Expression of equine herpesvirus type 1 glycoprotein gp14 in *Escherichia coli* and in insect cells: a comparative study on protein processing and humoral immune responses

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The extracellular portion (amino acids 1 to 844) of the equine herpesvirus type 1 (EHV-1) glycoprotein gp14, the homologue of gB of herpes simplex virus, was expressed in *Escherichia coli* and in insect cells using a recombinant baculovirus. Immunoblot analysis revealed that the recombinant *E. coli* expressed a fusion protein of Mr 135K which was composed of the truncated gp14 and the maltose-binding protein (MBP) provided by the vector and a 90K protein lacking the MBP moiety. Both proteins were sequestered within the cells in form of inclusion bodies. Infection of insect cells with the recombinant baculovirus resulted in the production of a 115K to 118K glycoprotein which was cleaved intracellularly into two subunits of Mr 55K and 63K to 65K. The cleaved subunits were secreted into the cell culture supernatant and formed disulphide-linked dimers of Mr 120K to 122K. The recombinant proteins produced in *E. coli* and in insect cells elicited EHV-1-specific antibodies in goats as demonstrated by Western blot analysis. The gp14 expressed in insect cells induced antibodies with virus-neutralizing activity. In contrast, the truncated gp14 expressed by *E. coli* failed to elicit neutralizing antibodies. The results suggest that post-translational modification of the EHV-1 gp14 may be important for the expression of epitopes necessary for the induction of neutralizing antibodies.

Equine herpesvirus type 1 (EHV-1) can cause a variety of clinical conditions in horses. In addition to causing infections of the respiratory tract, the virus is responsible for abortion in mares after fetal infection (Allen & Bryans, 1986). Central nervous system disorders have also been associated with EHV-1, although their pathogenesis is not fully understood (Edington et al., 1986; Allen & Bryans, 1986).

The EHV-1 glycoprotein gp14, one of the major envelope glycoproteins, has been genetically mapped and sequenced (Whalley et al., 1989). It has been shown to be the homologue of herpes simplex virus (HSV) glycoprotein B (gB) and is regulated as a β-γ class protein (Whalley et al., 1989; Sullivan et al., 1989). A model of gp14 processing proposes that a 980 amino acid polypeptide of Mr 118K is co-translationally N-glycosylated to generate a 138K precursor molecule. This precursor is then post-translationally cleaved into a 53K to 55K (58K) and a 75K to 77K subunit which form a disulphide-linked 145K heterodimer in the mature virion (Sullivan et al., 1989; Meredith et al., 1989). The gp14 is known to be an important target of the humoral immune response since several monoclonal antibodies (MAbs) directed against EHV-1 gp14 showed virus-neutralizing activity and conferred passive protection in the Syrian hamster model (Allen & Yeargan, 1987; Stokes et al., 1989; Shimizu et al., 1989). Attempts to express EHV-1 gp14 have been undertaken previously. The aminoterminal 50 amino acids of the protein expressed in *Escherichia coli* are sufficient for the detection of anti-EHV-1 antibodies in horse sera by an ELISA system (Sinclair et al., 1993). The expression of gp14 by recombinant vaccinia viruses led to the production of a highly cell-associated antigen which was very similar to authentic EHV-1 gp14. Immunization of laboratory animals with gp14-recombinant vaccinia viruses elicited EHV-1-specific antibodies and protected Syrian hamsters against lethal EHV-1 challenge (Bell et al., 1990; Guo et al., 1990).

In this study we have compared the expression of EHV-1 gp14 lacking the transmembrane and cytoplasmic domains in *E. coli* and insect cells. This communication contributes to further understanding of the post-translational modifications of EHV-1 gp14 with respect to its biological properties.
as described by Meyer et al. (1987) and the part of the gpl4 gene representing the extracellular region of the protein was amplified by a standard PCR (Saiki et al., 1988) from a cloned 5-kb Psrl fragment containing the entire gpl4 gene. Primer A (5'-acgtgaattc-ATGTCCCTGTTGCG 3') contained an inserted EcoRI site (bold) upstream of the start codon of the gpl4 gene and primer B (5'-acgtgaccttga-CGAAGCTATTCCAGATACG 3') was synthesized with two stop codons (italics) and a Psrl site (bold). The 2558 bp amplification product containing nucleotides 951 to 3482 of the EHV-1 gpl4 sequence (according to Whalley et al., 1989) was digested with EcoRI and Psrl, cloned into the prokaryotic expression vectors pMAL-c2 and pMAL-p2 (New England Biolabs; these vectors are designed for cytoplasmic and periplasmic expression, respectively) and the recombinant clones pCEP-8 and pPEP-7 were isolated. The PCR product was similarly cloned into the Autographa californica nuclear polyhedrosis virus (AcNPV) transfection vector pVL1393 (Invitrogen) resulting in the recombinant clone pVEP-5. Correct insertion of the gpl4 gene into the vectors was tested by restriction enzyme analysis and sequencing of the recombination sites (Sanger et al., 1977).

Recombinant pPEP-7 and pCEP-8 bacteria were grown in 5 ml Luria-Bertani broth to an OD at 600 nm of 0.45 and 1 ml was processed for SDS-PAGE and immunoblotting (Sambrook et al., 1989). The remaining 4 ml was induced with IPTG at a final concentration of 0.3 mM and after 1 or 2 h of incubation 0.5 ml aliquots were processed as described above. After induction with IPTG, a protein species of Mr 135K, correlating with the predicted Mr of the truncated gpl4-maltose binding protein (MBP) fusion product, and a smaller protein species of Mr 90K were detected in cell lysates of recombinant clones by immunoblot analysis. The proteins were reactive with both the convalescent horse serum 528/84 which was collected from a horse after experimental EHV-1 infection (H. Meyer, personal communication; Fig. 1) and with anti-HSV gB antiserum (kindly provided by H. P. Huemer; data not shown). Uninduced cells and cells transformed with wild-type (wt) plasmid did not react with the antiserum (Fig. 1). Incubation of the induced cell lysates with an anti-MBP antiserum (New England Biolabs) after immunoblotting demonstrated only one protein with an Mr of 135K (Fig. 1) suggesting that the translation initiation codon of EHV-1 gpl4 in addition to that of the MBP was also used for protein translation. Internal binding of mRNA to ribosomes which can occur in prokaryotic systems appears to be involved in the translation of the 90K protein since a Shine–Dalgarno homologous sequence three bases upstream of the MBP–gpl4 recombination site was found by computer analysis (Shine & Dalgarno, 1974). Moreover, the apparent Mr of 90K corresponded well to the predicted Mr (87K) of the unprocessed and truncated gp14. The recombinant proteins expressed by pCEP-8 and pPEP-7 were sequestered within the cytoplasm of E. coli in form of inclusion bodies. The inclusion bodies were isolated from IPTG-induced 11 cultures of pCEP-8-containing cells following standard procedures (Sambrook et al., 1989) and were checked for purity by SDS–PAGE and Coomassie brilliant blue staining or immunoblotting. They were shown to be composed almost exclusively of the 135K and 90K proteins (data not shown).

Wild-type AcNPV was propagated on Spodoptera frugiperda SF-9 cells essentially as described by Summers & Smith (1987). Recombinant baculovirus rAc17-11 was generated by cotransfection of SF-9 cells with linearized wt AcNPV DNA and pVEP-5 DNA using a transfection kit (Invitrogen). Recombinant rAc17-11 was plaque-purified and tested for gp14 expression by SDS–PAGE and immunoblotting. High Five cells (Invitrogen) were grown using serum-free SF-900 medium (Gibco BRL). Infection of High Five cells with rAc17-11 at an m.o.i. of 1 resulted in the expression of a 115K to 118K protein as demonstrated by immunoblot analysis using horse serum 528/84. The protein could be detected in rAc17-11-infected cells as early as 12 h post-infection (p.i.) and its observed Mr corresponded exactly to the value calculated for the gp14 lacking the transmembrane and cytoplasmic domains (Whalley et al., 1989). Further incubation of the
infected cells resulted in an increased production of the 115K to 118K protein until 36 h p.i. With the decrease of the 115K to 118K protein concentration in the cell lysates, two smaller protein species appeared and were specifically detected by horse serum 528/84 (Fig. 2). One of these proteins with an \( M_r \) of 63K to 65K could be demonstrated from 24 h p.i. onwards. At 48 h p.i., an additional band of \( M_r \) 55K was recognized by the 528/84 serum. The 115K to 118K protein species had completely disappeared at 72 h p.i. when only the smaller proteins could be detected in the cell lysates analysed. This suggested that the 115K to 118K precursor protein was post-translationally cleaved into two smaller subunits of 55K and 63K to 65K. These results are in agreement with the proposed model of gp14 processing in mammalian cells in which the precursor molecule is also cleaved into two subunits (Sullivan et al., 1989). The reason for the weak reaction of the 55K subunit is unknown. However, conformational epitopes which are not detectable by Western blotting or a masking of the authentic 53K to 55K subunit in EHV-1 virions resulting in a reduced exposure to the humoral immune response might explain this observation.

Immunoblot analysis of the cell culture supernatants with horse serum 528/84 revealed that both subunits were released into the cell culture supernatant from 48 h p.i. to 144 h p.i. (Fig. 2). The supernatants were also separated by SDS-PAGE in the absence of 2-mercaptoethanol (2-ME), revealing a protein species with an estimated \( M_r \) of 120K to 122K. This suggested the formation of a disulphide-linked complex and detailed analysis of this protein with increasing 2-ME concentrations (0% to 5%) demonstrated a gradual dissociation of the 120K to 122K complex into its component subunits (Fig 3). It is not known whether homo- or heterodimers are formed, but the formation of heterodimers has been described in mature EHV-1 virions (Sullivan et al., 1989) and the calculated sum of the \( M_r \)s of the 55K and 63K to 65K subunits exactly corresponds to the 120K to 122K complex demonstrated under non-reducing conditions. N-glycosylation of recombinant gp14 was inhibited by tunicamycin (Boehringer Mannheim). Treatment of rAc17-11-infected High Five cells with tunicamycin (0.1 to 5 \( \mu \)g per ml medium) 1 h after virus adsorption revealed that the \( M_r \) of the uncleaved precursor molecule was reduced from between 115K and 118K to 90K (Fig. 4), exactly corresponding to the \( M_r \) of the truncated gp14 expressed in E. coli. The 90K protein was detectable by Western blotting up to 168 h p.i. We could not confirm the reduced stability of EHV-1 gp14 in the presence of tunicamycin that has been discussed by Sullivan et al. (1989) who treated mammalian cells with tunicamycin after EHV-1 infection. This discrepancy could be due to divergent protein degradation processes in insect and mammalian cells. The cleavage of the precursor into the smaller subunits was highly reduced or even completely blocked in tunicamycin-treated insect cells and no release of recombinant gp14 into the cell culture supernatant could be demonstrated by immunoblot analysis at any time p.i. (Fig. 4). High Five insect cells infected with rAc17-11 (at an m.o.i. of 1) were also examined by
surface and intracellular flow cytometry (FACScan; Becton-Dickinson) using MAb 3F6 (kindly provided by G. P. Allen) and fluorescein isothiocyanate (FITC)-labelled goat-anti-mouse IgG (Sigma) according to standard protocols (Laffin & Lehmann, 1990). The intracellular immunofluorescence revealed almost identical numbers of labelled cells after rAc17-11 infection in either the presence (0.1 and 2.5 µg/ml) or absence of tunicamycin. At 72 h p.i., 54±9% of the cells showed a specific fluorescence with MAb 3F6 (four independent experiments) and no difference in signal intensity or morphology of infected cells was observed. Microscopic examination of infected cells stained by indirect (MAb 3F6) or direct immunofluorescence with FITC-labelled horse-anti-EHV-1 (Bioveta) confirmed a homogeneous distribution of recombinant gp14 within the cytoplasm in either the presence or absence of tunicamycin. In contrast, cell surface immunofluorescence analysis revealed that recombinant gp14 was detected on the surface in only 32±7% of cells in the presence of tunicamycin (four independent experiments) in relation to the scores obtained with rAc17-11-infected cells incubated without tunicamycin (100%). Moreover, the intensity of the fluorescence signal on the cell surface was reduced about 10-fold in tunicamycin-treated cells (data not shown). From these data we conclude that the stability and solubility of recombinant gp14 expressed in insect cells does not appear to be markedly impaired in the presence of tunicamycin. However, the cleavage of the precursor molecule into the subunits, the transport of recombinant gp14 to the cell surface and its release into the cell culture supernatant were greatly reduced. Hence these properties of recombinant gp14 appear, at least in insect cells, to be strongly dependent on glycosylation and/or post-translational modification of gp14.

To monitor immune responses to the recombinant gp14 proteins, two 6-week-old goats were immunized subcutaneously with either 100 µg of pCEP-8 inclusion body preparation (goats 1 and 2), or a lysate of 1×10⁶ sonicated High Five cells harvested at 48 h p.i. with rAc17-11 (goats 3 and 4), or 100 µg of rAc17-11-expressed gp14 purified from serum-free High Five cell supernatants (goats 5 and 6). The antigens were administered after emulsification in complete Freund's adjuvant at day 0, followed by two booster injections of antigen in incomplete Freund's adjuvant at days 20 and 40. Sera were collected at days 0, 14, 34 and 54 and were tested in immunoblots for their reactivity with purified RacH and Kentucky D (KentD) viral proteins separated

Table 1. NT titres of goat sera and their reactivity with EHV-1 proteins in Western blots

<table>
<thead>
<tr>
<th>Antigen†</th>
<th>pCEP-8 inclusions</th>
<th>rAc17-11 cells</th>
<th>rAc17-11 supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Goat 1</td>
<td>Goat 2</td>
<td>Goat 3</td>
</tr>
<tr>
<td>Day 0</td>
<td>&lt; 1:2</td>
<td>&lt; 1:2</td>
<td>&lt; 1:2</td>
</tr>
<tr>
<td>Day 14</td>
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<td>&lt; 1:2</td>
<td>1:4</td>
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<tr>
<td>Day 34</td>
<td>&lt; 1:2</td>
<td>&lt; 1:2</td>
<td>1:4</td>
</tr>
<tr>
<td>Day 54</td>
<td>&lt; 1:2</td>
<td>&lt; 1:2</td>
<td>1:16</td>
</tr>
<tr>
<td>EHV-1 proteins‡</td>
<td>75K</td>
<td>75K</td>
<td>75K/55K</td>
</tr>
</tbody>
</table>

* NT titres were determined for non-inactivated sera or inactivated sera supplemented with 10% guinea-pig complement.
† One-hundred µg of pCEP-8 inclusion body preparations, 100 µg of rAc17-11 cell culture supernatant, or 1×10⁶ rAc17-11-infected cells were used for each immunization.
‡ The M₀ of EHV-1 proteins detected in Western blots (data not shown) by goat sera collected at day 54 are indicated. No proteins were detected with sera collected at day 0.
by SDS-PAGE under reducing conditions. Whereas none of the preimmune sera reacted with RacH or KentD proteins, the sera from goats 1 and 2 collected at day 54 specifically recognized a 75K protein in both strains (Table 1) corresponding to the large subunit of gp14 in EHV-1 virions (Sullivan et al., 1989). In contrast, sera obtained from goats 3, 4, 5 and 6 at day 54 recognized both the 75K and 55K gp14 subunit in purified EHV-1 virions (Table 1). These results suggested that correct post-translational modification of gp14 is crucial for the immunogenicity of the smaller EHV-1 gp14 subunit.

For neutralization tests (NT), serum samples were used native or inactivated (30 min at 56 °C). In some experiments, guinea-pig complement was added to a final concentration of 10%. Serial serum dilutions (log2) were performed in triplicate in microtitre plates and RK-13 cells and 50 TCID50 RacH were used as indicators. Titres were defined as the highest serum dilution giving full protection. Serum samples from goats 1 and 2 (collected at days 0, 14, 34 and 54) did not show any virus-neutralizing activity in the NT. In contrast, neutralization titres of 1:16 to 1:32 were observed in native sera or in inactivated sera supplemented with guinea-pig complement from goats 3, 4, 5 and 6 collected on day 54 (Table 1). In inactivated serum samples of goats 3 to 6, titres of 1:4 to 1:8 could be demonstrated on day 54 in several independent assays. This indicated that neutralizing antibodies, which were in part dependent on complement, were elicited by the truncated gp14 expressed in insect cells but not by that produced in E. coli. The higher antibody titres in the presence of complement were not surprising since the activity of several MAbs against gp14 is also dependent on the addition of complement (Shimizu et al., 1989). The neutralizing antibody titres described here are in agreement with those obtained by Guo et al. (1990) after immunization of guinea-pigs with recombinant vaccinia virus expressing gp14, which was nonetheless capable of protecting against lethal EHV-1 challenge in Syrian hamsters.

In summary, by comparing the expression of EHV-1 gp14 in E. coli and in insect cells we have demonstrated that the correct post-translational modification of the protein is dependent on its expression in eukaryotic cells. The processing of recombinant gp14 expressed in insect cells was very similar to that described for authentic EHV-1 gp14 in mammalian cells (Sullivan et al., 1989). Both the immunogenicity of the smaller EHV-1 gp14 subunit and the induction of neutralizing antibodies appear to be dependent on post-translational modifications.

Owing to the deletion of the protein’s membrane anchor, the purification of the antigen from serum-free cell culture supernatants is easily achieved. This may be of great value since gp14 is a candidate antigen for inclusion in an EHV-1 subunit vaccine. The potential of recombinant gp14 in the protection of animals against EHV-1 challenge infection is currently under investigation.

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References


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