Co-expression of the Epstein–Barr virus BXLF2 and BKRF2 genes with a recombinant baculovirus produces gp85 on the cell surface with antigenic similarity to the native protein

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Glycoprotein H (gH) is a conserved herpesvirus gene product functionally implicated in the penetration of the virus into the host cell. Other human herpesviruses require an accessory glycoprotein named gL for the synthesis of mature gH. We constructed a series of recombinant baculoviruses to determine whether gL expression can overcome the constraints upon Epstein–Barr virus (EBV) gH (gp85) folding and transport in this expression system. When gH and gL were co-expressed some EBV gH was transported to the insect cell surface. Deletion of 27 amino acids from the gH carboxy terminus resulted in the secretion of an 80 kDa protein (gp85t) into the culture medium when it was expressed either in the presence or absence of gL and this protein could also be immunoprecipitated with E1D1. In contrast, gL was not secreted into the culture medium. Our results suggest that either co-expression of gH with gL or removal of the predicted transmembrane anchor sequence can overcome some of the constraints upon EBV gH expression in the baculovirus system.

Epstein–Barr virus (EBV) is a human herpesvirus associated with a number of important human cancers including undifferentiated nasopharyngeal carcinoma (NPC), Burkitt’s lymphoma, Hodgkin’s lymphoma and lymphomas of immuno-suppressed individuals (Rickinson, 1994). EBV is also the causative agent of infectious mononucleosis or glandular fever, a syndrome common in the West amongst individuals in which primary infection is delayed until adolescence. Understanding the molecular mechanism of infection is a primary goal in developing a preventative vaccine and research has focused upon the envelope glycoprotein gp340/220, the ligand responsible for binding the virus to CD21 the host cell receptor. This molecule has proved a good vaccine candidate and neutralizing antibodies to gp340 prevent infection of B cells in vitro and vaccination with this glycoprotein prevents the induction of tumours in cotton-top tamarins by EBV (Morgan, 1992). Glycoprotein 85 (gp85) is also a component of the EBV membrane antigen complex but is present in relatively minor quantities in virus and cell membranes making its characterization in EBV-transformed cell lines difficult. Nevertheless, gp85 has been identified as an essential protein for fusion between the virus and host cell envelopes during infection (Miller & Hutt-Fletcher, 1988; Haddad & Hutt-Fletcher, 1989).

gp85 is encoded by the EBV BXLF2 gene (Heineman et al., 1988) with homologues in all herpesviruses so far studied highlighting its functional importance. Expression of BXLF2 in recombinant eukaryotic expression systems has resulted in the synthesis of defective recombinant products (Heineman et al., 1988; Yaswen et al., 1993; Pulford et al., 1994). Similarly, other human herpesvirus gH proteins are also synthesized as defective products if expressed in heterologous systems in the absence of other viral proteins (Cranage et al., 1988; Gompels & Minson, 1989; Forrester et al., 1991; Roberts et al., 1991), but this aberrant expression can be overcome in mammalian cells when herpesvirus gH proteins are co-expressed with gL (Hutchinson et al., 1992). Homologues of the HSV-1 gL gene (UL1) have been identified for a number of other herpesviruses encoding glycoproteins which allow the normal folding, transport and processing of their gH counterparts (Kaye et al., 1992; Spaete et al., 1993; Liu et al., 1993; Forghani et al., 1994). The EBV BKRF2 gene has been identified as the EBV analogue of HSV-1 UL-1 (Yaswen et al., 1992).
Recombinant baculoviruses have been used to characterize a number of herpes virus glycoproteins including gH (Ghiasi et al., 1991; McGowan et al., 1994; Pulford et al., 1994). When EBV gH is expressed in the baculovirus system in the absence of other EBV gene products it is not recognized by gp85 MAb, and is not transported to the cell surface (Pulford et al., 1994). We set out to determine whether co-expression of BKRF2 with BXLF2 would allow EBV gH to be folded and transported to insect cells like the native protein and to determine whether removal of the predicted transmembrane anchor sequence of gH would enable this protein to be secreted.

A series of baculovirus plasmid transfer vectors was constructed to generate recombinant viruses capable of expressing truncated or full-length EBV gH and gL proteins either singularly or in tandem. The BKRF2 gene was generated as a PCR product using two synthetic oligonucleotide primers expressing truncated or full-length EBV gH and gL gene contained in pBXLF2-E5 (Pulford et al., 1994), were used to make a series of baculovirus transfer vectors. The gHt gene was subcloned into pVL1392 (Invitrogen) and the gL gene into pAcUW51 (Pharmingen) to generate recombinant viruses expressing only one foreign gene. In addition, the gH or the gHt genes were also inserted into pAcUW51 along with the gL gene to generate baculovirus dual expression vectors. In all these constructs the gH/gHt genes were driven by the polyhedrin promoter and the gL gene by the P10 promoter. Recombinant viruses were then generated using the Baculogold transfection kit (Pharmingen) according to the manufacturer’s instructions.

Fixed P3HR1 cells were used to demonstrate that gp85 MAb F-2-1 (Strnad et al., 1982) and E1D1 (Balachandran et al., 1987), affinity-purified with Protein-G, exhibited strong immunofluorescence with the P3HR1 EBV-transformed human B cell line (Fig. 1 a, b) demonstrating their suitability as reagents for the detection of native gp85. Although the gH and gL genes are derived from the B95-8 virus P3HR1 cells could be induced to greater levels of productive infection. Sequence differences or deletions are not known to be present in gH or gL coding regions. AAcgH–gL was the only recombinant virus that produced strong and specific immunofluorescence with F-2-1 (Fig. 1 c) and E1D1 (Fig. 1 d) demonstrating that co-expression of gH with gL was capable of generating some native product in insect cells. The expression of gH and gL were also assessed in fixed, 8195 (Pulford et al., 1994) (Fig. 1 e) and an EBV gL-specific antibody (anti-BK, Yaswen et al., 1993) (Fig. 1 f). Background fluorescence levels with the latter anti-peptide antibody were substantially higher than with either of the E1D1 or F-2-1 MAb. In addition, both F-2-1 (Fig. 1 g) and E1D1 (Fig. 1 h) also labelled the surface of live AAcgH–gL-infected cells demonstrating that this conformationally native structure was transported to, and anchored on, the insect cell membrane.

To quantify the relative reactivity of our recombinant virus constructs with the gp85 MAb E1D1 and F-2-1 we subjected live virus-infected insect cells to fluorescence-activated flow cytometry (Fig. 2 a, b). S9 cells were
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infected with wild-type or recombinant baculoviruses at an m.o.i. = 10 and incubated at 28 °C for 48 h. Cells were then washed twice in cold PBS, resuspended in 200 μl of PBS+5 % calf serum containing MAb and incubated on ice for 1 h. Samples were washed twice again, resuspended in 200 μl of PBS+5 % calf serum containing FITC-conjugated antibodies and incubated on ice for a further hour. Finally, cells were washed once more before analysis with a Becton Dickinson FACS and data were processed using PCLYSIS. Although AcgH- and AcgHt-gL-infected cells produced slightly more fluorescence than cells infected with a negative control virus (AcNPV) with both F-2-1 (Fig. 2a) and E1D1 (Fig. 2b), these small increases were insignificant compared to the large increases in fluorescence obtained with AcgH–gL-infected cells: 69 % and 87 % more AcgH–gL cells were fluorescent with F-2-1 and E1D1 respectively than with the negative control virus Autographa california nuclear polyhedrosis virus (AcNPV). These results were consistently observed and a negative control MAb 72A1 (Hoffman et al., 1980) specific for gp340 was negative for all recombinant baculoviruses (data not shown). None of the other recombinants produced more than 4 % more fluorescence than AcNPV with either MAb confirming that gH expression alone was insufficient for obtaining a native product and further demonstrating that these MAbs do not recognize recombinant gL produced in baculoviruses or at least that non-native gL is not recognized by the MAbs tested. F-2-1 may recognize a conformational epitope on gL which is only available when gL is combined with gH. The lack of immunoreactivity of AcgHt–gL-infected cells with these MAbs in this assay may be due to protein truncation inhibiting gHt folding. It is more likely that removal of the predicted transmembrane anchor sequence resulted in the secretion of soluble immunoreactive protein out of the cell. It is possible that the BKRF2 product might be anchored to membranes by the amino-terminal signal-like sequence as a type II membrane protein although this has not been found to be the case with other herpesvirus gL products.

To examine whether the recombinant products were synthesized as soluble or insoluble proteins in insect cells and to determine if gHt was secreted into the extracellular medium we prepared cell extracts and monitored them by Western blot. Baculovirus-infected Sf9 cells grown on SF900II serum free culture medium (Gibco) were harvested at 72 h p.i., cells were washed once in PBS, pelleted by centrifugation and were treated with lysis buffer (50 mM-Tris–HCl pH 7.5, 140 mM-NaCl, 1 % Triton X-100) on ice for 30 min to obtain a soluble protein extract. The cell debris and insoluble material were pelleted by centrifugation for 5 min at 5000 r.p.m., the supernatant (soluble protein fraction) was removed and the pellet resuspended in lysis buffer containing 1 % SDS (insoluble protein fraction) were separated by SDS–PAGE on 7 % gels and immunobotted with affinity-purified 8195 antibody to detect gH proteins (Fig. 3). The majority of gH synthesized by all the recombinant viruses was found in the insoluble protein fraction and was also susceptible to aggregation as previously observed (Pulford et al., 1994). The gH protein profile for each of the baculovirus insoluble protein fractions contained a smear of high molecular mass aggregates and a group of immunoreactive polypeptides ranging from 68 kDa to 84 kDa (Fig. 3a) that may correspond to incompletely glycosylated forms of the BXLF2 gene product. No decrease in the presence of
Fig. 3. Detection of recombinant gH from virus-infected cell fractions by Western blot. Sf9 cells were infected at an m.o.i. = 10 with wild-type (lane 1) or recombinant baculovirus for 72 h before cells and medium were harvested and insoluble (a), soluble (b) and extracellular (c) protein extracts prepared. Samples AcgH (lane 2), AcgHt (lane 3), AcgH-gL (lane 4), AcgHt-gL (lane 5) and AcgL (lane 6) were separated on 7% SDS-polyacrylamide gels and gH was detected on nitrocellulose electroblots with 8195 antibody (Pulford et al., 1994). (d) Tissue culture medium from AcgHt- (lanes 1 and 2) or AcgHt-gL- (lanes 3 and 4) infected cells was immunoprecipitated with E1D1 prior to detection of gHt by Western blot using antiserum #8195 made against bacterial gp85 fusion protein.

Insoluble gH was observed when recombinant viruses co-expressed gL. gHt had a maximum molecular mass of 80 kDa (Fig. 3a, lanes 3 and 5) and full-length gH a maximum molecular mass of 84 kDa (Fig. 3a, lanes 2 and 4). Soluble protein fractions all contained a faint 68 kDa protein band and some lower molecular mass products but only Sf9 cells infected with AcgH-gL contained a small amount of an 84 kDa form of gH (Fig. 3b, lane 4) which was absent from AcgH-infected cells (Fig. 3b, lane 2). This 84 kDa protein might represent soluble forms of gH generated as a result of gL co-expression. Previously, we had found small amounts of gH in the soluble fraction of AcgH-infected insect cells which was sensitive to aggregation on boiling (Pulford et al., 1994) and the presence of similar aggregates in some samples (Fig. 3b, lanes 2 and 3) suggested that these fractions may not be pure and could still contain insoluble protein contaminants.

Extracellular medium obtained from virus-infected cells expressing the gHt gene contained a discrete 80 kDa protein band that was absent from constructs with the full-length gH gene (Fig. 3c). This demonstrates that truncating the gH carboxy terminus from His-679 enables soluble forms of the protein to be secreted from insect cells in either the absence (Fig. 3c, lane 3) or presence (Fig. 3c, lane 5) of gL expression. The similar levels of gHt secreted by both the single and dual expression vectors indicated that expression of gL did not affect gHt secretion (Fig. 3c). In contrast, a truncated form of the HCMV gH was expressed as a defective
recombinant protein unless it was co-expressed with HCMV gL or with a truncated form of the fibroblast growth factor receptor (Spaete et al., 1993) indicating that other proteins are required for the folding and transport of this herpesvirus protein. Interestingly, secreted gHt did not undergo protein aggregation upon boiling suggesting that deletion of the transmembrane anchor membrane sequence offered more stability to soluble gHt. Alternatively, secretion might prevent soluble gHt reaching a critical intracellular concentration during its biosynthesis that might otherwise result in its aggregation.

Having established that some gHt was secreted from cells in the presence or absence of gL expression we set out to determine if these secreted products could be immunoprecipitated with gp85 MAb. Culture medium was freeze-thawed once, microcentrifuged for 20 min at 14000 r.p.m. to remove virus and debris and precleared by mixing with Protein-A-Sepharose beads (Sigma) at room temperature for 1 h. Supernatant was mixed with E1D1 at room temperature for 2 h then mixed with protein-A-Sepharose beads (Sigma) for 30 min and washed twice in PBS +1% Triton X-100; precipitated proteins were then boiled with gel sample buffer prior to Western blotting (Fig. 3d). gHt was immunoprecipitated with E1D1 (lanes 1 and 4) indicating that the truncated protein may resemble native gp85. Unexpectedly, gHt protein was also immunoprecipitated by E1D1 in the absence of gL (lane 1). Therefore, deletion of the EBV gH hydrophobic sequence allows native, conformation-dependent epitopes in the gH external domain to form in the absence of gL expression, a property also displayed by the human herpesvirus 6 truncated gH (Liu et al., 1993). Collectively, these results suggest that EBV gH might contain an endoplasmic reticulum retention signal within its last 27 carboxy-terminal amino acids whose influence can be eliminated by truncation or by interaction with a chaperone-like protein such as gL. Although E1D1 was capable of immunoprecipitating recombinant gHt, F-2-1 was not (data not shown). Previous studies using the EBV-positive lymphoid Akata cell line had demonstrated that F-2-1 recognized a complex of gH and gL with a third EBV glycoprotein called gp42 (Yaswen et al., 1993) offering some explanation for the lack of its activity with gHt. gp42 has now been identified as the product of the EBV open reading frame BZLF2 (Li et al., 1995). These authors found that the F-2-1 antibody was specific for gp42 and not for gp85 which is not supported by the results presented here where F-2-1 clearly binds specifically to a proportion of insect cells (as does E1D1) expressing only gH and gL from recombinant baculovirus (Fig. 2). A truncated form of HCMV gH was expressed as a defective recombinant protein unless it was co-expressed with either HCMV gL or with a truncated form of fibroblast growth factor receptor (Spaete et al., 1993) indicating that other apparently unconnected proteins can be directly or indirectly involved in the expression, folding and transport of this herpesvirus protein.

Recombinant gL was detected by immunoblot with anti-BKRF2 antibody (Fig. 4). The majority of EBV gL, like gH, was contained within the insoluble protein fraction indicating that over-expression of either product resulted in the synthesis of insoluble recombinant products despite their co-expression. Insoluble gL, unlike gH, did not aggregate (Fig. 4a). All the BKRF2-expressing viruses produced gL species of molecular mass 24 kDa, 20 kDa, 17 kDa and 13 kDa (Fig. 4a, lanes 1, 3 and 5) in this fraction. The BKRF2 gene codes for a protein with a predicted molecular mass of 15080 Da, which probably corresponds to the 13 kDa band observed by SDS-PAGE (Fig. 4a). Digestion with Endo H reduced all the gL species to a single 13 kDa protein (Fig. 4a, lanes 2, 4 and 6) demonstrating that the higher molecular mass species are different glycoforms of gL and probably represent proteins with an increasing number of the potential N-glycosylation sites occupied (Fig. 4a, lanes 1, 3, 5 and 7).

Densitometry of the gL blot (Fig. 4a) indicates that there is 2-fold more gL in AcgL (lane 1) than in AcgHt–gL (lane 5) and 5-fold more gL in AcgL (lane 1) than in AcgH–gL (lane 3) showing that the presence of both gHt and gH affects the amount of detectable gL but to different degrees. All the vectors expressed similar amounts of unglycosylated gL (13 kDa band) but the amounts produced by the dual expression vectors were always significantly lower. These results were consistently obtained and both gHt and gHt had this effect although it was more pronounced with gH.

Incubation of AcgL-infected cells with tunicamycin resulted in the production of a slightly smaller deglycosylated form of gL (Fig. 4a, lane 8) than was observed with Endo H digestion. This difference was probably a consequence of Endo H leaving an N-acetylgalactosamine glycan covalently bonded at each N-linked glycosylation site. Collectively, these results demonstrate that all insoluble BKRF2 products synthesized in insect cells are processed with high mannose sugars only, possibly as a result of being retained in the endoplasmic reticulum. Consequently, soluble gL was detected in AcgL extracts only (Fig. 4b, lane 1) probably because it expressed more recombinant gL (Fig. 4a, lane 1) than either of the dual expression vectors (Fig. 4a, lanes 3 and 5) but no gL was detected in the extracellular medium for any of the baculovirus vectors even after a 10-fold concentration of the culture medium (Fig. 4c, lane 5). This demonstrates that gL is not secreted from cells either independently or as a complex with gHt.
Despite gL not possessing a predicted carboxy-terminal transmembrane anchor sequence. As mentioned earlier anchorage by the putative signal sequence as a type II membrane protein seems unlikely since this has not been found to be the case with other herpesvirus gL products. The absence of EBV gL secretion was unexpected, however, because CMV gL has been reported as being secreted by a baculovirus vector (Spaete et al., 1993).

The non-secretion of recombinant gL and poor solubility of gH and gL in insect cells imply that other viral or cellular factors are important for the expression of these glycoproteins. If gp42 is a necessary third component in this complex or permits the stable interaction of EBV gH with gL (Li et al., 1995) then its absence from our expression system could be responsible for the failure to obtain gH–gL in the soluble cell fraction or gHt–gL complexes in the extracellular medium of insect cells infected with recombinant viruses. Co-expression of the EBV gH, gL and gp42 genes in a baculovirus system collectively might be the key for the production of soluble, antigenically native and fully processed forms of these recombinant EBV glycoproteins.

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References


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