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Oligosaccharide Structures Present on Asparagine-289 of Recombinant Human Plasminogen Expressed in a Chinese Hamster Ovary Cell Line†

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ABSTRACT: The oligosaccharide structures linked to Asn^{289} of a recombinant (r) variant ($R^{561}S$) human plasminogen (HPg) expressed in Chinese hamster ovary (CHO) cells, after transfection of these cells with a plasmid containing the cDNA coding for the variant HPg, have been determined. Employing high-performance anion-exchange liquid chromatography mapping of the oligosaccharide units cleaved from the protein by glycopeptidase F, compared with elution positions of standard oligosaccharides, coupled with monosaccharide compositional determinations and analyses of sequential exoglycosidase digestions and specific lectin binding, we find that considerable microheterogeneity in oligosaccharide structure exists at this sole potential N-linked glycosylation site on HPg. A variety of high-mannose structures, as well as bi-, tri-, and tetraantennary complex-type carbohydrate, has been found, in relative amounts of 1–25% of the total oligosaccharides. The complex-type structures contain variable amounts of sialic acid (Sia), ranging from 0 to 5 mol/mol of oligosaccharide in the different glycan structures. Neither hybrid-type moleucles, *N*-acetylglucosamine bisecting oligosaccharides, nor *N*-acetyllactosaminyl-repeat structures were found to be present in the complex-type carbohydrate pool in observable amounts. Of interest, a significant portion of the Sia exists an outer arm structures in an (α2,6) linkage to the penultimate galactose, a novel finding in CHO cell-directed glycosylation. We conclude that considerable differences occur in the N-linked carbohydrate structures of CHO cell-expressed HPg, in insect cell-expressed HPg [Davidson, D. J., Fraser, M. J., & Castellino, F. J. (1990) *Biochemistry 29*, 5584–5590], and in human plasma HPg [Hayes, M. L., & Castellino, F. J. (1979) *J. Biol. Chem. 254*, 8668–8671, 8672–8676, 8677–8680], with both species-specific glycosylation and also protein-directed events of importance in the oligosaccharide trimming and processing that result.

Human plasminogen ([Glu^1]Pg)[1] is the plasma protein zymogen of the enzyme plasmin ([Lys^{78}]Pm), a serine protease with fibrinolytic and fibrinogenolytic properties. [Glu^1]Pg exists in the circulation as a single-chain glycoprotein containing 791 amino acids (Forsgren et al., 1987; Malinowski et al., 1984; McLean et al., 1987; Sottrup-Jensen et al., 1978; Wiman, 1973, 1977) resolvable by affinity chromatography into two major variants (Brockway & Castellino, 1972), which are distinguishable by their respective extents of glycosylation at Asn^{289} (Castellino, 1983; Hayes & Castellino, 1979a–c). One form of the protein contains a biantennary complex oligosaccharide at Asn^{289}, and the other lacks N-linked oligosaccharide (Hayes & Castellino, 1979b), despite the presence of the required consensus tripeptide that directs N-based glycosylation at Asn^{289} (Powell & Castellino, 1983). Both forms of HPg contain O-linked oligosaccharide at Thr^{346} (Hayes & Castellino, 1979c).

We have shown previously that the cDNA for [Glu^1]Pg can be expressed in recombinant baculovirus-infected insect (*Spodoptera frugiperda*) cells (Whitefleet-Smith et al., 1989) and have determined that the Asn^{289}-linked oligosaccharides in the insect-expressed r-[Glu^1]Pg possess some differences from those found in the human plasma protein. In the insect-expressed r-HPg, both complex-type and high-mannose-type oligosaccharide units are present (Davidson et al., 1990a).

The carbohydrate of HPg influences the properties of this zymogen and its corresponding active enzyme. It has been found that HPg form 1 (Asn^{289} is glycosylated) binds differently to lysine-like activation effector molecules (Brockway & Castellino, 1972) and fibrin than HPg form 2 (aglycosyl at Asn^{289}), 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

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[1] Abbreviations: CHO, Chinese hamster ovary; HPg and HPm, refer generically to human plasminogen and human plasmin, respectively; [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; tPA, tissue plasminogen activator; r, recombinant; wt, wild type; NaDodSO$_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; SNA, *Sambucus nigra* agglutinin; WGA, wheat germ agglutinin; NDV, Newcastle disease virus; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; HPLC, high-performance liquid chromatography.

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et al., 1978). On the other hand, HPg form 2 is degraded by elastase more rapidly than HPg form 1 (Takada et al., 1988). In addition, carbohydrate side chains of HPg have been shown to be important in its interaction with cellular receptors (Gonzalez-Gronow et al., 1989). Recently, we found that removal of the carbohydrate side chains from [Glu^1]Pg influences the enzymatic activity of the plasminogen activator complex formed between streptkokinase and [Glu^1]Pg (Davidson et al., 1990b).

It is clear that cell and tissue types influence the nature of the glycosylation of proteins and that the glycosyltransferases that are involved in the synthesis of sugar chains are developmentally regulated, thus conferring further selective pressures on the nature of the oligosaccharides found on proteins [for reviews, see Parekh et al. (1989a) and Rademacher et al., (1988)]. This is also true in the case of [Glu^1]Pg, wherein it has been demonstrated that the high-mannose carbohydrate chains characteristic of insect cell glycosylation (Kornfeld & Kornfeld, 1985; Luckow & Summers, 1988) are also found as subpopulations in human [Glu^1]Pg, when its cDNA is expressed in insect cells (Davidson et al., 1990a). In addition, it appears as though the nature of the protein confers some direction to cell glycosylation pathways, since protein structure also plays a role in the nature of the glycosylation that occurs. Examples of such potentially important protein structural features are the following: specific polypeptide determinants that recognize appropriate glycosyltransferases (Kornfeld & Kornfeld, 1985; Roitsch & Lehle, 1989; Rutishauser et al., 1988; Smith & Pease, 1980); the position in the amino acid sequence that the consensus tripeptide required for glycosylation occupies (Pollack & Atkinson, 1983); the quaternary structure of the protein (Dahms & Hart, 1986); and the conformation of the polypeptide chain (Bause, 1983; Glabe et al., 1980; Ronin et al., 1981). That the nature of protein plays a role in directing its glycosylation is also true in the case of [Glu^1]Pg, since the recombinant human protein expressed in insect cells contains a significant amount of complex-type carbohydrate, a feature apparently not normally present in insect cell glycoproteins (Butters et al., 1981), or in glycoproteins from viruses used to infect insect cells and which utilize the insect glycosylation machinery (Hsieh & Robbins, 1984). The issue of protein structural-directed glycosylation is of paramount importance in expression of recombinant proteins in various cell lines because of the multiplicity of functions associated with the oligosaccharide moieties of proteins. We are undertaking a systematic detailed study of the glycosylation of recombinant wild-type and variant human plasminogens, resulting from expression of the appropriate cDNA in a variety of cell systems, in order to evaluate the contributions of the protein structure to the nature of the oligosaccharide structure(s) that ultimately result(s). The current investigation accomplishes this aim with CHO cells and provides a comparison of the oligosaccharide processing of the plasma protein with an invertebrate cell-expressed and CHO-expressed HPg.

Materials and Methods

Proteins. CHO-expressed recombinant [Glu^1]Pg was purified from appropriate cell culture media by affinity chromatography on Sepharose–lysine (Deutsch & Mertz, 1970), with batch elution of the protein at 50 mM EACA. The construction of the cDNA for this variant plasminogen has been described (Davidson et al., 1990b). CHO cells were transfected and the plasmid-containing cells selected and amplified in a manner similar to that previously reported (Goeddel et al., 1983).

Glycopeptidase F from *Flavobacterium meningosepticum* and biotinylated SNA were purchased from Boehringer Mannheim Biochemica (Indianapolis, IN). In addition, highly purified preparations of glycopeptidase F were provided by Professor T. V. Viswanatha (Waterloo, Canada).

The avidin–alkaline phosphatase complex was purchased from Bio-Rad (Richmond, CA).

Peroxidase-labeled WGA was obtained from the Sigma Chemical Co. (St. Louis, MO).

Exoglycosidases were obtained from the following sources: NAc-β-D-glucosaminidase (*Diplococcus pneumoniae*), β-galactosidase (*Diplococcus pneumoniae*), and neuraminidase (*Arthrobacter ureafaciens*), Boehringer Mannheim Biochemica; α-mannosidase (*Canavalia ensiformis*, Jack bean), α-L-fucosidase (bovine epididymis), β-mannosidase (*Turbo cornufus*), and NAc-β-D-glucosaminidase (*Canavalia ensiformis*, Jack bean), Sigma Chemical Co.; α-mannosidase (*Aspergillus phoenicis*), Oxford Glycosystems (Rosedale, NY). Reaction conditions for these enzymes were as suggested by the manufacturers and further detailed in a previous study (Parekh et al., 1989a).

Seed stocks of NDV (ATCC VR-109) were purchased from American Type Culture Collection (Rockville, MD) and infected into 10-day-old chick embryos, as described previously (Paulson et al., 1979). The allantoic fluid was collected; the virus was concentrated by centrifugation and resuspended in one-tenth of the original volume of allantoic fluid (Paulson et al., 1979). Aliquots of the resuspended virus were used as the source of NDV neuraminidase.

Oligosaccharide Methodology. Deglycosylation of CHO-expressed HPg with glycopeptidase F, oligosaccharide mapping by HPLC, isolation and complete enzymatic hydrolysis of oligosaccharides, and monosaccharide analyses were performed in our laboratory as described previously (Davidson et al., 1990a). All oligosaccharide standards were purchased from the Dionex Corp. (Sunnyvale, CA) or Oxford Glycosystems.

Lectin blotting of the protein was accomplished by NaDodSO$_4$/PAGE electrophoresis on 9% gels under nonreducing conditions (Laemmli, 1970), followed by visualization of the band with a lectin (SNA)-specific reaction to detect the presence of (α2,6)-linked Sia on the protein. The protein band(s) was (were) transferred to Immobilon-P (Millipore, Bedford, MA) membranes as described (Davidson et al., 1990a). After the free protein adsorption sites on the Immobilon-P sheet were blocked with a gelatin buffer containing 0.05 M Tris-HCl/0.15 M NaCl/1% (w/v) ElA-grade gelatin, pH 7.4, a solution of biotinylated SNA, consisting of 2.0 μg/mL protein in the above buffer, was added and allowed to incubate at 25 °C for 90 min. The membrane was washed with TBS–Tween buffer (0.05 M Tris–HCl/0.15 M phosphate/0.05% Tween-20, pH 7.4), and a solution of alkaline phosphatase labeled avidin in TBS was added. The bound lectin was visualized after addition of alkaline phosphatase substrate [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]. The reaction was terminated by washing with several changes of H_2O.

Sequential Exoglycosidase Digestions. The following exoglycosidases were added to purified oligosaccharides in different orders to samples in which the monosaccharide analyses provided tentative identifications of complex-type carbohydrate: *A. ureafaciens* neuraminidase [for determination of Sia(α2,3) and Sia(α2,6)Gal linkages]; NDV neuraminidase [for determination of Sia(α2,3) linkages]; *D. pneumoniae*

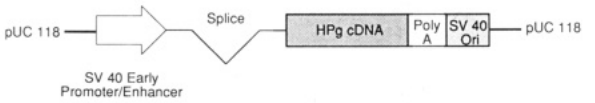

FIGURE 1: Expression vector for ($V^{475}A$, $R^{561}S$)-human plasminogen. The essential features of this pUC118-based expression vector are as follows: the SV 40 early promoter/enhancer for initiation of transcription; a chimeric intron, consisting of a 5′ splice sequence from the early immediate region promoter of human cytomegalovirus (Boshart et al., 1985), fused onto a synthetic 3′ splice; the entire coding sequence of [$V^{475}A$, $R^{561}S$]-human plasminogen; the poly(A) early polyadenylation sequence; and the SV 40 origin of replication.

β-galactosidase [for determination of Gal(β1,4)Man linkages]; Jack Bean NAc-β-D-glucosaminidase [for analysis of GlcNAc(β1,4)Man and GlcNAc(β1,2)Man linkages] or *D. pneumoniae* NAc-β-D-glucosaminidase [for analysis of GlcNAc(β1,2)Man linkages]; Jack bean α-mannosidase [for determination of Man(α1,2)Man, Man(α1,3)Man, and Man(α1,6)Man linkages]; *A. phoenicis* α-mannosidase [for determination of Man(α1,2)Man linkages]; *T. cornufus* β-mannosidase; bovine epididymis α-fucosidase. For oligosaccharides wherein total monosaccharide analysis indicated that they were present as high-mannose or truncated high-mannose structures, the following exoglycosidase additions were made: Jack bean or *A. phoenicis* α-mannosidase; *T. cornufus* β-mannosidase; bovine epididymis α-fucosidase; Jack bean NAc-β-D-glucosaminidase.

In every case, an aliquot of the total mixture was removed after each glycosidase addition in order to measure by HPLC (vide supra) the amount of the particular monosaccharide released from a known quantity of oligosaccharide substrate.

Results

A recombinant cleavage site resistant human plasminogen, r-R^{561}S-[Glu^1]Pg, has been constructed, inserted into an expression plasmid, as summarized in Figure 1, and expressed in CHO cells. Since the nucleotide sequence of the cDNA indicated that an amino acid replacement (Val for Ala) would be present in the expressed protein at amino acid position 475, the isolated cDNA was altered such that the Ala that has been found by amino acid sequence analysis to be clearly present at this position of the protein would also be in place in the r-[Glu^1]Pg. This latter change will not be referred to further in this paper.

The CHO-expressed recombinant protein was batch-eluted from a specific affinity chromatography column for HPg, was reactive in Western analyses toward a variety of polyclonal and monoclonal antibodies that recognize the human plasma protein, possessed the proper amino-terminal amino acid sequence, and generated highly active plasminogen activator complexes with equimolar levels of streptokinase (Davidson et al., 1990b). All of this information allows the conclusion to be reached that functional HPg is produced by these cells. The necessity for constructing a cDNA containing a mutation at the cleavage (activation) site of [Glu^1]Pg rests on our observations, as well as those of others (Busby et al., 1988), that demonstrate the difficulty of expression of wt-r-HPg in mammalian cells, due to the ubiquitous presence in such cells of plasminogen activators. These latter enzymes catalyze formation of the serine protease, HPm, which autodegrades. That this latter hypothesis is likely true is shown by the fact that activation-resistant r-R^{561}S-[Glu^1]Pg can be expressed in CHO cells (Davidson et al., 1990b) and in human 293 cells,[2]

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

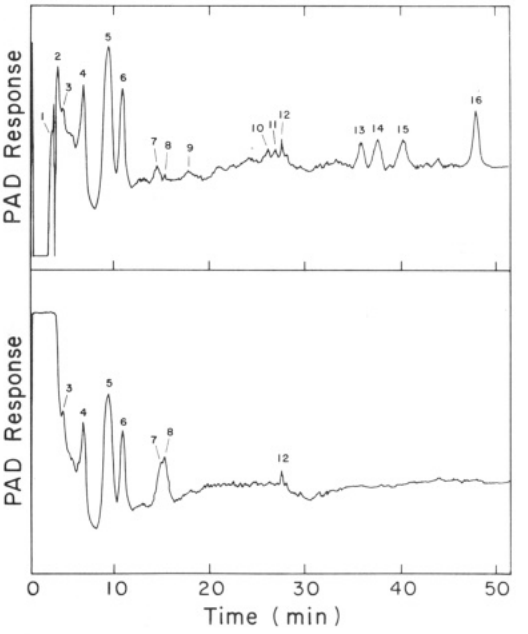

FIGURE 2: Anion-exchange liquid chromatographic mapping of the oligosaccharides released from CHO-expressed r-R^{561}S-human plasminogen after treatment with glycopeptidase F (top). Gradient 1, described in Davidson et al. (1990a), was employed to achieve the resolution. Bottom: As in top, except that the oligosaccharide pool was treated with *A. ureafaciens* neuraminidase. Gradient 2, described in Davidson et al. (1990a), was employed. The ordinate is expressed as the arbitrary scale response of the pulsed amperometric detector (PAD).

and that wt-r-HPg can be readily expressed in invertebrate cells that do not contain plasminogen activators (Whitefleet-Smith et al., 1989).

Due to the importance of the oligosaccharide side chains in defining the properties of HPg, and the inherent interest in examining cell- and protein-directed glycosylation, we have analyzed the nature of the oligosaccharides present on this recombinant protein, after release of the total N-linked oligosaccharides from the protein with glycopeptidase F. As evidenced by the lack of reactivity in blotting analysis of the protein with peroxidase-labeled WGA after glycopeptidase F treatment, it is clear that all of the N-linked oligosaccharide has been released from the protein by glycopeptidase F treatment. The HPLC fingerprint of the oligosaccharides present is shown in Figure 2 (top). That sialic acid is present on a variety of these components is demonstrated by the alteration of the retention times of several of the peaks after treatment of the oligosaccharide pool with neuraminidase, as seen in Figure 2 (bottom).

Preliminary identification of the structures of the peaks of Figure 2 was made after comparison of their elution times with a library of standard oligosaccharides. Figure 3 details the exact oligosaccharides with which the peaks of Figure 2 coeluted in HPLC analysis. As demonstrated by the data of Table I, the proposed structures are completely consistent with monosaccharide analysis of each isolated peak, after complete enzymatic hydrolysis of each sample.

The results obtained by sequential exoglycosidase treatment of each of the oligosaccharide pools are summarized in Table II. The sequences obtained completely agree with the structures proposed on the basis of the HPLC comparisons (Figure 2) of each r-HPg oligosaccharide with those of known standard structures. In addition, the data presented in Table III on digestion of the various oligosaccharides with *D. pneumoniae* NAc-β-D-glucosaminidase reveal the nature of some of the outer arm GlcNAc linkages. Specifically, initial

Table I: Monosaccharide Compositions of the Oligosaccharides Released from CHO-Expressed Recombinant Human Plasminogen

peak[a]	Sia[b]	Gal[c]	GlcNAc[c]	Man[c]	Fuc[c]
1			2.0	3.1	1.0
2			2.0	3.0	
3			4.0	2.8	1.0
4			2.0	4.8	
5		1.8	3.9	2.8	1.1
6		2.0	4.2	3.0	
7		3.0	4.8	2.9	
8		3.7	5.6	3.0	
9	1.0	2.0	3.8	2.8	
10	1.8	1.7	3.7	2.6	1.0
11	1.8	1.9	3.6	2.8	
12			2.0	8.8	
13	2.6	3.2	4.6	3.0	
14	3.3	3.6	5.7	3.0	
15	4.0	3.7	5.7	3.0	
16	4.7	3.9	5.8	3.1	

[a] The peak number corresponds to those in Figure 2 (top). [b] Cleaved from the oligosaccharide with neuraminidase (*A. ureafaciens*). [c] Complete digestion of the desialooligosaccharide with a clam liver glycosidase mixture (Davidson et al., 1990a).

digestion of pool 3 with this enzyme catalyzed release of 1.86 mol of GlcNAc/mol of oligosaccharide, supporting our claim that two terminal GlcNAc(β1,2)Man linkages are present in this component. It is known that this enzyme does not catalyze cleavage of GlcNAc(β1,4)Man or GlcNAc(β1,6)Man bonds and shows a strong preference for unhindered GlcNAc-(β1,2)Man linkages (Yamashita et al., 1981). Digestion of the oligosaccharides present in each of peaks 5–8 (Figure 2, top) with this same enzyme after β-galactosidase treatment, and of the oligosaccharides present in pools 9–11 and 13 (Figure 2, top) after neuraminidase and β-galactosidase digestions, yielded maximal amounts of 1.86–1.97 mol of GlcNAc/mol of each oligosaccharide, demonstrating that two GlcNAc(β1,2)Man linkages are present in all of these samples, in further agreement with each proposed structure. It is of interest to consider the same data obtained for treatment of the asialo-, agalacto peaks 14–16 with the *D. pneumoniae* NAc-β-D-glucosaminidase. With these samples, it would be expected that, again, 2 mol of (β1,2)-linked GlcNAc would be released. However, we obtained approximately half that amount in all cases. This is in complete accord with the study of Yamashita et al. (1981), wherein it was shown that the steric hindrance provided by the GlcNAc(β1,6)Man linkage inhibits the release of GlcNAc(β1,2) when linked to that same Man. These linkages are present in each of components 14–16. Thus, the fact that only one of the two GlcNAc(β1,2)Man bonds was disrupted after treatment with this enzyme is also in agreement with the structures proposed for oligosaccharides 14–16.

Treatment of the oligosaccharide from pools 5 and 6 (Figure 2, top) directly with *D. pneumoniae* galactosidase, which is specific for Gal(β1,4)GlcNAc linkages (Kobata, 1979), results in maximal liberation of approximately 2 mol of Gal/mol of each oligosaccharide, establishing the linkage positions of Gal in these components. Further, similar direct treatments of oligosaccharides from peaks 7 and 8, with *D. pneumoniae* galactosidase, as well as neuraminidase-treated peaks 9–11 and 13–16 with this same galactosidase yielded the expected amount of Gal. These results show that all outer arm Gal exists in (β1,4) linkage with GlcNAc.

The presence of (α1,2)-linked Man residues in peak 12 has been further verified with *A. phoenicis* α-mannosidase, which specifically cleaves Man in this linkage position (Yamashita et al., 1980). Treatment of this oligosaccharide with the above enzyme resulted in maximal release of 3.8 mol of Man/mol of oligosaccharide. Further, the oligosaccharide product of this reaction coelutes with the standard corresponding to peak 4 of Figure 2 (top). These observations are consistent with the structure proposed for peak 12 oligosaccharide.

The lectin blotting experiments of Figure 4 demonstrate that the CHO-expressed r-R^{561}S-[Glu^1]Pg is reactive with SNA (lane 4), a lectin that preferentially recognizes (α2,6)-linked Sia (Shibuya et al., 1987). This is an extremely important finding in CHO-based glycosylation mechanisms, the implications of which are discussed below. As seen from the gel of Figure 4, also reactive with SNA is affinity chromatography form 1 [Glu^1]Pg (lane 1), from human plasma, which is known to contain only Sia(α2,6)Gal-R linkages on its N-linked oligosaccharide (Hayes & Castellino, 1979b) and *Spodoptera frugiperda* expressed r-R^{561}S-[Glu^1]Pg, also known to contain this same N-based oligosaccharide linkage (Davidson et al., 1990a). Affinity chromatography form 2 of human plasma HPg, which does not possess N-linked oligosaccharide, but does contain mainly Sia(α2,3)Gal linkages on Thr^{346}-based oligosaccharide, with a small fraction of protein molecules containing Sia(α2,6)GalNAc (Hayes & Castellino, 1979c), does not react with SNA to the same extent as affinity form 1 (reaction only occurs at 10-fold higher concentrations of HPg form 1), as expected. A control experiment consisting of *A.*

Table II: Sequential Exoglycosidase Digestion of Oligosaccharides Released from CHO-Expressed Recombinant Human Plasminogen[a]

peak[b]	Sia[c]	Gal[d]	GlcNAc[e]	α-Man[f]	β-Man[g]	α-Fuc[h]	GlcNAc[e]
1				2.10	1.00	0.97	1.86
2				2.04	0.98		1.87
3			2.00	1.86	0.93	0.93	1.76
4				3.68	0.87		1.78
5		2.03	1.94	1.91	0.92	0.90	1.88
6		1.97	1.95	1.93	0.94		1.68
7		2.87	2.65	1.71	0.84		1.69
8		3.89	3.80	1.09	0.94		1.80
9	1.00	2.00	1.97	1.94	0.98		1.79
10	1.96	1.92	1.88	1.86	0.93	0.88	1.72
11	2.02	1.96	1.92	1.92	0.96		1.83
12				7.87	0.98		1.92
13	2.98	2.93	2.87	1.91	0.86		1.64
14	2.76	3.65	3.58	1.78	0.86		1.69
15	4.01	3.94	3.93	1.94	0.97		1.98
16	4.74	3.86	3.78	1.82	0.91		1.84

[a] For pools containing complex carbohydrate, the sequence of enzymes added was *c–h,e*. For high-mannose or truncated high-mannose pools, the sequence of addition was *e–h,e*. [b] The peak numbers correspond to those in Figure 2 (top). [c] Neuraminidase (*A. ureafaciens*). [d] β-1,4-Galactosidase (*D. pneumoniae*). [e] NAc-β-Glucosaminidase (Jack bean). [f] α-Mannosidase (Jack bean). [g] β-Mannosidase (snail). [h] α-Fucosidase (bovine epididymus).

Table III: Release of Terminal GlcNAc by *D. pneumoniae* *N*-Acetylhexosaminidase from Various Exoglycosidase-Treated Oligosaccharides Obtained from CHO-Expressed Recombinant Human Plasminogen

		GlcNAc (mol/mol)	
peak[a]	total GlcNAc[b]	theory[c]	actual[d]
1			
2			
3	2.0	2.0	1.86
4			
5	2.0	2.0	1.91
6	2.0	2.0	1.88
7	3.0	2.0	1.84
8	4.0	2.0	1.26
9	2.0	2.0	1.94
10	2.0	2.0	1.86
11	2.0	2.0	1.97
12			
13	3.0	2.0	1.89
14	4.0	2.0	0.97
15	4.0	2.0	0.92
16	4.0	2.0	0.88

[a] The peak numbers correspond to those in Figure 2 (top). [b] Total outer arm terminal GlcNAc residues. The appropriate oligosaccharides were treated with neuraminidase (*A. ureafaciens*) and β-galactosidase (Jack bean) when necessary to render them as terminal outer arm GlcNAc residues. [c] Outer arm GlcNAc residues predicted to be (β1,2)-linked to Man and subject to cleavage by *D. pneumoniae* *N*-acetylhexosaminidase. [d] Actual GlcNAc released by *D. pneumoniae* *N*-acetylhexosaminidase.

ureafaciens neuraminidase-treated r-R^{561}S-[Glu^1]Pg (lane 6) does not react with SNA, and glycopeptidase F treated r-R^{561}S-[Glu^1]Pg (lane 5) is also unreactive with this lectin. This latter observation is important, since it shows that O-linked oligosaccharide, if present on the CHO-expressed r-HPg and if it contains Sia(α2,6)Gal-R or Sia(α2,6)GalNAc-R, does not interfere with this assay. Additionally, the electrophoretogram of lane 7 shows that CHO-expressed r-tPA, which only contains Sia(α2,3)Gal linkages, is also unreactive with SNA (Spellman et al., 1989). Finally, as shown in lane 8, positive SNA blotting activity is present in r-R^{561}S-[Glu^{1}Pg] after release of (α2,3)-linked Sia from the protein with NDV neuraminidase. This further confirms the presence of (α2,6)-linked Sia on CHO-expressed r-R^{561}S-[Glu^{1}Pg].

Sialic acid linkages on many of the components were confirmed by use of NDV neuraminidase, in combination with *A. ureafaciens* neuraminidase. The results of these enzyme digests are summarized in Table IV. Treatment of peaks 9–11 with NDV neuraminidase did not lead to release of any Sia, whereas *A. ureafaciens* neuraminidase, added before or after treatment with NDV neuraminidase, led to liberation of all Sia present in these glycans (Tables II and IV). This indicates that in these cases all Sia is linked (α2,6) to Gal. Peak 13 is also proposed as an exact structure, since its retention time on HPLC matches exactly the standard oligosaccharide proposed for peak 13 (Figure 3). In addition, treatment of this oligosaccharide with NDV neuraminidase results in maximal release of 1.96 mol of (α2,3)-linked Sia per mole of glycan, and subsequent treatment with *A. ureafaciens* neuraminidase leads to release of an additional 0.9 mol of (α2,6)-linked Sia per mole of oligosaccharide. The exact positions of the (α2,6)-linked Sia in oligosaccharides 14–16 could not be identified unequivocally, but it is clear that $^2/_3$, $^1/_2$, and $^3/_5$ of the Sia, respectively in these glycans are present in (α2,6) linkages. While, on HPLC analysis, intact peak 16 did not align with any standard oligosaccharide structure that was available to us, the desialylated form did align with peaks 14 and 15, and with the standard asialo tetraantennary complex oligosaccharide standard. Its structure, except for the location of the fifth (α2,6) Sia, is thereby predicted with confidence.

Table IV: Establishment of the Sialic Acid Linkages in Oligosaccharides Released from CHO-Expressed Recombinant Human Plasminogen

		Sia (mol/mol) released with neuraminidases from	
peak[a]	total Sia[b]	NDV[c]	*A. ureafaciens*[d]
9	1.00	0	0.98
10	1.96	0	1.92
11	2.02	0	1.93
13	2.98	1.96	0.90
14	2.76	0.91	1.92
15	4.01	1.97	2.02
16	4.74	1.95	2.88

[a] The peak numbers correspond to those in Figure 2 (top). [b] Taken from Table II. Digested with *A. ureafaciens* neuraminidase. [c] Digested with NDV neuraminidase. [d] Peak from footnote *c*, reisolated and digested with *A. ureafaciens* neuraminidase.

Discussion

A central interest of our laboratory involves structure–function relationships of human coagulation and fibrinolytic proteins, and such questions surrounding human plasminogen have been the subject of long-term study in our group. In order to investigate regions of this protein that are responsible for its variety of binding interactions, a system for producing recombinant forms of human plasminogen has been developed in insect cells (Whitefleet-Smith et al., 1989). There exists on human plasminogen only one Asn residue (Asn^{289}) that is present as part of the tripeptide consensus sequence required for N-linked glycosylation. Since previous studies have suggested that glycosylation of this protein also plays a role in its function, we have investigated the oligosaccharide structures in the insect cell-expressed cDNA coding for HPg (Davidson et al., 1990a) and found that a variety of carbohydrate chains, including various high-mannose and biantennary complex carbohydrates, existed at this single potential N-linked glycosylation site. The presence of complex carbohydrate on proteins produced in insect cells, one chain of which is identical with the only oligosaccharide chain found in the human plasma-derived protein, was a novel finding of this former study. This suggested that, in addition to the nature of the cell type, the human protein played a role in directing its glycosylation pathways. Due to the great deal of current general interest in the role of oligosaccharide in protein function, and the fact that protein structure–function investigations occur with recombinant proteins expressed in foreign cell systems, it is necessary to possess knowledge of the determinants of glycosylation of proteins. In this regard, the improtance of the nature of the cell types used to express recombinant proteins and of protein structural features that may regulate its glycosylation processing pathways are reasonable starting points. Human plasminogen is an excellent protein with which to model such investigations, since it has only a single possible N-linked glycosylation site, since the carbohydrate structure of the plasma-derived protein is known, and since we are expressing the human cDNA in several different cell systems. In the current investigation, we have expressed the human protein in CHO cells and believed that it would be of great value to the questions raised above to compare its glycosylation with that of the human plasma-derived protein and a recombinant protein produced in invertebrate cells.

The oligosaccharide fingerprint (Figure 2), as revealed by HPLC analysis of the oligosaccharide released from the protein

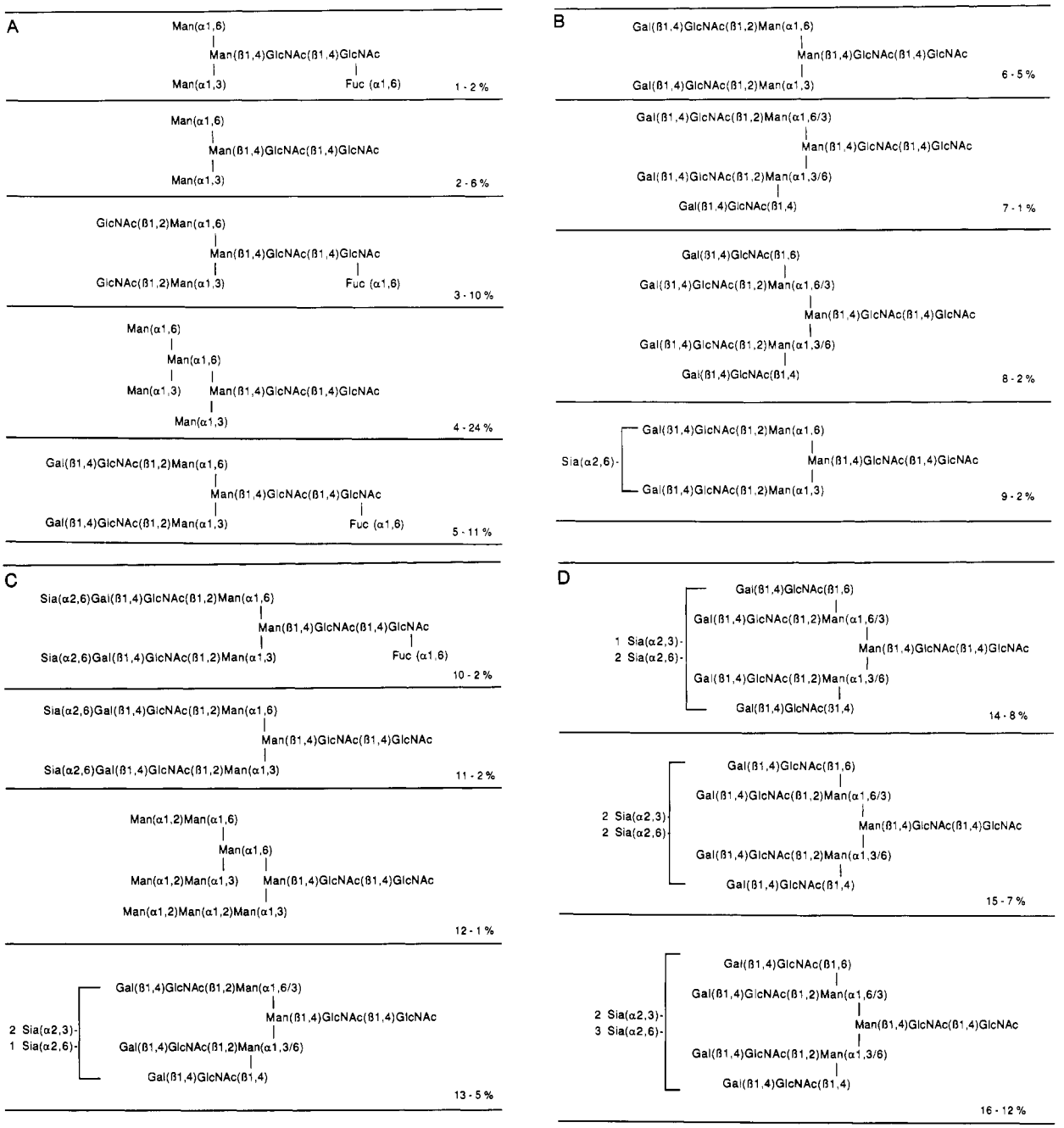

FIGURE 3: Structures of the oligosaccharides released from CHO-expressed r-R^{561}S-human plasminogen. The numbers of the pools correspond to their elution positions in Figure 2 (top).

by glycoprotein F, suggests that considerable microheterogeneity exists in the nature and extent of carbohydrate processing of CHO-expressed HPg. While other structures exist in the fingerprint shown in Figure 2, we have concentrated our structural identifications on components that are present at the level of at least 1% of the total oligosaccharide. From $NaDodSO_4$/PAGE of the purified protein, no detectable impurities were present (to a confidence level of less than 1%), and, thus, we believe that all oligosaccharides identified have been released from HPg. There is no manner of unequivocally proving this point, and our approach was to work with a rigorously purified protein. Analysis of the oligosaccharide fractions obtained from the recombinant protein shows that a mixture of various high-mannose and complex carbohydrate is present. All of these structures have been found in other glycoproteins. Interestingly, neither hybrid oligosaccharde, sulfated oligosaccharide, complex oligosaccharides with NAc-lactosaminyl repeats, nor oligosaccharide with bisecting GlcNAc has been found in significant amounts. This latter observation suggests that GlcNAc transferase III does not function in glycosylation of HPg in this particular system.

The structures of the fractions identified in Figure 3 are based upon their coelution on HPLC with standards of known structures, monosaccharide compositions, and sequential exoglycosidase analysis. Thus, the exact sequence of monosaccharides in each oligosaccharide fraction, as well as the anomeric configurations of the linkages, has been established. In addition, parallel sequential exoglycosidase cleavages of the oligosaccharides of Figure 3 with the standards with which they are associated, and reexamination of the HPLC elution properties of the oligosaccharides remaining after each step, suggested further that the standard structures and the HPg oligosaccharides were the same. Since the retention times of the oligosaccharides on the particular HPLC system employed are highly dependent on the exact linkages between monosaccharides (Basa & Spellman, 1990; Hardy et al., 1988;

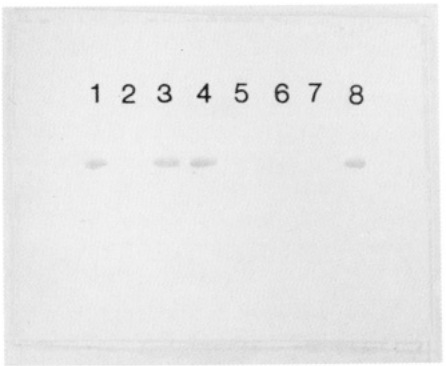

FIGURE 4: Lectin blotting of human plasminogens with biotinylated SNA. Lane 1, human plasma plasminogen affinity chromatography form 1; lane 2, human plasma plasminogen affinity chromatography form 2; lane 3, insect (*Spodoptera frugiperda*) expressed r-R^{561}S-human plasminogen; lane 4, CHO-expressed r-R^{561}S-human plasminogen; lane 5, glycopeptidase F treated CHO-expressed r-R^{561}S-human plasminogen; lane 6, *A. ureafaciens* neuraminidase-treated CHO-expressed r-R^{561}S-human plasminogen; lane 7, CHO-expressed recombinant tissue plasminogen activator; lane 8, NDV neuraminidase treated CHO-expressed r-R^{561}S-human plasminogen. Approximately 2–4 μg of protein was present in each lane.

Townsend et al., 1988), this approach provides extremely good initial evidence as to the linkage positions of the oligosaccharides. Further, for many of the high-mannose oligosaccharides (peaks 1, 2, 4, and 12, Figure 2, top), the monosaccharide linkages that we propose appear to be the only possibilities previously described for oligosaccharides of this class in mammalian cells. The same holds true for the Man(β1,4)GlcNAc(β1,4)GlcNAc[$\pm$Fuc(α1,6)] core structures for all vertebrate oligosaccharides identified herein. Assuming that mannosyl transferases that catalyze addition of mannose directly to glycoproteins are not present in CHO cells and that all N-linked glycosylation has occurred via a dolichol intermediate (Kornfeld & Kornfeld, 1985), it would follow that the high-mannose, trimmed high-mannose, down to $Man_3GlcNAc_2$($\pm$Fuc), and all core structures consist of the proposed monosaccharide linkages. Finally, all proposed outer arm (β1,2)-linked Gal residues, all proposed outer arm (β1,2)-linked GlcNAc, and all proposed (α1,2)Man linkages have been verified by experimentation with linkage-specific exoglycosidases.

The origin of several oligosaccharide components requires special comment, particularly peaks 1 and 2 (Figure 2, top). Normal trimming and processing pathways proposed for the $Glc_3Man_9GlcNAc_2$-protein in Golgi [for a review, see Yet et al. (1988)] do not predict that such structures can exist. However, an alternate pathway, wherein $Glc_3Man_5GlcNAc_2$-PP-dolichol is transferred to protein, has been proposed and, in fact, can become the major pathway under certain conditions of cell growth (Rearick et al., 1981). This alternate pathway also appears to be significant in glycosylation of hen oviduct ovomucoid (Yamashita et al., 1983). Assuming some level of participation of this pathway under normal conditions in CHO cells, a mechanism is allowed that accounts for the presence of peak 2 (Figure 2, top), after mannosidase action on the $Man_5GlcNAc_2$-protein that originated from the alternate pathway. Peak 1 can form upon assumption of the existence of an (α1,6)fucosyltransferase that accepts as substrates the oligosaccharide of peak 2, and/or $Man_5GlcNAc_2$-protein, the latter of which through mannosidase II action can lead to the fucosylated oligosaccharide structure of peak 1. While the oligosaccharide of peak 1 is unusual, it has been observed previously (Yamashita et al., 1990) and suggests that previously unrecognized processing pathways are present. The origin of the oligosaccharide of peak 3 can most likely be explained by glycosidase-catalyzed degradations of fully processed fucosylated biantennary complex carbohydrate, or incomplete synthesis of this latter structure. The pentamannosyl structure of peak 4 suggests that it did not originate from the alternate pathway, since the (α1,2)-linked Man residues that would be expected for the $Man_5GlcNAc_2$ oligosaccharide that arose from the alternate pathway are not present, as revealed by the absence of Man release with *A. phoenicis* α-mannosidase. Thus, peak 4 originated via degradative routes from high-mannose or complex oligosaccharides derived from the classical pathway.

The linkage positions of sialic acid are very relevant to glycosylation in CHO cells, and the results obtained allow more general conclusions to be reached on glycosylation of recombinant proteins. Except in the case wherein CHO cells were transfected with a plasmid incorporating the coding sequence for β-galactoside (α2,6)sialyltransferase, after which CHO membrane proteins showed Sia(α2,6)Gal-R linkages (Lee et al., 1989), CHO cell proteins normally contain outer chain terminal Sia(α2,3)Gal-R, and recombinant proteins expressed in these cells also display this latter type of linkage [as examples, see Kagawa et al. (1988), Parekh et al., (1989b), Sasaki et al., (1987), Spellman et al. (1989), and Takeuchi et al., (1988)]. It is clear from the HPLC coelution of CHO-expressed HPg oligosaccharides with standard structures, the retention times of which are very sensitive to the linkage positions of Sia (Townsend et al., 1989), that Sia(α2,6)Gal-R outer arm structures occur in the oligosaccharide components. This is further verified by the lectin (SNA) blotting of Figure 4, wherein it is seen that Sia(α2,6) linkages exist in the total oligosaccharide pool, and from our studies with NDV neuraminidase (Table III), showing that many of the oligosaccharide fractions are resistant to cleavage of some, or all, of their Sia. This latter enzyme processes strict specificity for Sia(α2,3)Gal linkages (Paulson et al., 1982). Thus, it is clear that CHO-expressed HPg contains a considerable amount of outer chain Sia(α2,6)Gal-R linkages, as is found with the human plasma-derived protein (Hayes & Castellino, 1979b). This indicates that β-galactoside (α2,6)sialyltransferase is present in CHO cells and is stimulated by structural and/or conformational determinants in HPg [or the β-galactoside (α2,3)sialyltransferase is inhibited by such structural determinants]. Whether such stimulation is at the level of transcription and/or translation of the transferase gene or is stimulated at the enzyme level by the plasminogen substrate is not known. The possibility exists that the particular CHO cells employed represent a mutant expressing galactosidase (α2,6)sialyltransferase activity, but this has been discounted after examination of proteins from the media and cell membranes of nontransfected CHO proteins by SNA blotting. These experiments[3] did not reveal the presence of Sia in (α2,6) linkages.

In conclusion, in agreement with studies with other proteins [as examples, see Kagawa et al. (1988) and Parekh et al. (1989a)], there is the obvious importance of cell specificity in dictating the glycosylation pattern of HPg. In addition, there is clear evidence that the HPg protein or gene structure incorporates within it the ability to contribute to determination of the nature of its glycosylation, and the ability to activate glycosyltransferases that are required for generation of (α2,6)-sialylated oligosaccharide, the sole Sia linkage in N-linked oligosaccharide of human plasma plasminogen (Hayes

[3] G. Hoover, unpublished experiments.

& Castellino, 1979b). These data suggest that a normally silent gene for expression of galactosidase (α2,6)sialyltransferase exists in CHO cells, which may be stimulated by transfected materials. That this may be a more general phenomena is suggested from insect cell expression of the cDNA for HPg. Here, the same biantennary complex oligosaccharide structure found in the human plasma protein was present as a major component of the HPg oligosaccharide (Davidson et al., 1990a). Such structures had not been reported previously for insect-derived proteins. Similarly, in the present paper, we find outer chain Sia(α2,6)Gal-R linkages in the CHO cell-expressed cDNA for HPg, whereas in CHO endogenous proteins and in recombinant proteins expressed in these cells, outer chain Sia(α2,3)Gal-R structures represent the major linkages. In addition, recent evidence has suggested that there is a transfection-based activation of an endogenous (α1,3)fucosyltransferase gene in CHO cells, that is normally silent (Potvin et al., 1990). Thus, it appears as though gene activation by transfected materials may be a general phenomenon, and we feel that we possess an excellent model system for investigation of the structural features present in a protein or gene that govern glycosylation.

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An Anti-Insect Toxin Purified from the Scorpion *Androctonus australis* Hector Also Acts on the α- and β-Sites of the Mammalian Sodium Channel: Sequence and Circular Dichroism Study†

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ABSTRACT: A new anti-insect neurotoxin, AaH IT4, has been isolated from the venom of the North African scorpion *Androctonus australis* Hector. This polypeptide has a toxic effect on insects and mammals and is capable of competing with anti-insect scorpion toxins for binding to the sodium channel of insects; it also modulates the binding of α-type and β-type anti-mammal scorpion toxins to the mammal sodium channel. This is the first report of a scorpion toxin able to exhibit these three kinds of activity. The molecule is composed of 65 amino acid residues and lacks methionine and, more unexpectedly, proline, which until now has been considered to play a role in the folded structure of all scorpion neurotoxins. The primary structure showed a poor homology with the sequences of other scorpion toxins; however, it had features in common with β-type toxins. In fact, radioimmunoassays using antibodies directed to scorpion toxins representative of the main structural groups showed that there is a recognition of AaH IT4 via anti-β-type toxin antibodies only. A circular dichroism study revealed a low content of regular secondary structures, particularly in β-sheet structures, when compared to other scorpion toxins. This protein might be the first member of a new class of toxins to have ancestral structural features and a wide toxic range.

Scorpion venoms are well-known sources of neurotoxins, which are small basic polypeptides endowed with one of the highest toxicity encountered so far in peptidic macromolecules (Miranda et al., 1970). In the last 20 years, considerable data on these macromolecules have been gathered. Sequence data on more than 40 different toxins showed structural similarities among scorpion toxins (Rochat et al., 1979). The most apparent feature was that the eight half-cystine residues were located in same position, which made it possible to predict that the four disulfide bridges would be in homologous positions in all toxins (Kopeyan et al., 1974). The first pharmacological studies of scorpion neurotoxins were limited to the observation of their effect after injection on test animals, i.e., mice. Therefore, the only kind of activity encountered was the so-called anti-mammal activity. Zlotkin et al. (1971) showed the presence, in the venom of *Androctonus australis* Hector, of a toxin only active in insects, which induced a contractional paralysis. It was the first member of a new class of toxins, which now are known as anti-insect toxins. Electrophysiological and binding studies showed that the pharmacological target of anti-mammal toxins was the voltage-dependent sodium channel of excitable cells (Catterall, 1980). Moreover, it was found that anti-mammal toxins could be classified into α- and β-type toxins, according to their pharmacological and electrophysiological activity, each type possessing a distinct binding site on the sodium channel (Jover et al., 1980; Couraud et al., 1982). It was observed that toxins from the New World were mainly β-type toxins, whereas toxins from the Old World were exclusively α-type toxins. The anti-insect toxins were, in turn, divided into two different pharmacological categories: contraction-inducing toxins, which caused rapid excitatory contraction paralysis in *Sarcophaga argyrostoma* fly larvae, and depressant toxins, which induced a slow depressant flaccid paralysis (Zlotkin et al., 1985). Each of these two types of toxins showed distinct electrophysiological properties, but they were capable of binding to a common site on the sodium

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