Oligosaccharide Processing in the Expression of Human Plasminogen cDNA by Lepidopteran Insect (*Spodoptera frugiperda*) Cells†

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ABSTRACT: A comparison has been made between the Asn^{289}-linked oligosaccharide structures of human plasma plasminogen and a recombinant human plasminogen, expressed in lepidopteran insect (*Spodoptera frugiperda*) cells, after infection of these cells with a recombinant baculovirus containing the entire human plasminogen cDNA. Using anion-exchange liquid chromatography mapping of the oligosaccharide units cleaved from the proteins by glycopeptidase F, compared with elution positions of standard oligosaccharide structures, coupled with monosaccharide compositional analysis, we find that the human plasma protein contained only bisialo–biantennary complex-type carbohydrate and asialo–biantennary complex carbohydrate, confirming earlier work published by this laboratory. The glycosylation pattern of the insect cell expressed recombinant human plasminogen showed considerable microheterogeneity, with identifiable high-mannose carbohydrate ($Man_9GlcNAc_2$) and truncated high-mannose oligosaccharide ($Man_5GlcNAc_2$, $Man_4GlcNAc_2$, and $Man_3GlcNAc_2$). Of major importance, approximately 40% of the oligosaccharide population consisted of complex carbohydrate (bisialo–biantennary), identical in structure with that of the human plasma protein. This is the first direct identification of complex carbohydrate in proteins produced in insect cells and demonstrates that trimming and processing of high-mannose carbohydrate into complex-type oligosaccharide can occur. Our data indicate that both normal and alternate pathways exist in these cells for incorporation and trimming of high-mannose oligosaccharides and that mannosidases, as well as galactosyl-, hexosaminidasyl-, and sialyltransferases are present, and/or can be induced, in these cells. From these observations, we conclude that amino acid sequences and/or protein conformational properties can control oligosaccharide processing events.

Human plasminogen ([Glu^1]Pg)[1] is a plasma-derived zymogen of the fibrinolytic and fibrinogenolytic enzyme, plasmin ([Lys^{78}]Pm), and exists in the circulation as a single-chain glycoprotein containing 791 amino acids (Wiman, 1973, 1977; Sottrup-Jensen et al., 1978; Malinowski et al., 1984; Forsgren et al., 1987; McLean et al.,1987), with Glu at its amino terminus ([Glu^1]Pg). This zymogen exists in plasma in the form of two major variants, separable by their differential affinities for lysine and its structural analogues (Brockway & Castellino, 1972), which differ in their extent of glycosylation at Asn^{289} (Castellino, 1983; Hayes & Castellino, 1979a–c). One form contains a biantennary complex oligosaccharide at Asn^{289}, and the other lacks carbohydrate at this position (Hayes & Castellino, 1979b), despite the presence of the required consensus tripeptide that directs N-based glycosylation, Asn^{289}-Arg-Thr, in both forms of the protein (Powell & Castellino, 1983). Both forms of HPg contain O-linked oligosaccharide at position Thr^{346} (Hayes & Castellino, 1979c).

We have shown previously that the cDNA for [Glu^1]Pg can be functionally expressed in a recombinant baculovirus infected insect (Sf) cell system (Whitefleet-Smith et al., 1989). This was an extremely important discovery since expression of the wt-human [Glu^1]Pg cDNA has not been accomplished in mammalian cells, due to the nearly ubiquitous presence of plasminogen activators in such cells. This latter situation results in conversion of HPg to HPm and degradation, perhaps by autolysis, of the HPm. Upon comparison of the properties of the two affinity chromatography carbohydrate variants of HPg and HPm, it has become clear that glycosylation plays a role in some of their functions. For example, HPg form 1 (Asn^{289} is glycosylated) binds more strongly to lysine-like activation effector molecules (Brockway & Castellino, 1972) and fibrin than HPg form 2 (aglycosyl at Asn^{289}) and is activated to HPm at a faster rate (Takada & Takada, 1983), and its corresponding HPm is more rapidly inactivated by its fast-acting plasma-derived inhibitor (Wiman et al., 1978). In addition, carbohydrate side chains of HPg have been shown to be important in binding to its cellular receptors (Gonzalez-Gronow et al., 1989).

Indirect evidence exists that Sf-expressed foreign genes only undergo the first step of N-linked glycosylation, i.e., the transfer of high-mannose oligosaccharide to the relevant Asn residue in proteins or peptides (Kornfeld & Kornfeld, 1985), with little additional further processing of carbohydrate (Luckow & Summers, 1988), although this has been recently challenged, again by indirect methodology (Jarvis & Summers, 1989). With the greatly increasing use of baculovirus/insect expression systems for mammalian proteins (Luckow & Summers, 1988), the particular relevance of such an expression system for the human [Glu^1]Pg cDNA (Whitefleet-Smith et al., 1989), and the important role of carbohydrate in the

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[1] Abbreviations: HPg, any form or variant of human plasminogen; HPm, any form or variant of human plasmin; [Glu^1]Pg, native human plasminogen with Glu, residue 1, at the amino terminus; [Lys^{78}]Pg, a proteolytically derived form of human plasminogen with Lys, residue 78, at the amino terminus; [Lys^{78}]Pm, human plasmin, which arises from [Glu^1]Pg by cleavage at the activation site Arg^{561}–Val^{562} and also at Lys^{77}–Lys^{78}; EACA, ϵ-aminocaproic acid; r, recombinant; wt, wild type; $DodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; Sf, *Spodoptera frugiperda* cells; WGA, wheat germ (*Triticum vulgaris*) agglutinin; CBL, castor bean (*Ricinus communis*) agglutinin (ricin); Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose.

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properties of HPg and HPm (vide supra), we believed it important to identify directly the nature of the N-linked carbohydrate present on the single Asn residue of HPg on which glycosylation is possible, with the expectation that more general conclusions on the glycosylation properties of this insect expression system could be revealed. This paper reports the most relevant aspects of such a study.

Materials and Methods

Proteins. Native human plasma [Glu^1]Pg, affinity chromatography form I, was purified according to the method of Deutsch and Mertz (1970) as modified by Brockway and Castellino (1972). *Spodoptera frugiperda* IPLB-SF21AE cells were maintained as monolayers in serum-free Excell 400 medium and infected with recombinant baculoviruses at multiplicities of 1–10 plaque forming units/cell. The recombinant baculoviruses, containing wt-[Glu^1]Pg and R^{561}E-[Glu^1]Pg, were constructed from cDNA clones of wt-[Glu^1]Pg and R^{561}E-[Glu^1]Pg (Whitefleet-Smith et al., 1989). Sf-expressed wt-[glu^1]Pg and R^{561}E-[Glu^1]Pg were purified as above from the culture media of the cells.

Restriction endonucleases were purchased from Promega (Madison, WI).

Glycopeptidase F (*Flavobacterium meningosepticum*) was purchased from Boehringer Mannheim Biochemica (Indianapolis, IN).

Peroxidase-labeled WGA and CBL were products of the Sigma Chemical Co. (St. Louis, MO).

Deglycosylation of HPg. The desired HPg preparation (1 mg/mL in 10 mM sodium phosphate, pH 7.4) was treated with glycopeptidase F (Tarentino et al., 1985) at a concentration of 2 units/mL. The reaction was allowed to proceed for 48–72 h at 37 °C. These conditions were found suitable for removal of the Asn^{289}-linked carbohydrate from all of the samples investigated in this study. The mixture was then subjected to centrifugation in molecular weight 10 000 cutoff (Centricon 10) microconcentrator tubes (Amicon, Danvers, MA) to separate the liberated oligosaccharide from the protein sample.

NaDodSO$_4$/PAGE (Laemmli, 1970) of the protein, followed by visualization of the bands with lectin-specific reactions, was employed to monitor the presence of GlcNAc-GlcNAc (with WGA) and β-D-Gal (with CBL) on the protein. In this procedure, protein samples were separated by DodSO$_4$/PAGE on 9% (w/v) gels under nonreducing conditions. The resolved protein bands were transferred to Immobilon-P (Millipore, Bedford, MA) membranes according to established procedures (Burnette, 1981) and then incubated at 37 °C for 1 h in 0.05 M Tris-HCl/0.15 M NaCl/1% (w/v) EIA grade gelatin (Bio-Rad, Richmond, CA), pH 7.4 (blocking buffer). Our exact conditions for transfer were 4 °C in 25 mM Tris-HCl/200 mM glycine/15% (v/v) methanol, pH 8.3, at 20 V for 12 h. After the free protein adsorption sites on the Immobilon-P sheet were blocked with the above gelatin buffer, a solution of peroxidase-labeled WGA or peroxidase-labeled CBL, containing 25 μg/mL of protein in blocking buffer, was added and allowed to incubate at 25 °C for 90 min. Bound lectin was visualized after addition of the peroxidase substrate, purchased as a kit from Bio-Rad (TMB peroxidase EIA substrate kit) and employed according to the instructions provided by the manufacturer.

Hydrolysis of Oligosaccharides. A clam (*Venus mercenaria*) liver extract containing all of the relevant glycosidases was provided by Professor Subhash Basu, of this department (Brown, 1985; Presper et al., 1986). This extract completely hydrolyzed the standard oligosaccharides of interest to this study (vide infra), purchased from the Dionex Corp. (Sunnyvale, CA), as well as a variety of glycolipids and synthetic *p*-nitrophenyl monosaccharyl substrates containing several different types of sugar linkages.[2]

Oligosaccharide Mapping. Resolution of oligosaccharide units liberated from the protein by glycopeptidase F was accomplished by employing a Dionex BIO LC liquid chromatography unit, an anion-exchange column, and pulsed amperometric detection (Hardy et al., 1988). In all cases, column flow rates of 1 mL/min were used at room temperature.

Two gradient procedures were utilized. In one (gradient 1), the sample to be analyzed was injected onto a column of Carbopac PA1 (4 mm × 250 mm) and resolved into its component oligosaccharides by employing gradient elution with solvents A (100 mM NaOH) and B (100 mM NaOH/500 mM NaOAc). Specifically, the sample was injected in a solvent containing 95% A/5% B (v/v), and the column was washed with this same solvent for 15 min. After this point, a linear gradient consisting of solvents 95% A/5% B (start solvent) to 80% A/20% B (limit solvent) was applied for an additional 15 min. Finally, another linear gradient consisting of 80% A/20% B (start solvent) to 60% A/40% B (limit solvent) was utilized to achieve complete resolution of the oligosaccharides.

In the second procedure (gradient 2), the sample was applied to the same column as above, equilibrated with a mixture (v/v) of 97% solvent A (100 mM NaOH)/3% solvent B (1 mM NaOAc). This same mixture was applied for 5 min, after which a linear gradient of 97% A/3% B (start solvent) to 50% A/50% B (limit solvent) was employed up to 35 min. The limit solvent was then continued for an additional 15 min. The flow rate was 1 mL/min at room temperature.

Preliminary identification of the oligosaccharide classification was made by comparing the elution times of the sample peaks with a library of standard *N*-acetyllactosaminic and oligomannosidic oligosaccharides, purchased from Dionex.

Monosaccharide Compositions of the Oligosaccharides. Complete resolution of the appropriate monosaccharides was accomplished by employing the same liquid chromatography system described above. In this case, the oligosaccharide component peak was obtained from the separation system described in the above section and adjusted with NaOAc to pH 4.0. The clam liver extract, containing all the relevant glycosidases, was incubated with the sample at 37 °C overnight. Various concentrations of the clam glycosidase mixture were added, and the results reported were based upon maximal amounts of the particular monosaccharide liberated. Blank samples consisted of solutions with both the oligosaccharide and the liver enzyme mixture eliminated from the incubation.

After enzymatic digestion of the oligosaccharides, the enzymes were removed by centrifugation in molecular weight 10 000 cutoff (Centricon 10) microconcentrator tubes. The solution was then lyophilized, and the monosaccharides were extracted into ethanol to remove salts, which interfere with subsequent liquid chromatography analysis (since sialic acid is the only monosaccharide incompletely extracted by this procedure, we do not report quantities of this component). The ethanol was then removed by evaporation, and the solids were redissolved in H_2O. The monosaccharides were completely resolved with use of a linear gradient of solvents A (200 mM NaOH) and B (1 mM NaOH). The sample was injected onto the above Carbopac PA1 (4 mm × 250 mm) column, in solvent B, and washed with this same solvent for 5 min. After

[2] Manuscript in preparation.

this time, a linear gradient was applied for 30 min with solvent B as the start solvent and a 1:1 (v/v) mixture of solvents A and B as the limit solvent. The column flow rate was 1 mL/min at room temperature.

Identification of the peaks was accomplished by comparison of the sample elution times with those of standard monosaccharides. Monosaccharide concentrations were obtained by comparison of sample peak areas with those of the standards, chromatographed at different concentrations.

Site-Directed Mutagenesis of the cDNA for HPg. In vitro mutagenesis was accomplished with the cDNA for human [Glu^1]Pg inserted in pUC119 (p119PN127.6) (McLean et al., 1987). The nucleotide sequence of p119PN127.6 suggested that the translated cDNA would possess a Val^{475} in place of the Ala^{475} obtained from direct amino acid sequence analysis of the human plasma protein. While this is probably a normal isoform of [Glu^1]Pg, we nonetheless wished to alter this Val to Ala, since this latter residue seems to be the more common amino acid at position 475. The mutagenic primer (the bold and underlined bases represent the mutations imposed) used to make the V^{475}A mutation was

5'-GTAACAGTGGT**IGC**CCTCTTGCCTC-3'

Plasmid DNAs were screened by using an *Eco*RI/*Bst*EII restriction endonuclease digest. Clones with the proper size fragments were sequenced (vide infra) over the region corresponding to amino acid positions 455–502.

The mutagenic primer used for construction of R^{561}E-[Glu^1]Pg was

5'-CCCCCCTACAACCT**C**CC**G**GGACATTTCTTCGG-3'

Plasmid DNAs were screened for the presence of the newly generated *Sma*I restriction endonuclease cleavage site.

Amino Acid Sequencing. Proteins were subjected to amino-terminal amino acid sequence analysis on a Porton Instruments gas-phase sequencer, after adsorption of the protein onto the peptide support discs. PTH amino acids were separated on a Beckman reverse-phase ODS column (5 μm, 4.6 mm × 250 mm), employing a Spectra-Physics HPLC system. The latter consisted of a Model 8800 ternary HPLC pump, a Model 8480 UV/vis detector, a Model 4270 recording integrator, and a PI2030 interface for on-line injections of the samples onto the HPLC column. Resolution of the 20 PTH amino acids was accomplished at 55 °C, under the following linear gradient conditions: 88% solution A (1 mL of glacial acetic acid/20 mL of tetrahydrofuran/0.05 mL of triethylamine/H_2O to 500 mL, pH adjusted to 4.10 with 3 N NaOH)/12% solution B (1% tetrahydrofuran in CH_3CN) as the start solvent, to 60% solution A/40% solution B (limit solvent) over a period of 24.5 min at a flow rate of 1 mL/min. Solution B was then continued for an additional 5.5 min at the same flow rate during which time the last four PTH amino acids were eluted.

DNA and Protein Analytical Methods. Oligonucleotides were synthesized by using phosphoramidite chemistry on a Biosearch (San Rafael, CA) Cyclone two-column DNA synthesizer. All reagents were purchased from this same source. The oligonucleotides were purified by using the Applied Biosystems (Foster City, CA) oligonucleotide purification cartridges. cDNAs were sequenced by the dideoxy technique (Sanger et al., 1977) with aid of the Sequenase reagent kit (United States Biochemicals, Cleveland, OH). Cell transfections were performed by the calcium phosphate method (Kingston, 1987).

Plasmid DNAs were purified by CsCl/ethidium bromide (EtBr) gradient centrifugation (Moore, 1987), with a Beckman (Palo Alto, CA) L5 65 preparative ultracentrifuge. We used vertical rotor (VTi.65.1) centrifugation for 7 h at 55 000 rpm, 15 °C, to separate the DNA bands. After the desired material was obtained from the centrifuge tube, EtBr was removed from the plasmid DNA by extraction into a solution of 2-propanol saturated with CsCl. The DNA was then dialyzed against a buffer of 1 mM Tris-HCl/0.1 mM EDTA, pH 7.1, prior to cell transfections.

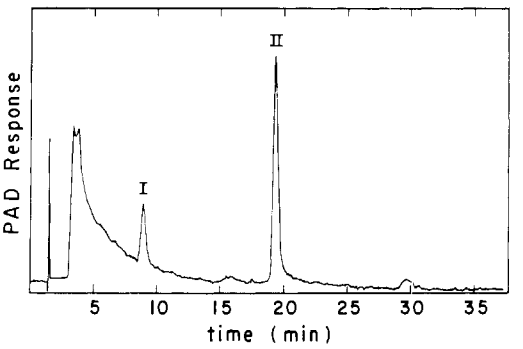


FIGURE 1: Anion-exchange liquid chromatographic resolution of the oligosaccharides released from human plasma plasminogen after treatment with glycopeptidase F. The ordinate is expressed as the arbitrary scale response of the pulsed amperometric detector (PAD). Gradient 1, described under Materials and Methods, was employed.

The cDNAs and cDNA fragments were purified by excising the appropriate bands after their electrophoretic separation on 1% agarose. Recombinant molecules were created by the method of Struhl (1985).

Single-strand plasmid DNAs were generated as described (Vieira & Messing, 1987), and site-specific mutagenesis was conducted according to published techniques (Kunkel et al., 1987).

Western Analysis. The proteins were separated by Dod-SO_4/PAGE, transferred to Immobilon-P, and blocked as described above. The blocking solution was then replaced with another containing 4 μg/mL of monoclonal murine anti-HPg (Whitefleet-Smith et al., 1989), in blocking buffer, and incubated at room temperature for 2 h with mixing. The filter was washed with three changes of 0.05% (v/v) Tween 20 in TBS at room temperature, over a 15-min period. It was next incubated with rabbit anti-mouse IgG–alkaline phosphatase conjugate (Sigma) in blocking buffer for 2 h at room temperature, with mixing, and then washed as above. Positive bands were visualized after incubations, at room temperature, with the substrate solution [16.5 mg of nitro blue tetrazolium/0.5 mL of 70% (v/v) aqueous DMF/8.5 mg of bromochloroindolyl phosphate in 1 mL of H_2O, which was added to 50 mL of 0.1 M Tris-HCl/0.1 M NaCl/0.005 M $MgCl_2$, pH 9.5].

Results

We have previously shown, by direct oligosaccharide sequencing of the N-linked oligosaccharide present on Asn^{289} of HPg, the exclusive presence of biantennary complex carbohydrate at various stages of sialyation (Hayes & Castellino, 1979b). We first wished to confirm these results, with the techniques used herein, to establish their efficacies for further work reported in this paper.

Figure 1 provides an anion-exchange liquid chromatography map of the oligosaccharides released from human plasma [Glu^1]Pg, affinity chromatography form 1 (Brockway & Castellino, 1972) by glycopeptidase F. That all oligosaccharide was released from the protein under our hydrolysis conditions was confirmed by DodSO_4/PAGE analysis of the resulting protein and visualization of the protein band by reactivity with

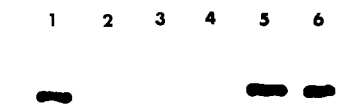


FIGURE 2: Staining of $DodSO_4$/PAGE electrophoretograms of various human plasminogens with peroxidase-labeled castor bean agglutinin. (Lane 1)Neuraminidase-treated human plasma [Glu^1]Pg affinity chromatography form 1; (lane 2) glycopeptidase F treated human plasma [Glu^1]Pg affinity chromatography form 1; (lane 3) glycopeptidase F treated Sf-expressed wild-type [Glu^1]Pg; (lane 4) glycopeptidase F treated Sf-expressed $R^{561}E$-[Glu^1]Pg; (lane 5) neuraminidase-treated Sf-expressed wild-type [Glu^1]Pg; (lane 6) neuraminidase-treated Sf-expressed $R^{561}E$-[Glu^1]Pg. A quantity of 8 μg of the various plasminogens was applied to the gel.

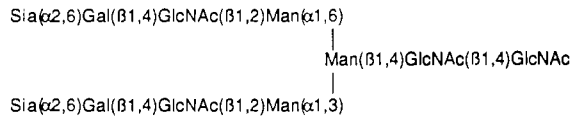
Table I: Monosaccharide Compositions of the N-Linked Oligosaccharides Released from Human Plasma Plasminogen by Glycopeptidase F

component	peak I, Figure 1	peak II, Figure 1[a]	bisialo–biantennary complex type[b]
Gal	2.0	2.0	2.2
Man	3.3	3.3	3.2
GlcNAc	3.9	3.9	4.0
Sia	nd[c]	nd[c]	2.0

[a] Pretreated with neuraminidase. [b] Commercial standard bisialo–biantennary oligosaccharide. [c] Not determined.

the specific peroxidase-conjugated lectins WGA and CBL. The neuraminidase-treated glycosylated protein reacted with both of these lectins, demonstrating the presence of both GlcNAc-GlcNAc and β-D-Gal, respectively, whereas the glycopeptidase F treated material did not display reactivity with either of these two lectins. An example of the results obtained with CBL is presented in Figure 2, and the results were similar to those obtained with WGA.

Identification of the peaks from the oligosaccharide map of human plasma HPg was accomplished by comparison of their elution times with a variety of standard oligosaccharides (employing gradient 1). Peak II (Figure 1) was found to coelute with the standard bisialo–biantennary complex-type oligosaccharide (P 105, Dionex), representing the known structure of the Asn^{289}-linked oligosaccharide of human plasma HPg, i.e.



Further confirmation of the structure of the peak II (Figure 1) oligosaccharide was accomplished by compositional analysis of a complete enzymatic hydrolysate of a neuraminidase-treated sample. As observed in Figure 3 and Table I, the monosaccharide components present are in complete accord with the requirements of the proposed oligosaccharide structure. The fact that the clam liver glycosidase extract completely hydrolyzed this complex-type oligosaccharide is evident from the data of Table I, wherein monosaccharide analysis of the standard bisialo–biantennary complex-type oligosaccharide yields the expected results. Another oligosaccharide component (peak I) was found in Figure 1. This material coelutes with asialo–biantennary complex-type oligosaccharide and with neuraminidase-treated peak II and has a monosaccharide composition virtually identical with that of asialo peak II, suggesting that a portion of peak II has not been fully sialyated or has become desialyated.

Figure 4 illustrates the anion-exchange liquid chromatography map (using gradient 2) of oligosaccharides released from

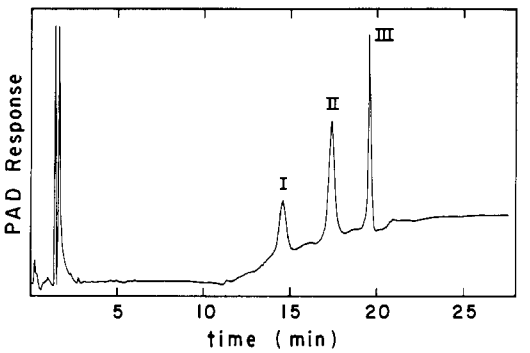


FIGURE 3: Monosaccharide analysis of the clam liver extract catalyzed hydrolysis of neuraminidase-treated peak II (Figure 1). The ordinate is expressed as the arbitrary scale response of the pulsed amperometric detector (PAD). The monosaccharides are Gal (peak I), GlcNAc (peak II), and Man (peak III).

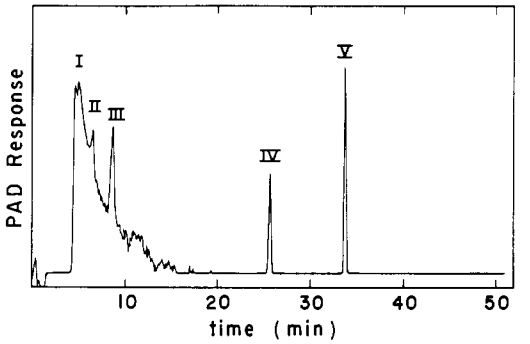
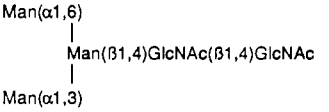


FIGURE 4: Anion-exchange liquid chromatographic resolution of the oligosaccharides released from Sf-expressed recombinant $R^{561}E$-human plasminogen after treatment with glycopeptidase F. The ordinate is expressed as the arbitrary scale response of the pulsed amperometric detector (PAD). Gradient 2, described under Materials and Methods, was employed.

Asn^{289} of $R^{561}E$-r-[Glu^1]Pg, expressed in Sf cells, after treatment with glycopeptidase F. This map was qualitatively very similar to that obtained from wt-r-Sf-expressed [Glu^1]Pg, but this wild-type protein was not further investigated since larger quantities of material were obtained from expression of the above $R^{561}E$ mutein. All remaining work described was conducted with $R^{561}E$-r-[Glu^1]Pg. Analysis of the neuraminidase-treated form of this protein with peroxidase-labeled CBL (Figure 2) demonstrated that at least a portion of the oligosaccharide population contained β-D-Gal, likely arising from complex-type carbohydrate. As seen in Figure 4, at least five resolvable oligosaccharide peaks were obtained by the mapping procedure described after treatment of this r-HPg with glycopeptidase F. Peak 1, at approximately 10% of the total oligosaccharide, coeluted with a standard $Man_3GlcNAc_2$ (GP18, Dionex), i.e.



peak III, at approximately 10% of the total oligosaccharide, coeluted with $Man_5GlcNAc_2$ (GP 11, Dionex), i.e.

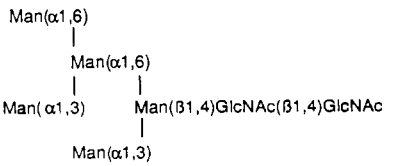


Table II: Monosaccharide Compositions of the N-Linked Oligosaccharides Released from Human Sf-Expressed Plasminogen by Glycopeptidase F

component	peak I	peak II	peak III	peak IV[a]	peak V	$Man_9GlcNAc_2$[b]
GlcNAc	2.0	2.0	2.0	4.1	2.0	2.0
Man	3.0	3.7	5.2	3.1	8.8	9.1
Gal	0	0	0	2.0	0	0

[a] Pretreated with neuraminidase. [b] Commercial standard $Man_9GlcNAc_2$.

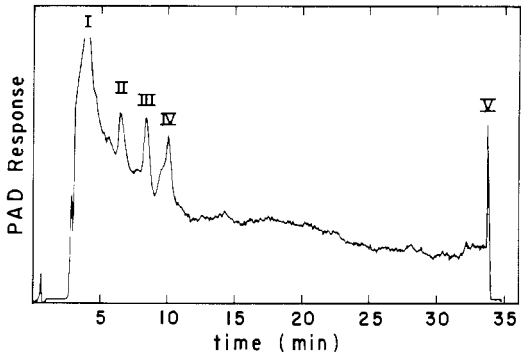
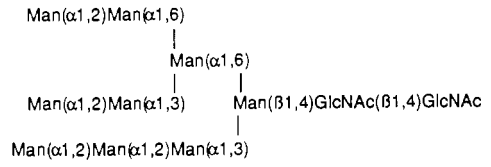


FIGURE 5: As in Figure 4, after treatment of the sample with neuraminidase.

and peak V, at approximately 30% of the total oligosaccharide, coeluted with $Man_9GlcNAc_2$, (GP 10, Dionex) i.e.



The monosaccharide compositions of each of these peaks, obtained after complete enzymatic hydrolysis, shown in Table II, are consistent with these structures. That complete hydrolysis of the high-mannose oligosaccharides occurred with the clam liver extract is seen from the compositional data obtained after hydrolysis of the $Man_9GlcNAc_2$ standard, by this enzyme mixture (Table II). While peak II, present at approximately 10% of the total oligosaccharide, did not align with an available standard oligosaccharide, it yielded the composition of $Man_4GlcNAc_2$ and presumably originated from processing of a high-mannose form. Peak IV, present at approximately 40% of the total oligosaccharide, coeluted with the bisialo–biantennary complex-type oligosaccharide found in human plasma HPg, and described above, and its monosaccharide composition (Table II) was in accord with that structure. When the chromatogram of Figure 4 was artificially enriched with peak II of Figure 1, peak IV was increased by the required amount, further confirming the identities of these two fractions. Upon treatment of this oligosaccharide pool with neuraminidase and reanalysis by oligosaccharide mapping, the profile presented in Figure 5 is obtained. When compared with the map shown in Figure 4, only peak IV shows a mobility shift, and its elution position corresponds to that of asialo–biantennary complex-type oligosaccharide. The monosaccharide composition of neuraminidase-treated peak IV (Figure 5), given in Table II, is consistent with the proposed structure.

The glycopeptidase F treated $R^{561}E$-r-[Glu^1]Pg did not react with peroxidase-labeled WGA, showing that deglycosylation was complete under our conditions and further demonstrating that the above oligosaccharides represent all of the major structures present.

Discussion

We have produced recombinant [Glu^1]Pg (Whitefleet-Smith et al., 1989) in insect (Sf) cells, which, by its physical and immunogical properties, zymogen characteristics, and activity after activation, is very similar to its human plasma counterpart. This suggests that the signal polypeptide is recognized and cleaved by these insect cells, that proper pairing of the 23 disulfide bonds occurs, and that the folding of the insect-expressed protein is similar to that of the plasma protein. Because the Sf-expressed r-[Glu^1]Pg has been produced by cells with probable differences in N-linked glycosylation machinery from human cells, and because of the role played by carbohydrate in many of the important properties of HPg and HPm, we described to directly assay the nature of the N-linked carbohydrate of HPg produced by the Sf cell system, after infection of these cells with a recombinant baculovirus containing the cDNA for [Glu^1]Pg. While some indirect evidence has been provided to the contrary (Jarvis & Summers, 1989), it is generally believed that only high-mannose carbohydrate is contained on recombinant proteins expressed in this baculovirus/insect system (Greenfield et al., 1988; Luckow & Summers, 1988; Wojchowski et al. 1987), suggesting that little processing of the original high-mannose oligosaccharide occurred in such cells. In addition to our specific interest in the nature of the N-linked oligosaccharide of Sf-expressed human plasminogen and plasminogen muteins, this protein provides an excellent vehicle with which to study general N-linked glycosylation properties by this increasingly employed system, since HPg contains only a single potential site of N-linked glycosylation, at Asn^{289} (Hayes & Castellino, 1979b). In our investigation, we have employed the $R^{561}E$ mutein of r-[Glu^1]Pg for detailed glycosylation studies, since it was expressed at higher levels than the corresponding wild-type protein, resulting in our being able to obtain larger quantities of material for this investigation. There were no qualitative differences in glycosylation patterns in the oligosaccharide maps of Sf cells expressed wt- and $R^{561}E$-r-[Glu^1]Pg.

Carbohydrate mapping of the glycopeptidase F released oligosaccharide from human plasma [Glu^1]Pg (Figure 1) reveals two major peaks, one (peak II, Figure 1) that coelutes with the standard bisialo–biantennary complex-type carbohydrate previously identified by carbohydrate sequence analysis as present on Asn^{289} of affinity chromatography form 1 of HPg (Hayes & Castellino, 1979b). The other peak (peak I) of Figure 1 coelutes with a standard asialo form of the above structure and with the above oligosaccharide after its treatment with neuraminidase. Figure 2 and Table I provide monosaccharide composition data of the two peaks of Figure 1. After treatment of peak II with neuraminidase, the compositions of peaks I and II are identical. This confirms previous work on the nature of the N-linked glycosylation of HPg (Hayes & Castellino, 1979b) and provides a reference point to investigate the glycosylation patterns of the recombinant insect expressed human cDNA for this protein.

As seen in Figure 3, the oligosaccharide map of $R^{561}E$-r-[Glu^1]Pg, after treatment of this protein with glycopeptidase F, is greatly different from that of the human plasma protein. Considerable microheterogeneity of glycosylation at Asn^{289} is obvious, with at least five oligosaccharide peaks present. From monosaccharide compositional analysis and comparison with standard oligosaccharides, it is clearly demonstrated that peaks I, II, III, and V are high-mannose oligosaccharide units, containing 3, 4, 5, and 9 mannose units, respectively, along with the requisite $(GlcNAc)_2$ structure. Peak IV, present at approximately 40% of the total oligosaccharide, coelutes with

the above bisialo–biantennary complex-type carbohydrate. Treatment of this oligosaccharide mixture with neuraminidase provides a map (Figure 4) displaying movement of only peak IV to a new position, which coelutes with the standard asialo–biantennary oligosaccharide. This demonstrates that sialyation can occur in these cells.

While precise structures have been proposed for these high-mannose oligosaccharides, on the basis of identity of anion-exchange properties under highly basic conditions with known structures, we realize that these exact structures have not been directly elucidated. We feel very confident of these structural assignments, due to the high sensitivity of the separation to the particular oligosaccharide structure (Hardy et al., 1988), the analysis of a good variety of standard structures, the very close chromatographic correlation between standard samples and the isolated oligosaccharides, and the agreement between the oligosaccharide assignments with the resulting monosaccharide compositions. In any event, the importance of this information is not affected by considerations of the exactness of the structural assignments of the high-mannose units, and those that have been proposed are very credible.

The results of this investigation have significant implications. First, as would be expected, the glycosylation pattern of the Asn^{289} residue of [Glu^1]Pg, obtained by expression of its cDNA in SF cells, is different from that of the human plasma protein. This observation needs to be taken into account if differences in properties of Sf-expressed r-HPg and human plasma HPg are found. In the latter case, biantennary complex carbohydrate is exclusively present, whereas in the former a variety of high-mannose oligosaccharides, as well as the biantennary complex oligosaccharide, are obtained. The data obtained also allow some general conclusions to be reached regarding foreign protein glycosylation in Sf cells and perhaps in similar cell types. It would appear that these cells possess the machinery to trim and process the $Man_9GlcNAc_2$ structure originally transferred to the relevant Asn residue on the protein by an oligosaccharyltransferase (Kornfeld & Kornfeld, 1985), since we have observed complex-type oligosaccharide in the total carbohydrate population. While the $Man_3GlcNAc_2$ structure is not usually observed as a component of mammalian cell proteins, and is not accommodated by the proposed steps in the ordinary pathway of oligosaccharide processing in mammalian cells (Kornfeld & Kornfeld, 1985), this oligosaccharide has been found in *Aedes albopictus* mosquito cells (Hsieh & Robbins, 1984), suggesting that Sf cells act in a similar fashion as other insect cells in trimming the high-mannose oligosaccharide, using a pathway separate from the usual mechanism. That such alternate pathways can exist, even in mammalian cells, has been proposed previously as a result of the discovery of the $Man_3GlcNAc_2$ pentasaccharide as a component of the sugar chains of hen ovomucoid (Yamashita et al., 1983). This alternate pathway may involve the transfer of $Glc_3Man_5GlcNAc_2$ (Chapman et al., 1980; Rearick et al., 1981), rather than $Glc_3Man_9GlcNAc_2$, to the protein acceptor groups, and the existence of such a pathway can account for the presence of $Man_4GlcNAc_2$ and $Man_3GlcNAc_2$ in the pool of oligosaccharides in Sf-expressed HPg. However, it is clear that the regular pathway must also be present in these cells, since $Man_9GlcNAc_2$ is an oligosaccharide component of Sf-expressed HPg.

Of major significance is the finding of the biantennary complex carbohydrate in the oligosaccharide population in the Sf-expressed [Glu^1]Pg, demonstrating that these cells possess the necessary glycosyltransferases to assemble complex carbohydrate from high-mannose precursors. Complex carbohydrate has not been shown previously to exist in this type of cell line and is apparently not a component of the N-linked glycosylation of endogenous proteins of *Aedes* aegypti cells (Butters et al., 1981). It is possible that amino acid sequences and/or conformational properties of the human protein can direct a portion of the trimming and further processing events in Sf cells, a conclusion in agreement with observations made as a result of studies of high-mannose oligosaccharide trimming in mosquito cells (Hsieh & Robbins, 1984) and in the nature of mammalian host cell processing of Sindbis virus glycoproteins (Hubbard, 1988). While a portion of the oligosaccharide on the Sf-expressed r-[Glu^1]Pg is of the high-mannose type, perhaps different cell growth conditions, and/or different harvesting procedures, would influence the glycosylation patterns and lead to larger amounts of the complex carbohydrate. Finally, sialyltransferase activity exists in these cells, since a bisialo–biantennary complex carbohydrate (peak IV, Figure 3) component of the oligosaccharide population has been found.

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REFERENCES

Brockway, W. J., & Castellino, F. J. (1972) *Arch. Biochem. Biophys. 151*, 194–199.
Brown, T. A. (1985) M.S. Dissertation, University of Notre Dame.
Burnette, W. H. (1981) *Anal. Biochem. 112*, 195–203.
Butters, T. D., Hughes, R. C., & Vischer, P. (1981) *Biochim. Biophys. Acta 640*, 672–686.
Castellino, F. J. (1983) *Bioscience 33*, 647–650.
Chapman, A., Fujimoto, K., & Kornfeld, S. (1980) *J. Biol. Chem. 255*, 4441–4446.
Deutsch, D. G., & Mertz, E. T. (1970) *Science 170*, 1095–1096.
Forsgren, M., Raden, B., Israelsson, M., Larsson, K., & Heden, L.-O. (1987) *FEBS Lett. 213*, 254–260.
Gonzalez-Gronow, M., Edelberg, J. M., & Pizzo, S. V. (1989) *Biochemistry 28*, 2374–2377.
Greenfield, C., Patel, G., Clark, S., Jones, N., & Waterfield, M. D. (1988) *EMBO J. 7*, 139–146.
Hardy, M. R., Townsend, R. R., & Lee, Y. C. (1988) *Anal. Biochem. 170*, 54–62.
Hayes, M. L., & Castellino, F. J. (1979a) *J. Biol. Chem. 254*, 8768–8771.
Hayes, M. L., & Castellino, F. J. (1979b) *J. Biol. Chem. 254*, 8772–8776.
Hayes, M. L., & Castellino, F. J. (1979c) *J. Biol. Chem. 254*, 8777–8780.
Hsieh, P., & Robbins, P. W. (1984) *J. Biol. Chem. 259*, 2375–2382.
Hubbard, S. C. (1988) *J. Biol. Chem. 263*, 19303–19317.
Jarvis, D. L., & Summers, M. D. (1989) *Mol. Cell. Biol. 9*, 214–223.
Kingston, R. E. (1987) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 1.8.1–1.8.3, Wiley, New York.
Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Luckow, V. A., & Summers, M. D. (1988) *Bio/Technology 6*, 47-55.
Malinowski, D. P., Sadler, J. E., & Davie, E. W. (1984) *Biochemistry 23*, 4243-4250.
McLean, J. W., Tomlinson, J. E., Kuang, W.-j., Eaton, D. L., Chen, E. Y., Gless, G. M., Scanu, A. M., & Lawn, R. M. (1987) *Nature (London) 330*, 132-137.
Moore, D. (1987) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 1.7.1-1.7.4, Wiley, New York.
Powell, J. R., & Castellino, F. J. (1983) *Biochemistry 22*, 923-927.
Presper, K. A., Concha-Slebe, I., De, T., & Basu, S. (1986) *Carbohydr. Res. 155*, 73-87.
Rearick, J. L., Chapman, A., & Kornfeld, S. (1981) *J. Biol. Chem. 256*, 6255-6261.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191-209.
Struhl, K. (1985) *BioTechniques 3*, 452-453.
Takada, A., & Takada, Y. (1983) *Thromb. Res. 30*, 633-642.
Tarentino, A. L., Gomez, C. M., & Plummer, T. H. (1985) *Biochemistry 24*, 4665-4671.
Vieira, J., & Messing, J. (1987) *Methods Enzymol. 153*, 3-11.
Whitefleet-Smith, J., Rosen, E., McLinden, J., Ploplis, V. A., Fraser, M. J., Tomlinson, J. E., McLean, J. W., & Castellino, F. J. (1989) *Arch. Biochem. Biophys. 271*, 390-399.
Wiman, B. (1973) *Eur. J. Biochem. 39*, 1-9.
Wiman, B. (1977) *Eur. J. Biochem. 76*, 129-137.
Wiman, B., Boman, L., & Collen, D. (1978) *Eur. J. Biochem. 87*, 143-146.
Wojchowski, D., Orkin, S. H., & Sytowski, A. J. (1987) *Biochim. Biophys. Acta 910*, 224-232.
Yamashita, K., Kamerling, J. P., & Kobata, A. (1983) *J. Biol. Chem. 258*, 3099-3106.

Peptide α-Helicity in Aqueous Trifluoroethanol: Correlations with Predicted α-Helicity and the Secondary Structure of the Corresponding Regions of Bovine Growth Hormone†

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ABSTRACT: The relationship between trifluoroethanol (TFE) enhancement of peptide α-helicity and protein secondary structure has been studied for a series of 11 peptides which span the complete primary sequence of bovine growth hormone (bGH). Ten of these peptides become increasingly α-helical as the solution concentration of TFE is increased. The amount of α-helicity developed by these peptides plateaus above 10 mol % TFE and ranges from 0 to 71%. The increased α-helicity, as determined by CD, closely correlates with the amount of α-helix predicted for eight of the eleven peptides analyzed ($r = 0.9$). Therefore, for this group of peptides, it appears that this technique can be used as a measure of α-helical propensity. Inclusion of the remaining three peptides in this analysis significantly lowers the correlation ($r = 0.6$). The reduced correspondence between TFE-enhanced and predicted α-helicity in this latter subset of peptides may be due to their relatively high hydrophobicity. In addition, the relevance of TFE-enhanced peptide α-helicity and the secondary structure of the corresponding protein regions was explored. Although the three peptides which form the largest amount of α-helicity in the presence of 10 mol % TFE correspond to α-helical regions of the protein, the overall correlation is significantly lower than is observed for the TFE-enhanced and predicted α-helicity. These findings suggest that the propensity of specific amino acid sequences for α-helix formation influences the amount of α-helicity which forms in corresponding protein sequences, but that other factors can modify this structure.

Bovine growth hormone (bGH),[1] a protein that stimulates multiple physiological responses leading to enhanced growth and lactation in vivo, is a single-domain, 191-residue protein that folds into a four antiparallel α-helix bundle (Abdel-Meguid et al., 1987). The folding mechanism of bovine growth hormone is consistent with framework and molten globule hypotheses (Kim et al., 1982; Ptitsyn, 1987; Brems et al., 1987a; Brems & Havel, 1989). These hypotheses suggest that portions of bGH should adopt native-like secondary structure in the absence of long-range interactions within the protein. However, previous studies of a large number of bGH fragments indicate that only bGH (96-133) contains stable secondary structure in aqueous solution (Chen et al., 1977; Brems et al., 1987b,c).

The low α-helical stability of bGH fragments in aqueous solvents is not unusual. A theoretical model which predicts

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[1] Abbreviations: BCA, bicinchoninic acid; bGH, bovine growth hormone; CD, circular dichroism spectroscopy; FAB-MS, fast atom bombardment mass spectrometry; Gdn·HCl, guanidine hydrochloride; HPLC, high-pressure liquid chromatography; TFE, trifluoroethanol; $[\theta]_{222}$, mean residue ellipticity at 222 nm.

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