Expression and structural characterization of a baculovirus ecdysteroid UDP-glucosyltransferase

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The baculovirus enzyme ecdysteroid UDP-glucosyltransferase (EGT) disrupts the hormonal balance of the insect host by catalysing the conjugation of ecdysteroids, the moulting hormones, with the sugar moiety from UDP-glucose or UDP-galactose. In this study, EGT has been overproduced using a recombinant Autographa californica nucleopolyhedrovirus and an antiserum has been raised against the purified protein. This antiserum was used to visualize the kinetics of expression of EGT by wild-type AcMNPV L-1 and by the overproducing recombinant virus. The inclusion of tunicamycin during these time-course experiments suggested that EGT is glycosylated. This was confirmed by Endo F treatment, which showed that glycosylation increased the apparent subunit molecular mass by approximately 11 kDa. These sugars do not appear to be required for enzyme activity. EGT activity invariantly elutes from gel-filtration columns as a single peak corresponding to a 260 kDa (±50 kDa) protein. This suggests that the enzyme is an oligomer of three to five subunits, since the subunit molecular mass is 56 kDa.

Introduction

One of the remarkable characteristics of baculoviruses is their ability to suppress the development of the infected insect host, preventing it from moulting or pupating (O'Reilly & Miller, 1989). We have shown that this developmental arrest is brought about by the action of the enzyme ecdysteroid UDP-glucosyltransferase (EGT) (O'Reilly & Miller, 1989). EGT catalyses the transfer of either glucose or galactose from their respective UDP-sugars to ecdysteroid molecules (O'Reilly et al., 1992a). The ecdysteroids are the family of steroid hormones which control moulting and pupation (reviewed in Coudron et al., 1981). Conjugation of sugars to these hormones inactivates them (Park et al., 1993).

A large number of baculovirus egt genes has now been identified and characterized in the genomes of both nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). Of these, most is known about the Autographa californica NPV (AcMNPV) egt gene (O'Reilly & Miller, 1989; reviewed in O'Reilly, 1995). AcMNPV egt is transcribed early in infection as two 5'-coterminal mRNAs that initiate 43 nt upstream of the start codon (O'Reilly & Miller, 1990). Transcript levels decline late in infection. These mRNAs are translated to give a proenzyme of 506 amino acids. An 18 amino acid signal sequence at the EGT N-terminal end directs it to the extracellular compartment. This signal sequence is cleaved from the mature protein (O'Reilly et al., 1992a). Under denaturing conditions, EGT has an apparent molecular mass of approximately 60 kDa (O'Reilly & Miller, 1990). The biochemistry of AcMNPV EGT has been studied extensively. Enzyme action on ecdysone and UDP-glucose results in the formation of ecdysone-22-O-β-D-glucoside (O'Reilly et al., 1991). It can accept sugars from either UDP-glucose or UDP-galactose with almost equal efficiency in vitro (Evans & O'Reilly, 1998). However, only ecdysteroid galactosides are formed during AcMNPV infection in vivo, presumably because UDP-galactose is the predominant UDP-sugar available to the enzyme in the insect host (O'Reilly et al., 1992a; Evans & O'Reilly, 1998). AcMNPV EGT can conjugate a range of ecdysteroids provided they possess a hydroxyl group at position C-22 (O'Reilly et al., 1992a). However, it displays very different specificities for different ecdysteroids showing, in particular, a marked preference for ecdysone and 3-dehydroecysone over 20-hydroxyecdysone, the most active form of the hormone in vivo (Evans & O'Reilly, 1998).

Baculovirus EGTs belong to the UDP-glycosyltransferase superfamily. All the members of this group conjugate small
lipophilic compounds with various sugars (O’Reilly & Miller, 1989; O’Reilly, 1995). The EGT amino acid sequence is particularly homologous to those of the mammalian UDP-glucuronosyltransferases (UDPGTs) (O’Reilly & Miller, 1989). The UDPGTs act in the lumen of the endoplasmic reticulum and play a central role in the detoxification and elimination of a wide range of endogenous and exogenous compounds (Burchell & Coughtrie, 1989; Meech & Mackenzie, 1997). The importance of these enzymes is highlighted by the fact that a number of serious pathologies are associated with lesions in UDPGT genes. For example, Crigler–Najjar syndrome, caused by mutation of the gene encoding bilirubin UDPGT, is characterized by the accumulation of toxic levels of bilirubin in the blood, resulting in death in early childhood (Erps et al., 1994). The mammalian UDPGTs have proved very difficult to study due to their close association with cell membranes (Puig & Tephly, 1986). In contrast, EGT is secreted, and thus is relatively simple to purify and characterize. Therefore, it represents a valuable model system for the characterization of the UDPGTs.

Whereas the biochemical properties of AcMNPV EGT have been studied in some detail, relatively little is known about the physical properties of the protein. In this study, we have overexpressed AcMNPV EGT using recombinant baculoviruses. An antiserum has been raised against the overexpressed protein and used to study the expression and post-translational modification of EGT.

Methods

**Cells and viruses.** Spodoptera frugiperda IPLB-SF21 cells (Vaughn et al., 1977) were maintained in TC100 medium (GIBCO) supplemented with 0.26% tryptose broth and 10% foetal calf serum (MBM Ltd), or in SF900 II serum-free medium (GIBCO). All virus stocks were generated, propagated and titred on SF21 cells in complete TC100 medium as described (O’Reilly et al., 1992b).

The viruses used are illustrated diagrammatically in Fig. 1 and were as follows:

(i) Wild-type (wt) AcMNPV L-1 (Lee & Miller, 1978).

(ii) vEGTDEL: an AcMNPV L-1 derivative lacking 1094 bp from within the egt gene (O’Reilly et al., 1991).

(iii) vVEGT: an occlusion-negative (occ−) virus containing a single egt gene under the control of a modified polyhedrin promoter. To generate this virus, the AcMNPV egt gene was amplified by PCR using the following primers (non-viral sequences added to facilitate cloning are italicized):

| EGT-4: 5’ CGAGATCTTTATCCGTTTGAAGC 3’ |
| EGT +1636: 5’ GCCGTAATCGTGAAGCTC 3’ |

The resulting product was cloned downstream of the modified polyhedrin promoter P\textsubscript{XIV} between the BglII and XhoI sites of the transfer plasmid pEVMXV (Wang et al., 1991) and cotransfected with vEGTDEL DNA into SF21 cells (O’Reilly et al., 1992b). Occ− plaques were amplified, since vVEGT has no polyhedrin gene.

(iv) vSynEVEGT: an occ− recombinant baculovirus containing two egt genes, one at its normal locus and one at the polyhedrin locus. To make this virus, the egt-containing SalI–BglII fragment of pUCBCPSB (O’Reilly & Miller, 1990) was cloned into the SalI and BamHI sites of pBluescript SKII+ (Stratagene). The insert was then removed using XhoI and SalI and ligated into the baculovirus transfer plasmid pSynXIV VI+X3 (Wang et al., 1991) downstream of P\textsubscript{synXIV}, a promoter composed of P\textsubscript{syn} and the synthetic promoter P\textsubscript{syn} arranged in tandem (P\textsubscript{Syn} VI+X3 also contains an unaltered polyhedrin gene). Recombination of the pSynXIV VI+X3 construct and the occ− parental virus vSynVEGT (Wang et al., 1991) generated the occ− virus vSynEVEGT.

**Assay of EGT activity.** EGT activity was assayed as described in Evans & O’Reilly (1998). Briefly, EGT was incubated at 37°C for 30 min in the presence of 20 μM N-acetyl glucosamine, 0.05 μCi [3H]UDP-glucose and 10 mM MgCl\textsubscript{2} (unless noted otherwise). The reaction was stopped and the product extracted using water-saturated butan-1-ol; 63% of the conjugate partitions into the organic phase, whereas unincorporated UDP-sugar remains in the aqueous phase. The amount of radioactivity in the organic phase was quantified and converted into the total amount of product formed/min.

**Gel filtration of EGT.** Gel filtration was performed as described previously (Evans & O’Reilly, 1998). The five fractions with peak EGT activity from a CM52 CM cellulose column (Evans & O’Reilly, 1998) were pooled and concentrated to a volume of about 4 ml; 1 ml of this concentrated was adjusted to 5% (v/v) glycerol and applied to a 20 ml/h to a calibrated Sephacryl S-300 HR column (Pharmacia) previously equilibrated in 100 mM potassium acetate, pH 5. Protein levels in the eluate were monitored by in-line recording of the A\textsubscript{280}. A 15 μl aliquot of each 3.3 ml fraction was assayed for EGT activity in a volume of 300 μl.

**Generation of anti-EGT antiserum.** EGT was purified using ion-exchange and gel-filtration chromatography of medium from cells infected with vSynEVEGT (Evans & O’Reilly, 1998). Five peak fractions from each of four gel filtration runs were pooled and concentrated to 5 ml using a YM30 ultrafiltration membrane mounted in an Amicon pressure cell, and then to about 2 ml using a Pall Macrosep centrifugal concentrator. This final volume, containing approximately 200 μg total protein (estimated with a Pierce BCA protein assay kit), was emulsified in Freund’s complete adjuvant and injected subcutaneously into a Halfpenny rabbit (Foxfield Farms). This process was repeated four times at 4 week intervals. For the final injection, the concentrate was emulsified in incomplete adjuvant instead. The serum (collected as described by Harlow & Lane, 1988) was tested for its response against EGT by immunoblotting and by inhibition of EGT activity (data not shown). For most experiments, the serum was first preadsorbed to acetone powders (Harlow & Lane, 1988), prepared from a mixture of uninfected and vEGTDEL-infected SF21 cells grown in SF900 II medium, to reduce background.

**Analysis of EGT expression.** SF21 cells (2×10\textsuperscript{6} per 60 mm dish) grown in complete TC100 were infected with virus at an m.o.i. of 10. After adsorption for 1 h, the inoculum was removed, and the cells reinfected with fresh complete TC100. At various times post-infection (p.i.), the cell culture medium (representing the extracellular fraction) and/or cell lysates were harvested as described (O’Reilly et al., 1992b). The cells were lysed in Triton X-100 detergent extraction buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris–HCl (pH 7.5), 1 mM MgCl\textsubscript{2}, 50 ng/ml leupeptin, 100 μg/ml PMSF and 1 μg/ml pepstatin A). Where noted, tunicamycin (Boehringer Mannheim) was used to investigate the N-linked glycosylation of EGT. For these experiments, the cells were reinfected with complete TC100 containing 5 μg/ml tunicamycin at least 24 h before harvesting.
Structural analysis of a baculovirus EGT

Fig. 1. Diagrammatic representation of the viruses used in this study. Coding regions are shown as shaded boxes with the arrow above indicating the direction of transcription. Promoters are represented as shaded arrowheads. The diagram is roughly to scale; as a reference point, map units have been shown on the wt AcMNPV L-1 diagram.

Glycosidase treatment. Pure EGT (910 ng) or cell supernatant (32.5 µl) was digested with 250 mU Endo F in 1 × glycosidase buffer (10 mM sodium phosphate, pH 7.2, 1%, v/v, NP40, 5 mM EDTA), in a total reaction volume of 50 µl. The digest was incubated at 37 °C for 24 h in the presence of 10 µg PMSF. As controls, identical digests were set up lacking Endo F. The reaction was stopped (except when EGT activity was to be subsequently quantified) by boiling in SDS–PAGE gel loading buffer. If EGT assays were to be subsequently performed, the reactions were placed at 4 °C until needed.

Immunoblotting and SDS–PAGE. Protein samples were electrophoresed through 10% SDS–polyacrylamide gels (Laemmli, 1970) and visualized either by staining with silver nitrate (Sambrook et al., 1989) or by immunoblotting. SDS–PAGE for subsequent immunoblotting was invariably done in non-reducing conditions, as the EGT antiserum was highly cross-reactive to the reducing agent β-mercaptoethanol. EGT migration was unchanged by the presence or absence of β-mercaptoethanol.

Immunoblotting was performed as described by Harlow & Lane (1988). Briefly, the proteins were transferred from an SDS–PAGE gel to Hybond-C Extra (Amersham) nitrocellulose membrane using a Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer cell. The membrane was blocked overnight at 4 °C and incubated for 1 h in diluted (1:2500) anti-EGT polyclonal antiserum at room temperature. Bound antibodies were detected using horseradish peroxidase-conjugated goat antiserum against rabbit IgG (Stratech) which was revealed using an ECL kit (Amersham).

Results

Recombinant viruses

Two recombinant baculoviruses that overproduce EGT, vEVEGT and vSynEVEGT, were constructed to facilitate generation of antiserum against the enzyme (Fig. 1). vSynEVEGT contains two copies of the egt gene, one at its normal locus, and one at the polyhedrin locus under the control

Fig. 2. EGT activity following infection with wt AcMNPV L-1, vEVEGT or vSynEVEGT for 6, 9, 12, 24, 48 and 72 h. EGT activity (mean ± standard deviation, n = 3) is shown as a percentage of wt AcMNPV L-1 EGT activity at 48 h p.i. The bolder error bars are for the vEVEGT data. The standard deviations in the L-1 data are very small when compared to the other data, and are barely visible on this graph. The inset is a close-up of 6–24 h p.i. (no error bars).
a modified polyhedrin promoter. vEVEGT has only a single copy of the \( egt \) gene. This copy is at the polyhedrin locus, but under the control of a different polyhedrin promoter to that in vSynEVEGT. The \( egt \) gene at its normal locus in vEVEGT has been deleted.

To compare the kinetics of EGT production by these viruses, a time-course was carried out monitoring EGT activity at 6, 9, 12, 24, 48 and 72 h p.i. following infection with vEVEGT, vSynEVEGT or wt AcMNPV L-1. EGT activity was detectable by 6 h p.i. with wt AcMNPV and increased through 12 h p.i. (Fig. 2). Thereafter, levels tended to plateau, only increasing marginally by 72 h p.i. EGT activity was also detectable by 6 h p.i. with vSynEVEGT. However, no plateau was observed. Instead, enzyme activity increased steadily to 72 h p.i. In contrast, following vEVEGT infection, EGT activity was undetectable until 24 h p.i. It then accumulated steadily to 72 h p.i. vSynEVEGT and vEVEGT gave rise to approximately seven times more active EGT than wt AcMNPV. Of the two recombinant viruses, vSynEVEGT produced more active enzyme on average, although this difference was quite small. Based on these observations, vSynEVEGT-infected cell supernatants harvested at 72 h p.i. were used as the starting material for the purification of EGT (Evans & O’Reilly, 1998). The purified protein was then used as immunogen for the generation of an anti-EGT antiserum.

**Kinetics of EGT expression during infection**

The anti-EGT antiserum was used to monitor EGT protein levels during infection of SF21 cells by wt AcMNPV. Immunoblot analysis was carried out on cell lysates and supernatants were harvested at 6, 9, 12, 24, 48 and 96 h p.i. Samples from vSynEVEGT- and vEGTDEL-infected cells were monitored in parallel as controls (Fig. 3). In the wt AcMNPV-infected cell lysates, EGT levels were very low at all time-points. Faint EGT-specific bands were visible on the original autoradiograph. However, the protein was clearly visible as a diffuse doublet of approximately 55 and 56 kDa in the extracellular fraction from 6 h p.i., and remained detectable throughout the time-course. As expected, EGT levels were much higher and intracellular EGT was easily observed following vSynEVEGT infections. Protein levels were low at early times p.i., but increased significantly later in infection with a peak at 48 h p.i. EGT was detectable in the extracellular fraction of vSynEVEGT-infected cells by 6 h p.i. and increased dramatically by 96 h p.i. Again, the enzyme was detected as a
doublet of 55 and 56 kDa. No EGT-specific bands were present in either the vEGTDEL intracellular or extracellular fractions.

Characterization of EGT glycosylation

The AcMNPV EGT amino acid sequence contains seven putative sites where N-linked glycosylation could occur (O'Reilly & Miller, 1990). To investigate whether glycosylation contributed to the heterogeneous appearance of EGT on SDS–PAGE gels, SF21 cells were infected with either wt AcMNPV or vSynEVEGT in the presence and absence of tunicamycin, an inhibitor of N-linked glycosylation. Tunicamycin treatment caused the EGT-specific bands at 55–56 kDa to disappear (Fig. 4). This effect was only partial in vSynEVEGT-infected samples treated with tunicamycin from 24–48 h p.i. (treatment iii). A novel band of 45 kDa appeared in all infected cell lysates upon treatment with tunicamycin. This was not detected in the supernatant fractions, indicating that it was not secreted. These data suggest that EGT is glycosylated and that the N-linked sugars increase the apparent subunit molecular mass by 11 kDa.

To confirm the conclusions drawn from the tunicamycin treatment, wt AcMNPV- and vSynEVEGT-infected cell supernatants (harvested at 24 h p.i.), and purified EGT, were treated with Endo F, a mixture of the enzymes endoglycosidase F and N-glycosidase F. These enzymes cleave N-linked oligosaccharides from glycoproteins at their point of attachment to the protein. The results (Fig. 5a, b) showed that Endo F caused a decrease in the apparent molecular mass of EGT by about 11 kDa in all three samples analysed.

To examine whether N-linked glycosylation is required for EGT activity, enzyme activity was assayed after incubation of purified protein in the presence or absence of Endo F (Fig. 5c). Samples were examined by SDS–PAGE to check that the digest had reached completion (data not shown), and the experiment was repeated twice to assure reproducibility. The results show no appreciable difference in the activity between glycosylated and deglycosylated EGT.

AcMNPV EGT also has four putative sites of O-linked glycosylation. To investigate whether the enzyme is modified by O-glycosylation, purified EGT was incubated in the presence or absence of the enzyme O-glycosidase, which catalyses the removal of O-linked sugars from the polypeptide backbone. The results of the digest showed no visible difference in apparent molecular mass of digested and nondigested EGT (data not shown).

Characterization of the native molecular mass of EGT

Gel-filtration chromatography was used to estimate the native molecular mass of AcMNPV EGT. EGT activity eluted from this column as a distinct and single peak corresponding to a 260 kDa protein (50 kDa) (Fig. 6). The gel-filtration fractions with EGT activity were blotted onto nitrocellulose membrane and probed with the anti-EGT antiserum. The band intensity on the resulting blot closely matched the values of EGT activity in each fraction, i.e. there was a single peak in immunopositivity, with the apex of this peak located in the samples from fractions 7 and 8 (Fig. 6). These data suggest that native EGT exists predominantly as an oligomer, possibly a
Fig. 5. Treatment of EGT with Endo F. (a) Immunoblot analysis of extracellular fractions from wt AcMNPV L-1 and vSynEVEGT infections (24 h.p.i.) incubated in the presence (+) or absence (−) of Endo F. The positions of EGT (glycosylated and unglycosylated forms) and molecular mass markers are shown. (b) Silver-stained SDS–PAGE gel of purified EGT incubated in the presence (+) or absence (−) of Endo F. The positions of the components of the Endo F mixture are also shown. (c) Effect of deglycosylation on EGT activity. Purified EGT was incubated in the presence (+) or absence (−) of Endo F and then assayed for EGT activity. EGT activity is shown in pmol ecdysone glucoside formed/min. An aliquot from each EGT treatment was previously checked for the completeness of the reaction on a silver-stained SDS–PAGE gel (data not shown).

Fig. 6. Gel-filtration chromatography of EGT. An aliquot of each fraction was assayed for EGT activity. EGT protein levels were also estimated in selected fractions by immunoblot analysis. The positions of the molecular mass markers and EGT are shown.

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discussion

The baculovirus egt gene represents a remarkable instance of a viral gene acting on the host at the organismal level to facilitate propagation of the virus. By allowing the virus to block host development, production of EGT results in a significant increase in the yield of progeny virus from an infected insect (O’Reilly & Miller, 1991; O’Reilly et al., 1998). This study represents part of our on-going efforts to understand in detail the function of this important enzyme. Here, we have generated a polyclonal antiserum to EGT and used it to explore the synthesis and processing of the protein.

The immunoblot analysis of EGT demonstrated that the protein is produced rapidly after infection by wt AcMNPV and secreted efficiently from the infected cells. This is in agreement with previous work that indicated that egt is transcribed as an early gene (O’Reilly & Miller, 1990). Transcript levels declined later in infection, whereas in this study protein levels remained relatively constant through the infection process. This suggests that EGT is relatively stable. There was a very good correspondence between EGT protein levels and enzyme activity during the time-course experiments (compare Figs 2 and 3), suggesting there is little post-translational control of EGT activity.
The kinetics of EGT production by the various viruses used in this study agreed well with the genome structures of the viruses. As discussed above, following infection by wt AcMNPV, egt is expressed as an early gene and EGT activity can be detected rapidly after infection. In contrast, in vEVGT, egt is expressed under the control of the very late polyhedrin promoter, and enzyme activity was not detected until 24 h p.i. However, EGT accumulated to much higher levels, reflecting the greater strength of the polyhedrin promoter. The recombinant vSynEVGT has two egt genes, one at its normal locus under the control of the egt promoter, and a second at the polyhedrin locus under the control of a polyhedrin-derived promoter. In this case, EGT was detectable rapidly after infection, with a substantial burst occurring later when the polyhedrin promoter became active (Fig. 2).

Immunoblot analysis revealed that the EGT protein migrates heterogeneously on SDS–PAGE gels. Typically, a doublet of approximately 55 and 56 kDa was observed but these bands were generally diffuse, suggesting each band represents multiple forms of the protein. These data are in agreement with previous findings following 35S pulse-labelling of AcMNPV-infected SF21 cells (O’Reilly & Miller, 1990). We have now shown that this heterogeneity is due to N-linked glycosylation of the protein. Each EGT monomer is modified by the addition of sugar residues that increase its apparent molecular mass by approximately 11 kDa. Tunicamycin treatment of infected cell lysates resulted in the synthesis of a single sharp EGT-specific band of 45 kDa. Thus, we suspect that EGT is not modified in any other way. N-Linked glycosylation of EGT does not appear to be important for the specific activity of the enzyme. However, it remains possible that glycosylation is important for other aspects of the enzyme’s function, such as stability. Several UDPGTs are known to be glycosylated with N-linked sugars and a number of the unglycosylated forms of these enzymes are also known to retain their catalytic abilities (Green & Tephly, 1989; Shepherd et al., 1989; Mackenzie, 1990; Dong et al., 1997).

It is well documented that post-translational modifications such as N-glycosylation are often not carried out efficiently in the late phases of a baculovirus infection as cellular functions begin to decline (reviewed in O’Reilly et al., 1992b). This was not observed in this case: EGT produced following vSynEVGT infection (predominantly produced late in infection) was indistinguishable from EGT produced during wt AcMNPV infection (produced early) in terms of its migration on SDS–PAGE gels (Fig. 3). Similarly, enzyme from both sources was glycosylated to the same extent, as indicated by the effects of tunicamycin and EndoF treatments (Figs 4 and 5). Thus, vSynEVGT infection of SF21 cells represents a valid expression system for the production of high levels of authentic EGT for further study.

The oligomeric state of native EGT was examined by gel-filtration chromatography. EGT protein and activity eluted from these columns as a single peak corresponding to a 260 kDa protein (± 50 kDa) (Fig. 6). Since the subunit molecular mass of EGT in denaturing conditions is approximately 56 kDa, we suspect that the active enzyme is an oligomer of between three and five subunits, possibly a tetramer. The subunits are not held together by disulphide bonds or indirect interactions between the sugar residues. We do not know whether oligomerization is necessary for enzyme activity. Many mammalian UDPGTs are also multimeric, consisting of one to six subunits (each monomer being typically 50–60 kDa), depending on the enzyme (Peters et al., 1984, 1986; Chowdhury et al., 1986; Bruni & Chang, 1995). For example, Peters et al. (1986) isolated rat liver UDPGT isozymes specific for p-nitrophenol and phenolphthalein which had molecular masses of 109 kDa (dimer) and 159 kDa (trimer) respectively. Chowdhury et al. (1986) isolated a UDPGT isozyme from rat liver which had an apparent native molecular mass of 310 kDa and monomer molecular mass of 52 kDa, suggesting that the enzyme was a hexamer.

This study has provided further evidence of the similarities between EGT and the mammalian UDPGTs. This reinforces our proposal that EGT represents a suitable model system for the study of the mammalian enzymes, which are themselves very hard to work with. The reagents developed in this study will facilitate both this work and the further characterization of baculovirus EGTS.

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