Expression of the Epstein–Barr virus envelope fusion glycoprotein gp85 gene by a recombinant baculovirus

David Pulford, Pauline Lowrey and Andrew J. Morgan*

Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

The gp85 envelope glycoprotein of Epstein–Barr virus (EBV) has a role in the molecular mechanism of infection, enabling fusion between the viral and host cell envelopes, a role in common with the homologous gH glycoproteins in other herpesviruses. A glutathione S-transferase bacterial fusion protein (GST85N-S) was generated, containing 178 amino acids from the C terminus of gp85 and including a known gp85 linear epitope. A panel of EBV-positive human antisera contained no antibodies to linear epitopes presented on the purified GST85N-S protein, indicating that primary protein structure in this region of gp85 is not a B cell target. This bacterial fusion protein was used to raise a rabbit monospecific polyclonal antiserum capable of detecting gp85 in a Western blot. The majority of recombinant baculovirus-expressed gp85 obtained from cell extracts prepared with SDS appeared on Western blots as heterogeneous high Mr protein aggregates and consistently included 84K, 81K and 70K bands. Recombinant gp85 aggregation was increased by boiling the sample prior to gel electrophoresis. The 84K and 81K proteins were completely sensitive to endoglycosidase H treatment, indicating that these glycosylated species did not undergo further post-translational processing. Immunofluorescence studies revealed that recombinant gp85 was not transported to the insect cell surface. It reacted only with antibodies recognizing denatured epitopes but not with antibody to native gp85. Therefore expression of the gene encoding gp85, BXLF2, alone in the baculovirus expression system is insufficient for the synthesis of a correctly transported, processed, folded and antigenically native form of recombinant gp85.

Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus infecting greater than 90% of the population worldwide and has been implicated in the causation of several important human tumours (zur Hausen, 1991). There are more than 80000 new cases per year of undifferentiated nasopharyngeal carcinoma (NPC; Parkin et al., 1984), which is the commonest cancer of males in southern China (Zeng, 1985). In addition, recent evidence suggests a link between EBV and certain Hodgkin’s lymphomas that are common in the West (Deacon et al., 1993; Young & Rowe, 1992). The molecular mechanism of EBV infection is therefore of considerable interest and to this end work has focused on the major envelope glycoprotein, gp340, principally in the context of vaccine development (Morgan, 1992). However, another viral envelope glycoprotein, gp85, is involved in the mechanism of fusion of the viral envelope with the host cell membrane and may be an important target of the immune system (Miller & Hutt-Fletcher, 1988). Monoclonal antibodies (MAbs) to gp85 can mediate virus neutralization, demonstrating its essential role in infection and complement-dependent infected cell cytolysis (Strnad et al., 1982). The 70K non-glycosylated precursor form of this protein is processed with simple and complex N-linked oligosaccharides (Edson & Thorley-Lawson, 1983; Strnad et al., 1983) to generate the mature 85K molecule. Experiments using octadecylrhodamine β-chloride-labelled EBV in a fluorochrome dequenching assay showed that gp85-neutralizing antibody does not prevent virus–cell binding but does inhibit membrane fusion (Miller & Hutt-Fletcher, 1988). In addition, artificial virosomes containing EBV membrane proteins are capable of fusing with B lymphocytes unless they are specifically depleted of gp85 (Haddad & Hutt-Fletcher, 1989).

The gene for gp85 has been mapped to the BXLF2 open reading frame (ORF) and is predicted to code for a 706 amino acid protein (Heineman et al., 1988). The glycoprotein has gH homologues in all three herpesvirus subfamilies (Cranage et al., 1988), which have all been implicated in virus penetration of the host cell. Expression of the BXLF2 gene in recombinant systems, like plasmid-transfected fibroblasts (Heineman et al., 1988) and recombinant vaccinia virus-infected cells (Yaswen et al., 1993) has resulted in expression of a recombinant gp85 that is not recognized by MAbs to the native molecule nor expressed on the cell surface. Similar observations have also been made with gH homologues
from other human herpesviruses (Cranage et al., 1988; Gompels & Minson, 1989; Forrester et al., 1991; Roberts et al., 1991) although the aberrant transport and folding of some of these glycoproteins may have been as a consequence of the expression systems adopted. Recent studies have shown that the gH of herpes simplex virus type 1 (HSV-1; Hutchinson et al., 1992; Roop et al., 1993), human cytomegalovirus (HCMV; Kaye et al., 1992; Spaete et al., 1993), human herpesvirus 6 (HHV-6; Liu et al., 1993a, b) and EBV (Yaswen et al., 1993) all appear to require an accessory protein, gL, to form a heterodimeric complex for their transport and expression.

A glutathione S-transferase (GST) bacterial fusion protein was constructed with a portion of the EBV BXLF2 gene to prepare a monospecific antibody to detect the gp85 molecule and to determine whether human EBV-positive antisera contain antibodies to a portion of the gp85 C terminus in which a known gp85 linear epitope exists (Oba & Hutt-Fletcher, 1988). A plasmid including the entire EBV strain B95-8 BXLF2 gene was constructed by cloning a 3.85 kbp Smal fragment from p31 (Griffin & Karran, 1983) and ligating it into the Smal site of pBluescript KS(−) (Stratagene). A 540 bp NheI-SstI DNA restriction fragment coding for a 178 amino acid stretch of gp85 from position 466 to 644 was excised from pS-2, end-repaired with T4 DNA polymerase and ligated into Smal-cut pGEX-3X (Smith & Johnson, 1988). A recombinant clone (pGST85N-S) containing the correctly orientated BXLF2 restriction fragment for fusion protein expression was identified by restriction endonuclease analysis and by dideoxy-nucleotide sequencing of plasmid DNA using GST synthetic oligonucleotide primer 5' GCATGCGCTT-GCAGGG 3'. The BXLF2 polypeptide sequence represented in pGST85N-S contains a short peptide sequence (position 482 to 488) conserved in HSV-1, HCMV and varicella-zoster virus and a potential hydrophobic fusion peptide sequence (518 to 533) identified by Oba & Hutt-Fletcher (1988).

Initially, only minor quantities of soluble fusion protein were synthesized in IPTG-induced Escherichia coli TG2 cells transformed with pGST85N-S, whereas the vast majority remained in the cell pellet (data not shown). Hartman et al. (1992) have shown that GST fusion proteins that are insoluble at 37 °C can nevertheless be produced as soluble proteins when induced at lower temperatures. We found that the best yields of soluble GST85N-S protein were achieved by inducing cells with 0.1 mM-IPTG at 25 °C with the HB101 E. coli strain and using a glutathione-Sepharose 4B column to purify the fusion protein (Ausubel et al., 1989). With this protocol it was possible to purify 2 to 4 mg of soluble recombinant protein per 1 dm³ of culture. The insolubility of this bacterial fusion protein might be a reflection of the high frequency (40%) of hydrophobic residues contained within the BXLF2 peptide. The major protein eluted by this procedure was a 50K species that

Fig. 1. Purification of GST85N-S antibody by affinity chromatography. Purified GST85N-S protein (lanes 3, 5, 7, 9 and 11) and wild-type GST protein (lanes 4, 6, 8, 10 and 12) were separated on a 12% SDS-polyacrylamide gel, electroblotted onto nitrocellulose (lanes 3 to 12) and probed with rabbit anti-V1 peptide antibody, rabbit 8195 anti-GST85N-S bleed-out serum, 8195 filtrate, 8195 eluate, or rabbit 14/5 anti-EBV serum. Blots were incubated with goat anti-rabbit alkaline phosphatase-conjugated IgG and developed with Fast Blue RR (Sigma) and α-napthyl phosphate (Sigma). Low Mr protein markers (Bio-Rad; lane 1) and purified GST85N-S protein (lane 2) were detected with Coomassie Brilliant Blue stain on a 12% SDS-polyacrylamide gel.
corresponded to the full length fusion protein and a 37K breakdown product (Fig. 1, lane 2) both of which reacted specifically with the V1 peptide antibody (Fig. 1, lane 3).

Purified GST85N-S fusion protein was used to raise a rabbit antiserum for detecting gp85. A pair of 10-week-old New Zealand white female rabbits were each subcutaneously immunized with 96 μg of purified GST85N-S prepared as an emulsion with Freund’s complete adjuvant. After 14 days, each rabbit received a second subcutaneous injection of antigen prepared with Freund’s incomplete adjuvant (FIA). Each rabbit was then boosted every month for 2 months further with antigen and FIA and antibody levels were monitored by Western blotting. After 3 months, rabbit antisera were found to react with purified GST85N-S and GST proteins by Western blot but also demonstrated some non-specific activity (Fig. 1, lane 5 and 6). Subsequently a two-step affinity purification protocol was employed using columns containing either purified GST or GST85N-S coupled to cyanogen bromide-activated Sepharose 4B (Sigma; Ausubel et al., 1989). Initially, antiserum from rabbit 8195 was depleted of GST antibodies by adsorption onto a GST column (Fig. 1, lane 7 and 8). The flow-through was then passed down a GST85N-S column, unbound proteins were washed away and fractions of purified GST85N-S antibodies were eluted. Using this approach a purified monospecific antibody was prepared that only reacted with the BXLF2 portion of the bacterial product (Fig. 1, lane 9) and showed no non-specific activity (Fig. 1, lane 10).

No antibodies were found to react with the purified GST85N-S protein by Western blot using a rabbit polyclonal anti-EBV serum 14/5 (North et al., 1980; Fig. 1, lane 11 and 12) indicating that the linear epitope(s) contained within this portion of the BXLF2 gene product may not be presented on the native gp85 molecule. To determine if these linear epitope(s) are dominant targets in humans we probed the GST85N-S (Fig. 2) and the wild-type GST purified proteins (data not shown) with an array of human gp85-positive and -negative antisera by immunoblotting. The purified fusion protein was detected with the V1 antibody and the purified 8195 antibodies but did not produce any reaction with human antiserum, diluted 1:25, from normal EBV-seropositive individuals, NPC and infectious mononucleosis (IM) patients or EBV-seronegative individuals (Fig. 2). This shows that any antigenic determinants within this portion of the native gp85 molecule must consist of discontinuous epitopes and that any linear epitopes, including the V1 epitope (Oba & Hutt-Fletcher, 1988), must be occluded in native gp85. Even so, some of the human and rabbit antibodies reacted with proteins of over 60K on the GST85N-S but not on the GST Western blots. These were probably E. coli protein contaminants and were detected because the GST85N-S protein was concentrated 50-fold to obtain an equivalent protein concentration to the GST protein, and because most individuals will have some antibodies to E. coli proteins.

Ghiasi et al. (1991) found that HSV-1 gH could be synthesized and expressed at the surface of insect cells by expressing the HSV-1 gH gene (UL22) with a recombinant baculovirus. In addition, antibodies raised in mice to this recombinant gH neutralized HSV-1 infectivity in vitro and mice vaccinated with the recombinant product developed delayed-type hypersensitivity but were not protected on challenge with HSV-1 (Ghiasi et al., 1992). In the light of these observations we constructed a recombinant baculovirus containing the EBV BXLF2 gene to determine if authentic EBV gp85 could also be synthesized in insect cells. The BXLF2 ORF was generated by PCR with a pair of synthetic oligonucleotide primers, #3 (5’ GCCCTCTAGAAGGATGC-AGTTGCTCTGT 3’) and #4 (5’ GCCCTCTAGATTA- CCTGCTGCGCATCTA 3’) that contained BXLF2 restriction sites. The PCR reaction utilized recombinant Vent DNA polymerase (New England Biolabs), rather than Taq, because Vent has a proofreading activity resulting in a lower error rate on DNA amplification. The PCR product was synthesized in a 100 μl reaction volume using a Perkin-Elmer DNA Thermal Cycler at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 3 min with p31 (Griffin & Karran, 1983) as a DNA template. The protocol was followed for 30 cycles. The 2157 bp PCR product was ligated into the Xbal site of pBluescript II KS(−) (Stratagene) to generate pWG5, but subsequent sequencing of this clone identified a single nucleotide substitution compared to B95-8 (Baer et al., 1984) from a G to an A at position 468 resulting in an amino acid change at residue 152 from an arginine to a histidine. To eliminate any possible phenotypic affect induced by the mutation we reconstructed the BXLF2 gene to contain the wild-type B95-8 sequence. A 383 bp EcoRI fragment was digested from pWG5, removing the 5’ end of the gene, and was ligated into pS-2 cut with EcoRI, to generate pBXLF2-E5. This arrangement produced a construct of the wild-type BXLF2 gene cloned with a cluster of unique restriction sites close to its 5’ end. A 2319 bp Xbal fragment containing the entire BXLF2 gene was excised from pBXLF2-E5 and was ligated into the Xbal site of pVL1392 (Invitrogen), a derivative of pVL941 (Lucknow & Summers, 1989), to generate the baculovirus transfer vector pVLBX-1. Reagents and procedures for maintaining the Spodoptera frugiperda insect cell line (S9) and for assaying and generating recombinant baculoviruses were provided in the Baculogold system (Pharmingen). Using this method, plasmid...
DNA was cotransfected with baculovirus DNA into SF9 cells by calcium phosphate precipitation and three individual recombinant virus clones (vAcEBgH) were isolated and found to have identical properties.

Synthesis of recombinant protein was monitored by Western blot. Virus-infected cells were washed in PBS and re-suspended in TNT buffer (50 mm-Tris–HCl pH 7.5, 140 mm-NaCl, 1% Triton X-100), incubated on ice for 30 min before debris and nuclei were pelleted by centrifugation (5000 r.p.m. for 5 min). Supernatants (cytoplasmic extracts) were collected and the pellets were re-suspended in an equal volume of TNT containing 1% SDS (nuclear extracts). The specificity of the 8195 antiserum to gp85 was demonstrated by its activity with native gp85 immunoprecipitated from B95-8 cells with the MAb F-2-1 (Fig. 3b, lane 1) in a Western blot. The affinity purified 8195 antiserum did not react with irrelevant proteins synthesized by a virus control (Fig. 3b, lane 2) and only detected products synthesized by vAcEBgH-infected SF9 cells (Fig. 3a, lanes 2 to 9) in Western blots. However, much of the recombinant protein generated by vAcEBgH-infected cells was observed in Western blots as a smear of higher M_r species that probably represent protein aggregates. Most recombinant protein was found in nuclear extracts prepared from vAcEBgH-infected cells (Fig. 3a, lanes 2, 4, 6 and 8) and was usually not observed in the cytoplasmic extracts (lanes 3, 5, 7 and 9), which suggests that the majority of recombinant gp85 was synthesized as insoluble product. Larger amounts of recombinant protein were found to enter both the stacking and resolving gels if the protein samples were not boiled prior to electrophoresis (lanes 6 to 9), demonstrating that boiling enhances protein aggregation. An 84K band was consistently observed in the cytoplasmic extracts of vAcEBgH-infected cells only when the sample was not

---

**Fig. 2. Reactivity of EBV-positive antisera to the GST85N-S bacterial fusion protein.** Purified GST85N-S protein was separated on a 12% SDS–polyacrylamide gel, electroblotted onto nitrocellulose and then probed with human antisera diluted 1:25 or purified rabbit antibodies diluted 1:50 (anti-GST & anti-GST85N-S) or 1:500 (VI). Human antisera were obtained from EBV-seronegative (including BIRD) and seropositive individuals including NPC and IM patients. Blots were incubated with secondary antibodies conjugated with horseradish peroxidase and detected with diaminobenzidine and NiCl₂ (Harlow & Lane, 1988).

<table>
<thead>
<tr>
<th>Sera</th>
<th>Rabbit</th>
<th>Human EBV-positive</th>
<th>NPC</th>
<th>IM</th>
<th>Human EBV-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GST</td>
<td>VI</td>
<td>BIRD</td>
<td>WUB</td>
<td>IM</td>
<td>NOSONI</td>
</tr>
<tr>
<td>Anti-GST85N-S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST85N-S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 97K
- 66K
- 45K
- 31K
- 21K
- 14K

3244  Short communication
boiled or reduced prior to electrophoresis (Fig. 3a, lane 7) indicating that small amounts of soluble misfolded protein might be synthesized by vAcEBgH. As well as high $M_r$ protein aggregates, 70K and 81 to 84K bands were also observed in nuclear extracts of vAcEBgH-infected cells (Fig. 3a, lane 2, 4, 6 and 8). The predominance of the recombinant product in the nuclear extracts of cells may mean that gp85 contains a nuclear membrane retention signal as has been demonstrated for the HSV-1 gB (Gilbert et al., 1994) or that the majority is misfolded product and requires strong denaturing conditions to be solubilized. The 70K and 81 to 84K bands resolved on gels probably correspond to unglycosylated and glycosylated forms of the polypeptide. To test this, the recombinant protein was deglycosylated with endoglycosidase H and was detected by Western blot with 8195 purified antibodies. Native gp85 from EBV-positive cells becomes partially resistant to endoglycosidase H upon maturation (Edson & Thorley-Lawson, 1983; Yaswen et al., 1993) which is indicative of further glycan processing beyond the endoplasmic reticulum. However, the 81 to 84K recombinant protein synthesized by vAcEBgH (Fig. 3c, lane 1) was reduced to 70K with endoglycosidase H (Fig. 3c, lane 2) confirming that the 70K component was probably an unglycosylated form of recombinant gp85. The presence of unglycosylated forms of gH in extracts from recombinant virus-infected Sf9 cells was also observed for HSV-1 (Ghiasi et al., 1991) and may represent translation of BXLF2 mRNA by free ribosomes in the cell cytosol as a consequence of insect cells having a lower fidelity for mammalian signal peptide sequences or it may represent protein that rapidly misfolds, resulting in potential $N$-linked glycosylation.
sites becoming inaccessible for processing. The observation that recombinant gp85 in the nuclear extract is glycosylated with simple sugars only is consistent with BXLFP recombinant products expressed in other eukaryotic systems (Heineman et al., 1988; Yaswen et al., 1993) and for the H3V-1 gH expressed by a recombinant baculovirus (Ghiasi et al., 1991). Although the lack of further glycan processing implies that recombinant gp85 was not transported beyond the endoplasmic reticulum. Some cell surface-expressed recombinant glycoproteins have been found to be largely processed with high mannose sugars when using the baculovirus system (Kuroda et al., 1990; Yeh et al., 1993). Overall levels of recombinant gp85 expression were lower and no 81 to 84K glycosylated protein was detected when vAcEBgH-infected cells were incubated with tunicamycin (Fig. 3c.

Fig. 4. Detection of native or denatured forms of gp85 in recombinant baculovirus-infected cells or EBV-positive P3HR1 cells by immunofluorescence. S9 cells infected for 30 h with recombinant baculoviruses containing the E. coli β-galactosidase gene (a) or the EBV BXLFP2 gene (b, c and d), and P3HR1 cells induced with 20 ng/ml phorbol 12-myristate 13-acetate for 72 h (e and f) were washed in PBS and were either incubated on ice with antibodies (d) or dried onto glass slides, fixed with 80% cold acetone and incubated with antibodies at 37°C (a, b, c, e and f). Cells were probed with purified 8195 antibody (a, b, d and e), V1 peptide antibody (c) or MAb F-2-1 (f) in PBS containing 5% calf serum for 1 h, washed three times in PBS, incubated with fluorescent isothiocyanate-conjugated antibodies in PBS containing 5% calf serum for 1 h, washed twice in PBS and examined by microscopy with u.v. illumination.
produced no fluorescence on cells infected with a recombinant baculovirus expressing the E. coli β-galactosidase gene (Fig. 4a) but strong fluorescence on vAcEBgH-fixed cells (Fig. 4b) confirming the suitability of this antibody for detecting denatured gp85 in a fluorescence assay. The V1 peptide antibody also produced a strong fluorescence with vAcEBgH-infected cells (Fig. 4c) confirming the availability of linear epitope(s) on the recombinant molecule. However, the absence of 8195 fluorescence with P3HR1 cells (Fig. 4e) indicates that linear epitopes contained in the GST85N-S fusion protein are not normally accessible in native gp85 because they are either occluded due to protein folding or because another protein shields these epitopes. EBV-positive P3HR1 cells fluoresced strongly when probed with the MAb F-2-1 raised to native gp85 (Strnad et al., 1982; Fig. 4f) confirming the presence of significant quantities of gp85 on these cells, but F-2-1 produced no reaction with vAcEBgH-infected cells (data not shown) indicating that the baculovirus gp85 does not have the conformation of the native EBV protein. No cell surface fluorescence was detected on vAcEBgH-infected cells using F-2-1 (data not shown), or 8195 purified antibody (Fig. 4d), by microscopy or fluorescent antibody flow cytometry (data not shown), indicating that the recombinant gp85 was not transported to the cell surface as a mature or immature folded protein. This lack of cell surface expression was also observed when BXLF2 was expressed in fibroblasts (Heineman et al., 1988) or recombinant vaccinia virus-infected cells (Yaswen et al., 1993). Therefore, expression of the BXLF2 gene alone in the baculovirus system does not provide the necessary components for synthesizing a correctly transported and folded form of recombinant gp85.

In a similar study, Ghiasi et al. (1991) observed that baculovirus-expressed HSV-1 gH was a significant portion of total cell protein, was glycosylated, migrated on gels with a similar Mr to the native protein and was transported to the cell surface. However it was not described as being an insoluble protein in non-ionic detergent nor as having a tendency to form aggregates. Therefore the EBV gH and the HSV-1 gH appear to have significantly different properties.

Both recombinant HSV-1 gH and EBV gp85 appear to require one or more other proteins in order to assume their native antigenic structure. Hutchinson et al. (1992) reported that the co-expression of another glycoprotein, gL, encoded by the UL1 gene of HSV-1, was required for normal processing, transport and antigenicity of gH and that gL complexes with gH to form a hetero-oligomer. In support of this result, Kaye et al. (1992) have also demonstrated that the UL75 gene, which codes for the HCMV gH product, forms a disulphide-linked complex with the UL115 gene product in a native conformation on the surface of recombinant vaccinia virus-co-infected cells. The UL115 gene of HCMV is a positional and a functional homologue of the HSV-1 UL1 gene and both of these genes appear to have the BKRF2 gene as a counterpart in the EBV genome (Kaye et al., 1992). Yaswen et al. (1993) demonstrated that a protein with a Mr of 25K was the product of the BKRF2 gene and it coprecipitated with gp85 along with a third unidentified 42K protein from EBV-infected cells with the MAb F-2-1. In addition, Liu et al. (1993b) demonstrated that the HHV-6 gH–gL complex contained three components, a 110K gH, a 32K gL and a minor 80K protein that may represent a modified form of gH or a third virus or cellular protein in the complex. The potential for proteins other than gL interacting with gH has also been highlighted by Spaete et al. (1993), who demonstrated that a truncated form of the human fibroblast growth factor receptor acted as an 'escort protein' for truncated HCMV gH when expressed in Chinese hamster ovary cells, resulting in glycan modification and transport of gH through the secretory pathway in the absence of gL.

Recently, Ghiasi et al. (1994) showed that the HSV-1 gH compared unfavourably with six other HSV-1 glycoproteins expressed individually in the baculovirus system, producing poor neutralizing antibody titres and not offering protection from a lethal HSV-1 challenge. However, Forrester et al. (1991) had previously shown that mice immunized with a recombinant vaccinia virus expressing the HSV-1 gH alone produced no neutralizing antibody and did not offer protection from a lethal HSV-1 challenge. Instead, a gH–gL complex was required to stimulate a neutralizing antibody response capable of protecting mice from the challenge (Browne et al., 1993). Although the baculovirus expression system cannot itself substitute for other factors necessary for correct transport and folding of the BXLF2 gene product, the simultaneous synthesis of the EBV BXLF2 and BKRF2 genes in insect cells may result in the generation of native recombinant gp85 that might prove to be a suitable immunogen for EBV-protective immune responses.

The authors would like to thank Isabella Aboderin for constructing the GST85N-S fusion protein vector and Dr L. Hutt-Fletcher for the use of the gp85 V1 peptide antibody. This work was funded by the Cancer Research Campaign of the U.K.

References


