themselves activate ATPase which is normally protected from the ionic environment and/or whether the energy process itself may induce a conformational change of the ATPase necessary for the expression of its activity remains unanswered.

Acknowledgment

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The Attachment Site of Carbohydrate in a Mouse Immunoglobulin Light Chain*

Fritz Melchers

ABSTRACT: An immunoglobulin light chain produced and secreted by a plasma cell tumor in mice (MOPC 46) is unusual in containing about 12% by weight of carbohydrate covalently attached to an asparagine or aspartic acid residue in the polypeptide chain. Glycopeptides from tryptic and chymotryptic digests of this light chain have been purified. Three forms each of the tryptic and the chymotryptic peptide, to which carbohydrate is attached, can be separated. Analysis of these glycopeptides give the unique sequence for all three forms:

carbohydrate

(Ser, Cys)-Arg-Ala-Ser-Gln-Asx-Ile-Ser-Asn-Asn-Leu-His-Trp-Tyr-Gln-Gln-Lys. Comparison of this to other sequences of mouse light chains (Gray, W., Dreyer, W., and Hood, L. (1967), *Science 155*, 465) suggests the attachment site of carbohydrate to be residue 28 within the variable part of the light chain using the numbering of mouse 41.

Immunoglobulins are glycoproteins formed of heavy chains of mol wt 55,000, and of light chains of mol wt 23,000. Carbohydrate is usually attached to a site in the carboxy-terminal half of the heavy chain, while the light chains seem not to contain carbohydrate (Fleischmann *et al.*, 1962; Edelman and Gally, 1964).

Recently we reported that the light chain produced and secreted by the mouse plasma cell tumor MOPC 46 does contain carbohydrate. The monomeric form of the light chain appears in three forms containing either two, one, or zero molecules of sialic acid (Melchers *et al.*, 1966). We show in this paper that, from tryptic or chymotryptic digests of the three forms of the light chain, three tryptic or three chymotryptic glycopeptides can be obtained. We have determined the amino acid

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to which the carbohydrate group is attached and can localize this attachment point within the variable portion of the light chain (Hilschmann and Craig, 1965; Gray *et al.*, 1967).

Materials and Methods

The plasma cell tumor, MOPC 46, was given to us by Dr. Michael Potter, National Institutes of Health, Bethesda, Md., in passage 30 and is now in passage 56. Light chain was purified, as described previously (Melchers *et al.*, 1966), from the urine of Balb/C mice carrying this tumor subcutaneously. Light chain labeled with either [^{14}C]glucose or [^{3}H]leucine was prepared by incubation of a cell suspension in Eagle's medium without added glucose or leucine, respectively. Of the [^{14}C]glucose incorporated, 85–90% is found in the carbohydrate portion of the light chain (F. Melchers and E. S. Lennox, manuscript in preparation). After purification, the protein was reduced, treated with iodoacetamide or ethylenimine, then digested with trypsin or chymotrypsin.

Trypsin was the twice trichloroacetic acid precipitated enzyme from Worthington previously incubated for 24 hr in 0.16 N HCl at 37° to diminish chymotryptic activity (Wittmann, 1962). Tryptic digestion was done for 16 hr at 37° in 0.1 M triethylammonium carbonate (pH 8.5) in a 1:100 w/w ratio of enzyme to protein or in a 1:10 w/w ratio of enzyme to peptide.

Chymotrypsin (Boehringer) was pretreated with 10^{-3} M 1-chloro-3-tosylamido-7-amino-2-heptanone in 0.025 M ammonium acetate (Shaw *et al.*, 1965). Tryptic activity of the treated enzyme on the synthetic substrate benzoylarginine ethyl ester was about 2% that of an equivalent weight of trypsin. Chymotryptic digestion was done in 0.05 M ammonium acetate (pH 8.0)–10^{-2} M calcium acetate at 37° for 15 min, 2 hr, or 16 hr in a 1:50 w/w ratio of enzyme to protein or in a 1:15 w/w ratio of enzyme to peptide.

Digestion with Pronase (Calbiochem) was done at 37° for 48 hr in 0.05 M ammonium acetate (pH 8.0)–10^{-2} M calcium acetate. Enzyme amounting in total to a 1:5 w/w ratio enzyme to peptide was added in equal portions at 0, 20, and 40 hr.

Mercuripapain (Sigma Chem. Co.) was activated by 10^{-2} M mercaptoethanol. The glycopeptides were digested in 0.05 M potassium phosphate buffer (pH 7.5) at 37° for 2 hr with activated papain in a 1:50 ratio of enzyme to glycopeptide.

Aminopeptidase M (Röhm und Haas, Darmstadt, Germany) and carboxypeptidases A-DFP and B-DFP (Worthington) were used in a 1:50 enzyme to glycopeptide ratio in H_2O adjusted to pH 7.8. The reactions were carried out at 37° and stopped by adjusting the pH to 2 with acetic acid. The reaction mixture was analyzed on the amino acid analyzer for released amino acids.

Paper electrophoresis of peptides was carried out at pH 6.1 (pyridine–glacial acetic acid–H_2O, 10:1:69, v/v) or pH 2.1 (formic acid–glacial acetic acid–H_2O, 2:8:90, v/v) under cooled Varsol/1 (Humble Oil Co.). Paper chromatography of peptides was done in system A (1-butanol–pyridine–glacial acetic acid–H_2O, 488:756:152:604, v/v; Weigert and Garen, 1965). For chromatography and electrophoresis Whatman No. 3MM paper was used. Peptides were eluted from paper with pH 6.1 electrophoresis buffer diluted 1:10 and desalted by lyophilization. Hydrolysis of peptides was carried out *in vacuo* for 24 hr at 110° in constant-boiling HCl previously flushed with nitrogen. Assay of amino acids was done with the Beckman 120 C amino acid analyzer, using a range expander to give full-scale deflection for 0.1 optical density unit. Glucosamine was assayed by the method of Walborg *et al.* (1963) using the amino acid analyzer.

Neutral hexoses were detected by the procedure of Dubois *et al.* (1956) modified as follows. Toluene was used instead of phenol in the reaction mixture, giving an absorption spectrum of the color developed with a maximum around 460 mμ (fucose λ_{max} 459.5 mμ (ϵ 1.35 × 10^3), galactose λ_{max} 464.5 mμ (ϵ 1.90 × 10^3), and mannose λ_{max} 465.5 mμ (ϵ 2.75 × 10^3). ϵ of the MOPC 46 glycopeptide was calculated from the neutral hexose composition of the light chain (Melchers *et al.*, 1966) to be 2.13 × 10^3.

Glycopeptides were located on paper by analysis of ninhydrin-positive spots for their glucosamine content on the amino acid analyzer. Since the glycopeptides contain tryptophan and tyrosine (see Results), they could also be detected by their ultraviolet fluorescence under an ultraviolet lamp. Glycopeptides were located in Dowex column eluates by the modified Dubois method for the determination of neutral hexoses.

Digestion with neuraminidase was carried out in 0.05M sodium acetate (pH 5.5)–10^{-2} M $CaCl_2$ using 100 receptor-destroying units/2 mg of glycopeptide. Neuraminidase preparated from *Cholera vibrio* was a gift from Dr. S. Fazekas de St. Groth.

Stepwise removal of amino acids from the N terminus of the glycopeptides was performed using the three-cycle form of the phenyl isothiocyanate procedure of Edman (Blombäck *et al.*, 1966) as modified by Doolittle *et al.* (1967). The resulting phenylthiohydantoin amino acids were identified by thin-layer and paper chromatography in the usual systems D and E of Edman and Sjöquist (1956) and in the chloroform–formic acid (96:5, v/v) system described by Randerath (1963). After each of the first three steps of the Edman procedure, the amino acid composition of the residual peptide was determined on the amino acid analyzer.

Radioactive samples were counted in Bray's (1960) solution containing 4% Cab-O-Sil in a Nuclear-Chicago scintillation counter. Radioactivity on paper was detected by stapling the paper to Eastman Kodak Blue Brand X-Ray film and developing the film after suitable periods of exposure.

Results

The Purification of Glycopeptides from Tryptic and Chymotryptic Digests of the Light-Chain Protein. Purification of small amounts of glycopeptides (100–500 nmoles) could be achieved by two-dimensional chromatography and electrophoresis at pH 6.1. This yielded

TABLE I: Composition of Purified Tryptic and Chymotryptic Glycopeptides.[a]

	Purification on Paper						Purification on Columns						
	Major Tryptic Peptides			Minor Tryptic Peptides			Chymotryptic Peptides				Tryptic Peptides		
	T_I	T_{II}	T_{III}	T'_I	T'_{II}	T'_{III}	C_I 15 min	C_{II} 15 min	C_{III} 15 min	C_{II} 16 hr	T_I	T_{II}	T_{III}
Cys							1.0	1.0	0.8	0.2			
Asp	2.8	2.7	2.7	3.4	3.2	3.4	3.0	3.3	3.1	3.0	3.1	3.3	3.2
Ser	1.9	1.8	1.9	2.1	2.2	2.2	3.1	3.3	3.0	2.2	2.1	2.3	2.1
Glu	2.7	2.6	2.7	3.3	3.2	3.1	1.2	1.3	1.2	0.8	3.2	3.4	3.3
Ala	1.1	0.8	0.9	1.0	0.9	0.9	1.0	1.1	1.1	1.1	1.1	1.1	1.0
Ile	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Leu	1.1	0.9	1.0	1.1	0.9	1.0	1.0	1.0	0.9	1.1	1.1	1.0	1.0
Tyr	0.8	0.8	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.1	0.7	0.8	0.7
Glucosamine[b]	2.4	2.4	2.5	2.7	2.6	2.7	2.5	2.6	2.6	2.5	2.8	2.7	2.9
Lys	1.1	0.9	1.0	1.1	1.0	0.9					1.0	1.1	1.0
His	0.9	0.8	0.9	0.9	0.8	0.9	1.0	1.0	0.9	1.0	0.9	1.0	0.9
Arg							1.0	1.1	1.2	0.3			
Trp[c]	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)

[a] Given in number of residues per isoleucine residue assuming one Ile residue per glycopeptide. [b] A total of 6 moles of glucosamine has been found per mole of MOPC 46 light chain (Melchers *et al.*, 1966). Under the conditions of hydrolysis used for amino acid analysis, approximately 50–60% of the glucosamine is destroyed. [c] Determined by the method of Smith (1953).

three glycopeptides which were further purified by electrophoresis at pH 2.1. Figure 1 shows the purification of the glycopeptide from a tryptic digest of reduced and aminoethylated light chain. The glycopeptides from chymotryptic digests had mobilities in chromatography and electrophoreses nearly identical with those of the tryptic glycopeptides. Table I gives the amino acid composition, Table II the recoveries in amino acids and neutral hexoses of the three purified glycopeptides from tryptic and chymotryptic digests. From each of the three major tryptic glycopeptides, α, β, and γ, a minor component could be separated by electrophoresis at pH 2.1 (Figure 1), which had an amino acid composition and glucosamine content very similar to the major glycopeptides (Table I). The reason for the difference in electrophoretic mobility between the major and minor glycopeptides is not known. A possible explanation could be the deamination of asparagines or glutamines within the amino acid sequence during the purification procedure rendering the glycopeptide more negative. Deacetylation of *N*-acetylglucosamine residues of the glycopeptide would change the charge of the peptide to more positive values and can therefore not be taken as an explanation.

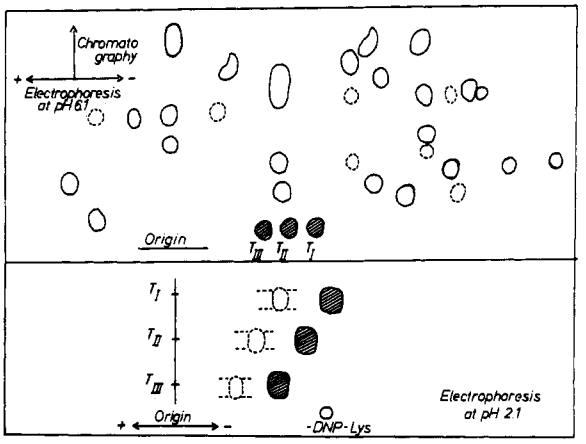


FIGURE 1: Purification of glycopeptides α, β, and γ from a tryptic digest of aminoethylated MOPC 46 light chain on Whatman No. 3MM paper. After chromatography and electrophoresis at pH 6.1 glycopeptides α, β, and γ were located by their strong ultraviolet absorption, eluted from paper, and reapplied onto paper for electrophoresis at pH 2.1. Other peptides were spotted by developing the paper with 0.03% ninhydrin.

Larger amounts of glycopeptides (10–50 μmoles) were prepared by column chromatography on Dowex 1-X2 and purified subsequently on Dowex 50-X4 (Figure 2). Glycopeptides I, II, and III had identical mobilities in chromatography and electrophoreses to those α, β, and γ obtained by separation on paper (Figure 1) and showed identical amino acid composition (Table I).

The amounts of neutral hexoses and glucosamine recovered in the glycopeptides (Table II) indicate that possible other points of attachment for carbohydrate do not contain more than 10% of the total amount of neutral hexoses and glucosamines. The amounts of glycopeptides recovered from the glycoprotein show that at least 85% of the total population of MOPC 46 light chains have a full carbohydrate complement.

From the analyses of the glycopeptides from tryptic and chymotryptic digests, the following arrangement

TABLE II: Recoveries of Purified Tryptic and Chymotryptic Glycopeptides.

MOPC 46 Protein	Purification on Paper		Purification on Column
	Tryptic Digest	Chymotryptic Digest	Tryptic Digest
Total input of MOPC 46 protein	11 mg = 487 nmoles[a]	11 mg = 487 nmoles[a]	1 g = 44.5 μmoles[a]
Total recovery, nmoles (%)	428 (88)	426 (87.5)	41.6 (94)
Neutral hexoses in MOPC 46 protein			
Total input (μmoles)	4.87[b]		445[b]
Total recovery, μmoles (%)	4.60,[b] 95		405, 91

[a] Assuming one Ile residue per glycopeptide. [b] Assuming a molecular weight of 22,500 and 1 mg = 1.5 OD_{280}.

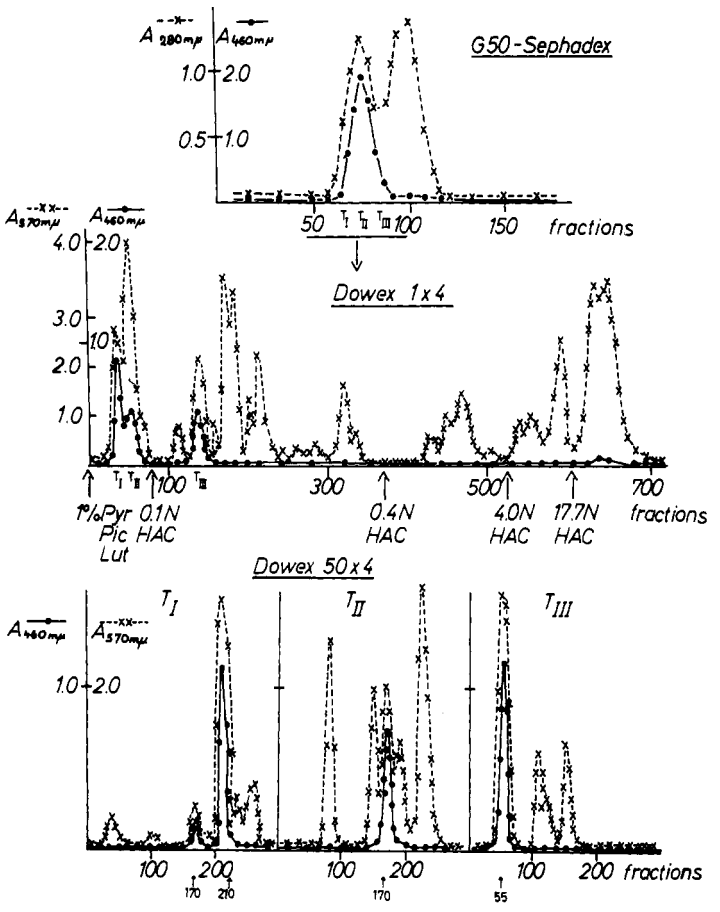


FIGURE 2: Purification of glycopeptides I–III from a tryptic digest of 500 μmoles of alkylated MOPC 46 light chain. The soluble tryptic peptides were separated according to their molecular weight on a 1 × 200 cm Sephadex G-50 column in 0.05 M NH_4OH (a, aliquot of approximately 250 mg of digest). Peptides were measured by their $A_{280\ m\mu}$ due to their ultraviolet absorption, neutral hexose content by $A_{460\ m\mu}$ of the toluene–H_2SO_4 reaction. Peptides containing hexose-positive material (fractions 51–90 from G-50 Sephadex) were chromatographed on a 0.9 × 100 cm Dowex 1-X2 (200–400 mesh) column at 37° (b). A pH gradient similar to that described by Hilschmann and Craig (1965) was used for elution. Peptides containing neutral hexoses were detected by the toluene–H_2SO_4 reaction ($A_{460\ m\mu}$). Peptide fractions I–III containing neutral hexoses were rechromatographed individually on 0.9 × 100 cm Dowex 50-X4 (200–400 mesh) columns. The fractions were applied to 0.2 M pyridine acetate (pH 3.1) and the columns were washed with the same buffer. Around fraction 100 a linear gradient of 325 ml each of 0.2 M pyridine acetate (pH 3.1) and 0.7 M pyridine acetate (pH 5.8) was started resulting in a salt and pH gradient. Peptides were detected as described for the Dowex 1-X2 column chromatography. The volume of fractions of all chromatographies was 3.6 ml. All peptide fractions were desalted by flash evaporation and lyophilization.

for the amino acids around the attachment point of carbohydrate can be deduced: (Ser, Cys)-Arg-(Asx_2, Asx-carbohydrate, Ser_2, Glu_3, Ala, Leu, Ile, Tyr, His, Trp)-Gln-Gln-Lys.[1]

The Attachment Site of the Carbohydrate Group to the Light-Chain Protein. In order to obtain the amino acid to which the carbohydrate group of MOPC 46 light chain is attached, a mixture of glycopeptides T_I, T_{II}, and T_{III} containing [^{14}C]carbohydrate and [^{3}H]leucine was digested with pronase. The digest was separated on a column of G-10 Sephadex (Figure 3). A 10-nmole aliquot of tryptic glycopeptide gave 30,000 cpm of ^{3}H and 2200 cpm of ^{14}C. Carbohydrate as indicated by ^{14}C radioactivity (fraction I) was well separated from [^{3}H]leucine (fraction II). Pronase was removed from fraction I by passage over G-25 Sephadex in 0.05 M ammonium acetate (pH 5.5). Fractions with ^{14}C radioactivity were pooled and lyophilized.

[1] Asx (Glx) is used whenever it has not been determined whether aspartic acid or asparagine (glutamic acid or glutamine) is present in the polypeptide chain. Peptides designated T are glycopeptides obtained by trypsin digestion of reduced and aminoethylated MOPC 46 light chain, peptides C are glycopeptides obtained from the same protein by chymotrypsin digestion. Peptides designated TC result from chymotryptic digestion of T; CT result from tryptic digestion of C.

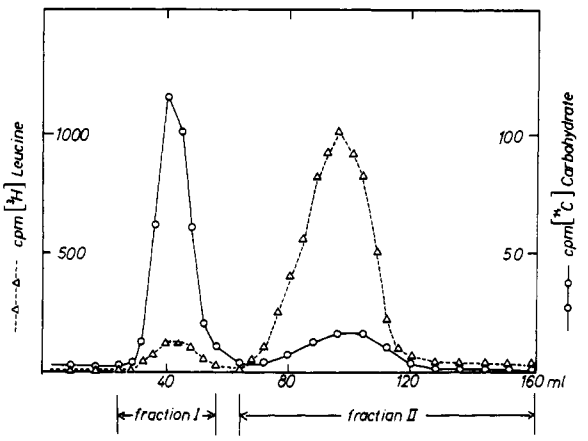


FIGURE 3: Separation on G-10 Sephadex column of Asx-[^{14}C]carbohydrate (—○—○—) from [^{3}H]leucine (—△—△—) released from MOPC 46 light-chain tryptic glycopeptide by 48-hr Pronase digestion. Column was 2.9 × 11 cm; buffer was 0.025 M ammonium acetate (pH 8).

Table III shows the amino acid composition of fraction II, and of fraction I after purification over G-25 Sephadex. These results indicate that the amino acid to which carbohydrate is covalently linked is asparagine or aspartic acid.

The Amino Acid Sequence around the Attachment Site of Carbohydrate. A mixture of the glycopeptides T_I, T_{II}, and T_{III} was digested with chymotrypsin while a mixture of the glycopeptides C_I, C_{II}, and C_{III} was digested with trypsin. The digests were separated on paper in two dimensions by chromatography and electrophoresis at pH 6.1 (Figure 4a,b). Table IV gives the amino acid compositions of the separated peptides obtained by digestion of the individual glycopeptides T_I, T_{II}, and T_{III}, and C_I, C_{II}, and C_{III}. Peptides CT_I and TC_I, CT_{II} and TC_{II} as well as CT_{II} and TC_{II} have identical electrophoretic mobilities. All six peptides show the same amino acid composition and contain carbohydrate (Table IV). This indicates that all six peptides might have the same amino acid sequence and differ in their carbohydrate composition having either two, one, or no sialic acids as was found for the different monomer bands of the whole protein.

The tyrosine values obtained from hydrolysates of the peptides C, C^1T, and T^1C decreased with increasing length of chymotryptic digestion (*e.g.*, for peptide C^1T: 15 min gave 7.7 nmoles, 2 hr gave 5.7 nmoles, and 16 hr gave 1.7 nmoles). This indicates that chymotrypsin splits preferentially at tyrosine, more slowly at tryptophan, and that the sequence within the glycopeptide is -Trp-Tyr-.

Longer chymotryptic digests of whole reduced and alkylated light chain also showed a decrease in the serine, cysteine, and arginine values in peptide C. Instead of three distinct peptide spots, a smear of glycopeptides appeared; 2-hr digestion gave cysteine and arginine values which amounted to approximately 20% of the isoleucine value in the glycopeptide and lowered the amount of serine in the glycopeptide from 3 to 2.3. After digestion for 16 hr, cysteine and arginine were present in amounts less than 10% of Ile. This is probably due to a contamination of tryptic activity in our chymotrypsin preparations, which would split off peptide T^1C (Ser, Cys)-Arg, leaving a mixture of C and T^2C, both probably in their three forms and all with slightly different electrophoretic mobilities. The leucine position within the glycopeptide seems to be resistant to chymotrypsin.

TABLE III: Composition and ^{3}H to ^{14}C Radioactivity of Fractions I and II from G-10 Sephadex.

	Fraction I		Fraction II	
	10^{-9} Moles	No. of Residues	10^{-9} Moles	No. of Residues
Asp	7.6	1	10.7	2
Ser	0.7	0.1	9.9	2
Glu	0.9	0.2	13.8	3
Gly[a]	1.9	0.3	1.0	0.1
Ala	0.3	0.1	5.5	1
Ile	0.7	0.1	4.5	1
Leu	0.3	0.1	4.3	1
Tyr	0.2	0.1	3.5	1
Glucosamine[b]	22.5	3+	0.4	0.1
Lys	0.2	0.1	4.7	1
His	0.3	0.1	5.0	1

	Counts per Minute		Recovery (%)
[^{14}C]Carbohydrate	1,500	280	70
[^{3}H]Leucine	2,000	26,000	93

[a] Glycine is probably a contamination eluted from Whatman No. 3MM paper. [b] Under the conditions of hydrolysis used for amino acid analysis, approximately 50–60% of the glucosamine is destroyed. Tryptophan was not determined.

TABLE IV: Compositions of CT and TC Peptides.[a]

	Peptides Derived from Tryptic Glycopeptides by Treatment with Chymotrypsin (15 min)						Peptides Derived from Chymotryptic Glycopeptides by Treatment with Trypsin (16 hr)					
	$(C^1T)_I$	$(C^1T)_{II}$	$(C^1T)_{III}$	$(C^2T)_I$	$(C^2T)_{II}$	$(C^2T)_{III}$	$(T^1C)_I$	$(T^1C)_{II}$	$(T^1C)_{III}$	$(T^2C)_I$	$(T^2C)_{II}$	$(T^2C)_{III}$
Cys							0.7	0.7	0.7			
Asp	2.9	3.1	2.8							3.1	2.7	2.9
Ser	2.1	2.2	1.9				0.9	1.1	0.9	2.0	1.8	1.9
Glu[b]	1.1	0.8	0.9	2.2	2.2	2.3				1.0	0.9	1.0
Ala	1.0	0.9	0.8							1.0	0.9	1.0
Ile	1.0	1.0	1.0							1.0	1.0	1.0
Leu	1.0	0.9	0.9							1.0	1.0	1.0
Tyr	0.7	0.7	0.6							0.8	0.8	0.7
Glucosamine[c]	3.0	2.6	2.9							3.1	2.7	3.1
Lys				1.2	1.0	1.0						
His	0.9	0.9	0.9							0.9	0.8	0.9
Arg							1.1	1.0	1.1			
Trp[d]	(1)	(1)	(1)							(1)	(1)	(1)

[a] Number of residues per isoleucine residue assuming one Ile residue per glycopeptide. [b] Glutamine was identified by paper chromatography in system A after Pronase digestion. [c] A total of 6 moles of glucosamine has been found per mole of MOPC 46 light chain (Melchers *et al.*, 1966). Under the conditions of hydrolysis used for amino acid analysis, approximately 50–60% of the glucosamine is destroyed. [d] Determined by the method of Smith (1953).

Stepwise degradation from the N terminus was carried out with all three forms of the glycopeptide from a tryptic digest. Edman degradations from the N terminus proceeded well to leucine (step 9) with glycopeptide T_I. For three more steps thereafter, no phenylthiohydantoin amino acid could be detected in either the benzene or the water phase.

Degradation step 4 did not seem to modify the residual peptide obtained after step 3, for neither did the composition of the water phase change, nor was there any detectable phenylthiohydantoin amino acid released into the benzene phase. Since steps 5–9 of the Edman degradation released phenylthiohydantoin amino acids into the benzene phase, it was surmised that the carbohydrate group of glycopeptide T_I does not interfere with the formation and subsequent release of phenylthiohydantoin amino acids. Of the amino acids found in T_I (Table I) only phenylthiohydantoin-His or phenylthiohydantoin-Asx-carbohydrate could be expected to stay in the water phase. The attached carbohydrate might keep phenylthiohydantoin-Asx-carbohydrate from entering the benzene phase. Upon analysis of the water phase after step 4, it was found that the undegraded glycopeptide T_I had a very similar mobility in electrophoreses at pH 2.1 (Doolittle *et al.*, 1967) to standard phenylthiohydantoin-His and could also be stained for histidine. No reference substance was available for phenylthiohydantoin-Asx-carbohydrate. The phenylthiohydantoin amino acid released from glycopeptide T_I at step 4 of the Edman degradation could therefore not be identified.

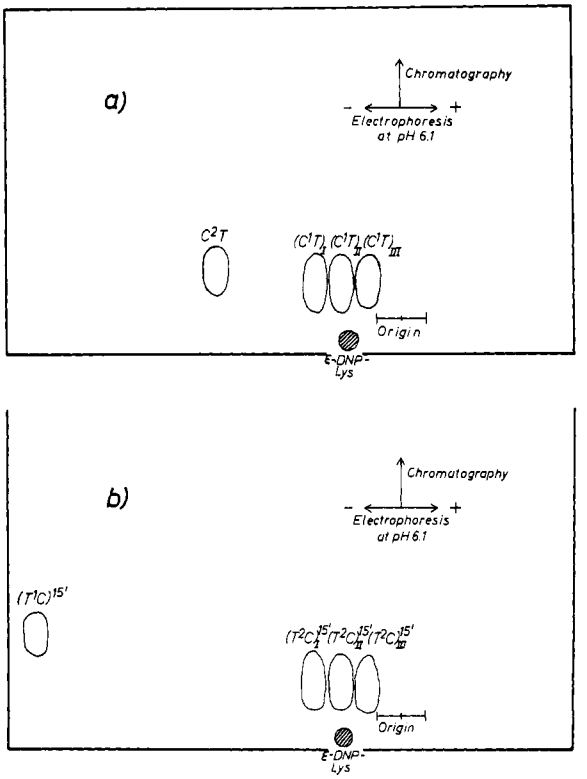


FIGURE 4: Separation of (a) CT peptides and (b) TC peptides by chromatography and electrophoresis at pH 6.1. Peptides were spotted by developing the chromatogram with 0.03% ninhydrin.

TABLE V: Digestion of Glycopeptides T_{II} and T_{III} with Aminopeptidase and Carboxypeptidases A and B.[a]

	Aminopeptidase[b]		Carboxypeptidase A[b]		Carboxypeptidase B[b]			
					T_{II}		T_{III}	
Glycopeptide:	T_{II}	T_{III}	T_{II}	T_{III}				
Digestion Time:	24 hr	24 hr	4 hr	4 hr	1 hr	4 hr	1 hr	4 hr
Amide[c]								
Asn			++	++	(+)	(+)	(+)	(+)
Ser	<0.1	<0.1	7.1 ++	6.6 ++	0.76 (+)	1.33 (+)	0.69 (+)	1.49 (+)
Gln			++	++	++	++	++	++
Ala	<0.1	<0.1	1.1	1.2	<0.1	<0.1	<0.1	<0.1
Ile	<0.1	<0.1	0.9	1.1	<0.1	<0.1	<0.1	<0.1
Leu	<0.1	<0.1	1.1	0.8	0.21	0.29	0.18	0.27
Tyr	<0.1	<0.1	1.0	0.9	0.38	0.46	0.33	0.40
Glucosamine	<0.1	<0.1						
Lys	<0.1	<0.1	1.0	1.0	0.8	1.0	0.8	1.1
His	<0.1	<0.1	0.9	0.8	0.23	0.33	0.20	0.26

[a] Tryptophan was not determined. Glycopeptide input per experiment was 8.4 nmoles; values in the table are nmoles per nmole of input glycopeptide. [b] The position of Asx-carbohydrate in analyses with the amino acid analyzer is not known. All analyses are therefore lacking the value for Asx-carbohydrate. [c] On the amino acid analyzer glutamine and asparagine appear together with serine as amides. For the identification of these three amino acids an aliquot of the digest was analyzed by chromatography in system A. Semiquantitative analysis is indicated in the table.

Similar to glycopeptide T_I, glycopeptides T_{II} and T_{III} did not release a phenylthiohydantoin amino acid into the benzene phase at step 4. In contrast to glycopeptide T_I, however, the amino acid composition of glycopeptides T_{II} and T_{III} did not change on three more degradations, nor was there any phenylthiohydantoin amino acid detected in the benzene or the water phases. One difference between these glycopeptides has been found in the amount of sialic acid attached to the carbohydrate group. Glycopeptide T_{III} contains two, T_{II} one, and T_I no sialic acid (F. Melchers, in preparation). After neuraminidase digestion of T_{II} and T_{III}, the degradation steps proceeded well to leucine (step 9). Steps 10–12, as in the case of T_I, did not release any detectable amino acid.

From these experiments it was not yet clear whether Asx-carbohydrate is located between glutamine and isoleucine, and histidine is located between leucine and tryptophan-tyrosine, or whether the reverse is true.

Exopeptidases were tried on glycopeptides T_{II} and T_{III} to establish the positions of Asx-carbohydrate and histidine within the glycopeptide. The specificities of the three exopeptidases used (aminopeptidase and carboxypeptidases A and B) had been established with reference peptides of Dr. Wittmann.

Aminopeptidase did not release any amino acid from T_{II} and T_{III} within 24 hr. Carboxypeptidase A digested T_{II} and T_{III} within 4 hr down to their amino acids. Carboxypeptidase B preferentially released the carboxy-terminal lysine. More slowly it acted in 4 hr to approximately 40% on the residual peptide. The results are summarized in Table V. From the results with carboxypeptidase B it can be surmised that histidine is located between leucine and (tryptophan)-tyrosine, consequently placing Asx-carbohydrate between glutamine and isoleucine within the glycopeptide.

Another way to establish the position of Asx-carbohydrate and histidine within the glycopeptides was to digest T_I, T_{II}, and T_{III} with papain. Each digest was separated on Bio-Gel P-2 (Figure 5) and the carbohydrate-containing first fraction of the column analysed for its amino acid composition. Table VI summarizes the results. In all three glycopeptides T_I, T_{II}, and T_{III}, Asx-carbohydrate appears together with alanine, serine, glutamine, and isoleucine, while the carboxy-terminal part of the peptides with leucine, histidine, tyrosine, and lysine has been lost by the digestion with papain. These results independently show that Asx-carbohydrate has to be placed between glutamine and isoleucine, while histidine is located

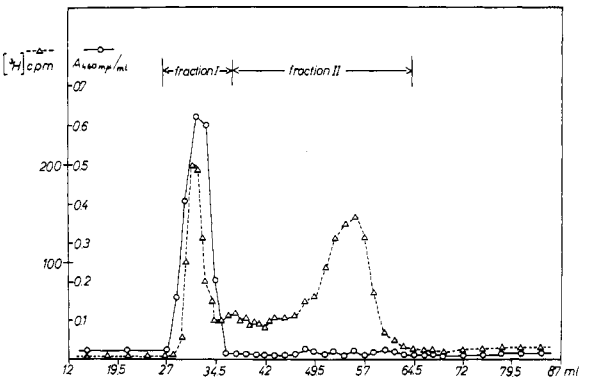


FIGURE 5: Chromatography of a 2-hr papain digest of glycopeptide T_I on a 0.8 × 50 cm Bio-Gel P-2 column in H_2O. (—○—○—) $A_{460\ m\mu}$; toluene–H_2SO_4 assay for neutral hexoses; (—△—△—) counts per minute of [^{3}H]-leucine.

TABLE VI: Analysis of Glycopeptides Obtained by Papain Digestion of the Glycopeptides T_I, T_{II}, and T_{III}.

Glycopeptide:	T_I	T_{II}	T_{III}
Input:	6.5 nmoles with 13,400 cpm of [^{3}H]Leucine	7.5 nmoles with 10,200 cpm of [^{3}H]Leucine	6.0 nmoles with 5200 cpm of [^{3}H]Leucine
% [^{3}H]leucine in			
fraction I = undigested	26	30	22
fraction II = digested	74	70	78
(cpm in fraction I/cpm in fraction II; see Figure 5)			
Amino Acids Recovered in Fraction I (nmoles/nmole input of glycopeptide)			
Asp	1.72 (1.0, 0.35, 0.35)	1.85 (1.0, 0.42, 0.42)	1.70 (1.0, 0.35, 0.35)
Ser	1.77 (1.0, 0.77)	1.90 (1.0, 0.90)	1.70 (1.0, 0.7)
Glu	1.36 (1.0, 0.36)	1.48 (1.0, 0.48)	1.20 (1.0, 0.2)
Ala	0.72	0.85	0.69
Ile	0.99	1.10	0.89
Leu	0.29	0.34	0.25
Tyr	0.20	0.30	0.24
Glucosamine	2.60	2.75	2.51
His	0.21	0.26	0.18
Lys	0.18	0.25	0.21

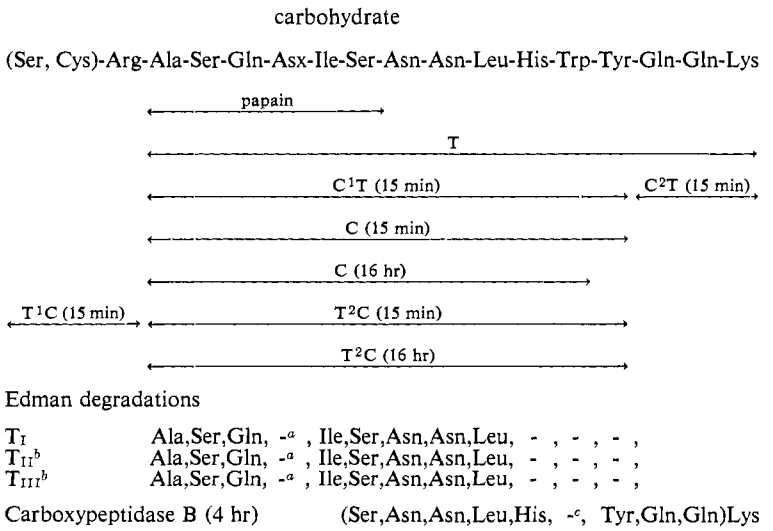
between leucine and (tryptophan)-tyrosine. Furthermore these results show that Asx-carbohydrate is located in all three glycopeptides T_I, T_{II}, and T_{III} at the same position. We conclude that T_I, T_{II}, and T_{III} must have the same amino acid sequence. They therefore differ only in their content of sialic acid within the carbohydrate group.

An explanation for the resistance to Edman degradations at the Asx-carbohydrate position, when sialic acid is present in the carbohydrate group, cannot be given at present.

Figure 6 summarizes the experimental evidence from which the amino acid sequence around the attachment site of carbohydrate is derived.

The amino acid sequence in Figure 6 is different than the one published previously by us (Melchers and Knopf, 1967). It has been corrected for the positions of Asx-carbohydrate and histidine within the glycopeptide. The sequence agrees with data obtained by Coleman *et al.* (1967).

Comparison with Known Amino Acid Sequences in κ-Type Light Chains. Two complete amino acid sequences of mouse κ-type light chains are known (Gray *et al.*, 1967). Since the light chain secreted by the



[a] No phenylthiohydantoin amino acid was detected in the benzene phase. For the analysis of the water phase, see text. [b] After neuraminidase digestion. [c] Not determined.

FIGURE 6: For details, see text. → indicates Edman degradation steps. ← indicates carboxypeptidase B digestion.

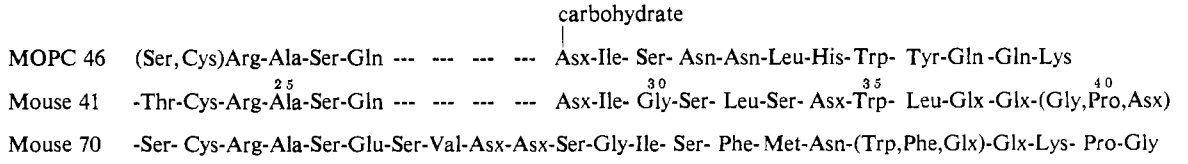


FIGURE 7: Suggested alignment of the glycopeptide from MOPC 46 light chain in the variable portion of the protein by comparison with two κ-type light chains from mouse (Gray *et al.*, 1967).

MOPC 46 plasma cell tumor is κ type (Liebermann *et al.*, 1965; F. Melchers and E. S. Lennox, manuscript in preparation), we can try to place the glycopeptide in the sequence.

It is apparent immediately that it cannot belong to the carboxy-terminal invariant part of κ-type light chain. Further supporting evidence that the MOPC 46 protein is indeed a κ-type light chain is the fact that eleven of the expected thirteen tryptic peptides from the invariant part of a reduced and aminoethylated κ-type light chain have been found (P. M. Knopf, personal communication).

By comparison with the amino acid sequences of the variable parts of the two known κ-type light chains (Gray *et al.*, 1967), the glycopeptide from MOPC 46 can be aligned best with a corresponding sequence between positions 22 and 39 (mouse 41) (Figure 7).

It should be noted that the attachment of carbohydrate appears to be at position 28, in a region where a high degree of variability of amino acid sequences has been observed in human light chains (Putnam *et al.*, 1967).

Discussion

Why is MOPC 46 light chain exceptional in having carbohydrate attached while other light chains of very similar sequence do not? We discuss several possibilities. (1) It is crucial whether position 28 be occupied by aspartic acid or asparagine. (2) The short sequence examined around residue 28 may be sufficient for recognition by the enzymes that attach carbohydrate. (3) In most cases, the enzymes themselves are lacking. (4) The presence of carbohydrate is more common than thought. These possibilities are not, of course, complete or mutually exclusive.

We have made no attempt to ascertain the chemical bond between the carbohydrate group and the polypeptide chain other than to show that the linkage is to aspartic acid or asparagine. Other glycoproteins with one carbohydrate group per 200–500 amino acids (α_1-glycoprotein) (Eylar, 1962; Satake *et al.*, 1965), ovalbumin (Johansen *et al.*, 1961; Nuenke and Cunningham, 1961), γ-globulin (Rosevear and Smith, 1961), also have aspartic acid or asparagine as the connecting amino acid within the polypeptide chain. In those cases it was shown that the carbohydrate–peptide bond links C_1 of the sugar to C_3 of aspartic acid or asparagine. This might occur either by linking the β-carboxyl group of aspartic acid to the 1-amino-group of 2-deoxy-2-acetamidoglucosylamine, or by linking asparagine through its β-amido group to the 1-hydroxyl group of 2-deoxy-2-acetamidoglucose (Marks *et al.*, 1963; Yamashina *et al.*, 1963; Tsukamoto *et al.*, 1964; Bogdanov *et al.*, 1964). It seems more likely to go *via* the latter pathway, *i.e.*, *via* UDP-*N*-acetylglucosamine to asparagine. In this case MOPC 46 light chain might contain a characteristic sequence near the asparagine at position 28 recognizing the carbohydrate attaching enzyme.

Comparison of the MOPC 46 light-chain glycopeptide to the sequences of other mouse light chains does reveal differences that could give it a recognition sequence around position 28. Also, if the attachment of sugar occurs onto asparagine within the polypeptide chain, as we discussed above, it might be that a change from aspartic acid to asparagine at position 28 has been needed to complete the requirements for the attachment. It is interesting in this connection to compare glycopeptides from other proteins.

Since the carbohydrate of the light chain is very similar in composition to that of heavy chains of γG-globulin (Melchers and Knopf, 1967), it is of interest to examine sequences around the carbohydrate attachment point of heavy chains (Nolan and Smith, 1962; Rothfus and Smith, 1963; Press, 1964; Hill *et al.*, 1966). Amino acid sequences around the carbohydrate attachment point have also been studied in several other glycoproteins (Plummer and Hirs, 1964; Jamieson, 1965; Satake *et al.*, 1965; Lis *et al.*, 1966; Radhakrishnamurthy and Berenson, 1966; Haschemeyer *et al.*, 1966). In addition partial amino acid sequences of glycopeptides from two heavy chains of myeloma IgG-globulins from Balb/c mice have been determined (Melchers and Knopf, 1967). The comparison, however, does not reveal conclusively amino acid sequences around the attachment point common also to MOPC 46 which could serve as the recognition site for carbohydrate attaching enzymes.

Neuberger and Marshall (1968) have put forward the idea that a sequence Asn-X-$^{\text{Ser}}_{\text{Thr}}$ could be the recognition site for an enzyme attaching glucosamine to glycoproteins, where X can be any amino acid. The sequence found for MOPC 46 light chain would support their hypothesis.

Little is known of the enzymes that are responsible for fixing carbohydrate to protein. By studies that we have now begun, we should be able to tell whether the cells that secrete light chain without carbohydrate lack the enzymes while MOPC 46 plasma cells are special in having a full complement.

Evidence that carbohydrate on light chains is more common than previously thought is accumulating (Marshall and Potter, 1966; Edmundson, 1966; Hood,

1967). It will be especially interesting to see how widespread the occurrence is, what structural features are required, and what role it might play in light-chain function.

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