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Factors affecting recombinant Western equine encephalitis virus glycoprotein production in the baculovirus system

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Abstract

In an effort to produce processed, soluble Western equine encephalitis virus (WEEV) glycoproteins for subunit therapeutic vaccine studies, we isolated twelve recombinant baculoviruses designed to express four different WEEV glycoprotein constructs under the transcriptional control of three temporally distinct baculovirus promoters. The WEEV glycoprotein constructs encoded full-length E1, the E1 ectodomain, an E26KE1 polyprotein precursor, and an artificial, secretable E2E1 chimera. The three different promoters induced gene expression during the immediate early (*ie1*), late (*p6.9*), and very late (*polh*) phases of baculovirus infection. Protein expression studies showed that the nature of the WEEV construct and the timing of expression both influenced the quantity and quality of recombinant glycoprotein produced. The full-length E1 product was insoluble, irrespective of the timing of expression. Each of the other three constructs yielded soluble products and, in these cases, the timing of expression was important, as higher protein processing efficiencies were generally obtained at earlier times of infection. However, immediate early expression did not yield detectable levels of every WEEV product, and expression during the late (*p6.9*) or very late (*polh*) phases of infection provided equal or higher amounts of processed, soluble product. Thus, while earlier foreign gene expression can provide higher recombinant glycoprotein processing efficiencies in the baculovirus system, in the case of the WEEV glycoproteins, earlier expression did not provide larger amounts of high quality, soluble recombinant glycoprotein product.

Keywords

Baculovirus; insect cell; glycoprotein; promoters; alphavirus; Western equine encephalitis virus

Introduction

The baculovirus expression vector system (BEVS)¹ is widely used for recombinant protein production [1-3]. The advantages of the BEVS include its ability to provide eukaryotic post-translational modifications and high levels of foreign gene expression. In addition, recombinant proteins produced using the BEVS are safe and effective for use in humans, as demonstrated by the recent FDA approval of two BEVS-derived products [4, 5].

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¹**Abbreviations used:** BEVS, baculovirus expression vector system; DB, Laemmli protein disruption buffer; *ie1*, immediate early-1; *polh*, polyhedrin; PSFM, Protein Sciences Formulary Medium; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WEEV, Western equine encephalitis virus.

The promoter used to drive foreign gene expression by most baculovirus vectors is derived from a baculoviral *polyhedrin* (*polh*) gene. Baculovirus *polh* genes are classified as “very late” because they are expressed during the very late phase of infection, which occurs well after the onset of viral DNA replication. Baculovirus *polh* promoters are strong and can provide high levels of foreign gene expression in conjunction with a virus-encoded transcription complex [1]. However, baculoviruses also have other temporally regulated promoters that can be used to drive foreign gene expression during insect cell infections. In fact, previous studies have shown that BEVs encoding foreign genes under the control of immediate early (e.g., *ie1*) or late (e.g., *p6.9*) promoters can provide higher efficiencies of recombinant protein glycosylation and secretion and can yield products with higher specific activities, as compared to BEVs expressing the same genes under the control of the very late *polh* promoter [6-10]. These apparent advantages have been attributed to the fact that the *ie1* and *p6.9* promoters are active earlier in infection and drive foreign gene expression at lower levels than the *polh* promoter, which has led to speculation that using these promoters circumvents problems of host secretory pathway saturation and/or loss of function that might occur during baculovirus infection [7, 8, 10, 11].

The alphaviruses, including Sindbis virus, Chikungunya virus, Ross River virus, and Western, Eastern, and Venezuelan equine encephalitis viruses, are a group of arthropod-borne viruses that are considered to be emerging human pathogens and potential biological weapons [12-14]. Unfortunately, neither alphavirus vaccines nor alphaviral-specific antiviral drugs are currently available. However, the alphavirus E2 and E1 envelope glycoproteins, which are the viral attachment and fusion proteins, respectively, are excellent subunit therapeutic vaccine candidates. These glycoproteins are initially synthesized as part of a large structural polyprotein precursor comprised of capsid, E3, E2, 6K, and E1 (Fig. 1A). After the capsid protein self-excises, the rest of the polyprotein enters the secretory pathway, where it is cleaved into pE2 (an E3 and E2 precursor), 6K, and E1 by signal peptidase. pE2 heterodimerizes with E1 and is later cleaved into E3 and E2 by furin. The E2 and E1 glycoproteins, each of which is *N*-glycosylated at two sites, are displayed as a trimer of E2E1 heterodimers on the cell surface and incorporated into progeny virions during the budding process.

The overall purpose of this study was to produce soluble, recombinant forms of the Western equine encephalitis virus (WEEV) E1 and E2 glycoproteins for subunit therapeutic vaccine studies. We chose the BEVs as a recombinant protein production platform due to its ability to provide high yields, eukaryotic glycosylation, and for its relative biosafety. In the interest of optimizing the yield of high-quality, processed, soluble glycoprotein products, we isolated twelve different baculoviruses encoding four different WEEV glycoprotein constructs (Fig. 1A) under the transcriptional control of three temporally distinct baculovirus promoters including *ie1*, *p6.9* and *polh* (Fig. 1B). We then compared the amounts and quality of WEEV glycoprotein products obtained with each vector. The results revealed that the nature of the WEEV glycoprotein construct and the timing of its expression both influenced the amounts and quality of recombinant product obtained. Generally, expression during the immediate early or late phases of baculovirus infection reproducibly provided more efficient protein processing, as compared to expression during the very late stage of infection. However, earlier foreign gene expression did not offer a real advantage for any WEEV glycoprotein construct tested, as expression during later phases reproducibly yielded at least as much processed, soluble product as expression during earlier phases of infection.

Materials and methods

Cells and cell culture

Sf9 cells were maintained in shake flask cultures in TNM-FH media containing 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO) and 0.1% Pluronic F-68 (Sigma Aldrich, St. Louis, MO) as described previously [15]. *ExpresSF+*® cells, a *Spodoptera frugiperda* cell line obtained from Protein Sciences Corporation (Meriden, CT), were maintained at densities of $0.4 - 8.0 \times 10^6$ cells per ml in shake flask cultures in Protein Sciences Formulary Medium (PSFM). SfSWT-6 cells (H. Mabashi-Asazuma, C. Geisler, and D.L. Jarvis, unpublished), a glycoengineered Sf9 cell subclone with properties similar to those of SfSWT-3 cells [16], were maintained in shake flask cultures in ESF921 media (Expression Systems; Woodland, CA) at densities of $1.5-8.0 \times 10^6$ cells per ml.

Isolation of AcIE1GT and Ac6.9GT baculovirus destination vectors

AcGT is a new Gateway® baculovirus destination vector that was isolated using a transfer plasmid called pAcGT-TV, as will be described elsewhere (J. Aumiller, C. Geisler, and D.L. Jarvis, unpublished). AcIE1GT and Ac6.9GT are two new Gateway® baculoviral destination vectors that were isolated using transfer plasmids called pAcIE1GT-TV and pAc6.9GT-TV, respectively. In general, these transfer plasmids were constructed by replacing the *polh* promoter in pAcGT-TV with the *ie1* promoter/*hr5* enhancer or *p6.9* promoter elements, respectively. Briefly, the *polh* promoter consisting of 128 bp from the upstream *EcoRV* site to the translational start site and some flanking sequences were deleted from pAcGT-TV by digestion with *BbvCI* and *PshAI*, and then the deleted fragment was replaced with a PCR amplification product containing the flanking sequences and unique *FseI* and *AscI* sites in place of the promoter. The resulting plasmid, which was called pAcGT-TV(-promoter), was the target for insertion of PCR products containing the *ie1* promoter/*hr5* enhancer or *p6.9* promoter elements flanked by *FseI* and *AscI* sites, which had been amplified from pIE1HR4 [10] or BAKPAK6 [17], respectively. The plasmids resulting from insertion of the *FseI-AscI* fragments containing the *ie1hr5* or *p6.9* control elements were designated pAcIE1GT-TV and pAc6.9GT-TV, respectively. All of the primer sequences used to produce the amplification products described above are shown in Table 1.

pAcIE1GT-TV and pAc6.9GT-TV were subsequently used to isolate the AcIE1GT and Ac6.9GT baculovirus destination vectors (Fig. 1B) via a standard allelic transplacement method [2, 3] with BestBac 2.0 ($\Delta v\text{-cath/chit}$) linearized viral DNA (Expression Systems) as the target for homologous recombination. Recombinant viruses produced in this way gained an *E. coli lacZ* marker, which enabled identification by their blue plaque phenotypes in the presence of X-gal. Blue plaque recombinants were plaque-purified once, amplified in Sf9 cells, and then genomic DNA was extracted from each virus stock, as described previously [3].

Isolation of baculovirus expression vectors encoding WEEV glycoproteins

Sequences encoding full-length E1, the E1 ectodomain, the E26KE1 polyprotein, or an E2E1 chimera (Fig. 1A) were PCR-amplified using a cDNA clone of the McMillan strain of WEEV [18] as the template and the primers shown in Table 1. In each case, the forward primer added a CACC for directional cloning into pENTR™/D-TOPO® (Invitrogen, Carlsbad, CA), as well as a sequence encoding a Tobacco etch virus cleavage site, and the reverse primer added a stop codon. The E2E1chim sequence encoded the E2 ectodomain (amino acids 1-363) fused to the E1 ectodomain (amino acids 1-408 of E1) through a (Gly-Gly-Gly-Gly-Ser)₃ peptide linker. Identification of the WEEV E2 and E1 ectodomains was based on structures determined by cryoelectron microscopy of Sindbis virus [19].

The E1, E1ecto, E26KE1, and E2E1chim PCR products were cloned into pENTR™/DTOP0® (Invitrogen), error-free clones were identified by sequencing, and each was digested with *NotI*, repaired with Klenow, and re-ligated to place the coding sequence in-frame with the start codon located in the AcIE1GT, Ac6.9GT and AcGT destination vectors. This yielded the final transfer plasmids encoding the four different WEEV glycoprotein constructs, which were used to produce recombinant baculoviruses by *in vitro* recombination with the *Bsu36I*-linearized AcIE1GT, Ac6.9GT, and AcGT baculovirus destination vector DNAs (Fig. 1B), as described in the BaculoDirect™ (Invitrogen) manual. The recombination reactions were used to transfect Sf9 cells and the transfected cells were cultivated in the presence of ganciclovir for three days to select against the parental destination vectors, which contained the Herpes simplex virus 1 thymidine kinase gene (Fig. 1B), as described in the BaculoDirect™ manual. Recombinant baculoviruses lost the thymidine kinase negative selection marker and also lost the *E. coli lacZ* identification marker and, therefore, were able to replicate in the presence of ganciclovir and could be identified by their white plaque phenotypes. These viruses were plaque purified once, amplified in Sf9 cells, and the virus stocks were titered by plaque assays as described previously [2].

Expression of recombinant WEEV glycoproteins

ExpresSF+® cells were seeded into six well plates at a density of 1×10^6 cells/well in PSFM. The cells were then mock-infected with PSFM or infected at a multiplicity of infection of 3 plaque-forming units/cell with the BEVs specified in the relevant Figures and Figure legends. After a 1-hr adsorption period, the inocula were removed and replaced with 1 ml of PSFM. At the times post-infection specified in the relevant Figures and Figure legends, the cells were scraped into the media and pelleted by low speed centrifugation. The supernatants were collected and mixed with 5X Laemmli disruption buffer (DB, [20]) and the cell pellets were washed once with PBS and then lysed by resuspension in 500 μ l of 1X DB with shearing by three passages through a 27.5 gauge needle. Samples of the cell supernatants (10%) and pellets (2%) were analyzed by SDS-PAGE [20] and western blotting [21], with anti-penta-His (Invitrogen) or anti-Sindbis M-21190 (Centers for Disease Control, Atlanta, GA) as the primary antisera and rabbit anti-mouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) as the secondary antiserum. Immunoreactive proteins were visualized using a standard alkaline phosphatase-based color reaction [22]. Relative immunostaining intensities were estimated by scanning the blots on a flatbed scanner and quantitating the bands using the Bio-Rad Quantity One software package.

For solubility assays, *expresSF+*® cells were infected with various BEVs for 72 hr, and then divided into two equal aliquots. One aliquot was lysed with 1X DB to extract total proteins, as described above, while the other was treated with extraction buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1% NP-40) for 10 min on ice and then clarified for 5 min at 16,000 g in a microcentrifuge. The supernatant (soluble fraction) was removed and mixed with 5X DB, whereas the pellet (insoluble fraction) was lysed by resuspension in 1X DB with shearing by three passages through a 27.5 gauge needle. Aliquots (2%) of each fraction were then analyzed by SDS-PAGE and western blotting with anti-penta-His as the primary antiserum, as described above.

Analysis of recombinant WEEV protein glycosylation

To analyze recombinant protein glycosylation, *expresSF+*® or SfSWT-6 cells were infected with the appropriate BEVs, as described above, except the SfSWT-6 cell growth medium was supplemented with 200 μ M tetra-acetylated *N*-acetylmannosamine (Dextra, Reading, UK), which is a sialic acid precursor needed to support recombinant glycoprotein sialylation. At appropriate times post-infection, the cells were pelleted, extracted with

glycoprotein denaturation buffer (0.5% SDS, 40 mM dithiothreitol), and the cell-free supernatants were mixed with 10X glycoprotein denaturation buffer. Each sample was boiled for 3 min, the SDS was sequestered by adding 1% NP-40, and the extracts were then treated overnight at 37°C with buffer alone, peptide: N-glycosidase F (PNGase F; New England Biolabs, Ipswich, MA) or endo- β -N-acetyl-D-glucosaminidase H (EndoH; New England Biolabs), according to the manufacturer's instructions. After treatment, the proteins were analyzed by SDS-PAGE and western blotting, as described above.

Results and Discussion

Design and isolation of BEVs encoding different WEEV glycoprotein constructs under the control of various baculovirus promoters

The overall goal of this project was to produce high quality, co- and post-translationally processed, soluble WEEV glycoproteins for therapeutic vaccine studies. We used a modified Gateway® (Invitrogen) baculovirus destination vector called AcGT, which was produced for another project in our lab, to produce recombinant BEVs encoding several different forms of the WEEV glycoproteins under the transcriptional control of the *polh* promoter. However, we also considered that those BEVs would express the WEEV glycoprotein constructs under the control of the *polh* promoter, which is only active during the very late phase of infection. Previous studies had suggested that it might be advantageous to express foreign genes encoding secretory pathway proteins earlier, using promoters from baculovirus genes expressed during earlier phases of infection [6-10]. Thus, we constructed two additional modified Gateway® (Invitrogen) baculovirus destination vectors, designated AcIE1GT and Ac6.9GT, which could be used to produce recombinant BEVs encoding foreign genes under the control of the *ie1* or *p6.9* promoters, respectively. The strategy used to isolate these new baculovirus destination vectors is described in detail in Materials and methods and the key features of AcGT, AcIE1GT, and Ac6.9GT are shown in Fig. 1B.

Another important consideration in this project was the nature of the WEEV glycoprotein constructs to be expressed. In the final analysis, we designed four different constructs encoding full-length E1, the E1 ectodomain, the E26KE1 polyprotein precursor, or an E2E1 chimera (Fig. 1A). E1 was chosen because previous studies had shown that it can be used as a subunit vaccine to provide 100% protection in a mouse model [23, 24] and it can potentially provide cross-protection against multiple alphaviruses [24-26]. The E26KE1 polyprotein was chosen because in its native form, it is proteolytically processed to yield the E2E1 heterodimer complex, which might induce a more effective immune response than E1 alone [23, 24, 27]. Previous studies had indicated that polyprotein precursors derived from other alphaviruses could be processed when expressed under *polh* control in the baculovirus system [23, 28]. However, the polyprotein processing efficiencies had not been carefully examined and a high efficiency would be required to produce large amounts of the E2E1 heterodimer. Thus, we also chose to design a novel construct encoding an E2E1 chimera that would not require proteolytic processing. This construct consisted of the E2 and E1 ectodomains fused through a flexible peptide linker [(Gly-Gly-Gly-Gly-Ser)₃]. Similar chimeric constructs were used previously to produce recombinant hepatitis C virus proteins in both the baculovirus and *E. coli* expression systems and, in the case of the baculovirus system, soluble protein with appropriate structure was obtained [29, 30]. The linker sequence connecting the two glycoprotein ectodomains was based on a peptide commonly used to fuse V_H and V_L chains in engineered single chain Fv antibodies [31].

Expression and analysis of full-length E1

Our initial experiments focused on the expression of full-length WEEV E1 using BEVs encoding this construct under the transcriptional control of the *ie1*, *p6.9*, or *polh* promoters.

Western blotting analysis showed that insect cells infected with AcIE1-E1 produced no detectable immunoreactive E1 at any time examined (Fig. 2A). In contrast, cells infected with Ac6.9-E1 or Ac-E1 accumulated two strongly immunoreactive forms of E1 by 48 and 72 hr post-infection, with slightly higher amounts produced by the former, which was confirmed by densitometry (Fig. 2A). The size of the smaller of these two immunoreactive proteins was consistent with the predicted size (of ~55 kDa) of the unglycosylated, tagged E1 protein without its signal peptide. Thus, this protein appeared to be an unglycosylated form of E1, while the larger one was likely to be its *N*-glycosylated product.

We confirmed this interpretation by treating these proteins with endoglycosidases that specifically remove *N*-glycans, followed by western blotting analysis. The results showed that the endoglycosidase treatments had no impact on the smaller, but increased the mobility of the larger immunoreactive protein (Fig. 2B). Thus, the smaller and larger immunoreactive proteins were, indeed, unglycosylated and *N*-glycosylated forms of E1. The buffer controls included in these experiments revealed that cells infected with Ac6.9-E1 and Ac-E1 actually contained at least three major forms of E1. Endoglycosidase treatment collapsed these three proteins into a single band that comigrated with the smallest product (Fig. 2B), which indicated that three major forms of E1 had been produced with two, one, or no *N*-glycans, from largest to smallest. Together, these results indicated that *N*-glycosylation site utilization and/or *N*-glycan processing was incomplete when full-length E1 was produced with BEVs containing the *p6.9* or *polh* promoters.

We subsequently examined the solubility of the full-length E1 protein produced by these vectors and found that virtually none of this protein could be extracted with a nonionic detergent (Fig. 2C, upper panel). In addition, virtually none of this protein could be detected in the resolving gel when it was examined by SDS-PAGE under non-reducing conditions (Fig. 2C, lower panel), suggesting that it had formed large disulfide-linked aggregates that failed to enter the gel.

Overall, these results indicated that none of the BEVs encoding the full-length WEEV E1 construct would be useful for our therapeutic vaccine studies. Expressing this construct under the control of the *ie1* promoter yielded no detectable product, while expressing it under the control of the *p6.9* and *polh* promoters yielded only malformed, insoluble products, which were inefficiently *N*-glycosylated and/or had inefficiently processed *N*-glycans. Thus, the nature of the WEEV glycoprotein construct was the major factor blocking our initial efforts to produce a high quality recombinant protein preparation.

Expression and analysis of the E1 ectodomain

Our next series of experiments focused on expression and secretion of the E1 ectodomain using BEVs encoding this construct under the transcriptional control of the three different baculovirus promoters. Western blotting analysis showed that all three BEVs induced production and secretion of the immunoreactive E1 ectodomain in *expresSF*⁺ cells during a three day time course of infection (Fig. 3A). No detectable immunoreactive product was extracted from cells infected with the *ie1*-based BEVs (Fig. 3A, top panel), but the cell-free media contained a strongly immunoreactive protein of about the expected size starting at 24 hr, with a plateau at 48 hr post-infection (Fig. 3B, lower panel), indicating that the E1 ectodomain had been very efficiently secreted. In contrast, two major immunoreactive forms of the E1 ectodomain were extracted from cells infected with Ac6.9-Electo or Ac-Electo for 48 or 72 hr, with an increase in the amounts of accumulated protein from 48 to 72 hr post-infection (Fig. 3A, top panel). The intracellular immunoreactive protein bands were very broad, perhaps reflecting heterogeneous *N*-glycosylation, and the size of the smaller band in the Ac-Electo-infected cells was consistent with the predicted size of the unglycosylated, tagged E1 ectodomain without its signal peptide (~49 kDa). The cell free

media from Ac6.9-E1ecto- and Ac-E1ecto-infected cells also contained a strongly immunoreactive protein of about the expected size at 48 and 72 hr postinfection, with little or no increase from 48 to 72 hr post-infection in either case (Fig. 3A, lower panel). Hence, the E1 ectodomain also had been secreted, albeit less efficiently, as compared to cells infected with the BEVs encoding this protein under *ie1* control. Densitometric analysis indicated that the approximate efficiencies of WEEV E1 ectodomain secretion were 100% for *ie1*, ~50% for *p6.9*, and ~33% for the *polh* promoter. Thus, the efficiency of recombinant WEEV E1 ectodomain secretion was inversely proportional to the timing of foreign gene expression, which was consistent with previous conclusions on the impact of earlier expression on glycoprotein secretion in the baculovirus-insect cell system [6-10].

Endoglycosidase analyses were performed to more directly examine glycosylation of the E1 ectodomains produced by insect cells infected with the three different BEVs. The results showed that both PNGase-F and EndoH reduced the sizes of the intracellular proteins, whereas only PNGase-F reduced the sizes of the extracellular proteins (Fig. 3B). EndoH can remove only relatively immature, unprocessed *N*-glycans [32], while PNGase-F can remove both unprocessed and processed *N*-glycans [33] from glycoprotein products. Thus, the EndoH-sensitivity of the intracellular E1-ectodomain proteins showed that their *N*-glycans were less extensively processed than those on the secreted products and indicated that the intracellular forms had not been transported to the Golgi apparatus. A close comparison of the broadly immunoreactive E1 ectodomain bands observed in insect cells infected with the *p6.9*- and *polh*-based BEVs showed that the latter was much more diffuse and encompassed proteins of about the same size as the deglycosylated product (Fig. 3B, top panel). This suggested that *N*-glycosylation site utilization was less efficient when the E1 ectodomain was produced in insect cells infected with the *polh*-based, as compared to the *p6.9*-based BEV. Nevertheless, the overall results indicated that all three of the BEVs encoding the WEEV E1 ectodomain could be used for our therapeutic vaccine studies. Insect cells infected with the *ie1*-based BEV processed the E1 ectodomain protein most efficiently and produced about as much of this protein as the other two BEVs. Interestingly, while insect cells infected with the *p6.9* and *polh*-based BEVs processed the E1 ectodomain far less efficiently, they still produced about as much high quality, soluble, *N*-glycosylated, and secreted product as the *ie1*-based BEV. Thus, in the case of this particular WEEV glycoprotein construct, earlier expression offered no practical advantage for recombinant glycoprotein production in the baculovirus-insect cell system.

Expression and analysis of the E26KE1 polyprotein

Subsequently, we examined expression of the E26KE1 polyprotein by BEVs encoding this WEEV construct under the control of the three different baculovirus promoters. Western blotting analysis showed that insect cells infected with AcIE1-E26KE1 contained no detectable anti-penta-His immunoreactive proteins, while those infected for 48 or 72 hr with Ac6.9-E26KE1 or Ac-E26KE1 contained two major immunoreactive proteins (Fig. 4A, upper panel). The sizes of the larger and smaller of these latter two proteins were about the same as the expected sizes of the E26KE1 polyprotein precursor (~106 kDa) and proteolytically processed E2 and E1 products (~47-52 kDa), respectively. These results suggested that the large polyprotein precursor had been produced and cleaved, at least in part, to generate the E2E1 heterodimer complex, which dissociated into the E2 and E1 protein subunits during SDS-PAGE under reducing conditions. However, this was a tentative conclusion because the anti-penta-His probe could only detect the polyprotein precursor and E2 product, which contained the *N*-terminal 8XHIS tag, and not the E1 product, which had no tag. Therefore, we performed another western blotting analysis with anti-Sindbis virus antiserum, which recognizes both WEEV glycoproteins, as the primary antibody. The results showed that this antiserum also reacted with the larger protein in

Ac6.9-E26KE1 and Ac-E26KE1-infected cell extracts, as expected of the WEEV polyprotein precursor, and produced a more diffuse signal in the same region as the smaller protein detected with anti-penta-His (Fig. 4A, lower panel), suggesting that this latter region included both E2 and E1, which are similar in size (52 and 47 kDa, respectively). These results supported our original conclusion that insect cells infected with Ac6.9-E26KE1 or Ac-E26KE1, but not AcIE1-E26KE1, produced the E26KE1 polyprotein precursor and that a subpopulation was processed into E2 and E1. This result was expected because proteolytic processing of several viral polyprotein precursors expressed under *polh* control was previously demonstrated in the baculovirus-insect cell system [28, 34-37]. However, it was surprising to find that neither the WEEV polyprotein nor its processing products could be detected by western blotting when this construct was expressed under the transcriptional control of the *iel* promoter. Perhaps less surprisingly, we found that the polyprotein was more efficiently processed when expressed during the late phase of infection with the *p6.9* promoter than when it was expressed during the very late phase of infection with the *polh* promoter (about 85% vs. 50% respectively, as determined by densitometry). In the final analysis, however, both of these BEVs yielded about the same amounts of processed, immunoreactive E2 and E1 products.

We also assessed solubility and *N*-glycosylation of the WEEV polyprotein precursor and products obtained with the three different BEVs. The polyprotein precursor produced in Ac6.9-E26KE1- or Ac-E26KE1-infected cells was not extractable with a nonionic detergent, but roughly equal amounts of the E2 and E1 products, representing about half of the totals in each case, could be extracted from cells infected with either virus using this same detergent (Fig. 4B). Treatment with PNGase-F reduced the sizes of both the WEEV polyprotein and E2 produced in Ac6.9-E26KE1- or Ac-E26KE1-infected cells (Fig. 4C, upper panel), indicating that these proteins were both *N*-glycosylated when expressed under the control of the *p6.9* and *polh* promoters. To more closely examine E2 glycosylation, we used infected cell extracts from SfSWT-6 cells, which are glycoengineered to produce elongated, mammalian-type *N*-glycans (H. Mabashi-Asazuma, C. Geisler, and D.L. Jarvis, unpublished), for endoglycosidase digestions and western blotting analyses. We expected that SfSWT-6 cells would further elongate the E2 *N*-glycans and enhance resolution of the endoglycosidase digestion products. The results showed that EndoH treatment of the Ac-E26KE1-infected cell extract yielded two products in the E2 region (Fig. 4C, lower panel). The smaller one co-migrated with the completely deglycosylated product obtained by PNGase F treatment († in Fig. 4C, lower panel), and the larger migrated just behind the smaller one (* in Fig. 4C, lower panel). As noted above, EndoH can remove only relatively immature, partially processed *N*-glycans, while PNGase-F can remove both partially and fully processed *N*-glycans. Thus, these results indicated that the Ac-E26KE1-infected insect cells produced two different E2 glycoforms, one containing only relatively immature, EndoH-sensitive *N*-glycans and the other containing a mixture of both relatively immature, EndoH-sensitive and more highly processed, EndoH-resistant *N*-glycans. In contrast, EndoH treatment of the E2 from Ac6.9-E26KE1-infected SfSWT-6 cells yielded only one product, which comigrated with the larger one obtained with the E2 from Ac-E26KE1-infected cells (Fig. 4C, lower panel). These results showed that the *N*-glycans on the E2 product derived from the WEEV polyprotein expressed under the control of the *p6.9* promoter were more efficiently processed than those on the same product expressed under the control of the *polh* promoter.

Together, these results indicated that the BEVs encoding the WEEV polyprotein under *p6.9* or *polh* control, but not the BEV encoding the polyprotein under *iel* control, would be useful for our therapeutic vaccine studies. Both the *p6.9*- and the *polh*-based BEVs produced the polyprotein precursor, which was *N*-glycosylated and proteolytically processed, at least in part, to yield the E2 and E1 products. The *N*-glycans on these products also were processed

when expressed using these two BEVs. As with the E1 ectodomain, expression under the control of the *p6.9* promoter provided more efficient protein processing, including proteolytic cleavage and *N*-glycan processing, but in the final analysis, about the same amounts of processed WEEV glycoprotein products were obtained when the polyprotein was expressed under the control of either the late or very late promoter.

Expression and analysis of the E2E1 chimera

In a final set of experiments, we examined the expression of an artificial, secretable E2E1 chimeric protein (Fig. 1A) by BEVs encoding this construct under the transcriptional control of the three different baculovirus promoters. Western blotting analysis with the anti-penta-His antibody showed that immunoreactive proteins of about the expected size (91 kDa) were produced and secreted by cells infected with each of the E2E1chim viruses (Fig. 5A). Insect cells infected with AcIE1-E2E1chim contained no detectable immunoreactive proteins, while those infected with Ac6.9-E2E1chim contained one and those infected with Ac-E2E1chim contained two major immunoreactive proteins (Fig. 5A, top panel). The results of endoglycosidase treatments and western blotting analyses showed that the single immunoreactive protein in Ac6.9-E2E1chim-infected and the larger of the two immunoreactive proteins in Ac-E2E1chim-infected cells were *N*-glycosylated (Fig. 5B, top panel). These results also showed that the smaller of the two proteins in Ac-E2E1chim-infected cells was unglycosylated. Thus, more efficient *N*-glycosylation was once again observed when a WEEV glycoprotein construct was expressed during the late phase of infection under *p6.9* control, as compared when it was expressed during the very late phase of infection under *polh* control.

The efficiency of secretion also was inversely proportional to the timing of expression, as observed for E1ecto. All of the detectable immunoreactive protein was secreted from cells infected with AcIE1-E2E1chim and densitometry showed that ~62% was secreted from cells infected with Ac6.9-E2E1chim (Fig. 5A, lower panel). In this case, however, the densitometric analysis showed that cells infected with Ac-E2E1chim also secreted ~42% of the detectable immunoreactive protein and produced much more secreted E2E1chim than cells infected with AcIE1-E2E1chim and slightly more than cells infected with Ac6.9-E2E1chim (Fig. 5A, lower panel). The secreted chimeric protein produced by cells infected with any of the three BEVs was resistant to EndoH and sensitive to PNGase-F (Fig. 5B, lower panel), indicating that it contained more highly processed *N*-glycans. Thus, in the case of the chimeric E2E1 protein, a conventional BEV encoding this product under the control of the *polh* promoter was the best choice for recombinant glycoprotein production. Insect cells infected with the *ie1*-based BEV *N*-glycosylated and secreted the chimeric protein most efficiently, but yielded much lower amounts of extracellular protein relative to the other two BEVs. Cells infected with the *p6.9*-based BEV glycosylated the chimeric protein efficiently and secreted it less efficiently, but yielded much higher levels of extracellular protein, as compared to the *ie1*-based BEV. Cells infected with the *polh*-based BEV glycosylated the chimeric protein least efficiently, but secreted the glycosylated subpopulation efficiently and ultimately yielded the highest levels of soluble, extracellular protein.

Conclusions

The overall purpose of this study was to produce WEEV glycoproteins for therapeutic vaccine studies using the BEVs. To determine how we might best achieve this goal, we assessed the expression of four different WEEV glycoprotein constructs under the transcriptional control of three temporally distinct baculovirus promoters. From previous experience and information available in the literature, we expected that recombinant glycoprotein processing, including *N*-glycosylation, proteolytic cleavage, and secretion, would be more efficient when WEEV constructs were expressed during earlier phases of

baculovirus infection. As expected, we reproducibly observed higher efficiencies of glycoprotein processing when the WEEV E1ecto and E2E1chim constructs were expressed during earlier phases of infection. However, despite the increased processing efficiencies, we also found that expression of these constructs earlier in infection offered no true practical advantage, as we reproducibly obtained about the same or higher amounts of processed, soluble product when these constructs were expressed during later phases of infection. Furthermore, expression of the E26KE1 polyprotein construct under *ie1* control yielded no detectable immunoreactive proteins, while its expression under *p6.9* or *polh* control yielded easily detectable levels of proteolytically cleaved and *N*-glycosylated products. Thus, for this construct, expression earlier in infection offered absolutely no advantage for recombinant glycoprotein production in the BEVS. Finally, expression of the full-length WEEV E1 construct yielded either no detectable (under *ie1* control) or insoluble (under *p6.9* or *polh* control) immunoreactive products, which underscored the fact that the nature of the construct is also an important factor influencing recombinant glycoprotein production in the BEVS. In summary, the results of this study demonstrated that the timing of expression and the nature of the construct both have an impact on recombinant glycoprotein production in the BEVS and, while expression during earlier phases of infection can provide more efficient processing, this approach does not necessarily provide higher yields of high quality, processed, soluble products.

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- New baculovirus destination vectors with temporally distinct promoters.
- Parallel analysis of glycoprotein production with different promoters.
- Earlier promoters provide higher glycoprotein processing efficiencies.
- Polyhedrin promoter provides equal or higher yields of processed proteins.

Fig. 1.
(A) Diagram of the WEEV structural polyprotein and the WEEV glycoprotein constructs designed for this study. The latter included constructs encoding full-length E1, the E1 ectodomain (amino acids 1-408), the E26KE1 polyprotein, and an artificial, secretable E2E1 chimera containing the E2 and E1 ectodomains (amino acids 1-363 and 1-408, respectively) connected by a flexible (Gly-Gly-Gly-Gly-Ser)₃ linker. Each construct included an *N*-terminal Tobacco etch virus (TEV) protease cleavage site for removal of the 8XHIS and StrepII affinity purification tags. **(B)** Baculovirus destination vectors. This diagram shows the key features of the modified Gateway® (Invitrogen) baculovirus destination vectors used in this study. AcIE1GT, Ac6.9GT, and AcGT are destination vectors designed for recombinant protein expression under the transcriptional control of baculovirus *ie1*, *p6.9*, or *polh* promoters, as indicated. The diagram also shows other key genetic features of these new destination vectors, which facilitate the isolation of BEVs encoding the gene of interest and the expression and purification of recombinant proteins. These include: the honey bee mellitin signal peptide (HBM SS); an octahistidine affinity purification tag (8XHIS); the

Strep II affinity purification tag (Strep II); the lambda phage *att* sites for *in vitro* recombination with entry clones encoding the gene of interest (*attR1* and *attR2*); the Herpes simplex virus Type 1 thymidine kinase gene for negative selection of parental viruses with ganciclovir (TK); the *E. coli lacZ* gene encoding β -galactosidase for blue-white screening of recombinant baculovirus plaques (*lacZ*); and a unique *Bsu36I* site for linearization of the destination vectors to enhance the efficiency of recombinant baculovirus isolation. The diagram also shows that the baculoviral DNA used to isolate the new destination vectors was BestBac 2.0 (Expression Systems), which lacks the AcMNPV cathepsin and chitinase genes.

Fig. 2.

BEV-mediated expression of full-length WEEV E1. **(A)** *ExpresSF*⁺ cells were mock-infected (mock) or infected with the parental Ac6.9GT destination vector (neg), AcIE1-E1 (*ie1*), Ac6.9-E1 (*p6.9*), or Ac-E1 (*polh*), and then the cells were extracted with DB at the indicated times post-infection and the extracts were analyzed by western blotting with anti-penta-His as the primary antibody. **(B)** *ExpresSF*⁺ cells were infected for 72 hr as in (A), extracted with glycoprotein disruption buffer, and then the extracts were treated with buffer alone (-), PNGase F (P), or EndoH (E) and analyzed by western blotting with anti-penta-His as the primary antibody. **(C)** *ExpresSF*⁺ cells were infected for 72 hr as in (A) and equal aliquots were extracted with either DB (T) or extraction buffer containing NP-40 (S). The insoluble material obtained after treatment with extraction buffer was pelleted and extracted

with DB (P) and each fraction was analyzed by reducing (top panel) or non-reducing (lower panel) SDS-PAGE followed by western blotting with anti-penta-His.

Fig. 3. BEV-mediated expression of WEEV E1ecto. **(A)** *ExpresSF*⁺ cells were mock-infected (mock) or infected with the parental Ac6.9GT destination vector (neg), AcIE1-E1ecto (*ie1*), Ac6.9-E1ecto (*p6.9*), or Ac-E1ecto (*polh*), and then the cells were pelleted at the indicated times post-infection and the cell-free media were harvested. The cell pellets (top panel; IC) and cell-free media (lower panel; XC) were then mixed with DB and samples of each were analyzed by western blotting with anti-penta-His as the primary antibody. **(B)** *ExpresSF*⁺ cells were infected for 72 hr and cells (top panel; IC) and cell-free media (lower panel; XC) were isolated. Samples of each were mixed with glycoprotein disruption buffer, treated with buffer alone (-), PNGase F (P), or EndoH (E), and analyzed by western blotting with anti-penta-His as the primary antibody.

Fig. 4.

BEV-mediated expression of WEEV E26KE1. **(A)** *ExpresSF*⁺ cells were mock-infected (mock) or infected with the parental Ac6.9GT destination vector (neg), AcIE1-E26KE1 (*ie1*), Ac6.9-E26KE1 (*p6.9*), or Ac-E26KE1 (*polh*), and then the cells were extracted with DB at the indicated times post-infection and the extracts were analyzed by western blotting with anti-penta-His (top panel) or anti-Sindbis (lower panel) as the primary antisera. **(B)** Infections, protein extractions, and immunoblotting with anti-penta-His were performed as described in the legend to Fig. 2C. **(C)** *ExpresSF*⁺ or SfSWT-6 cells were infected for 72 hr as in (A), extracted with glycoprotein disruption buffer, and then the extracts were treated

with buffer alone (-), PNGase F (P), or EndoH (E) and analyzed by western blotting with anti-penta-His as the primary antibody.

Fig. 5. BEV-mediated expression of E2E1chim. **(A)** *ExpresSF*® cells were mock-infected (mock) or infected with the parental Ac6.9GT destination vector (neg), AcIE1-E2E1chim (*ie1*), Ac6.9- E2E1chim (*p6.9*), or Ac-E2E1chim (*polh*), and then the cells were pelleted at the indicated times post-infection and the cell-free media were harvested. The cell pellets (top panel; IC) and cell-free media (lower panel; XC) were then mixed with DB and samples were analyzed by western blotting with anti-penta-His as the primary antibody. **(B)** *ExpresSF*® cells were infected for 72 hr and cells (top panel; IC) and cell-free media (lower panel; XC) were isolated. Samples of each were mixed with glycoprotein disruption buffer, treated with buffer alone (-), PNGase F (P), or EndoH (E), and analyzed by western blotting with anti-penta-His as the primary antibody.

Table 1

Primers used to generate transfer plasmids pAcIE1GT-TV, pAc6.9GT-TV, and entry clones for isolation of BEVs encoding WEEV glycoproteins.

Primer Name	Sequence (5' -> 3')
PROSWAP P2 SP1	cgcgccttaaaatcggccgccatcagcaactatatattgatag
PROSWAP P2 SP2	cgcgccttaaaatcggcc
PROSWAP P2 ASP1	ctggcaactgcaagggtctc
PROSWAP P1 SP1	gcaacttacctcgggatgg
PROSWAP P1 ASP1	ccggccgaattttaaggcgcgcctgatctcaccatgaaattttg
PROSWAP P1 ASP2	ccggccgaattttaaggcg
IE1HR5 PROSWAP SP1	atggccggccggctcgtatgtgtgtgg
IE1HR5 PROSWAP SP2	atggccggccggctcgtat
IE1HR5 PROSWAP ASP1	atggcgcgcgggtcactgtgtgttcacgat
IE1HR5 PROSWAP ASP2	atggcgcgcgggtcactgt
pp6.9 PROSWAP SP1	atggccggccggtaccaaattccgttttcgac
pp6.9 PROSWAP SP2	atggccggccggtaccaaatt
pp6.9 PROSWAP ASP1	atggcgcgcgggtttaattgtgtaatttatgtagc
pp6.9 PROSWAP ASP2	atggcgcgcgggtttaattgtg
E1 For1	caccaagagaacctgtattccaagggttcgaacatgcgaccact
E1 For2	caccaagagaacctgtat
E1 Rev1	ctatctacgtgtgtttataagc
E1ecto Rev1	ctagttccaagatgttttggaac
E26KE1 For1	caccaagagaacctgtattccaaggcagcattaccgatgacttcac
Chimera_E2ecto For	caccaagagaacctgtattcc
Chimera_E2ecto Rev1	actaccaccaccagagccgccgcccatgccgatgataatagtgga
Chimera_E2ecto Rev2	actaccaccaccagag
Chimera_E1ecto For1	ggtggtggtgtagtgaggaggaggatcattcgaacatgcgaccactgt
Chimera_E1ecto For2	ggtggtggtgtagtgaggaga
Chimera_E1ecto Rev	ctagttccaagatgttttggaac