correspond in size to the glycosylated form of gp110 at 110 kDa (Gong et al., 1987). However, all the carbohydrates could be removed using endoH which results in a decrease of the molecular mass to 93 kDa, corresponding to the molecular mass of the unmodified form of gp110 predicted from its amino acid composition. These data demonstrate that the predominant form of gp110 possesses only high-mannose type glycosylation and that it is not further modified by Golgi enzymes regardless of the EBV strain or species of host cell. Further analysis of gp110 expressed by induced B95-8 cells was carried out by treating immunoprecipitates with O-glycanase plus sialidase which would remove O-linked carbohydrate residues if present. The molecular mass of the immunoprecipitated gp110 remained unaltered (Fig. 1c, O+S), indicating the absence of O-linked oligosaccharides. As both high-mannose modification and O-linked addition of oligosaccharides take place in the Golgi compartment the data imply that gp110 is located in a pre-Golgi compartment.

**gp110 expressed by vaccinia virus recombinants**

Since the level of expression of gp110, even in EBV positive lymphoid cell lines induced to replicate virus, is low its labelling and detailed analysis were problematical and we therefore chose to express EBV gp110 at higher levels using recombinant vaccinia viruses. Two basic recombinants were derived which express the full-length gp110, vgpl10 and vgpl10/7.5. The only difference between these two recombinants is that vpg110 expresses gp110 from a strong vaccinia, late structural gene promoter and vgpl10/7.5 expresses the
gene from a promotor that is constitutive, being active at early and late times after vaccinia infection.

The recombinant vaccinia virus vgp110 was initially characterized by immunofluorescence using the anti-gp110 sera generated against E. coli derived products. All three sera generated (anti-S15, anti-D9 and anti-P12) reacted with vgp110 infected Ramos EBV negative lymphoid cells and not with control vgp110/rev infected cells (data not shown). Comparison of the molecular mass of products immunoprecipitated with anti-S15 antiserum from induced B95-8 cells (Fig. 2a, lane 1) and cells infected with vgp110 (Fig. 2a, lane 4) show a protein exhibiting the same gel mobility at 110 kDa.
Fig. 3. Subcellular localization and modification of gp110. (a) Immuno-electron microscopy of vaccinia recombinant infected RK13 cells. RK13 cells infected with vgp110 at 10 p.f.u. per cell for 18 h before being fixed, embedded, sectioned and labelled with a combination of anti-S15 antiserum and 5 nm diameter colloidal gold. The sections were silver enhanced, counterstained and analysed using the transmission electron microscope. Arrows indicate examples of particles at the nuclear membrane. (b) RK13 (lanes 1, 2, 7 and 8) HeLa (lanes 3 and 4) or MRC-5 (lanes 5 and 6) cells infected with vgp110 were labelled with [35S]methionine for 4 h at 12 h p.i. Cells were lysed and proteins immunoprecipitated with rabbit anti-S15 antiserum and either run directly on 7% SDS-polyacrylamide gels (lanes 1, 3, 5 and 7) or treated with endoglycosidase H (lanes 2, 4, 6 and 8). An autoradiograph is shown and molecular masses of marker proteins run in parallel are shown in kDa.

which was deglycosylated to the same extent by endoH (Fig. 2a, lanes 2 and 3).

To characterize the vaccinia recombinants further, immunoprecipitations at 4 h and 12 h post-infection were carried out (Fig. 2b). Proteins expressed by both the gp110 recombinant viruses – vgp110 (Fig. 2b, lanes 1–4) and vgp110/7.5 (Fig. 2b, lanes 5–8) – were found to be sensitive to endoH both before and after vaccinia virus DNA replication. This indicates that glycosylation of the recombinant gp110 is not altered by higher levels of expression or by synthesis at late times in the vaccinia virus infectious cycle. Under all conditions investigated gp110 was reduced in size by endoH to approximately 93 kDa corresponding to the size of the endoH digestion product of gp110 expressed by B95-8 cells. The short labelling times used here might make it difficult to visualize higher molecular mass gp110 related molecules; however, none were
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Fig. 4. Analysis of vgp110Δ. (a) RK13 cells infected with vgp110 (lanes 1–3), vgp110Δ (lanes 4–6) or vgp110/rev (lanes 7–9) were labelled with [35S]methionine at 7 h post-infection for 8 h in the absence (lanes 1, 2, 4, 5, 7 and 8) or presence of tunicamycin (lanes 3, 6 and 9). Cells were lysed and immunoprecipitated with anti-S15 antiserum. Immunoprecipitates of the cells treated with tunicamycin were analysed directly on SDS–polyacrylamide gels whereas proteins immunoprecipitated from untreated cells were either run directly on the gels (lanes 1, 4 and 7) or treated with endoglycosidase H before electrophoresis. An autoradiograph is shown and molecular masses of marker proteins run in parallel are shown in kDa. (b) RK13 cells infected with vgp110 (lane 2) or vgp110Δ (lanes 3 and 4) were lysed with 0–5% NP40 and mixed with an equal volume of protein loading buffer with (lane 4) or without 2-mercaptoethanol (lanes 2 and 3), boiled and electrophoresed through 7% SDS–PAGE gels followed by transfer to nitrocellulose. Molecular masses of marker proteins (lane 1) are indicated in kDa. Western blots were probed with anti-S15 antiserum and visualized by incubation with phosphatase-conjugated anti-rabbit IgG and Fast BCIP/NBT buffered substrate (Sigma). The position of the gp110 and gp110Δ monomers (M) and dimers (Di) are indicated.

observed when labelling was carried out for 8 h (see Fig. 4) even if the autoradiographs were overdeveloped.

gp110 in non-lymphoid cell lines

EBV DNA and proteins can be found in a number of epithelial pathologies including nasopharyngeal carcinoma and hairy oral leukoplakia and it has long been postulated that nasopharyngeal epithelial cells may be the productive source of EBV found in saliva. We were therefore interested to analyse the glycosylation and transport of gp110 in non-lymphoid epithelial cell lines on the basis that variation in gp110 may have a role in the cell tropism of the virus. Immunofluorescence analysis (data not shown) suggested a general cytoplasmic subcellular localization of gp110 in many cells with significant amounts also located at the nuclear membrane of both RK13 (rabbit kidney epithelial cells) and HeLa recombinant infected cells. Both cell lines gave similar patterns of staining with all three antisera. To examine the localization of gp110 more closely we undertook immuno-electron microscopy of vaccinia recombinant infected RK13 cells, which showed that the majority of immunoreactive gp110 is present at the nuclear envelope with a significant amount being detected in the cytoplasm (Fig. 3a). Deglycosylation of immunoprecipitated gp110 from recombinant infected epithelial cells (Fig. 3b) further reinforced the notion that gp110 was located in a pre-Golgi compartment as only the immature high-mannose (endoH sensitive) form of gp110 was present. Thus gp110 appears to behave similarly in both lymphoid and epithelial cells and unlike HSV gB expressed by vaccinia recombinants (Cantin et al., 1987; Lin et al., 1996), gp110 is not expressed to any significant level at the cell surface.

gp110 deletion and chimaeric molecules

In order to analyse further the transport and processing of gp110 we generated a vaccinia recombinant (vgp110Δ) expressing a truncated version of gp110 containing the first 577 aa of the mature protein but lacking the membrane anchor and two recombinants expressing chimaeric molecules. Fig. 4 shows the results of immunoprecipitation with anti-S15 antiserum on vaccinia recombinant infected cells expressing the full-length gp110 (lanes 1–3), the truncated version of the gene (lanes 4–6) or a control, vgp110/rev, containing the gp110 gene in an antisense orientation with respect to the 11K promoter (lanes 7–9). It is clear from the biochemical analysis that deglycosylation with endoH and inhibition of glycosylation with tunicamycin give a similar sized product indicating that both the full-length gp110 and the truncated gp110 have not passed through the Golgi. This was somewhat
unexpected as removal of the predicted transmembrane regions of many other molecules allows secretion. We concentrated the supernatant from labelled vgp110Δ infected cells 5-fold but were still unable to demonstrate any secreted protein that could be immunoprecipitated with anti-S15 antisera (data not shown). It is possible that this deletion affected secondary structure to an extent which would not allow transport whereas other smaller, or even larger, deletions may have been transported. We attempted to assess the higher order structure of our mutant by analysing the ability of gp110Δ to form SDS resistant dimers in a manner similar to that shown for other herpesvirus gB homologues (Ali, 1990; Billstrom & Britt, 1995; Laquerre et al., 1996; Lin et al., 1996). Fig. 4(b) shows Western blots of extracts of vgp110 and vgp110Δ infected cells using anti-S15 antiserum. Dimers that formed when 2-mercaptoethanol was absent from the cell lysis buffer were heat and SDS resistant as evidenced by the presence of an immunoreactive protein at around 220 kDa (Fig. 4b, lane 1).

Interestingly, again in contrast to HSV gB where aa 628–653 (Laquerre et al., 1996) are essential in dimer formation, dimers could be formed even upon deletion of the 286 3′-terminal aa of gp110 which includes the region corresponding to the HSV dimerization region and the putative membrane anchor. This implies to some extent that the secondary structure of EBV gp110 was maintained in the deletion allowing disulphide bonds to be formed.

Recombinants expressing gp110 related chimaeric molecules containing either the 5′, 571 aa of gp110 including the signal sequence and the 3′, 171 aa of EBV gp340 encompassing the membrane anchor (vgp110/340) or containing the 5′, 736 aa of EBV gp340 covering the signal sequence and the internal repeat region of gp340 and the 3′, 286 aa of gp110 which includes the putative membrane anchor of gp110 (vgp340/110) were constructed. Immunofluorescence analysis using anti-S15 antisera on cells infected with the deletion mutant vgp110Δ and the chimaeras vgp340/110 and vgp110/340 indicated that the recombinant proteins were predominantly located in the cytoplasm and at the nuclear membrane (data not shown). 3′ Terminal deletion mutants of gp340 similar in size to the gp340 aa retained in vgp340/110 and vgp110/340 indicated that the recombinant proteins were predominantly located in the cytoplasm and at the nuclear membrane (data not shown). 3′ Terminal deletion mutants of gp340 similar in size to the gp340 aa retained in vgp340/110 and vgp110/340 indicated that the recombinant proteins were predominantly located in the cytoplasm and at the nuclear membrane (data not shown). 3′ Terminal deletion mutants of gp340 similar in size to the gp340 aa retained in vgp340/110 and vgp110/340 indicated that the recombinant proteins were predominantly located in the cytoplasm and at the nuclear membrane (data not shown).

The carbohydrate modifications to these proteins were...
analysed by immunoprecipitation of labelled proteins from vaccinia recombinant infected cells with a set of deglycosylating enzymes (Fig. 5). In summary, all proteins were completely deglycosylated with endoH indicating that the carbohydrate modification was of the immature high-mannose form. This was further confirmed by the finding that sialidase treatment followed by O-glycosidase had no effect on the mobility of the recombinant or wild-type gp110 related proteins indicating the absence of O-linked glycosylation. The activity of the enzyme preparations was confirmed by incubation with immunoprecipitated gp340 which was sensitive to the enzymes used. This pattern of glycosylation is consistent with the previous immunofluorescence based observations that the proteins have not passed through the Golgi.

As can be seen from Fig. 5, N-glycanase treated gp110/A (a) or the chimaeric protein gp110/340 (c) produced slightly larger proteins than those treated with endoH. If both enzyme digests were complete, N-glycanase would be expected to leave an unmodified polypeptide core of a lower molecular mass than the product of endoH treatment which would leave single GlcNAc groups attached to asparagine residues. However, N-glycanase is known to require a minimum length of N-N-acetyllactosamyl oligosaccharide chains for efficient deglycosylation (Chu et al., 1986); therefore, this result may be explained if short oligosaccharide units are attached to some of the asparagine residues of gp110 resulting in incomplete removal of carbohydrates by N-glycanase.

Discussion

Our results confirm that in B95-8 marmoset cells EBV gp110 is predominantly localized within the cell to the ER/nuclear membrane despite gB from members of the alphaherpesviruses being transported to the cell surface and incorporated into virions. We show that the glycosylation of gp110 is exclusively N-linked and that gp110 from other virus strains behaves in a similar fashion as does gp110 expressed in human cells of both lymphoid and epithelial origin. Like other members of the herpesvirus gB family, gp110 forms SDS and heat resistant dimers implying similar secondary structure motifs between family members (Highlander et al., 1991; Qadri et al., 1991). Classic ER retention signals or nuclear targeting signals are absent from gp110. Our studies with a deletion mutant and several chimaeras suggest that signals at both the 5’ and 3’ ends of the molecule target gp110 to the ER/nuclear membrane. In contrast, HSV gB when truncated similarly is secreted, suggesting that only the 3’ end of that molecule is primarily responsible for its interaction with cellular membranes.

Herpesviruses acquire their primary envelope while budding out from the nuclear membrane and it has been suggested that the precursor forms of herpesvirus glycoproteins might be included into the viral envelope at the nuclear membrane and then modified during trafficking of the virion through the cytoplasm (Poliquin et al., 1985; Ali et al., 1987; Torrisi et al., 1992). However, data do not support this hypothesis since pseudorabies virus and HSV have been reported to be assembled in two stages including primary envelopment at the nuclear membrane and de-envelopment at the ER followed by secondary envelopment within the Golgi apparatus (Browne et al., 1996). Also, in the case of varicella-zoster virus mature forms of glycoproteins have been shown to be incorporated into virions in the trans-Golgi network (Gershon et al., 1994; Zhu et al., 1995), which is likely to be the true place for the final assembly of all the herpesviruses. Both alpha- and betaherpesvirus gB homologues present on the viral particle are actively involved in penetration of the virus into the cell since anti-gB antibodies directed against gBs can neutralize these viruses (Cranage et al., 1986; Highlander et al., 1988; Massaer et al., 1993) and gB mutants can attach but cannot penetrate into the cell (Cai et al., 1988a, b).

The apparent absence of gp110 from the EBV virion and the inability of anti-gp110 antibodies to neutralize virus suggests that despite collinear homology and similar structural motifs EBV gp110, in contrast to HSV gB, is not required for entry into the cell. It was postulated some time ago that this would be the case (Misra et al., 1988) on the basis that such a high level of gB protein conservation is unlikely to be consistent with a role in membrane fusion because subfamilies of herpesviruses differ drastically in their cell type restriction. Therefore, gBs might be multifunctional proteins playing different roles as fully glycosylated components of the viral particle (of alpha- and betaherpesviruses) and high-mannose glycosylated forms at the nuclear membrane of the infected cell. Recent reports of an EBV deletion mutant lacking gp110 (Herrold et al., 1996; Lee & Longnecker, 1997) showed that gp110 was required for infectious virus to be produced and this would be consistent with a role for gp110 in virus maturation or release from the nuclear envelope. C-terminal truncations of gp110 (lacking 16, 41 or 56 aa) are unable to complement the gp110 deletant virus. Interestingly, only the deletion lacking 16 aa appears to be retained in the wild-type intracellular location larger deletions give some cell surface fluorescence. Electron microscopic studies on the mutant virus should elaborate on whether virus lacking gp110 can exit from the nucleus. The mechanism of glycoprotein transport from ER to the nuclear envelope as well as the function which viral glycoproteins might play there remain uncertain.

A further remaining enigma is that mutants of HSV lacking the gB gene can still form extracellular virions implying that gB, unlike gp110, is not required in steps other than the entry of virus into the cell. This raises the intriguing possibility that the gammaherpesviruses have modified the site of action of gB requiring membrane fusion at the nuclear envelope instead of the cell surface as for other herpesviruses and implying that another HSV gene product achieves this function. Alternatively, EBV gp110 may simply have evolved a different function. In conclusion, despite the structural relatedness of...
gp110 and gB they seem to have developed different biological functions possibly due to differences in glycosylation and subcellular localization.

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