Structures of the Asparagine-289-Linked Oligosaccharides Assembled on Recombinant Human Plasminogen Expressed in a *Mamestra brassicae* Cell Line (IZD-MBO503)†

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ABSTRACT: In this report, we have fortified and extended a previous investigation [Davidson, D. J., Fraser, M. J., & Castellino, F. J. (1990) *Biochemistry 29*, 5584–5590] in which we demonstrated for the first time that lepidopteran insect (*Spodoptera frugiperda*) cells (IPLB-SF-21AE) were capable of assembling N-linked complex oligosaccharide on a human protein (plasminogen), the cDNA of which had been inserted into these cells via recombinant DNA technology with a baculovirus vector. In order to investigate whether a more general capability of lepidopteran insect cells to produce complex oligosaccharide existed, and to identify the chemical nature of the types of oligosaccharides that such insect cells were able to assemble, we have infected *Mamestra brassicae* (IZD-MBO503) cells for 48 h with a recombinant (r) baculovirus containing the [R^{561}E]human plasminogen (HPg) cDNA and characterized the nature of the glycopeptidase F (GF) released N-linked oligosaccharides contained on Asn^{289} of the r-HPg expressed by these cells. We found that approximately 63% of the total N-linked oligosaccharides were of the complex type, with bisialo–biantennary (28%), asialo–biantennary (7%), fucosylated bisaialo–biantennary (25%), and fucosylated asialo–biantennary (3%) oligosaccharides representing the major complex-type carbohydrate species. The remainder of the oligosaccharides were of the high-mannose type, with $(mannose)_9(N\text{-acetylglucosamine})_2$ (22%), $(mannose)_5(N\text{-acetylglucosamine})_2$ (13%), and $(mannose)_3(N\text{-acetylglucosamine})_2$ (2%) representing the major oligosaccharides observed. Investigations with r-HPg expression in another lepidopteran insect cell line, *Manduca sexta* (CM-1), also clearly demonstrated that (α2,6)-linked sialic acid was present on the purified protein, suggesting that the ability of insect cells to assemble complex-type oligosaccharide on r-HPg is general in nature. These studies demonstrate that, despite the observations that endogenous insect cell proteins apparently do not contain N-linked complex oligosaccharide, the glycosyltransferase genes required for assembly of such structures are present in these cells and are capable of being utilized under proper conditions. The resulting alteration of oligosaccharide processing appears to be a broad property of lepidopteran insect cells, which is effected in this case by infection with a recombinant baculovirus containing the cDNA for HPg. This system may serve as a general probe for elucidation of some of the regulatory factors governing protein glycosylation.

Human plasminogen (HPg)[1] is a plasma glycoprotein that is the inactive precursor of the fibrinolytic and fibrinogenolytic serine proteinase, HPm. The activation of HPg occurs consequent to cleavage of a single peptide bond between residues R^{561} and V^{562}, a reaction catalyzed by a variety of plasminogen activators (Robbins et al., 1967). HPg is a single-chain protein containing 791 amino acids in known sequence (Wiman, 1973, 1977; Sottrup-Jensen et al., 1978; Malinowski et al., 1984, Forsgren et al., 1987). Its cDNA has been cloned and sequenced (Malinowski et al., 1984; Forsgren et al., 1987) and expressed in a fully functional form in insect cells (Whitefleet-Smith et al., 1989; Davidson et al., 1990a). It has been difficult to date to effect similar expression of r-HPg in mammalian cells due to the nearly ubiquitous presence of plasminogen activators in these cells, which leads to activation of r-HPg and subsequent autodegradation of r-HPm (Busby et al., 1988). However, the cDNA for an activation-resistant variant of HPg, [R^{562}S]r-HPg, has been expressed in CHO cells (Davidson et al., 1990b) and in human kidney 293 cells.[2]

The HPg present in human plasma exists as two glycoforms, separable by affinity chromatography (Brockway & Castellino, 1972). One form contains a single site of N-linked glycosylation on N^{289} and one site of O-linked oligosaccharide at T^{346}. The second glycoform contains only the T^{346}-based O-linked oligosaccharide (Hayes & Castellino, 1979a–c), despite the presence in this form of the same consensus tripeptide sequence for N-linked glycosylation at N^{289} as exists in the first affinity chromatography resolved form of HPg (Powell & Castellino, 1983). We have investigated the nature of the N-linked glycosylation of wild-type r-HPg and [R^{562}E]r-HPg produced in *Spodoptera frugiperda* (IPLB-SF-21AE) cells (Davidson et al., 1990a). Considerable microheterogeneity was present in the GF-released oligosaccharide pool. It was found that approximately 60% of the total N-linked oligosaccharide was of the high-mannose type, as expected in insect cells, but that

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[1] Abbreviations: HPg, any form or variant of human plasminogen; HPm, any form or variant of human plasmin; EACA, ϵ-aminocaproic acid; r, recombinant; wt, wild type; $DodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; SF, *Spodoptera frugiperda*; MB, *Mamestra brassicae*; CM, *Manduca sexta*; SNA, *Sambucus nigra* agglutinin; NDV, Newcastle disease virus; GF, glycopeptidase F; WGA, wheat germ (*Triticum vulgaris*) agglutinin; NAc, *N*-acetyl; Sia, sialic acid; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; p.i., postinfection; HPAEC, high-pH, anion-exchange chromatography.

[2] D. J. Davidson, unpublished experiments.

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approximately 40% was the same bisialo–biantennary oligosaccharide found in the plasma protein (Davidson et al., 1990a). This was the first investigation wherein it was shown that insect cell glycosylation machinery existed for assembly of N-linked complex-type oligosaccharide on proteins. In addition, we have characterized the nature of the glycosylation of [R^{561}S]r-HPg produced in CHO cells and found, again for the first time, that sialic acid linked (α2,6) to outer arm Gal was present (Davidson & Castellino, 1990). To that time, only Sia(α2,3)Gal outer arm linkages were observed in CHO cell proteins. Similarly, in another study, transfective procedures stimulated an apparently endogenous latent (α1,3)-fucosyltransferase gene in CHO cells (Potvin et al., 1990), again demonstrating that activation of a glycosyltranferase, not previously recognized in these cells under normal conditions, could occur.

From the above results, we believe that a variety of unutilized glycosyltransferase for N-linked complex-type oligosaccharide assembly in insect cells, and for a β-galactoside (α2,6)-sialyltransferase and an (α1,3)-fucosyltransferase in CHO cells, can become functional as a result of infection and transfection, respectively. In our cases, this process can be accomplished by insertion into the cells, via a vector, the cDNA coding for HPg. We wished to learn whether this enhanced utilization of glycosyltransferases was a general property of insect cells infected with this plasmid, in order to investigate specific features of cell-specific glycosylation of r-HPg and to ascertain whether a system could be made available to assist in addressing factors that govern glycosylation of proteins at the level of glycosyltransferase utilization. The pertinent results are summarized in this paper.

Materials and Methods

Proteins. Native [Glu^1]Pg was isolated from fresh human plasma by affinity chromatography on Sepharose–lysine. Glycoform 1 was employed herein (Brockway & Castellino, 1972). SF-21AE-expressed [R^{561}E]r-HPg[3] was obtained as published (Whitefleet-Smith et al., 1989; Davidson et al., (1990a). Rabbit anti-human HPg polyclonal antibodies were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Glycopeptidase F from *Flavobacterium meningosepticum* and biotinylated SNA [recognizes Sia(α2,6)Gal/GalNAc linkages] were purchased from Boehringer Mannheim Biochemicals. Peroxidase-labeled WGA [recognizes GlcNAc-(β1,4)GlcNAc linkages] was obtained from the Sigma Chemical Co. (St. Louis, MO). The avidin–alkaline phosphatase complex employed for visualization of biotinylated lectin blots was purchased from Bio-Rad (Richmond, CA).

Exoglycosidases were obtained from the following sources: NAc(β1,2)-D-glucosaminidase (*Diplococcus pneumoniae*), (β1,4)-D-galactosidase (*D. pneumoniae*), and (α2,3/6/8)-D-neuraminidase (*Arthrobacter ureafaciens*), from Boehringer Mannhemi Biochemicals; (α1,2/3/6)-D-mannosidase (*Canavalia ensiformis*, jack bean), (α1,6>2/3/4)-L-fucosidase (bovine epididymis), (β1,2)-D-mannosidase (*Turbo cornufus*), and NAc(β1,2/3/4/6)-D-glucosaminidase (*C. ensiformis*, jack bean), from the Sigma Chemical Co.; and (α1,2)-D-mannosidase (*Aspergillus phoenicis*), from Oxford GlycoSystems (Rosedale, NY). The reaction conditions required for these enzymes were as suggested by the manufacturers and by a previous study (Parekh et al., 1989). NDV (α2,3)-D-neuraminidase was prepared and utilized in our laboratory as previously described (Davidson & Castellino, 1991).

Cell Lines. IPLB-SF-21AE cells (Vaughn et al., 1977) were obtained from Dr. Eliot Rosen (Notre Dame, IN). CM-1 (Eide et al., 1975) and IZD-MBO503 (Weiss et al., 1981) cells were donated by Dr. W. F. Hink (Columbus, OH).

[R^{561}E]r-HPg Expression in Insect Cells. Mamestra brassicae (IZD-MBO503) cells were maintained as monolayers in serum-free Excell 400 medium and infected with recombinant baculoviruses at multiplicities of 4 plaque-forming units/cell. Infections were allowed to proceed for a time of 48 h. The method of insertion of the cDNA for wtr-[Glu^1]Pg into the plasmid, pAV6, and for obtaining the recombinant baculovirus for subsequent infection of the insect cells, has been described (Whitefleet-Smith et al., 1989). The same method was used to insert the variant plasminogen, [R^{561}E]r-HPg. After the desired infection time, the conditioned medium was collected and the r-HPg was purified by affinity chromatography on a Sepharose–lysine column (Deutsch & Mertz, 1970; Brockway & Castellino, 1972).

The same procedure was employed for expression of [R^{561}E]r-HPg in all cell lines employed in this investigation.

Oligosaccharide Methodology. Deglycosylation of r-HPg with GF; isolation and complete enzymatic hydrolysis of oligosaccharides; monosaccharide analyses; and lectin visualization of Western blots of r-HPg were performed in our laboratory as described previously (Davidson & Castellino, 1990; Davidson et al., 1990a). Lectin blotting of the protein and sequential exoglycosidase digestions were peformed as described previously (Davidson & Castellino, 1990).

For oligosaccharide mapping by HPAEC, the methodology described by Davidson et al. (1990a) was employed. The sample was applied to a Carbopac PA1 (4 mm × 250 mm), equilibrated with a mixture (v/v) of 50% solvent A (200 mM NaOH)/47% solvent B (H_2O)/3% solvent C (1 M NaOAc). This same solvent was applied for 15 min, after which a linear gradient of the equilibration solvent (start solvent) to 50% A/25% B/25% C (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 the Dionex Corp. (Sunnyvale, CA) or Oxford GlycoSystems. The relative abundance of each glycoform in the oligosaccharide pool was determined from dose–response curves with each of the glycans subsequently identified.

DNA Methodology. The mutation of the cDNA for wtr-HPg to that which would translate into [R^{561}E]r-HPg, and the screening procedure for the cells containing this transformant, has been described (Davidson & Castellino, 1990). All other appropriate companion methods for manipulating DNA, as used in our laboratory, are as published previously (Davidson & Castellino, 1990).

Results

Nearly complete oligosaccharide structures that are contained on Asn^{289} of [R^{561}E]r-HPg expressed in IZD-MBO503 lepidopteran insect cells have been elucidated by a combination of monosaccharide compositional analyses, HPAEC mapping with a variety of standard oligosaccharides, and quantitative sequential exoglycosidase digestions with several linkage-

[3] [R^{561}E]r-HPg is a variant of HPg, produced by site-directed mutagenesis. The variant protein contains an R → E mutation at sequence position 561, leading to its resistance to activation to HPm. This is the form of r-HPg used in this paper. While this may be repeated for emphasis in the paper, any deviation from this nomenclature will be specified.

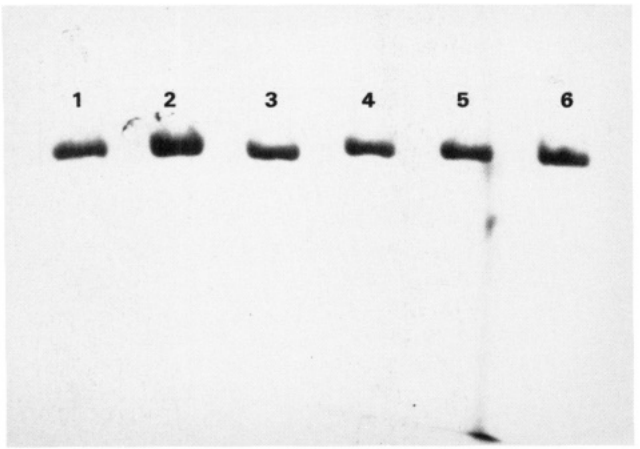

FIGURE 1: Gel electrophoretic analysis of the purified r-HPg preparations employed. Nonreduced $DodSO_4$/PAGE gels stained with Coomassie brilliant blue dye. Lane 1, human plasma HPg, affinity chromatography glycoform 1; lane 2, SF-21AE-expressed [R^{561}E]-r-HPg, 48 h, p.i.; lane 3, CM-1-expressed [R^{561}E]r-HPg, 48 h, p.i.; lane 4, CM-1-expressed [R^{561}E]r-HPg, 72 h, p.i.; lane 5, MBO503-expressed [R^{561}E]r-HPg, 48 h, p.i.; lane 6, GF-treated MBO503-expressed [R^{561}E]r-HPg, 48 h, p.i. Approximately 5 μg of each protein was applied in each lane.

specific enzymes. In all cases, this cell line was infected with a recombinant baculovirus containing the entire coding sequence for HPg for a time period of 48 h, at which point morphological examination of the cells revealed that little cell death had occurred (<5%, estimated). The resulting r-HPg was purified from the culture media by Sepharose–lysine affinity chromatography. This protein was obtained in a high state of purity as evidenced by the single protein bands obtained in $DodSO_4$/PAGE analysis, as shown in Figure 1. Concomitant Western antibody analysis of a blot of the $DodSO_4$/PAGE gels of Figure 1 demonstrated that each band was reactive against a polyclonal rabbit anti-HPg antibody (data not shown). Each r-HPg was treated with GF until the time at which WGA-dependent staining of Western blots of the r-HPg (Davidson & Castellino, 1990; Davidson et al., 1990a) revealed the virtual complete absence of the reducing-terminal GlcNAc(β1,4)GlcNAc structure, concomitantly demonstrating the absence of all N-linked olgiosaccharide on the protein.

Following isolation of the total carbohydrate pool of the r-HPg (Davidson & Castellino, 1990; Davidson et al., 1990a), individual oligosaccharides have been resolved by HPAEC and the sample contained in each of the peak tubes of the individual pools was hydrolyzed by using a clam exoglycosidase mixture (Davidson & Catellino, 1990; Davidson et al., 1990a), after desialyation (when applicable) with *A. ureafaciens* neuraminidase. The resulting monosaccharide composition was then determined by HPAEC (Davidson & Castellino, 1990; Davidson et al., 1990a). Tentative structures were proposed for each of the resolved glycans, based on comparative HPAEC retention times of standard oligosaccharides with those of the samples tested, as well as the agreement of the proposed structures were the monosaccharide compositions.

Individual exoglycosidases were then incubated with the sample, and the quantity of the released monosaccharide was determined by HPAEC. The remaining oligosaccharide was reisolated for compositional analysis and for the next addition of specific exoglycosidase. This procedure was repeated until only GlcNAc from the reducing end of the oligosaccharide remained. In all cases, the proposed standard oligosaccharide was treated in parallel with the sample, and retention times of the oligosaccharides remaining after exoglycosidase cleavage

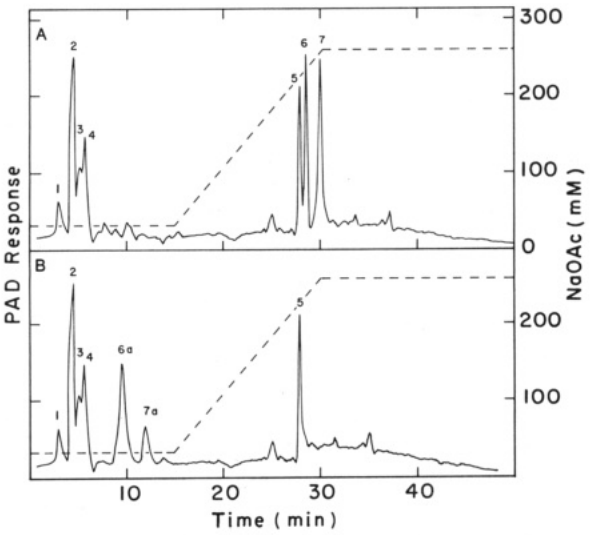

FIGURE 2: HPAEC mapping of the oligosaccharides released from MBO503-expressed [R^{561}E]r-HPg (48 h, p.i.) after treatment with GF (A). The gradient described under Materials and Methods was employed to effect the resolution. In this gradient, the NaOH concentration remained constant, at 100 mM, while the concentration of NaOAc changed as indicated on the right ordinate. (B) As in (A), except the the GF-released oligosaccharide pool was treated with *A. ureafaciens* neuraminidase prior to HPAEC analysis. The fractions from (B) that show changes in migration after *A. ureafaciens* neuraminidase treatment are referred to with the fraction number, followed by "a". The left oridinate is expressed as the arbitrary scale response of the pulsed amperometric detector (PAD).

were compared. This assured that the sample and standard were indeed the same throughout all steps of the digestion.

This approach to elucidation of oligosaccharide structures of various r-HPg samples has been detailed previously (Davidson & Castellino, 1990; Davidson et al., 1990a) and is dependent upon the very high resolving power of HPAEC. The utility of HPAEC for the purpose of oligosaccharide structural determinations has been amply documented (Hardy et al., 1988; Townsend et al., 1988; Spellman et al., 1989; Basa & Spellman, 1990; Davidson & Castellino, 1990; Davidson et al., 1990a).

Figure 2A illustrates the HPAEC fingerprint of the N-linked oligosaccharides released from r-HPg expressed in IZD-MBO503 cells, at a time of 48 h, p.i., using a viral multiplicity of 4. After treatment of the entire oligosaccharide pool with *A. ureafaciens* neuraminidase, pools 6 and 7 underwent chromatographic shifts (yielding components 6a and 7a in Figure 2B), suggesting that sialic acid was removed from the oligosaccharide. Clearly, at least these two fractions represented complex-type oligosaccharide. Since less than optimal resolution of oligosaccharides 2–4 of Figure 2A was obtained by the gradient employed, these peaks were pooled and readsorbed to the same HPAEC column, and a NaOH gradient was applied that would result in greater resolution of peaks 2–4. The HPAEC profile and the gradient employed are displayed in Figure 3. Clearly, peaks 2–4 were completely resolved by this procedure.

The peak tube of each fraction was first desialyated with *A. ureafaciens* neuraminidase, and the amount of Sia released was determined. Next, the asialo oligosaccharide was subjected to monosaccharide compositional analysis after complete digestion with the clam exoglycosidase mixture. The data obtained are listed in Table I and represent the optimal amounts of monosaccharides obtained, based upon the determination of the temporal release of monosaccharides with the clam enzyme mixture. Since the Gal that is present on N-linked oligosaccharides can only originate from complex-

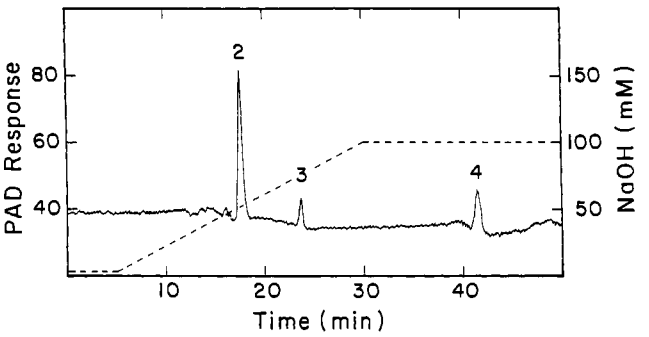

FIGURE 3: HPAEC rechromatography of fractions 2–4 from Figure 2A. The solvent system applied was as follows: 1 mM NaOH for 5 min; a linear gradient of 1 mM NaOH (start solution) to 100 mM NaOH (limit solution) for an additional 25 min; and 100 mM NaOH for an additional 20 min. The column flow rate was 1 mL/min.

and hybrid-type structures, it is clear that peaks 3 and 4, in addition to 6 and 7, contain complex- and/or hybrid-type carbohydrate. Hybrid-type structures were ruled out by the compositional analysis and subsequent studies with exoglycosidases (vide infra). Thus, fractions 3, 4, 6, and 7 are complex-type glycans and represent 63% of the total oligosaccharide mixture.

Each sample was subjected to sequential exoglycosidase quantitative analysis, in parallel with a standard oligosaccharide representing its structure, the prediction of which was based upon HPAEC retention times and monosaccharide compositional analysis. The particular structures ultimately proposed for each of the seven components of Figure 2A are summarized in Figure 4, and these form the basis for the comparisons detailed below.

Since the monosaccharide compositional analysis (Table I) of fractions 1 and 2 of Figure 2A showed the presence of only Man and GlcNAc, we employed only exomannosidases and exoglucosaminidases for sequential analyses. Initial treatment of each sample with *T. cornufus* β-mannosidase or jack bean

Table I: Monosaccharide Compositions of the Oligosaccharides Released from IZD-MBO503-Expressed Recombinant Human Plasminogen at 48 h Postinfection

fraction[a]	Sia[b]	Gal[c]	GlcNAc[c]	Man[c]	Fuc[c]
1 (2)			2.00	2.98	
2 (13)			2.00	5.12	
3 (3)		2.00	3.78	2.93	0.84
4 (7)		2.00	4.00	2.96	
5 (22)			2.00	8.76	
6 (25)	2.00	2.13	4.02	2.89	1.00
7 (28)	2.06	2.00	3.84	3.10	

[a] The fraction numbers (percent of total oligosaccharide) correspond to those in Figures 2A and 3. The monosaccharide compositions of replicate analyses were reproducible within 5%. [b] Cleaved from the oligosaccharide with neuraminidase (*A. ureafaciens*). [c] Complete digestion of the desialo oligosaccharide with a clam liver glycosidase mixture (Davidson et al., 1990a).

NAc-β-glucosaminidase did not result in release of monosaccharide. Digestion of samples 1 and 2 with jack bean α-mannosidase led to release of approximately 2 and 4 mol of α-linked Man/mol of glycan, respectively, none of which was present in (α1,2)-linkage, since no reaction was observed upon treatment of the same original samples with *D. pneumoniae* (α1,2)-mannosidase (Table II). The oligosaccharide remaining subsequent to α-mannosidase-catalyzed hydrolysis was then treated with *T. cornufus* β-mannosidase, and in each case approximately 1 residue of β-linked Man/mol of oligosaccharide was obtained (Table II), leaving a component present that comigrated on HPAEC with a disaccharide of structure GlcNAc(β1,4)GlcNAc. The latter compound was completely hydrolyzed with jack bean NAc-β-glucosaminidase, providing approximately 2 mol of GlcNAc/mol of disaccharide (Table II). Structural assignments were based upon this analysis, and upon comigration of each sample on HPAEC at each stage of the exoglycosidase hydrolysis with the standards represented by structures 1 and 2, respectively, of Figure 4.

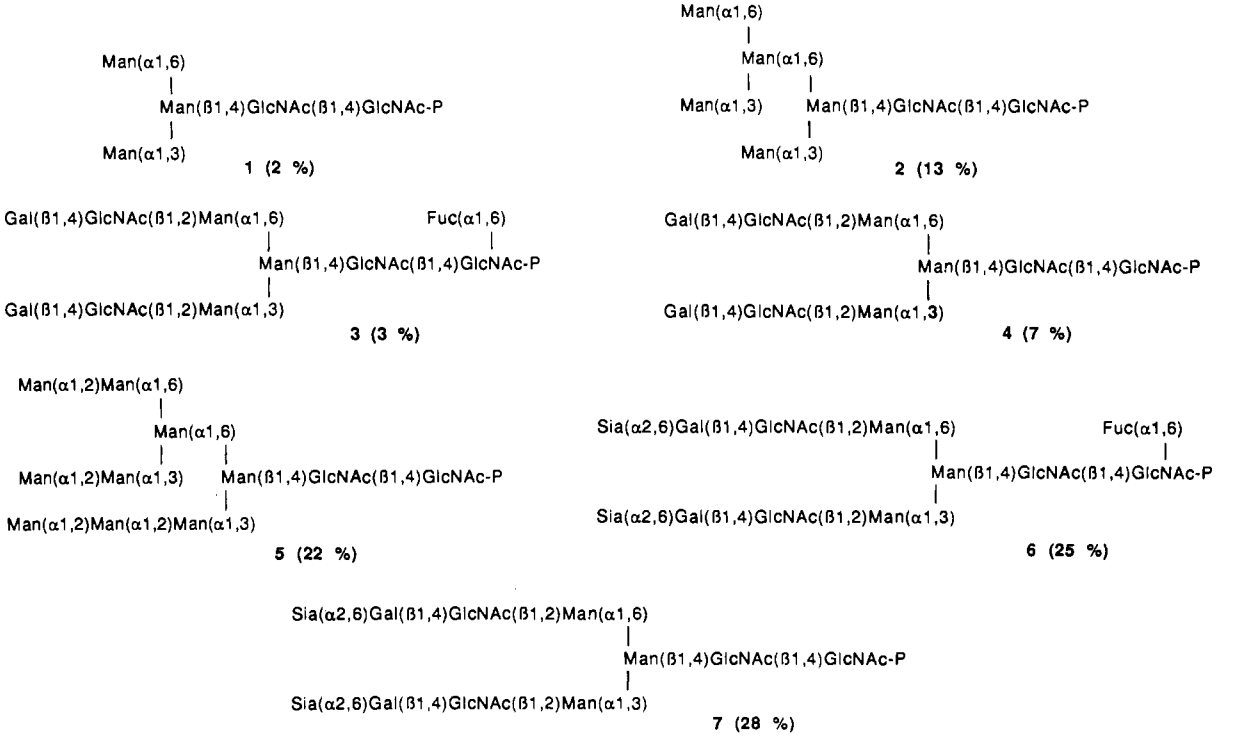

FIGURE 4: Structures of the oligosaccharides released from MBO503-expressed [R[561]E]r-HPg. The numbers of the oligosaccharides correspond to their elution positions in Figure 2A, and the percentages indicated refer to the relative abundances of each glycan in the total r-HPg pool. P refers to the site of attachment of protein to the oligosaccharide.

Table II: Sequential Exoglycosidase Digestion of Oligosaccharides Released from IZD-MBO503-Expressed Recombinant Human Plasminogen at 48 h Postinfection[a]

fraction[b] (structure)	Sia[c] (α2,3)/R^c	Gal[d]	GlcNAc (β1,2)/R^e	α-Man (α1,2)/R^f	β-Man[g]	α-Fuc[h]	GlcNAc[i]
1 (1)				0/2.03	0.98		2.01
2 (2)				0/3.89	0.95		1.96
3 (3)		2.06	1.86/0	0/1.98	0.97	1.04	2.10
4 (4)		1.99	2.12/0	0/1.93	0.96		1.94
5 (5)				4.08/3.94	0.94		1.91
6 (6)	0/2.16	1.94	1.92/0	0/1.97	0.97	0.94	1.98
7 (7)	0/2.02	2.00	1.98/0	0/1.98	1.00		1.97

[a] For pools containing complex carbohydrate, the sequence of enzymes added was c–i. For high-mannose or truncated high-mannose pools, the sequence of addition was e–i. Replicate analyses agreed to within 5%. [b] The fraction numbers correspond to those in Figure 2A (the structures are from Figure 4). [c] Neuraminidase (NDV)/followed by neuraminidase (*A. ureafaciens*). This provides the number of (α2,3)-linked Sia residues/followed by the number (R) of α-Sia residues in linkages other than (α2,3). [d] β-1,4 Galactosidase (*D. pneumoniae*). [e] NAc-β-glucosaminidase (*D. pneumoniae*)/followed by NAc-β-glucosaminidase (jack bean). This provides the number of (β1,2)-linked GlcNAc residues/followed by the number (R) of β-GlcNAc residues in linkages other than (β1,2). [f] α-Mannosidase (*D. pneumoniae*)/followed by α-mannosidase (jack bean). This provides the number of (α1,2)-linked Man residues/followed by the number (R) of α-Man residues in linkages other than (α1,2). [g] β-Mannosidase (snail). [h] α-Fucosidase (bovine epididymus). [i] NAc-β-glucosaminidase (jack bean).

The monosaccharide compositions (Table I) of fractions 3 and 4 (Figure 2A) demonstrated that each was a complex-type oligosaccharide and differed by the presence of one residue of Fuc on the oligosaccharide of fraction 3. Each was unreactive with all exoglycosidases employed, except *D. pneumoniae* (β1,4)-galactosidase, which resulted in liberation of approximately 2 mol/mol of (β1,4)-linked Gal (Table II). In each case, the remaining oligosaccharide was reactive only with jack bean NAc-β-glucosaminidase, which catalyzed release of approximately 2 mol of β-linked GlcNAc/mol (Table II). Essentially identical amounts of GlcNAc were obtained after treatment of these samples with *D. pneumoniae* (β1,2)-glucosaminidase (Table II), demonstrating that all GlcNAc released at this step was present in (β1,2)-linkages. The remaining oligosaccharides were reactive with jack bean α-mannosidase, yielding approximately 2 mol of α-linked Man/mol, none of which was present in an (α1,2)-linkage, as revealed by the lack of reactivity of the same samples with *D. pneumoniae* (α1,2)-mannosidase (Table II). The samples that remained after this treatment were reactive with *T. curnufus* β-mannosidase, yielding approximately 1 mol of β-linked Man/mol (Table II). Following this, the oligosaccharide from fraction 3 (Figure 2A) contained only $(GlcNAc)_2$, and the oligosaccharide from fraction 4 (Figure 2A) contained $(GlcNAc)_2Fuc$. This latter glycan was treated with bovine epididymus α-fucosidase, an enzyme displaying preference for cleavage of (α1,6)-linked Fuc.[4] This resulted in release of approximately 1 mol of Fuc/mol at this step (Table II). Finally, only $(GlcNAc)_2$ remained for each oligosaccharide, and approximately 2 mol/mol of this monosaccharide was liberated after hydrolysis with jack bean β-glucosaminidase (Table II). On the basis of the above results, the structures of fractions 3 and 4 of Figure 2A were assigned to standard structures 3 and 4, respectively, of Figure 4. These standards were analyzed by exoglycosidase digestion in parallel with peaks 3 and 4 in all steps and showed the same behavior.

The monosaccharide composition of fraction 5 of Figure 2A demonstrated that it was composed of $(Man)_9(GlcNAc)_2$ (Table I). Approximately 8 mol of Man/mol of oligosaccharide was released upon treatment with jack bean α-mannosidase, four of which were present in (α1,2)-linkages, as revealed by analysis of the original sample with *D. pneumoniae* (α1,2)-mannosidase (Table II). The sample remaining after removal of all α-Man, of composition $(GlcNAc)_2Man$, was treated with *T. cornufus* β-mannosidase, resulting in liberation of approximately 1 mol/mol of β-Man (Table II). Following this, approximately 2 mol of GlcNAc/mol of disaccharide was obtained upon hydrolysis of the remaining sample with jack bean NAc-β-glucosaminidase. This, plus the parallel behavior of standard sample 5 (Figure 4) in all analyses, allowed fraction 5 of Figure 2A to be assigned to standard structure 5 of Figure 4.

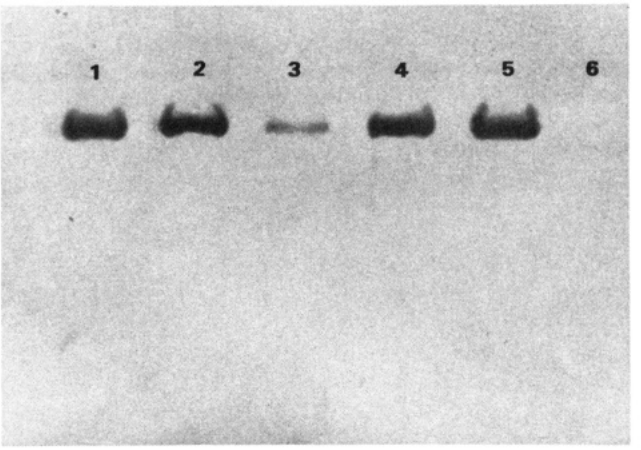

FIGURE 5: Lectin blotting of human plasminogens with biotinylated SNA and visualization with avidin–alkaline phosphatase/nitroblue tetrazolium. Lane 1, human plasma HPg, affinity chromatography glycoform 1; lane 2, SF-21AE-expressed [R^{561}E]r-HPg, 48 h, p.i.; lane 3, CM-1-expressed [R^{561}E]r-HPg, 48 h, p.i.; lane 4, CM-1-expressed [R^{561}E]r-HPg, 72 h, p.i.; lane 5, MBO503-expressed [R^{561}E]r-HPg, 48 h, p.i.; lane 6, GF-treated MBO503-expressed [R^{561}E]r-HPg, 48 h, p.i. The proteins (ca. 4 μg) were electrophoretically transferred to to Immobilon P prior to staining procedures.

Fractions 6 and 7 of Figure 2A possessed the same oligosaccharide compositions as fractions 3 and 4, respectively, except for the presence of 2 mol of Sia/mol in each (Table I). After desialylation, both fractions displayed the same exoglycosidase behavior and HPAEC retention times as fractions 3 and 4 (Table II). Intact peaks 6 and 7 possessed the same HPAEC retention properties as standard samples 6 and 7, respectively, of Figure 4, and these assignments were made. Conformation of the Sia(α2,6)Gal linkage was obtained with the aid of NDV neuraminidase, which is specific for the Sia(α2,3)Gal linkage. No reaction of the original fractions 6 and 7 (Figure 2A) was obtained with this enzyme (Table II), thus proving the absence of this linkage. This, plus the reactivity of IZD-MBO503-expressed r-HPg on Western blots of the r-Pg stained with the Sia(α2,6)Gal-specific lectin, SNA, shown in Figure 5, and the comigration of fractions 6 and 7 (Figure 2A) with standard samples 6 and 7 of Figure 4, re-

[4] Oxford GlycoSystems technical bulletin.

spectively, provided additional evidence for the (α2,6)-Sia linkage proposed.

In order to attempt to demonstrate the generality of the observations that lepidopteran insect cells were capable of assembling complex-type oligosaccharide or r-HPg, we have expressed this protein in a variety of lepidopteran insect cell lines (Hink et al., 1990), employing the same recombinant baculovirus/HPg vector system as for SF-21AE (Whitefleet-Smith et al., 1989; Davidson et al., 1990a) and MBO503 (this study) cell expressions. Gel electrophoretic properties of the purified [$R^{562}E$]r-HPg from CM-1 cells are presented in Figure 1 and demonstrate that it is similar in this regard to HPg from human plasma and r-HPg preparations from SF-21AE and MBO503 cells. Shown in Figure 5 is the SNA blot of purified r-HPg produced in CM-1 cells (72 h, p.i.). Clearly, (α2,6)-linked Sia is present in this r-HPg, demonstrating that assembly of complex-type oligosaccharides on lepidopteran insect expressed r-HPg is a broad phenomenon. In confirmation of this view, a similar observation regarding the presence of (α2,6)-linked Sia has been made with r-HPg produced in the same transfer vector (72 h p.i.) in two other *S. frugiperda* cell lines, viz., SF-9 and SF-1254 (data not shown). In all of these cases, the GF-treated r-HPg did not display reactivity in the SNA blot assays. This not only validates the above conclusions but also shows that if Sia-containing O-linked oligosaccharide is present on the proteins, the linkage of Sia is other than (α2,6).

Of great interest is the observation of Figure 5, demonstrating that r-HPg produced in CM-1 cells at 48 h p.i., shows much less (α2,6)-linked Sia than the r-HPg produced in these same cells at 72 h, p.i. Thus, the exact nature of oligosaccharide assembly on r-HPg appears to be dependent upon the time of exposure of the cells to recombinant baculovirus/HPg vector, suggesting a temporal alteration in the glycosylation machinery available in such cells, after this infective procedure.

Discussion

Previous work from this laboratory has demonstrated conclusively that *S. frugiperda* (IPLB-SF-21AE) cells are capable of assembling N-linked complex oligosaccharide on r-HPg expressed in these cells, after their infection with a recombinant baculovirus containing the cDNA for this protein (Davidson et al., 1990a). That same investigation was the first to show that insect cells must contain the glycosyltransferase genes necessary for production of the required enzymes and also led to the conclusion that direct or indirect activation at the gene or protein level, somehow induced by infection with this species recombinant baculovirus, was necessary for utilization of the appropriate glycosyltransferases. Also, the possibility exists that this infective process stimulated processing mannosidases, thus making available appropriate r-HPg substrates for the glycosyltransferases responsible for catalyzing assembly of complex-type glycans. Prior to our investigation, referred to above, it was generally accepted that only high-mannose type oligosaccharide was assembled on endogenous insect proteins (Butters et al., 1981) and on recombinant proteins that utilized insect cell glycosylation machinery [Luckow and Summers (1988) and references therein]. More recently, however, other investigators have begun to infer, by more indirect qualitative methods, that some degree of processing of high-mannose oligosaccharide may occur on recombinant proteins expressed in insect cells (Jarvis & Summers, 1989).

In order to determine whether the ability of insect cells to assemble N-linked complex oligosaccharide was specific to the cell type employed previously (IPLB-SF-21AE) or was a more general previously unrecognized phenomenon, we chose to elucidate the oligosaccharide structures placed on Asn^{289} or r-HPg, the cDNA of which was inserted in the same recombinant baculovirus vector, after expression of this protein from another lepidopteran insect cell line. We selected *M. brassicae* (IZD-MBO503) cells for this purpose, since these cells were found to be capable of production of relatively high levels of r-HPg consequent to infection with a recombinant baculovirus carrying the human cell derived cDNA for HPg (Hink et al., 1990). On the basis of HPAEC mapping of the oligosaccharides released by GF catalysis from N^{289} of IZD-MBO503-expressed r-HPg, monosaccharide compositions of the isolated oligosaccharides, lectin blotting, and sequential exoglycosidase digestions, we were able to determine the structures of the major glycans released, the sum of which represented at least 95% of all N-linked oligosaccharide on r-HPg. Considerable microheterogeneity of oligosaccharides was found to be present at this position in r-HPg, with biantennary complex oligosaccharide, at various degress of sialylation and fucosylation, constituting a total of 63% of the oligosaccharide released from this protein. Additionally, at least three high-mannose oligosaccharides, two of which underwent extensive trimming, constituted the major components of this class of glycans, totaling approximately 37% of the oligosaccharides released from IZD-MBO503-expressed r-HPg, under the infective and cell growth conditions chosen. The exact oligosaccharide structures proposed for each component are provided in Figure 4.

The oligosaccharides obtained do not represent unusual structures and were clearly defined. However, some pertinent observations can be made on the basis of the structures identified. First, to our knowledge, this investigation is the first to identify the presence of a latent fucosyltransferase gene in insect cells, as revealed by the presence of Fuc on structures 3 and 6 of Figure 4. The Fuc placed on r-HPg is exclusively in (α1,6)-linkage in the cases described here, and we are not certain as to the existence of an (α1,3)-fucosyltransferase gene in these cells. Interestingly, we did not observe fucosylated oligosaccharide in the glycan pool of IPLB-SF-21AE cell expressed r-HPg (Davidson et al., 1990a). Since r-HPg expressed in IZD-MBO503 cells (this study) and in CHO cells (Davidson & Castellino, 1990) is capable of accommodating fucosylated oligosaccharide, its absence in IPLB-SF-21AE cells is not due to the nature of the substrate (r-HPg) provided, but likely represents a deficiency of the fucosyltransferase or its inability to be employed under the conditions selected. Next, on the basis of analysis of the oligosaccharides assembled on r-HPg, we cannot comment directly on the presence of the alternate oligosaccharide processing pathway (Rearick et al., 1981) in IZD-MBO503 cells. The $(Man)_5(GlcNAc)_2$ (structure 2, Figure 4) oligosaccharide does not contain (α1,2)-linked Man, thereby suggesting it arose from trimming of the $(Man)_9(GlcNAc)_2$ (structure 5 Figure 4) that originated from the classical pathway. The glycan, $(Man)_3(GlcNAc)_2$ (structure 1, Figure 4), could have arisen from further trimming of $(Man)_5(GlcNAc)_2$ originating from the classical pathway, or alternate pathway, if the latter exists in these cells.

Of interest regarding the complex-type oligosaccharide, the Sia present on the biantennary complex glycans is in (α2,6)-linkage to Gal, exactly as is the case in the oligosaccharide structure of human plasma HPg (Hayes & Catellino, 1979b) and in the biantennary complex oligosaccharide of SF-21AE-expressed r-HPg (Davidson et al., 1990a). The Sia(α2,3)Gal structure was not found in the biantennary oligosaccharide pools released from r-HPg expressed in IZD-

MBO503 cells and SF-21AE cells under the conditions chosen. While some Sia(α2,3)Gal outer arm linkages were found in r-HPg expressed in CHO cells (Davidson & Castellino, 1990), none of this was present in biantennary oligosaccharides. Clearly, the presence of (α2,6)-linked Sia on biantennary oligosaccharides of r-HPg appears to be a general property and suggests that a specific determinant is present on the protein, and/or the cDNA, which activates and allows utilization of the appropriate (α2,6)-sialyltransferase.

The investigations reported herein fortify considerably and extend a previous report from this laboratory (Davidson et al., 1990a), which demonstrates the capability of lepidopteran insect cells to assemble complex-type oligosaccharide under special conditions. In our work, these conditions were met as a consequence of infection with a recombinant baculovirus, containing a cDNA for a human protein (HPg) that in its natural biosynthetic environment would possess complex-type oligosaccharide. Some unique feature of the baculovirus/HPg construction is present that specifies assembly of complex-type glycan on r-HPg, since similar infective procedures with mammalian proteins in similar vectors apparently did not result in assembly of complex-type oligosaccharide on the relevant recombinant protein [reviewed in Luckow and Summers (1988)]. However, we show herein that before conclusions of this latter type are reached in the future, some attention must be paid to the times of infection of insect cells with the recombinant baculovirus expression vectors employed.

In conclusion, on the basis of analysis of the oligosaccharide structures present on the substrate, r-HPg, it is clear that otherwise undetected glycosyltransferases can be utilized in insect cells and that invertebrate cells can accomplish carbohydrate processing previously believed to occur only in higher order species. This seems to be a broad property of, at the least, lepidopteran insect cells. However, these glycosyltransferases, and/or the high-mannose type processing mannosidases, must be activated, and whether this occurs at the gene and/or protein level cannot be ascertained from current knowledge. The fact that CM-1 cells show an increase in the amount of (α2,6)-linked Sia on r-HPg (Figure 5) with time of infection with the recombinant baculovirus further suggests that utilization of the appropriate tranferases or mannosidases occurs, that these enzymes are not immediately available, and/or that the r-HPg substrate is not immediately suitable for use with the glycosyltransferases. This enhanced employment of glycosyltransferases or mannosidases cannot be a result of the presence of the protease, HPm, since all r-HPg expressed for the purpose of oligosaccharide analyses, in all cell systems that we use, contains an $R^{561}S/E$ mutation, thus providing resistance to activation to HPm. In addition to these investigations that demonstrate the activation of glycosyltransferases in insect cells, at least two other examples of activation of a normally silent glycosyltransferase gene resulting from incorporation of a foreign DNA into a cell have been published. Specifically, (α2,6)-sialyltransferase (Davidson & Castellino, 1990) and (α1,3)-fucosyltransferase activities (Potvin et al., 1990) were observed in CHO cells, after transfective procedures. Such activities had not been observed previously in these cells. It is highly important to understand further the basis of this type of control of protein processing, since such knowledge will allow manipulation of the nature of protein glycosylation, a consideration, at the least, very pertinent to generation of properly processed recombinant proteins.

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Role of the Hexapeptide Disulfide Loop Present in the γ-Carboxyglutamic Acid Domain of Human Protein C in Its Activation Properties and in the in Vitro Anticoagulant Activity of Activated Protein C†

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ABSTRACT: In order to examine whether the structural integrity of the hexapeptide disulfide loop (residues 17–22), present in the γ-carboxyglutamic acid (γ) domain of human protein C (PC), and common to all vitamin K dependent coagulation proteins, is necessary for its anticoagulant properties, we employed recombinant (r) DNA technology to generate two important variants that would address this issue. One such mutein contained aspartic acid for γ-residue substitutions at sequence positions 19 and 20 ([γ^{19}D, γ^{20}D]r-PC) in the light chain of the mature protein, and the other possessed a serine for cysteine substitution at position 22 ([C^{22}S]r-PC of the same light chain. A subpopulation of molecules of these mutant proteins, containing the maximum levels of γ-residues in each, has been purified by fast-protein anion-exchange liquid chromatography and affinity chromatography on an anti-human PC column. A study of the kinetic characteristics of the inhibition by Ca^{2+} of the thrombin-catalyzed activation rates of these variants, and the corresponding stimulation by Ca^{2+} of the thrombin/thrombomodulin-catalyzed activation rates of the same recombinant PC molecules, demonstrated that higher concentrations of Ca^{2+} were required to display these effects, when compared to wild-type (wt) r-PC and human plasma PC. This suggested that the kinetically relevant Ca^{2+} site responsible for these effects on activation of PC, and known to be present in another domain of PC, was affected by both mutations in the γ-domain. The recombinant PC variants were converted to their activated forms ([γ^{19}D, γ^{20}D]r-APC and [C^{22}S]r-APC) and assayed for their Ca^{2+}-dependent anticoagulant activities. Each of these molecules displayed less than 1% of the activity of the wtr-APC in the activated partial thromboplastin time of PC-deficient plasma and similar low activity, relative to their wt counterpart, toward inactivation of purified human coagulation factor VIII. These results suggest that γ-residues at positions 19 and 20 of PC and APC, as well as the intact disulfide loop structure encompassing residues 17–22 of these proteins, are of some importance to the Ca^{2+}-dependent kinetics properties of PC activation and of great importance to the anticoagulant properties of APC. Coupled with a previous study demonstrating that the pair of γ-residues at positions 6 and 7 of the γ-domain of APC is similarly important to its anticoagulant properties [Zhang, L., & Castellino, F. J. (1990) *Biochemistry 29*, 10828–10834], this work suggests that Ca^{2+} interaction(s) essential for the anticoagulant functions of APC is influenced by different regions of the γ-domain.

Protein C (PC)[1] is a member of the class of vitamin K dependent plasma proteins, sharing amino acid sequence homology with other zymogens of this type. The biological role of the activated form of PC (APC), resides in its ability to maintain the fluid state of blood, and this enzyme functions in this manner by at least two different mechanisms. First, its anticoagulant properties are a consequence of its ability to proteolytically inactivate, by specific limited cleavage reactions, coagulation cofactors, factor V (f-V) and factor Va (f-Va) (Kisiel et al., 1977) as well as factor VIII (f-VIII) and factor VIIIa (f-VIIIa) (Vehar & Davie, 1980), in reactions that are stimulated by Ca^{2+}, phospholipid (Kisiel et al., 1977), and protein S (Walker, 1980). Second, APC functions as a fibrinolytic agent, likely due to its ability to inactivate inhibitors of plasminogen activators (Sakata et al., 1985; Taylor & Lockhart, 1985; van Hinsberg et al., 1985). Maximal activation of PC occurs at the endothelial cell surface and results from limited proteolysis, catalyzed by thrombin, along with

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[1] Abbreviations: PC, human plasma protein C; APC, activated human protein C; [γ^{19}D, γ^{20}D]r-PC(APC); a recombinant protein C (or activated protein C) containing aspartic acid residues substituted for γ-carboxyglutamic acid residues at positions 19 and 20 of the protein C amino acid sequence; [C^{22}S]r-PC(APC), a recombinant protein C (or activated protein C) containing a serine substituted for cysteine at position 22 of the protein C amino acid sequence; TM, thrombomodulin; ATIII, antithrombin III; γ, γ-carboxyglutamic acid; βOH-D, β-hydroxyaspartic acid; r, recombinant; wt, wild-type; DodSO$_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; FPLC, fast-protein liquid chromatography; HPLC, high-performance liquid chromatography; MAb, monoclonal antibody; Fmoc, [(9-fluorenylmethyl)-oxy]carbonyl; S2366, L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide.

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