Carbohydrate Structures of Recombinant Soluble Human CD4 Expressed in Chinese Hamster Ovary Cells†

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ABSTRACT: Infection of T-lymphocytes and macrophages by human immunodeficiency virus (HIV) is mediated by the binding of the HIV envelope glycoprotein to the cell-surface receptor glycoprotein CD4. A soluble, recombinant CD4 molecule (rCD4), produced by expression of a truncated CD4 gene in Chinese hamster ovary (CHO) cells [Smith et al. (1987) *Science 238*, 1704–1707], is in clinical trials as a potential therapeutic agent in the treatment of acquired immunodeficiency syndrome (AIDS). In the present study, the structures of the Asn-linked oligosaccharides of soluble rCD4 have been elucidated. The rCD4 molecule has two potential sites for N-glycosylation, Asn-271 and Asn-300. Tryptic glycopeptides containing either of the sites were purified by reversed-phase HPLC, and their oligosaccharides were released enzymatically. The structures of the oligosaccharides were determined by methylation analysis, high-pH anion-exchange chromatography, fast-atom bombardment mass spectrometry, and ^{1}H NMR spectroscopy at 500 MHz. Asn-271 was found to carry diantennary *N*-acetyllactosamine-type ("complex") oligosaccharides, of which 8% were asialo, 55% were monosialyl, and 37% were disialyl. Approximately 18% of these structures contained fucose $\alpha(1\rightarrow6)$ linked to the reducing GlcNAc residue. Two different hybrid structures were found to account for 34% of the oligosaccharides attached to Asn-300. The remainder of the oligosaccharides attached to Asn-300 were diantennary *N*-acetyllactosamine-type, of which 10% were asialo, 61% were monosialyl, and 29% were disialyl. Approximately 9% of the hybrid structures and 40% of the *N*-acetyllactosamine structures at Asn-300 were found to contain fucose $\alpha(1\rightarrow6)$ linked to the innermost GlcNAc residue.

T-Lymphocytes are divided into two major classes, which are distinguished by the cell-surface glycoproteins CD4[1] and CD8 (Reinherz et al., 1980; Fitch, 1985). The glycoproteins CD4 and CD8 play a role in the recognition of MHC antigens. In general, CD4-positive T-cells interact with cells of the immune system that bear class II MHC antigens on their surfaces (Swain, 1983). The CD4 molecule has also been shown to play a key role in the infection of CD4-positive T-cells by HIV. The envelope glycoprotein of HIV, gp120, binds with high affinity to CD4 (McDougal et al., 1986), and this binding is believed to mediate entry of the virus into CD4-positive cells (Maddon et al., 1986). Antibodies to CD4 have been shown to be capable of blocking HIV infection and syncytium formation in vitro (Dalgleish et al., 1984; Klatzmann et al., 1984; McDougal et al., 1985).

The CD4 molecule comprises a 370 amino acid extracellular domain that has significant sequence homology with the variable and joining regions of immunoglobulin heavy chains, a 21 amino acid transmembrane domain and a 38 amino acid cytoplasmic domain (Maddon et al., 1986). The extracellular domain has six cysteine residues that form three intrachain disulfide bonds (Carr et al., 1989; Harris et al., 1990) and two potential sites for N-glycosylation as recognized by the consensus sequence Asn-Xaa-Ser/Thr (Kornfeld & Kornfeld, 1985).

König et al. (1988) have examined the role of glycosylation in obtaining expression of CD4 on the cell membrane of human acute lymphoblastic leukemia cells. They found that treatment with the glycosylation inhibitor tunicamycin caused a 76% decrease in CD4 surface expression. The difference in electrophoretic mobility between CD4 grown in the presence or absence of tunicamycin indicated that these cells glycosylate both of the potential N-glycosylation sites. These investigators also carried out expression of full-length CD4 in CHO cells (König et al., 1989). On the basis of susceptibility to endo H, endo F, and PNGase F, they concluded that the predominant carbohydrate structures were asialo diantennary *N*-acetyllactosamine-type structures. The carbohydrates of CD4 do not appear to be directly involved in binding HIV gp120 (Fenouillet et al., 1989; Ibegbu et al., 1989).

Recently several soluble forms of CD4, in which the transmembrane and cytoplasmic domains were deleted, have been produced by expression in CHO cells (Smith et al., 1987; Deen et al, 1988; Fisher et al., 1988) and in insect cells (Hussey et

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[1] Abbreviations: AIDS, acquired immune deficiency syndrome; CCSD, complex carbohydrate structure database; CD, cluster differentiation antigen; CHO, Chinese hamster ovary; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; endo H, *endo*-β-*N*-acetylglucosaminidase H (*endo*-glycosidase H); EH-1 and EH-2, oligosaccharides released by endo H; FAB-MS, fast-atom bombardment mass spectrometry; GLC-MS, gas–liquid chromatography/mass spectrometry; HIV, human immunodeficiency virus; HPAE, high-pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; MHC, major histocompatibility complex; NeuAc, *N*-acetylneuraminic acid (sialic acid); PAD, pulsed amperometric detection; PNGase F, peptide:*N*-glycosidase F; Q0, Q1, and Q2, fractions separated by neutral-pH anion-exchange chromatography on Mono Q; Q+F, Mono Q separated oligosaccharides that contain a fucosyl group; Q−F, Mono Q separated oligosaccharides that lack the fucosyl group; rCD4, recombinant soluble CD4 receptor; rt-PA, recombinant tissue plasminogen activator; TFA, trifluoroacetic acid; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

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al., 1988). These molecules differ in their amino-terminal residues and in their carboxy-terminal truncation sites, but all have been demonstrated to be capable of blocking HIV infection and syncytia formation in vitro. Several of these molecules (Smith et al., 1987; Fisher et al., 1988; Deen et al., 1988) are currently in clinical trials to evaluate their potential as therapeutic agents in the treatment of AIDS.

Carr et al. (1989) examined the protein and carbohydrate structures of one version (Deen et al., 1988) of a soluble rCD4 molecule. Through a combination of FAB-MS and methylation analyses, they concluded that both of the potential N-glycosylation sites were glycosylated and that both carried diantennary N-acetyllactosamine-type oligosaccharides containing zero to one residue of fucose and zero to two residues of NeuAc.

Harris et al. (1990) have reported structural characterization of a similar CHO-expressed rCD4 molecule [that described by Smith et al. (1987)]. This work included assignment of the three intrachain disulfide bonds and demonstration that both potential N-glycosylation sites are glycosylated. Tryptic mapping of enzymatically deglycosylated rCD4 revealed that Asn-300 carried a mixture of endo H resistant and endo H susceptible structures whereas all of the structures attached to Asn-271 were endo H resistant (Harris et al., 1990). The goal of the present study has been the detailed elucidation of the carbohydrate structures of CHO-expressed rCD4, including the distribution of structures at each of the two glycosylation sites. The rCD4 molecule used in this study is the same as that studied by Harris et al. (1990).

Experimental Procedures

Materials. Recombinant soluble human CD4 was purified from cell culture supernatants of CHO cells transfected with an expression vector containing cDNA coding for a soluble form of CD4, truncated after Pro-368 (Smith et al., 1987). TPCK–trypsin was from Worthington. PNGase F and endo H were purchased from Genzyme. Glycopeptidase A was purchased from ICN Immunologicals.

Standard Methods. Reduction and S-carboxymethylation, digestion with TPCK–trypsin, oligosaccharide composition analysis, and methylation analysis were carried out as described (Spellman et al., 1989). Amino acid analysis conditions were as described by Harris et al. (1990).

Isolation of Tryptic Glycopeptides. A sample (55 mg) of reduced and S-carboxymethylated rCD4 was treated with TPCK–trypsin. Glycopeptides were isolated by reversed-phase HPLC using a Hewlett-Packard Model 1090M HPLC system equipped with a Vydac C_{18} column (0.46 × 25 cm; 5-μm particle size). Solvent A was 0.16% aqueous TFA and solvent B was acetonitrile containing 0.16% TFA. The column was preequilibrated in 100% A at a flow rate of 1 mL/min. After a 3-min hold, peptides were eluted with a linear gradient from 0 to 50% B in 75 min. The same chromatography conditions were used to monitor deglycosylation experiments and to recover oligosaccharides released by glycosidase digestion.

PNGase F Digestion. The Asn-271-containing glycopeptide (530 nmol) was reconstituted in 4.0 mL of 0.25 M sodium phosphate buffer, pH 8.6, containing 0.02% NaN_3 and 10 mM EDTA. PNGase F (N-Glycanase; 100 manufacturer's units in 0.4 mL) was added and the sample incubated for 20 h at 37 °C.

Endo H Digestion. The Asn-300-containing glycopeptide (960 nmol) was reconstituted in 4.0 mL of 50 mM sodium phosphate buffer, pH 6, containing 0.02% NaN_3. Endo H (4 units in 0.1 mL of the same buffer) was added and the sample incubated for 20 h at 37 °C.

Glycopeptidase A Digestion. The endo H treated Asn-300-containing glycopeptide was reconstituted in 2.0 mL of 50 mM sodium phosphate buffer, pH 6, containing 0.02% NaN_3. Glycopeptidase A (4 milliunits in 0.4 mL of the same buffer) was added, and the sample incubated for 74 h at 37 °C.

Neutral-pH Anion-Exchange Chromatography. Neutral-pH anion-exchange separations of oligosaccharides were carried out on a Dionex BioLC system equipped with a Mono-Q column (HR 5/5; Pharmacia Fine Chemicals). The column was preequilibrated with water at a flow rate of 1 mL/min. Oligosaccharides were eluted with a linear gradient from 0 to 0.45 M sodium acetate in 20 min and detected by pulsed amperometry (Dionex PAD II). A solution of sodium hydroxide (0.05 M) was mixed at a flow rate of 0.7 mL/min with the effluent stream to facilitate detection. The column effluent was desalted by using on-line ion suppression as described (Spellman et al., 1989).

High-pH Anion-Exchange Chromatography. The HPAE separations were carried out on a Dionex BioLC system equipped with an AS6 column (0.4 × 25 cm; Dionex). Solvent A was 0.1 M NaOH and solvent B was 0.1 M NaOH containing 0.5 M sodium acetate. The column was preequilibrated in 5% B at a flow rate of 1 mL/min. After a 2-min hold, oligosaccharides were eluted by using a two-stage linear gradient from 5 to 15% B in 5 min and from 15 to 40% B in 25 min. All other chromatography and detection conditions have been described (Spellman et al., 1989).

^1H NMR Spectroscopy. ^1H NMR spectroscopy, at 500 MHz, was performed on a Bruker AM-500 spectrometer interfaced with an Aspect-3000 computer. Experimental details were as described (Green et al., 1988; Spellman et al., 1989). The probe temperature was 27 °C. Chemical shifts (δ) are expressed in parts per million downfield from internal DSS, measured by reference to internal acetone (δ 2.225 in ^2H_2O at 27 °C) or free acetate (δ 1.908 in ^2H_2O at p^{2}H 6–8 and 27 °C), with an accuracy of 0.002 ppm.

FAB Mass Spectrometry. FAB mass spectra were acquired on a JEOL HX110HF/HX110HF tandem mass spectrometer operated in a normal two-sector mode. FAB was performed with 6-keV xenon atoms (10-mA emission current), and data were acquired over a mass range of 400–2000 amu.

Results

Isolation of Tryptic Glycopeptides. A sample (55 mg) of rCD4 was reduced, S-carboxymethylated, and subjected to trypsin digestion. An aliquot (0.1 mg) of the resulting mixture of tryptic peptides was fractionated by reversed-phase HPLC (Figure 1). The glycopeptides were identified by amino acid analysis; they were homogeneous in their peptide portions. The Asn-300- and Asn-271-containing glycopeptides comprised residues 300–312 (NLTCEVWGPTSPK) and 252–279 (KLPLHLTLPQALPQYAGSGNLTACEAK), respectively (Harris et al., 1990). Because of carbohydrate heterogeneity, each glycopeptide appears as a multiplet in Figure 1. The peaks representing the two potential glycosylation sites, Asn-271 and Asn-300, were pooled from a total of 11 replicate reversed-phase HPLC tryptic maps (5 mg each) for carbohydrate analysis.

Glycosidase Susceptibilities of Isolated Glycopeptides. Aliquots of the isolated glycopeptides were treated with glycosidases of differing specificities to gain information about the types of oligosaccharides at each site and to optimize conditions for complete deglycosylation. A shift to increased reversed-phase HPLC retention time is observed upon deglycosylation of a glycopeptide. The glycosidases used were

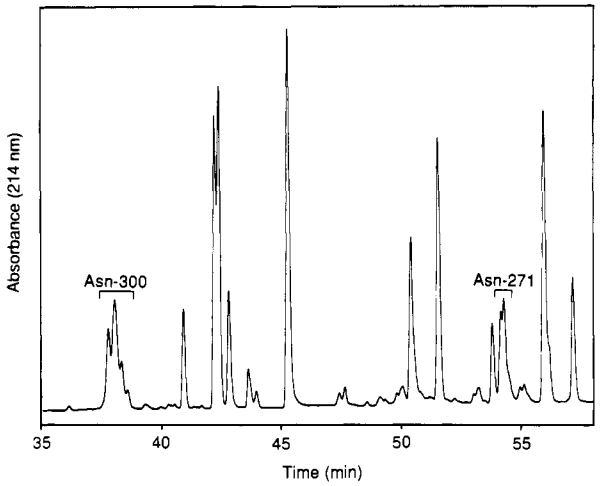


FIGURE 1: Detail of the reversed-phase HPLC tryptic map of rCD4. Chromatography conditions were as described under Experimental Procedures. Multiplets are observed for the glycopeptides containing "Asn-271" and "Asn-300" at the elution positions indicated.

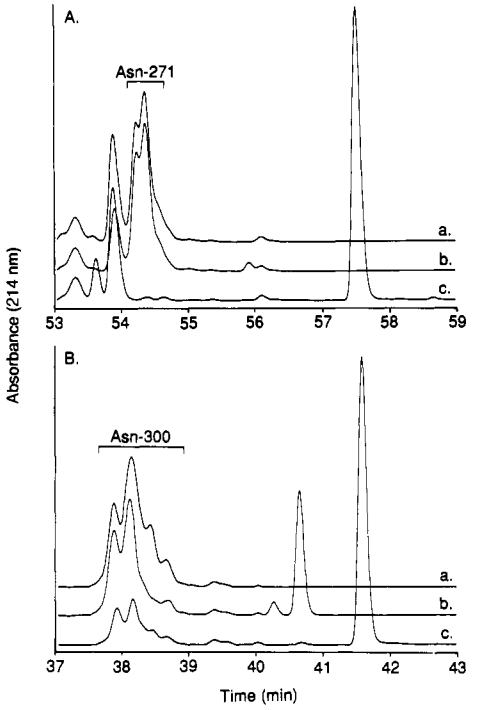


FIGURE 2: Reversed-phase HPLC chromatograms, monitoring the susceptibility of isolated rCD4 glycopeptides to different glycosidases. (A) Asn-271 glycopeptide: (a) no glycosidase; (b) endo H treated; (c) PNGase F treated. (B) Asn-300 glycopeptide: (a) no glycosidase; (b) endo H treated; (c) glycopeptidase A treated.

endo H, which cleaves between the proximal GlcNAc residues of high mannose and hybrid-type N-linked structures (Tai et al., 1977); PNGase F, which breaks the β-aspartamido-glucosaminyl bond of all known N-linked oligosaccharides (Tarentino et al., 1985); and glycopeptidase A, which, like PNGase F, has a broad oligosaccharide specificity but which can act more efficiently than PNGase F on glycopeptides with a short peptide chain (Maley et al., 1989).

The Asn-271-containing glycopeptides showed no significant susceptibility to endo H (Figure 2A, trace b) but were completely deglycosylated by PNGase F (Figure 2A, trace c). This result indicated that all of the oligosacchardies attached to Asn-271 are of the *N*-acetyllactosamine type. The peaks in Figure 2A that were unaffected by PNGase F treatment (i.e., those eluting between 53 and 54 min, trace c) were identified

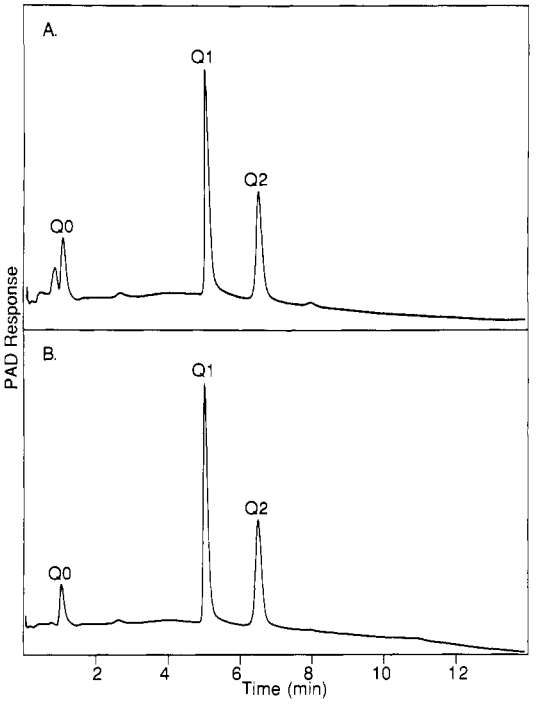


FIGURE 3: Neutral-pH anion-exchange chromatography (Mono Q) of *N*-acetyllactosamine oligosaccharides. (A) Oligosaccharides released from Asn-271 by PNGase F. (B) Oligosaccharides released from Asn-300 by Glycopeptidase A. The Asn-300-containing glycopeptide had been treated with endo H and repurified by reversed-phase HPLC prior to the glycopeptidase A treatment.

by amino acid analysis and found to be nonglycosylated peptides that copurified with the Asn-271 glycopeptide in the reversed-phase HPLC tryptic map.

Asn-300, the second potential N-glycosylation site of rCD4, is the amino-terminal residue of a tryptic peptide (see above). Therefore, the Asn-300-containing glycopeptides would not be predicted to be good substrates for PNGase F (Tarentino et al., 1985). This was confirmed in pilot experiments (results not shown). Treatment with glycopeptidase A, however, resulted in nearly complete deglycosylation (Figure 2B, trace c). Endo H treatment caused partial deglycosylation of the Asn-300-containing glycopeptide (Figure 2B, trace b), indicating that approximately one-third of the structures attached to Asn-300 were high mannose and/or hybrid-type oligosaccharides. Endo H treatment of the Asn-300-containing peptide gave rise to two distinct later-eluting peaks (retention times of 40.2 and 40.6 min; Figure 2B, trace b). The source of this heterogeneity was determined by FAB-MS to be partial fucosylation of the GlcNAc residue left attached to the peptide. The pseudomolecular ion observed for the peak at 40.2 min was m/z 1839.0 (NLTCEVWGPTSPK + GlcNAc + Fuc), while that observed for the peak at 40.6 min was m/z 1692.8 (NLTCEVWGPTSPK + GlcNAc). The area ratio of the two peaks in Figure 2B, trace b, indicated that ~9% of the endo H susceptible structures attached to Asn-300 were fucosylated at the innermost GlcNAc residue.

Isolation and Characterization of the Oligosaccharides Attached to Asn-271. A sample (530 nmol) of the isolated Asn-271-containing glycopeptide was treated with PNGase F, and reversed-phase HPLC was used to purify the released oligosaccharides from the residual peptides (results not shown). The PNGase F released oligosaccharides isolated from Asn-271 were separated by neutral-pH anion-exchange chromatography (Figure 3A) into subfractions that were neutral (Q0; 8% of total area), monosialyl (Q1; 55%), and disialyl (Q2;

Table I: Methylation Analysis of Oligosaccharide Pools

residue	normalized molar ratio						
	Asn-271			Asn-300			
	Q0[a]	Q1[a]	Q2[a]	EH[b]	Q0[a]	Q1[a]	Q2[a]
fucitol							
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	0	0.1	0.2	0	0.3	0.3	0.3
mannitol							
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	0	0	0	1.9[c]	0.5[c]	0	0
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	1.1	1.7	2.1	1.0	1.4	1.6	2.0
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	0	0	0	0.5	0	0	0
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1	1	1	1.9	1	1	1
galactitol							
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	1.5	0.8	0.7	0.2	1.5	0.8	0.5
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	0	1.0	1.6	1.0	0	1.0	1.9
2-(*N*-methylacetamido)-2-deoxyglucitol							
1,3,5,6-tetra-*O*-methyl (4-mono-*O*-acetyl)	0.8	0.8	0.9	1	0.6	0.6	0.6
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	0	0.3	0.2	0	0.2	0.6	0.5
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	2.5	3.0	3.0	1.0	2.4	2.4	3.2

[a] Normalized on 2,4-di-*O*-methylmannitol. [b] Normalized on 1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol. [c] Quantitation of this residue is unreliable because of its coelution with contaminating 2,3,4,6-tetra-*O*-methylglucitol.

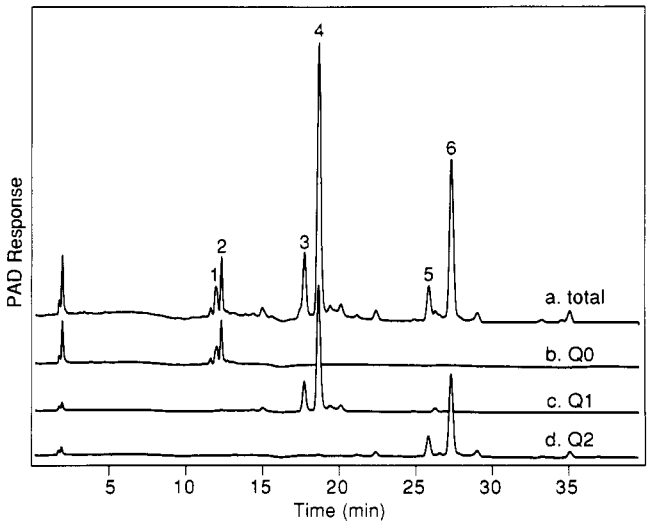


FIGURE 4: HPAE chromatography of *N*-acetyllactosamine oligosaccharides released from Asn-271 by PNGase F. (a) Unfractionated oligosaccharides; (b) neutral fraction from Mono Q; (c) monosialyl fraction from Mono Q; (d) disialyl fraction from Mono Q. The chromatography and detection conditions are described under Experimental Procedures.

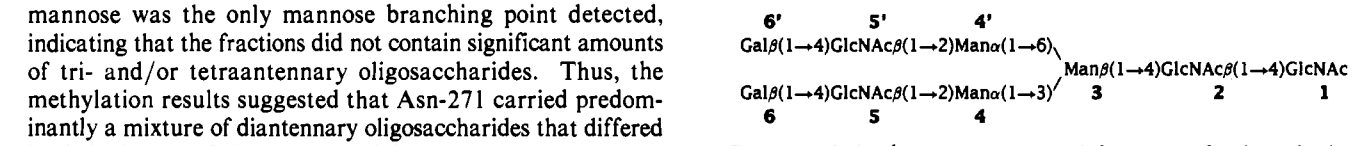
37%) oligosaccharides. A trace (<1% of total area) of a trisialyl subfraction was also detected (elution time of 8 min in Figure 3A).

The subfractions Q0, Q1, and Q2 were subjected to methylation analysis (Table I), HPAE chromatography (Figure 4), and 500-MHz ^{1}H NMR spectroscopy (Figure 5 and Table II) to elucidate the structures of the oligosaccharide components of each mixture. The methylation analysis results (Table I) revealed, inter alia, the following structural elements: galactose was found to be terminal or 3-substituted, suggesting that sialic acid, when present, was attached exclusively to the 3-position of galactose; prereduced GlcNAc was found to be 4-substituted or 4,6-disubstituted, indicating partial fucosylation at the 6-position of the residue; 3,6-disubstituted mannose was the only mannose branching point detected, indicating that the fractions did not contain significant amounts of tri- and/or tetraantennary oligosaccharides. Thus, the methylation results suggested that Asn-271 carried predominantly a mixture of diantennary oligosaccharides that differed in their degree of sialylation and fucosylation.

The HPAE chromatographic analyses were consistent with the conclusion that Asn-271 carried a mixture of diantennary oligosaccharides. Figure 4 shows HPAE chromatograms obtained with the total pool of oligosaccharides liberated from Asn-271 (trace a) and of the subfractions prepared by neutral-pH anion-exchange chromatography (Q0–Q2: traces b–d). The total oligosaccharide pool was resolved by HPAE chromatography into six major components, with elution positions characteristic of neutral (peaks 1 and 2), monosialyl (peaks 3 and 4), and disialyl (peaks 5 and 6) oligosaccharides. The peak at 35 min in Figure 4a corresponds to the trace of trisialyl triantennary oligosaccharides (see above). The major separation is based on the number of residues of sialic acid, as was confirmed by HPAE analysis of the isolated subfractions, in which the neutral subfraction Q0 contained peaks 1 and 2 (trace b), the monosialyl Q1 contained peaks 3 and 4 (trace c), and the disialyl Q2 contained peaks 5 and 6 (trace d). The elution positions of peaks 1 and 2 corresponded to those of authentic standards of fucosylated and nonfucosylated asialo diantennary *N*-acetyllactosamine oligosaccharides Q0+F and Q0–F, respectively, while those of peaks 5 and 6 corresponded to fucosylated and nonfucosylated disialyl diantennary oligosaccharides Q2+F and Q2–F (Basa & Spellman, 1990). Chromatographic standards were not available for the monosialyl oligosaccharides (peaks 3 and 4). However, desialylation of these oligosaccharides shifted their retention times to those of peaks 1 and 2; as a result, we conclude that peaks 3 and 4 are fucosylated and nonfucosylated monosialyl diantennary oligosaccharides Q1+F and Q1–F, respectively.

To complete the primary structure determination of the oligosaccharides released from Asn-271 by PNGase F, the fractions Q0, Q1, and Q2 were each subjected to ^{1}H NMR spectroscopy at 500 MHz. The chemical shifts of the structural reporter groups of the individual oligosaccharides present in the three Mono Q separated fractions are compiled in Table II. The structural reporter group regions of the 500-MHz ^{1}H NMR spectra of fractions Q1 and Q2 are shown in Figure 5.

The NMR data confirmed that all Asn-271-released oligosaccharides have in common the reducing diantennary nonasaccharide structural element denoted Q0–F:



Several of the ^{1}H NMR spectral features of this reducing nonasaccharide have been reported (Bayard et al., 1983; Debeire et al., 1983; Samor et al., 1986; Bendiak & Cumming,

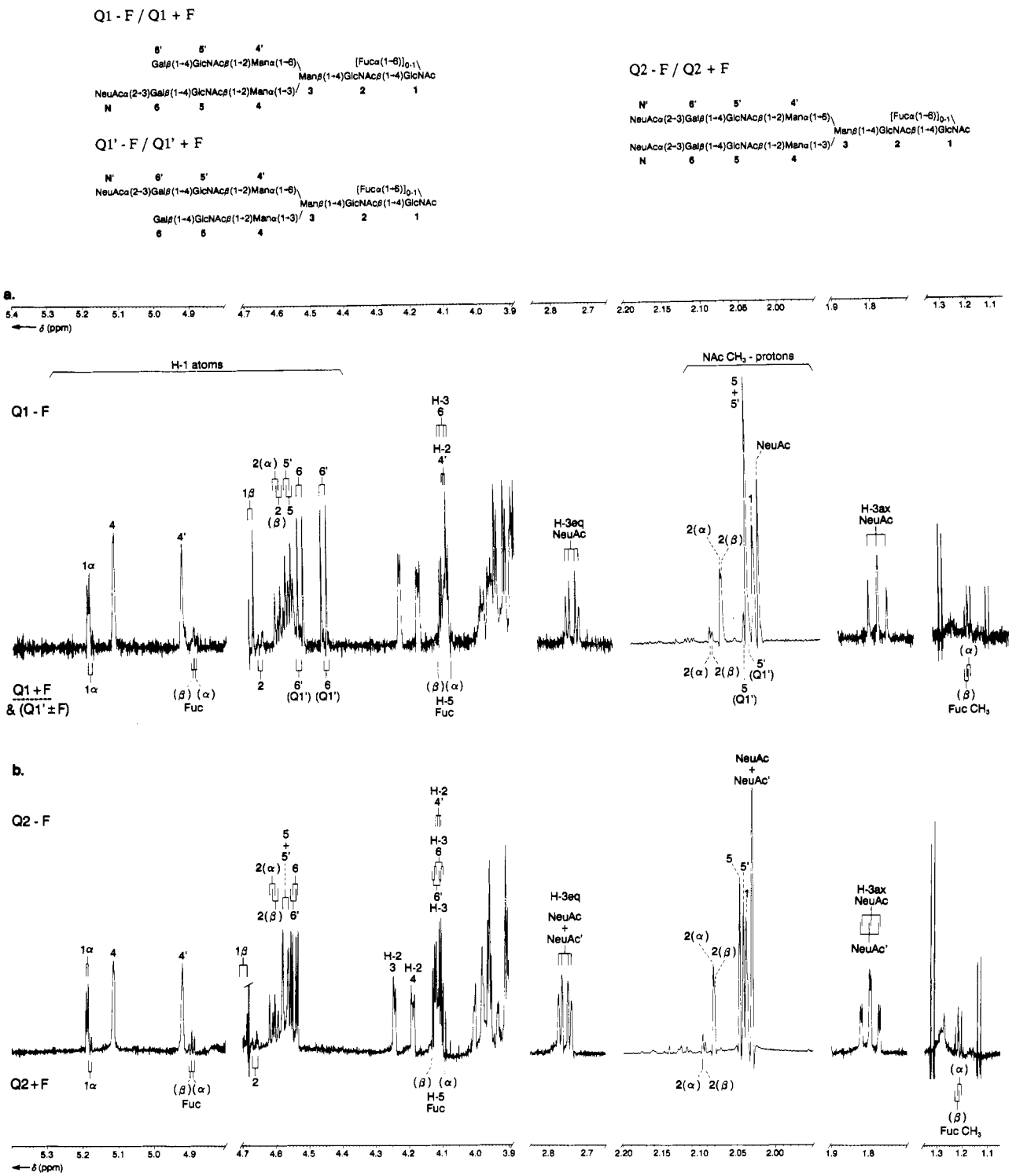


FIGURE 5: Structural reporter group regions of the 500-MHz ^1H NMR spectra (^2H_2O, p^2H 6, 27 °C) of the Mono Q separated mono- and disialyl oligosaccharides released from Asn-271 of rCD4 by PNGase F. (a) Fraction Q1, containing compounds Q1-F, Q1+F, Q1′-F, and Q1′+F in the ratio of 72:18:8:2; (b) fraction Q2, containing compounds Q2–F and Q2+F in the ratio of 4:1. The bold numbers above and below the spectra refer to the corresponding glycosyl residues in the structures (see also Table II). Assignments above the signals in the spectra refer to the major component in each mixture. The positions of the signals for the minor component(s) in the mixture are indicated below the spectra only when they differ from those for the major one. The NAc CH_3 singlets are shown at a different intensity than the other sections of the spectra.

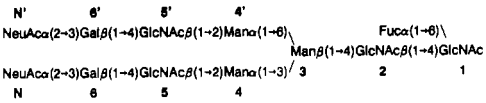
1986; Brockhausen et al., 1988; Spellman et al., 1989). For instance, the diantennary type of branching of the oligosaccharides was evident from the set of chemical shifts of the Man H-2 atoms [compare Table II with Spellman et al. (1989)].

Fraction Q0 was found to contain the fucosylated (Q0+F) and nonfucosylated (Q0–F) nonasaccharide in the ratio of 1:9. The presence of the Fuc residue in $\alpha(1\rightarrow6)$ linkage to the reducing GlcNAc-1 residue in ~10% of the molecules manifested itself in the NMR spectrum of the oligosaccharide by (i) the appearance of (low intensity) structural reporter group signals of the Fuc residue itself and (ii) influences on the chemical shifts of a small part of the reporter group signals of GlcNAc-1 and -2. The signals of Fuc H-1 were observable at δ 4.89 and 4.90 (for the α- and β-anomers of the reducing oligosaccharide Q0+F), those for H-5 at δ 4.095 (α) and 4.13

Table II: ^{1}H Chemical Shifts of Structural Reporter Groups of Constituent Monosaccharides for Oligosaccharides Released from Asn-271 of Recombinant Soluble Human CD4

reporter group	residue[a]	anomer of oligosaccharide	chemical shift[b] (ppm) in[c] Q0+F	Q0−F	Q1+F	Q1−F	Q1′±F[d]	Q2+F	Q2−F
H-1	GlcNAc-1	α	5.186	5.191	5.183	5.190	5.190	5.182	5.191
		β	4.70	4.70	4.696	4.696	4.696	4.697	4.697
	Fucα(1→6)	α	nd		4.889		*d*	4.893	
		β	nd		4.896		*d*	4.900	
	GlcNAc-2	α	nd	4.615	4.660	4.614	4.614	4.663	4.613
		β	nd	4.606	4.660	4.605	4.605	4.667	4.603
	Man-3	α,β	nd	nd	4.77	4.77	4.77	4.77	4.77
	Man-4	α,β	5.121	5.121	5.119	5.119	5.119	5.118	5.118
	Man-4′	α,β	4.927	4.927	4.928	4.928	4.926	4.924	4.924
	GlcNAc-5	α,β	4.585	4.585	4.575	4.575	4.583	4.573	4.573
	GlcNAc-5′	α,β	4.585	4.585	4.583	4.583	4.575	4.573	4.573
	Gal-6	α,β	4.469	4.469	4.544	4.544	4.467	4.544	4.544
	Gal-6′	α,β	4.474	4.474	4.474	4.474	4.550	4.550	4.550
H-2	Man-3	α,β	4.248	4.248	4.247	4.247	4.247	4.246	4.246
	Man-4	α,β	4.190	4.190	4.191	4.191	4.191	4.190	4.190
	Man-4′	α,β	4.110	4.110	4.108	4.108	4.108	4.114	4.114
H-3	Gal-6	α,β	nd	nd	4.113	4.113	nd	4.113	4.113
	Gal-6′	α,β	nd	nd	nd	nd	4.113	4.118	4.118
$H\text{-}3_{ax}$	NeuAc	α,β			1.796	1.796		1.796	1.796
	NeuAc′	α,β					1.799	1.800	1.800
$H\text{-}3_{eq}$	NeuAc	α,β			2.757	2.757		2.759	2.759
	NeuAc′	α,β					2.757	2.759	2.759
H-5	Fucα(1→6)	α	4.097		4.10			4.10	
		β	nd		4.13			4.13	
CH_3	Fucα(1→6)	α	1.209		1.210			1.212	
		β	1.220		1.220			1.222	
NAc	GlcNAc-1	α,β	2.039	2.039	2.039	2.039	2.039	2.039	2.039
	GlcNAc-2	α	2.095	2.082	2.096	2.082	2.082	2.096	2.082
		β	2.095	2.082	2.093	2.080	2.080	2.093	2.081
	GlcNAc-5	α,β	2.051	2.051	2.048	2.048	2.051	2.048	2.048
	GlcNAc-5′	α,β	2.049	2.049	2.048	2.048	2.045	2.043	2.043
	NeuAc	α,β			2.031	2.031		2.032	2.032
	NeuAc′	α,β					2.031	2.032	2.032

[a] The numbering system used for denoting glycosyl residues in the rCD4 Asn-271 oligosaccharides is as follows:



[b] Data were acquired at 500 MHz for neutral solutions of the compounds in ^2H_2O at 27 °C; nd = not determined. [c] Oligosaccharides were released by PNGase F; for complete structures, see Table IV. Structures are schematically illustrated in the table heading by using a short-hand symbolic notation [compare Spellman et al. (1989)]; ■ = GlcNAc; ● = Gal; ○ = Man; ▲ = NeuAc; □ = Fuc. The peripheral unit on the left corresponds to the glycosyl residues 5–6, the unit on the right to the 5′–6′ glycosyl residues. Dashed lines over the symbolic structures denote oligosaccharides analyzed within mixtures of two or more structures by NMR spectroscopy. [d] Structure Q1′ denotes a monosialyl diantennary oligosaccharide having its sialic acid residue attached to Gal-6′. Due to the relatively low abundance of Q1′ in the mixture with Q1, it could not be unequivocally inferred from the NMR spectrum whether compound Q1′ is fucosylated at GlcNAc-1. Chemical shifts here are listed for the oligosaccharide without fucose. Assuming the same ratio of fucosyl and non-fucosyl structures for Q1′ as for Q1 (i.e., 1:4), the structures Q1′+F and Q1′−F constitute 2% and 8% of the total mixture, respectively (compare to Table IV).

(β), and those for the CH_3 protons at δ 1.21 (α) and 1.22 (β); the assignment within each pair to the α- and β-anomer of Q0+F is based on the intensity ratio [$\alpha:\beta \simeq 2:1$; compare Spellman et al. (1989)]. Extending the chitobiose core by Fucα(1→6) at GlcNAc-1 apparently affected the chemical shifts of GlcNAc-2 H-1 (Δδ 0.055 ppm) and the GlcNAc-2 NAc protons (Δδ 0.013 ppm; Spellman et al., 1989), of GlcNAc-2, and of GlcNAc-1 H-1 in the α-anomer (Δδ −0.008 ppm). The latter effect has not been reported before; it became apparent in this study and was useful for determining the ratios of fucosylated and nonfucosylated compounds in the mixture Q0, and also for Q1 and Q2 (see below), along with the intensity ratio of the NAc signals of GlcNAc-2 at δ 2.096/2.093 for the fucosylated and δ 2.082/2.080 for the nonfucosylated compound (compare Table II).

Fractions Q1 and Q2 were found to contain monosialyl and disialyl diantennary oligosaccharides, respectively (see Table II). The sialylated oligosaccharides were extensions of the (fucosyl-)nonasaccharide (Q0±F), with one and two NeuAc residues attached to terminal Gal. NeuAc was found to be attached in α(2→3) linkage to Gal, as seen by the pair of NeuAc $H\text{-}3_{ax}$ and $H\text{-}3_{eq}$ signals (δ 1.80 and 2.76, respectively; Table II).

Fraction Q1 was found to be a mixture of four components, which differ from each other in the branch location of the NeuAc residue and/or the presence of Fucα(1→6). The branch location of the NeuAc residue was determined from the subtle differences in chemical shift between the NeuAc $H\text{-}3_{ax}$ signal in the C-3-linked (δ 1.796) versus the C-6-linked branch (δ 1.799) and the Gal H-1 signals (δ 4.544 versus 4.550 for sialyated Gal-6 versus Gal-6′), in conjunction with the downfield shift effect (Δδ −0.003 ppm) on the NAc signals of GlcNAc-5 and -5′ upon sialylation (Spellman et al., 1989). The ratio of structures with sialic acid in the C-3 versus C-6 branch (Q1±F versus Q1′±F) was 95:5, on the basis of the intensity ratios of the Gal-6 and Gal-6′ H-1 signals (at δ 4.544/4.474 versus 4.467/4.550). This ratio is confirmed by that of the NAc signals at δ 2.051, 2.048, and 2.044 (see Figure 5a). The ratio of fucosylated (Q1+F and Q1′+F) to nonfucosylated (Q1−F and Q1′−F) structures is 1:4, as deduced from the intensities of the GlcNAc-2 NAc signals at δ 2.096/2.093 versus 2.082/2.080 and from the intensities of

the GlcNAc-1 H-1 (α) signals at δ 5.183 versus 5.190 (see Figure 5a). Combination of the two ratios enabled us to determine the relative abundances of the four components in fraction Q1 (see Table IV).

The ^{1}H NMR spectrum of fraction Q2 (Figure 5b) showed that the disialyl fraction comprises two components that differ in the presence or absence of the fucosyl group at C-6 of GlcNAc-1. The major (>80%) component, Q2-F, is the di-α(2→3)-sialyl diantennary oligosaccharide without Fuc. The minor component in the mixture (Q2+F) has the additional Fuc residue in the core, as compared to Q2-F; its abundance was estimated to be $\simeq$20% from the intensity ratio of the GlcNAc-2 NAc signals at δ 2.096/2.093 compared to those at δ 2.082/2.080.

As described above, a trace of trisialyl oligosaccharides was also detected (elution times of 8 min in Figure 3A and 35 min in Figure 4a). This fraction was not present in quantities sufficient for methylation analysis or NMR spectroscopy. After desialylation and α-fucosidase digestion (Basa & Spellman, 1990), HPAE chromatographic analysis indicated that the trisialyl fraction comprised a mixture of 2,4-branched and 2,6-branched triantennary oligosaccharides ± fucose (results not shown).

The structures of the oligosaccharides released from Asn-271 and their relative abundances are summarized in Table IV. In general, the relative amounts listed there were calculated from the PAD detector response in the HPAE analyses and verified by integration of the ^{1}H NMR spectra. In instances where HPAE chromatography did not resolve two components (e.g., for monosialyl compounds Q1 and Q1′, differing in the branch location of sialic acid) the relative abundances were calculated from the NMR spectra.

Isolation and Characterization of the Oligosaccharides Attached to Asn-300. In preliminary experiments (Figure 2; Harris et al., 1990), Asn-300 had been found to carry a mixture of endo H susceptible and endo H resistant oligosaccharides. To isolate oligosaccharides for structure elucidation, a sample (960 nmol) of the purified Asn-300-containing glycopeptides was first treated with endo H. At the conclusion of digestion, reversed-phase HPLC was used to recover the released oligosaccharides (which were detected by HPAE chromatography of aliquots of the reversed-phase HPLC column fractions) and the endo H resistant portion of the glycopeptide (not shown). The endo H resistant Asn-300 glycopeptide was then treated with glycopeptidase A to release the remaining (*N*-acetyllactosamine-type) oligosaccharides, which were also recovered by reversed-phase HPLC. Reversed-phase HPLC analysis of aliquots from the digestion mixture was used to confirm that the glycopeptidase A digestion had gone to completion (not shown).

The endo H released oligosaccharides were subjected to methylation analysis (Table I), HPAE chromatography (Figure 6), and 500-MHz ^{1}H NMR spectroscopy (Figure 8a and Table III). The most notable aspect of the methylation analysis results (Table I) was the presence of terminal, 3-substituted, and 2-substituted mannose residues together with 3-substituted galactose residues. These results suggested that the endo-H-released oligosaccharides were hybrid-type structures. HPAE chromatographic analysis (Figure 6) and ^{1}H NMR spectroscopy (Table III) provided additional evidence that the endo H susceptible structures attached to Asn-300 were hybrid-type oligosaccharides. HPAE chromatography resolved the sample into two major components (EH-1 and EH-2), with retention times appropriate for monosialyl oligosaccharides. In addition, these compounds

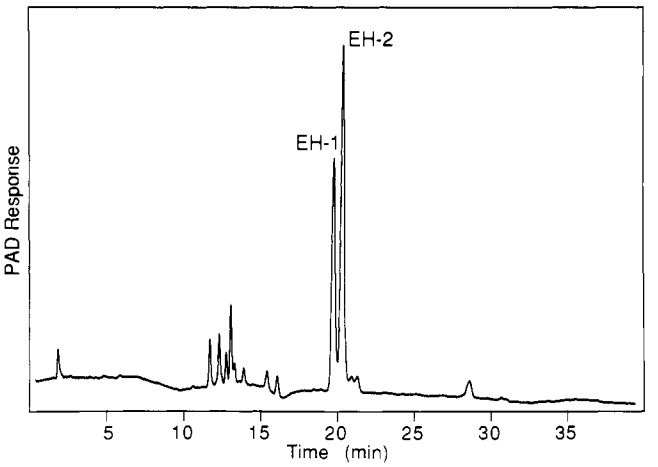


FIGURE 6: HPAE chromatography of oligosaccharides released from Asn-300 by endo H. Chromatography and detection conditions were as described under Experimental Procedures.

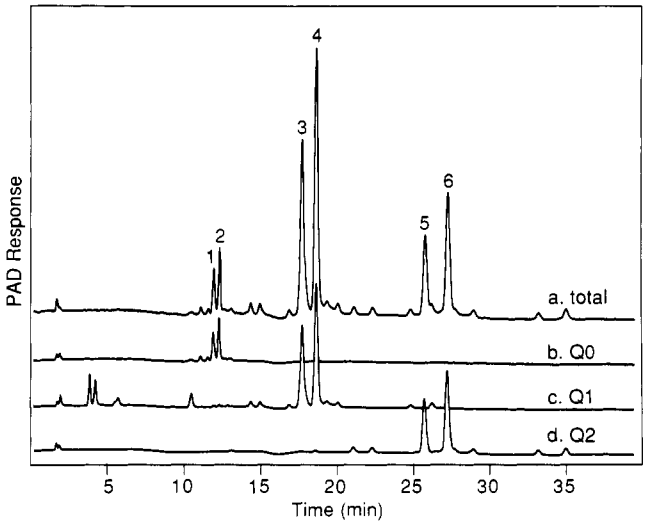
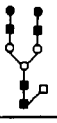
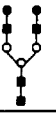
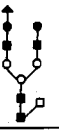
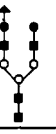
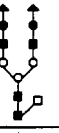


FIGURE 7: HPAE chromatography of *N*-acetyllactosamine oligosaccharides released from Asn-300 by glycopeptidase A. The glycopeptide had been treated with endo H and then repurified by reversed-phase HPLC prior to treatment with glycopeptidase A: (a) unfractionated oligosaccharides; (b) neutral fraction from Mono Q; (c) monosialyl fraction from Mono Q; (d) disialyl fraction from Mono Q.

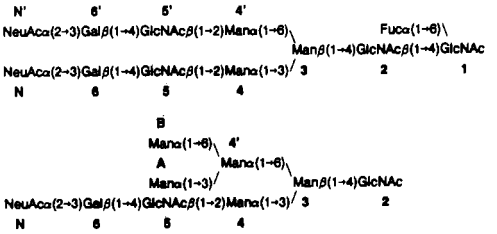
eluted with the same retention times as two hybrid-type oligosaccharides released by endo H from rt-PA (Spellman et al., 1989), with the earlier eluting peak (EH-1) corresponding to NeuAcGalGlcNAc$_2$Man$_4$ and the later eluting (EH-2) to NeuAcGalGlcNAc$_2$Man$_5$ (see Table IV for the detailed structures).

The oligosaccharides EH-1 and EH-2 were analyzed by 500-MHz ^{1}H NMR spectroscopy, in a mixture. The NMR spectrum of the mixture (Figure 8a) was found to be a superposition of the spectra of two hybrid-type oligosaccharides with structures EH-1 and EH-2 (see Table IV) in the ratio of 2:3. Those oligosaccharides have been isolated previously from another recombinant glycoprotein, rt-PA, and characterized in a pure state by 500-MHz ^{1}H NMR spectroscopy (Spellman et al., 1989). The chemical shifts of the structural reporter groups of compounds EH-1 and EH-2 isolated from rCD4 (see Table III) are in full agreement with those reported for the compounds isolated from rt-PA (Spellman et al., 1989). The ratio in which EH-1 and EH-2 occurred in the mixture of endo H released oligosaccharides from Asn-300 of rCD4 was deduced from the intensity ratio of the H-1 signals of the

Table III: ^{1}H Chemical Shifts of Structural Reporter Groups of Constituent Monosaccharides for Oligosaccharides Released from Asn-300 of Recombinant Soluble Human CD4

			chemical shift[b] (ppm) in[c]								
			EH-1	EH-2	Q0+F	Q0−F	Q1+F	Q1−F	Q1′±F[d]	Q2+F	Q2−F
reporter group	residue[a]	anomer of oligosaccharide									
H-1	GlcNAc-1	α			5.180	5.188	5.182	5.188	5.188	5.181	5.189
		β			4.70	4.70	4.695	4.695	4.695	4.699	4.696
	Fucα(1→6)	α			4.889		4.889		*d*	4.892	
		β			4.895		4.897		*d*	4.900	
	GlcNAc-2	α	5.253	5.248	4.66	4.612	4.665	4.613	4.613	4.665	4.612
		β	4.72	4.72	4.66	4.603	4.669	4.603	4.603	4.665	4.602
	Man-3	α,β	4.78	4.78	4.77	4.77	4.78	4.78	4.78	4.763	4.763
	Man-4	α,β	5.124	5.122	5.119	5.119	5.120	5.120	5.120	5.116	5.116
	Man-4′	α,β	4.898	4.877	4.925	4.925	4.928	4.928	4.928	4.922	4.922
	Man-A	α	5.094	5.079	*f*						
		β	5.123	5.105	*f*						
	Man-B	α,β		4.914	*f*						
	GlcNAc-5′	α,β	4.576	4.576	4.581	4.581	4.573	4.573	4.582	4.572	4.572
	GlcNAc-5′	α,β			4.581	4.581	4.582	4.582	4.573	4.572	4.572
	Gal-6	α,β	4.545[e]	4.545[e]	4.467	4.467	4.544	4.544	4.468	4.546	4.546
	Gal-6′	α,β			4.473	4.473	4.473	4.473	4.547	4.551	4.551
H-2	Man-3	α	4.256	4.256	4.247	4.247	4.248	4.248	4.248	4.245	4.245
		β	4.242	4.242	4.247	4.247	4.248	4.248	4.248	4.245	4.245
	Man-4	α,β	4.197	4.202	4.190	4.190	4.191	4.191	4.191	4.189	4.189
	Man-4′	α,β	4.136	4.146	4.108	4.108	4.106	4.106	4.106	4.112	4.112
	Man-A	α	4.050	4.050	*f*						
		β	4.067	4.067	*f*						
	Man-B	α,β		3.987	*f*						
H-3	Gal-6	α,β	4.114	4.114	nd	nd	4.115	4.115	nd	4.112	4.112
	Gal-6′	α,β			nd	nd	nd	nd	4.115	4.117	4.117
$H\text{-}3_{ax}$	NeuAc	α,β	1.796	1.796			1.797	1.797		1.796	1.796
	NeuAc′	α,β							1.797	1.800	1.800
$H\text{-}3_{eq}$	NeuAc	α,β	2.758	2.758			2.758	2.758		2.758	2.758
	NeuAc′	α,β							2.758	2.758	2.758
H-5	Fucα(1→6)	α			4.095		4.095			4.095	
		β			4.130		4.135			4.136	
CH_3	Fucα(1→6)	α			1.207		1.210			1.211	
		β			1.220		1.221			1.222	
NAc	GlcNAc-1	α,β			2.037	2.037	2.039	2.039	2.039	2.039	2.039
	GlcNAc-2	α	2.044	2.044	2.095	2.080	2.096	2.082	2.082	2.096	2.082
		β	2.044	2.044	2.091	2.080	2.093	2.080	2.080	2.092	2.080
	GlcNAc-5	α,β	2.050	2.050	2.050	2.050	2.047	2.047	2.051	2.047	2.047
	GlcNAc-5′	α			2.050[g]	2.050	2.047	2.047	2.045	2.043	2.043
		β			2.045[g]	2.045	2.047	2.047	2.045	2.043	2.043
	NeuAc	α,β	2.031	2.031			2.031	2.031		2.031	2.031
	NeuAc′	α,β							2.031	2.031	2.031

[a] The numbering system used for denoting glycosyl residues in the rCD4 Asn-300 oligosaccharides is as follows:



[b] Data were acquired at 500 MHz for neutral solutions of the compounds in ^2H_2O at 27 °C; nd = not determined. [c] Oligosaccharides EH-1 and EH-2 were released by endo H, the other compounds by glycopeptidase A. For complete structures of the compounds, see Table IV. Structures are schematically illustrated in the table heading by using a short-hand symbolic notation [compare Spellman et al. (1989)]; ■ = GlcNAc; ● = Gal; ○ = Man; ▲ = NeuAc; □ = Fuc. The peripheral unit on the left corresponds to the glycosyl residues 5–6, the unit on the right to the 5′–6′ glycosyl residues. [d] Structure Q1′ denotes a monosialyl diantennary oligosaccharide having its sialic acid residue attached to Gal-6′. Due to the relatively low abundance of Q1′ in the mixture with Q1, it could not be unequivocally inferred from the NMR spectrum whether compound Q1′ is fucosylated at GlcNAc-1. Chemical shifts here are listed for the oligosaccharide without fucose. Assuming the same ratio of fucosyl and non-fucosyl structures for Q1′ as for Q1 (i.e., 2:3), the structures Q1′+F and Q1′−F constitute 2% and 3% of the total mixture, respectively (compare Table IV). [e] A small portion (approximately 5%) of the mixture of EH structures had Gal-6 not sialylated. The H-1 doublet for Gal-6 in those structures was observed at δ 4.47. See Figure 8a. [f] The mixture Q0 may contain a small proportion of glycopeptidase A released hybrid structures; this is suggested by the occurrence of H1/H2 signals for Man-A at δ 5.091/4.051 and for Man-B at δ 4.918/3.99. It seems probable that these hybrid structures are fucosylated at GlcNAc-1; that might explain why they were less susceptible to endo H. [g] Tentative assignment, implying an anomerization effect on the NAc signal of GlcNAc-5′. The interpretation is based on the relative intensities of the signals at δ 2.050 and 2.045.

α-anomer of the reducing GlcNAc-2 (at δ 5.253 and 5.248, respectively) and also from the ratio of the H-1 signals of Man-A in the α-anomer of the respective oligosaccharides (at δ 5.094 and 5.079) (see Figure 8a).

As was described above, FAB-MS analysis of the peptide portion of the endo H digestion products revealed that approximately 9% of the endo H susceptible structures carried fucose attached to the reducing GlcNAc residue. We have no indication at this time whether the fucose residues are evenly distributed between the two hybrid structures characterized here or whether one of the structures is more heavily fucosylated than the other.

The endo H resistant oligosaccharides released from Asn-300 by glycopeptidase A were subfractionated by neutral-pH anion-exchange chromatography (Figure 3B) and resolved into neutral (Q0; 10%), monosialyl, (Q1; 61%), and disialyl (Q2;

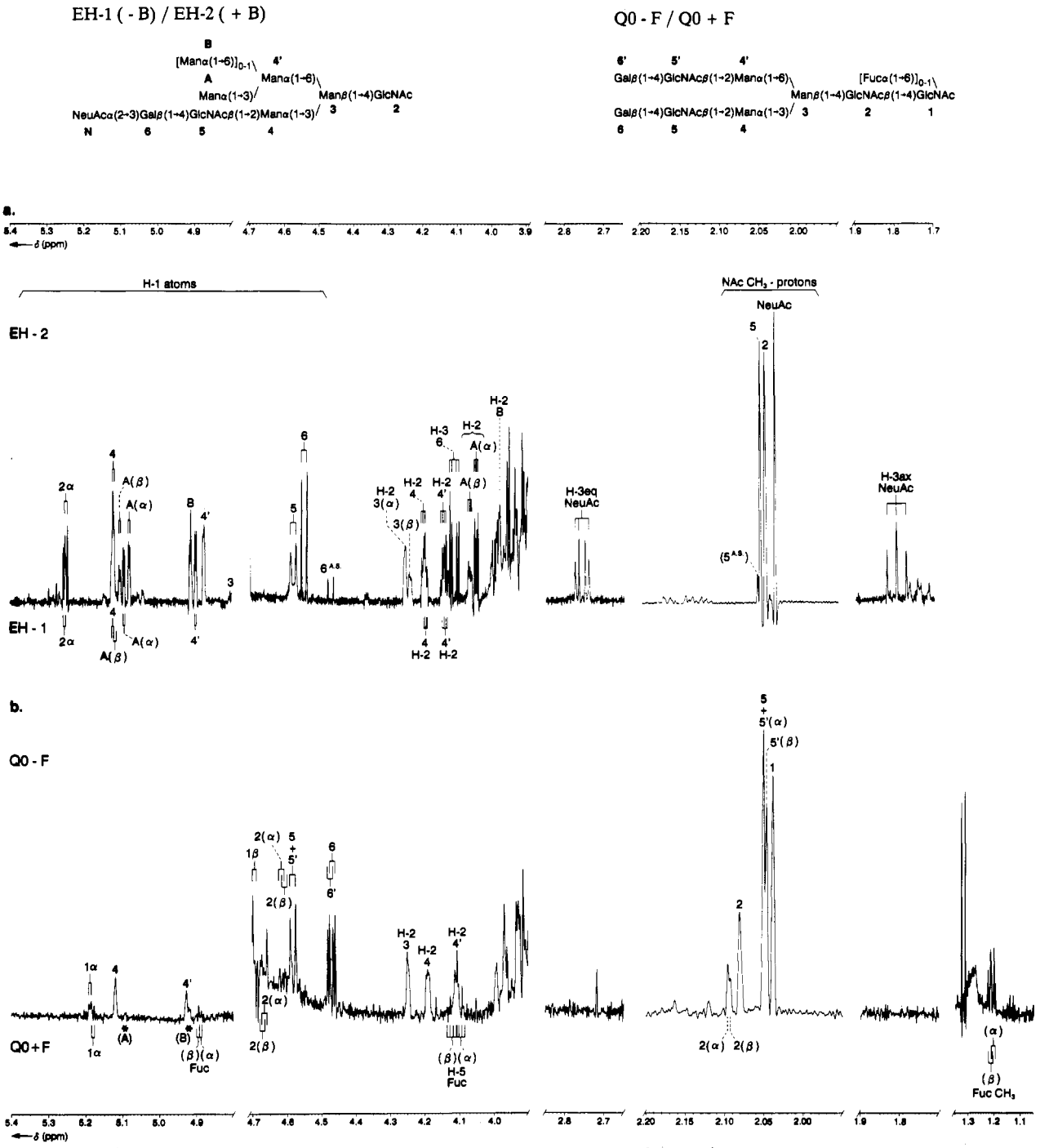
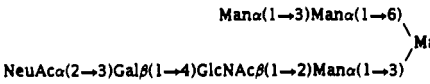
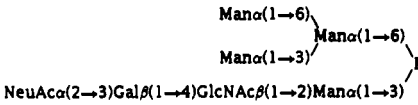
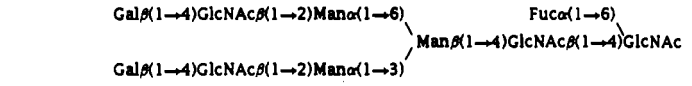
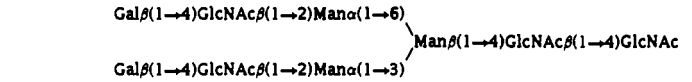
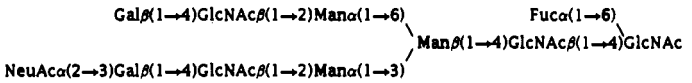
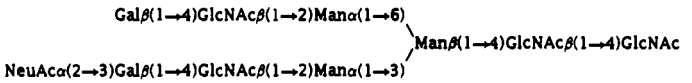
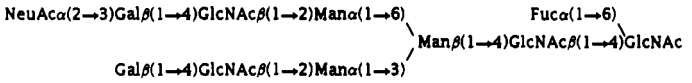
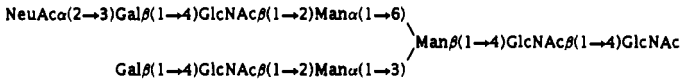
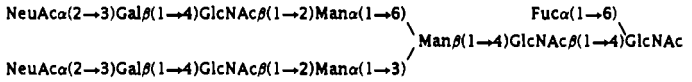
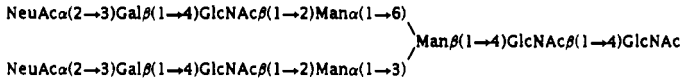


FIGURE 8: Structural reporter group regions of the 500-MHz ^1H NMR spectra (^2H_2O, p^2H 6, 27 °C) of the hybrid-type and neutral *N*-acetyllactosamine-type oligosaccharides released from Asn-300 of rCD4. (a) Fraction EH, released by endo H, containing compounds EH-1 and EH-2 in the ratio of 2:3; (b) fraction Q0, released by glycopeptidase A, containing compounds Q0–F and Q0+F in the ratio of 3:2. The bold numbers above and below the spectra refer to the corresponding glycosyl residues in the structures (see also Table III). Assignments above the signals in the spectra refer to the major component in each mixture. The positions of the signals for the minor component(s) in the mixture are indicated below the spectra only when they differ from those for the major one. The NAc CH_3 singlets are shown at a different intensity than the other sections of the spectra. The signals marked by A.S. in trace a indicate the presence of minute amounts (<3%) of asialo EH-1 and/or EH-2 in the mixture. The signals marked by asterisks in trace b are tentatively attributed to Man-A and Man-B H-1 signals, implying that a minute amount (~2%) of mixture Q0 might consist of neutral (endo H resistant) hybrid-type structures ending in reducing (fucosyl) chitobiose.

29%) fractions. The oligosaccharide components of these fractions were characterized by methylation analysis (Table I), HPAE chromatography (Figure 7), and 500-MHz ^1H NMR spectroscopy (Table III and Figure 8b), as described above for the *N*-acetylactosamine-type oligosaccharides released from the other glycosylation site. Like those isolated from Asn-271, the *N*-acetyllactosamine-type structures isolated from Asn-300 were found to consist of a mixture of diantennary structures differing in their degree of sialylation and fucosylation. Again, fractions Q0, Q1, and Q2 contained two, four, and two components, respectively. Like those released from Asn-271, the *N*-acetyllactosamine-type oligosaccharides released from Asn-300 contained a trace of trisialyl triantennary oligosaccharides (elution times of 8 min in Figure 3B and 35 min in Figure 7a). Once again, HPAE analysis of these oligosaccharides after desialylation and α-fucosidase treatment indicated that they consisted of 2,4-branched and 2,6-branched triantennary oligosaccharides ± fucose (results not shown).

Table IV: Structures of the Asn-Linked Oligosaccharides Isolated from rCD4

oligosaccharide	structure	rel abundance at Asn-271	rel abundance at Asn-300
EH-1		--	13.6
EH-2		--	20.6
Q0+F		0.8	2.9
Q0-F		6.8	3.6
Q1+F		9.5	16.3
Q1-F		43.2	21.5
Q1'+F		0.5	0.9
Q1'-F		2.3	1.1
Q2+F		6.7	7.3
Q2-F		30.2	11.9

The structures of the oligosaccharides released from Asn-300 and their relative abundances are summarized in Table IV.

Discussion

The carbohydrate structures of soluble rCD4 expressed in CHO cells have been determined and are summarized in Table IV. Asn-271 carries exclusively *N*-acetyllactosamine-type structures comprising fucosylated and nonfucosylated forms of asialo, monosialyl, and disialyl diantennary structures. The same eight *N*-acetyllactosamine-type structures accounted for 66% of the carbohydrates at Asn-300 but were present in different relative abundances. Approximately 40% of the *N*-acetyllactosamine-type structures attached to Asn-300 were found to contain fucose $\alpha(1\rightarrow6)$ linked to the reducing GlcNAc residue, whereas only 18% of the structures attached to Asn-271 were fucosylated. Among the monosialyl *N*-acetyllactosamine-type structures isolated from both glycosylation sites, some 80% were sialylated in the Man$\alpha(1\rightarrow3)$ branch versus 20% in the Man$\alpha(1\rightarrow6)$ branch. Both glycosylation sites were also found to carry a trace (<1%) of trisialyl oligosaccharides. HPAE analysis after desialylation indicated that, at both sites, the trisialyl structures consisted of a mixture of 2,4-branched and 2,6-branched triantennary oligosaccharides with and without fucose. Traces (<1%) of mono- and disialyl triantennary structures were also detected by HPAE of desialylated aliquots of fractions Q1 and Q2 from both glycosylation sites (results not shown).

In addition to *N*-acetyllactosamine-type structures, Asn-300 was found to carry hybrid structures that accounted for approximately one-third of the carbohydrates attached to this position. Our identification of the hybrid-type oligosaccharides EH-1 and EH-2 at Asn-300 confirms and extends the earlier observation (Harris et al., 1990) that some of the structures at this glycosylation site are endo H susceptible. FAB-MS analysis of the endo H digestion products of the Asn-300 glycopeptide revealed that 9% of the hybrid structures were fucosylated on the reducing GlcNAc residue (presumably by substitution of its 6-position). When fucosylation is taken into

account, there are potentially four different hybrid structures attached to Asn-300. Fucosylated hybrid structures have been identified previously as glycosylation products of human fibroblasts grown in the presence of swainsonine (Arumugham & Tanzer, 1983; Bozon et al., 1986) and as a major component of the Asn-linked oligosaccharides of bovine interphotoreceptor retinol binding protein (Taniguchi et al., 1986). It has also been demonstrated previously that fucosylated hybrid structures remain susceptible to endo H digestion (Arumugham & Tanzer, 1983; Bozon et al., 1986).

The oligosaccharides attached to Asn-271 and Asn-300 in rCD4, listed in Table IV, have been obtained and characterized previously from other glycoproteins, either as such (reducing oligosaccharides) or in the form of glycopeptides or reduced oligosaccharides.[2] While the *N*-acetyllactosamine-type structures are quite common in natural and recombinant glycoproteins, the hybrid structures are rare. Structures EH-1 and EH-2 have been reported before for recombinant gp120 (Mizuochi et al., 1988) and rt-PA (Spellman et al., 1989), both expressed in CHO cells.

Carr et al. (1989) have reported partial elucidation of the carbohydrate structures of another CHO-expressed soluble CD4 molecule. From the results of FAB-MS and methylation analyses, they concluded that both Asn-271 and Asn-300 carried nearly identical families of diantennary *N*-acetyllactosamine-type structures containing zero to one residues of fucose and zero to two residues of sialic acid. Minor components (possibly triantennary oligosaccharides) with three sialic acid residues were also detected. Our results are in agreement with those of Carr et al. concerning the prevalent *N*-acetyllactosamine-type oligosaccharides at the two glycosylation sites of CHO-expressed rCD4. Our use of ^{1}H NMR spectroscopy has enabled us, further, to assign anomeric configurations to the glycosyl residues of all structures and to determine the extent of sialylation of the two branches of the monosialyl *N*-acetyllactosamine-type structures. This study demonstrates that high-field ^{1}H NMR spectroscopy is an invaluable method, when used in conjunction with methylation analysis, HPAE, and FAB-MS, for complete and reliable structure determination of the carbohydrate structures of (recombinant) glycoproteins.

The most notable difference between our results and those of Carr et al. is our identification of the hybrid oligosaccharides (EH-1 and EH-2, Table IV) that account for one-third of the carbohydrate attached to Asn-300 of the material examined here. The reason for this difference is not clear. The difference may arise from the different analytical methods used in the two studies (Carr et al. did report the presence of terminal Man in the methylation analysis of their material, which was not accounted for in the structures that they reported). It is also possible that the differences in carbohydrate structures are real and arise from the slightly different polypeptides (different N- and C-termini) studied by Carr et al. and by us or from differences in cell-culture conditions, purification methods, or other environmental influences (Goochee & Monica, 1990).

[2] The CCSD identification numbers (Doubet et al., 1989) for the oligosaccharides are as follows: EH-1, 2805.08024EA2; EH-2, 2853.0903241E; Q1+F, 2911.0B032B4E; Q1-F, 2872.0A02F11F; Q1′+F, 2917.0B038D7D; Q2+F, 2928.0C030C28; Q2-F, 2893.0B022F46. The CCSD number is a unique identifier for the given structure. Structures Q0+F, Q0-F, and Q1′~F are not listed in the latest CCSD release (CCSD0002, Aug 1990). These structures have, however, been characterized previously, in the form of either glycopeptides or reduced oligosaccharides.

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Registry No. EH-1, 118045-68-4; EH-2, 118071-47-9; Q0+F, 78392-81-1; Q0-F, 71496-53-2; Q1+F, 113799-69-2; Q1-F, 113799-70-5; Q1′+F, 121283-16-7; Q1′-F, 118074-19-4; Q2+F, 93375-83-8; Q2-F, 93395-38-1; L-asparagine, 70-47-3.

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Control of Insulin Receptor Autophosphorylation by Polypeptide Substrates: Inhibition and Stimulation by Interaction with the Catalytic Subunit†

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ABSTRACT: Autophosphorylation of purified insulin receptor, in the absence of insulin, was stimulated by selected polypeptide substrates. In the presence of 1 μM insulin these peptides inhibited autophosphorylation. Stimulation was observed with reduced [*S*-(carboxamidomethyl)cysteinyl]lysozyme (RCAM-lysozyme) and three peptides generated by CNBr cleavage, V8 proteinase digestion, and/or chemical modification. We also generated two peptide substrates from RCAM-lysozyme which did not stimulate receptor autophosphorylation and were very weak inhibitors. As a control peptide, the simple substrate angiotensin inhibited receptor autophosphorylation in the absence or presence of insulin. However, stimulatory peptide, but not insulin, significantly shifted the concentration dependence for inhibition by angiotensin. The stimulatory peptides also increased autophosphorylation of the cloned cytoplasmic domain of the kinase [R-BIRK; Villalba, M., Wente, S. R., Russell, D. S., Ahn, J., Reichelderfer, C. F., & Rosen, O. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7848]. Therefore, stimulation occurs by interaction with the cytoplasmic process of the β-subunit and not through interaction with the insulin binding α-subunit of the native receptor. Autophosphorylation was analyzed by mapping ^{32}P-labeled tryptic phosphopeptides from the β-subunit and from R-BIRK. Nearly identical phosphopeptide maps were found, comparing first, basal R-BIRK and basal native receptor, second, peptide- and insulin-stimulated native receptor, and third, peptide-stimulated R-BIRK and insulin-stimulated native receptor. Therefore, R-BIRK functions as a basal-state enzyme and can be stimulated in an insulin-like manner. On the basis of these observations, stimulation by insulin and by peptides yields similar functional results, but by apparently different mechanisms.

The insulin receptor is a protein-tyrosine kinase with a native $\alpha_2\beta_2$ disulfide-linked structure. The α-subunit binds insulin, and the β-subunit bears the protein kinase activity. Insulin stimulates autophosphorylation of the β-subunit in intact cells (Klein et al., 1986; Kohanski, et al., 1986; Pang et al., 1985). After insulin-stimulated autophosphorylation in vitro, exogenously added polypeptide substrates are phosphorylated at an increased rate, compared to the aporeceptor (Rosen et al., 1983; Herrera & Rosen, 1986; Tornqvist & Avruch, 1988).

Autophosphorylation is an intrinsic reaction of the insulin receptor. A large body of evidence supports a biological role for autophosphorylation, and possibly for substrate phosphorylation as well [these properties are reviewed comprehensively by Rosen (1987)]. There is also evidence that challenges this view (Simpson & Hedo, 1984; Debant et al., 1989; Hawley et al., 1989; Baron et al., 1989; Soos et al., 1989) or suggests alternative pathways not dependent on protein kinase activity (Kelly et al., 1986; Saltiel & Cuatrecassas, 1986; Saltiel et al., 1986; Witter et al., 1988).

A major issue concerning the autophosphorylation reaction is the mechanism of insulin activation. To delineate a formal

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