Comparative Analysis of Glycopeptides Derived from Human Platelet Membrane Glycoprotein Ib†

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ABSTRACT: Although several purification procedures have been reported for platelet glycocalicin and macroglycopeptide, compositional data suggest that contamination with tightly associated peptide fragments is a continuing problem. This, together with the lack of a reliable estimate of molecular weights, has delayed a clear resolution of the relationship of intact platelet membrane glycoprotein Ib and these proteolytically derived glycopeptides. A new procedure was developed for purification of both macroglycopeptide and glycocalicin from human platelet plasma membranes. It consists of ion-exchange chromatography on diethylaminoethyl-Sephacel, lectin affinity chromatography on wheat germ agglutinin coupled to Sepharose, and gel filtration chromatography under denaturing conditions and avoids exposure of these sialylated glycoproteins to acidic conditions. Electrophoretic evidence for the purity of macroglycopeptide and glycocalicin prepared by this procedure was obtained by Laemmli (1970) [Laemmli, U. K. (1970) *Nature (London) 227*, 680–685] sodium dodecyl sulfate–polyacrylamide gel electrophoresis of samples radiolabeled by sequential sodium metaperiodate oxidation and borotritide reduction. Electrophoresis gave apparent molecular weights of 108 000 and 118 000 for macroglycopeptide and glycocalicin, respectively. However, sedimentation equilibrium centrifugation experiments using the meniscus-depletion method in 6 M guanidine hydrochloride established the weight-average molecular weights of macroglycopeptide and glycocalicin as 59 700 and 105 600, respectively. The molecular weight determinations are the first by a primary physical method for platelet macroglycopeptide and glycocalicin and, together with compositional analyses, permitted calculation of the compositions of the two glycopeptides in terms of residues per molecule, which is consistent with the derivation of macroglycopeptide from glycocalicin by proteolysis.

The major human platelet membrane glycoprotein, glycoprotein Ib, of molecular weight (M_r)[1] 170 000 has been shown to consist of two externally oriented disulfide-linked glycopeptide subunits (Ibα of M_r 143 000 and Ibβ of M_r 22 000) by Phillips & Agin (1977). Glycoprotein Ib is greatly reduced or absent in Bernard–Soulier syndrome, a hereditary giant platelet syndrome associated with defective platelet adhesion and defective hemostasis (Nurden & Caen, 1975; Jamieson et al., 1979; McGregor et al., 1980; Nurden et al., 1981; Clementson et al., 1982; Peterson et al., 1982). Immunochemical evidence has been advanced (Solum et al., 1980) that glycoprotein Ibα contains within it glycocalicin, which is a single-chain glycopeptide of M_r 148 000 liberated from the platelet membrane upon platelet lysis (Okumura & Jamieson, 1976). Solum et al. (1977) observed that freeze–thawing platelets in the presence of EDTA prevented the solubilization of glycocalicin. By the use of inhibitors of the calcium-dependent intracellular neutral platelet protease (Phillips & Jakábová, 1977), Solum et al. (1980) were able to retard the appearance of glycocalicin in the supernatant of freeze–thawed platelets.

Glycocalicin has been purified (Okumura et al., 1976; Hagen et al., 1980) and its composition determined (Okumura et al., 1976). Treatment of glycocalicin with TPCK–trypsin yielded a glycopeptide of M_r 118 000, apparently identical with that previously purified from proteolytic digests of intact platelets which had been termed macroglycopeptide (Pepper & Jamieson, 1970; Barber & Jamieson, 1971). Despite immunochemical evidence (Okumura et al., 1976) of identity between macroglycopeptide derived from glycocalicin and from intact platelets, reported differences in composition indicate significant impurity of at least one of these preparations.

We describe here a new purification procedure for glycocalicin and macroglycopeptide and describe the characterization of these glycopeptides, including their molecular weights as determined by sedimentation equilibrium centrifugation.

Experimental Procedures

Materials. All chemicals were of the highest purity commercially available. Deionized water, acids, and organic solvents were glass distilled prior to use. Brij 58, ovalbumin, soybean trypsin inhibitor, and ribonuclease A were from Sigma. Glycine, *N*-succinimidyl 3-(4-hydroxyphenyl)-propionate, and bovine serum albumin were from Calbiochem. TPCK–trypsin, β-galactosidase, and α-chymotrypsinogen were from Worthingfton. The following proteins were gifts: myosin heavy chain (Dr. Dixie W. Frederiksen) and human α_1-acid glycoprotein (Dr. Karl Schmid, Boston University School of Medicine). TRI-SIL silylation reagent was from Pierce. Guanidine hydrochloride was from Heico. Alditol acetates derived from fucose, mannose, glucose, galactose, GlcNAc, and GalNAc were purchased from Regis. Chromatographic media were from Pharmacia and Bio-Rad. Ultrafiltration equipment and XM100A membranes were from Amicon. Electrophoresis equipment and chemicals were from Bio-Rad.

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[1] Abbreviations: Brij 58, poly(oxyethylene) 20 cetyl ether; DEAE-Sephacel, diethylaminoethyl-Sephacel; EDTA, ethylenediaminetetraacetic acid; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; M_r, estimated molecular weight; NANA, *N*-acetylneuraminic acid; PAS, periodic acid–Schiff base; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK–trypsin, tosylphenylalanyl chloromethyl ketone treated trypsin; WGA, wheat germ agglutinin; Tris, tris(hydroxymethyl)aminomethane.

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Gel Electrophoresis. Samples in 1% (w/v) SDS were reduced by the addition of β-mercaptoethanol to 5% (v/v) with boiling for 5 min. Electrophoresis was conducted in the presence of 0.1% (w/v) SDS in 7.5% polyacrylamide gels according to the procedure of Laemmli (1970). Protein was detected by staining with Coomassie Blue by the method of Fairbanks et al. (1971). Glycoprotein was detected by the PAS staining technique as described by Segrest & Jackson (1972). Standard marker proteins and their molecular weights were as follows: myosin (heavy chain), 220 000; β-galactosidase (*Escherichia coli*), 116 000; bovine serum albumin, 68 000; ovalbumin, 44 600; chymotrypsinogen A, 25 800; ribonuclease A, 13 700.

Radiolabeling Techniques. Proteins with free amino groups were labeled with ^{125}I by using the acylation technique of Bolton & Hunter (1973). Glycoproteins containing sialic acid were labeled with periodic acid oxidation followed by reduction with tritiated borohydride according to Van Lenten & Ashwell (1971). Macromolecular-bound tritium fractionated by SDS–PAGE was solubilized and counted as described by Basch (1968).

Preparation of Crude Macroglycopeptide. Starting material for these preparations consisted of expired (≥72 h on a rotary shaker at 22 °C) human platelet concentrate units from volunteer donors prepared for clinical use. Platelets of multiple blood types and from multiple donors were pooled after being completely freed of erythrocytes by differential centrifugation as described by Barber & Jamieson (1970). After the platelets were washed by three suspensions and centrifugations in 1 mM EDTA–10 mM Tris-HCl (pH 7.5) with 0.15 M NaCl, they were subjected to TPCK–trypsin treatment as described by Pepper & Jamieson (1970). Crude macroglycopeptide as trypsin supernatant was frozen at −30 °C until required.

Preparation of Crude Glycocalicin. The glycerol–lysis method of barber & Jamieson (1970) was followed with one modification. To minimize further proteolysis, the glycerol-loaded platelets were lysed in 0.25 M sucrose and 0.01 M Tris-HCl (pH 7.5) containing 1 mM EDTA, 5 mM *p*-aminobenzamidine dihydrochloride, and 10 mM *N*-ethylmaleimide. After centrifugation of this lysate at 63500*g* for 3 h at 4 °C onto a cushion of 27% sucrose, the supernatant remaining above the platelet membranes banded at the interface was frozen at −30 °C as crude glycocalicin. Membranes were washed by centrifuging at 105000*g* for 1 h at 4 °C through the lysis buffer and also frozen.

Dialysis. Frozen supernatant was thawed at 4 °C; solid Brij 58 was added to a concentration of 0.1% (w/v). The sample was then dialyzed against 0.05 M glycine (pH 9) with 0.15 M NaCl, 1 mM EDTA, 0.1% (w/v) Brij 58, and 0.02% (w/v) NaN_3 for 48 h with four changes of solution.

Ion-Exchange Chromatography. Initial chromatography was on a 2.6 × 14 cm column of DEAE-Sephacel equilibrated with 0.05 M glycine (pH 9), 0.15 M NaCl, and 0.1% (w/v) Brij 58 at 4 °C. Crude supernatant containing either glycocalicin or macroglycopeptide was pumped onto the column at approximately 19 mL/h. After the absorbance at 280 nm declined to base line, bound material was eluted with 2 bed volumes of the above buffer containing 0.4 M NaCl. Eluted material was detected by the absorbance at 280 nm and pooled (100 mL).

Affinity Chromatography. Wheat germ agglutinin was coupled to cyanogen bromide activated Sepharose 4B by mixing for 12 h at 4 °C in 0.1 M sodium bicarbonate (unreacted sites were blocked with ethanolamine). It was poured into a 1 × 10 cm column and equilibrated with 0.05 M glycine (pH 9), 0.40 M NaCl, and 0.1% (w/v) Brij 58. Pooled material partially purified by ion-exchange chromatography was pumped onto the WGA–Sepharose at about 19 mL/h at 4 °C. The column was then washed with its equilibration buffer until the absorbance at 280 nm of the emerging solution was identical with that of the applied buffer. Bound glycoprotein material was then eluted by changing to an identical buffer with the exception that it contained 0.1 M GlcNAc.

Ultrafiltration. Material eluted from WGA–Sepharose was concentrated by ultrafiltration under pressurized nitrogen using Amicon stirred cells and XM100A membranes (nominal molecular weight limit of 100 000). Simultaneously, the buffer's salt concentration was raised to 1 M by adding multiple aliquots of 0.05 M glycine (pH 9), 1 M NaCl, and 0.1% (w/v) Brij 58. The final volume from the last cycle of concentrating was either 5 or 10 mL (i.e., 1% or 2% of the bed volume of the next column).

Gel Filtration. After ultrafiltration, affinity-purified material was subjected to gel permeation chromatography on a 1.8 × 188.5 cm column of Sephacryl S-300 at 4 °C. Sample materials were allowed to enter the gel by gravity flow; then the column was closed, and its equilibation buffer, 0.05 M glycine (pH 9), 1 M NaCl, and 0.1% (w/v) Brij 58, was pumped through the column at about 19 mL/h while 1-mL fractions were collected. Emerging peaks were located by use of the absorbance at 280 nm, pooled, and concentrated to 1.5 mL (approximately 1% of the final column's bed volume) by ultrafiltration as described above.

Gel Filtration under Denaturing Conditions. Samples applied to this final column were made 6 M in guanidine hydrochloride by the addition of the dry solid and incubated at 37 °C for 24 h prior to being chromatographed. The final column used was a 0.9 × 217 cm Sepharose 4BCL matrix in 6 M guanidine hydrochloride and 0.01 M potassium phosphate (adjusted to pH 6.4 with phosphoric acid) which ran at 6 mL/h under gravity flow from a 160-cm pressure head. This column was operated at near 20 °C and monitored by means of a flow cell with a 1-cm optical path which provided a record of the absorbance at 230 nm. Eluate was collected into individually weighed plastic cups in about 1-mL volumes.

Pooled materials obtained from this column were freed of guanidine hydrochloride by exhaustive dialysis against deionized water, dialyzed against 0.1 M ammonium bicarbonate, and lyophilized.

Amino Acid Analyses. Samples were hydrolyzed in 6 N HCl in sealed ampules evacuated <25 μmHg at 110 ± 1 °C for either 24 or 48 h (Moore & Stein, 1963). Analysis of hydrolyzed samples was by automated ion-exchange chromatography essentially as decribed by Moore & Stein (1963). Average values from the two timed determinations were reported for all of the amino acids except serine and threonine; for these two, the value reported was that from extrapolation to zero hydrolysis time (Hirs et al., 1954).

Sialic Acid Cleavage and Purification. Preferential cleavage of NANA (Codington et al., 1976) from glycoproteins was obtained by treatment with dilute aqueous acid (0.05 N sulfuric acid, 80 °C, 60 min) in glass test tubes previously washed in hot sulfuric acid. After cleavage of sialic acids had proceeded for 1 h, the sample tubes were rapidly cooled to near 0 °C and adjusted to pH 7 with barium carbonate.

The neutral solutions of sialic acids and asialoglycoproteins were next dialyzed against 10 mL of Dowex AG 1-X8 (formate form) beads in 25 mL of water (neutral pH) for 48 h at 4 °C. At the end of that period, the retentates were lyophilized into ampules which had been acid washed as previ-

ously described for amino acid analysis in order to be used for the determination of the neutral and amino sugars. The Dowex beads bearing sialic acid for each sample were transferred to small disposable columns, rinsed with 20 mL of water (neutral pH), and then eluted with 3 bed volumes of 1 N formic acid (Schauer, 1978). All operations were performed at 4 °C. The pooled eluates were freed of formic acid by lyophilization. Samples of 5 mg of 98% NANA subjected to the 80 °C hydrolysis and the Dowex fractionation procedure were used as standards.

Quantitation of Sialic Acids. Samples of NANA were converted to their trimethylsilyl ethers by reaction with hexamethyldisilazane and chlorotrimethylsilane in pyridine according to Sweeley et al. (1963). The residue was taken up in chloroform and analyzed quantitatively by gas chromatography with a Perkin-Elmer Sigma 1 gas chromatography equipped with a 20-m SP-1000 glass capillary column (Supelco, Inc.) operated isothermally at 209 °C. A flame ionization detector was used.

Determination of Neutral and Amino Sugars. Asialoglycoproteins prepared as described previously were subjected to a mixed acid hydrolysis as described by Hellerqvist et al. (1972). After addition of 50 μg of inositol as an internal standard, monosaccharides were converted to alditol acetates as previously described (Hellerqvist et al., 1972). Alditol acetates were dissolved in ethyl acetate and filtered through a small column of activated charcoal in order to remove amino acids, and ethyl acetate was removed by evaporation under dry nitrogen.

The resulting mixtures of alditol acetates were redissolved in chloroform and analyzed quantitatively by gas chromatography with a Perkin-Elmer Sigma 1 gas chromatograph equipped with a 10-m OV-225 glass capillary column (Supelco, Inc.). Peak areas were converted into nanomoles of alditol acetate by comparison with a standard sample of human α_1-acid glycoprotein (Bürgi & Schmid, 1961). Since the complete primary structures of the five asialo N-linked oligosaccharides of human α_1-acid glycoprotein are known (Fournet et al., 1978), electronically integrated alditol acetate peak areas derived from 5 mg of α_1-acid glycoprotein were converted into nanomoles and used as conversion factors for the peak areas of the samples. Retention times for all alditol acetates were authenticated by comparison with purified standards purchased from Regis.

Analytical Ultracentrifugation. Macroglycopeptide and glycocalicin (each from 150 units of outdated platelet-rich plasma) were purified as described above. These two lyophilized samples were dissolved in 100 μL of 6 M guanidine hydrochloride containing 0.01 M potassium phosphate (pH 6.4) and dialyzed overnight against 2 L of the same buffer. Sedimentation equilibrium experiments were carried out by using the meniscus-depletion method (Yphantis, 1964) at 27 687 rpm at 20 °C in a Beckman Model E ultracentrifuge.

Densitometry. The density of 6 M guanidine hydrochloride and 0.01 M potassium phosphate (pH 6.4) was determined with a precision densitometer (Mettler-Paar, Model DMA-02D) as described by Kratky et al. (1973).

Partial Specific Volume. Partial specific volumes were calculated for macroglycopeptide and glycocalicin in 6 M guanidine hydrochloride according to the method of Lee & Timasheff (1979) from the amino acid and sugar compositions determined as described above.

Results

Purification of Macroglycopeptide. The crude supernatant of a trypsin digest of human platelets exhibited a single band, centered about an M_r of 107 000, when analyzed with SDS–PAGE and PAS staining. Multiple Coomassie Blue stained bands were evident. Progress during the course of the purification was assessed by SDS–PAGE and parallel staining of duplicate samples by PAS and Coomassie Blue. Purification of macroglycopeptide involved sequential ion-exchange chromatography on DEAE-Sephacel, affinity chromatography on WGA–Sepharose, and both nondenaturing and denaturing gel filtration chromatography as described under Experimental Procedures. Figure 1 displays the elution pattern of the final column used. Compositional analyses indicated that outdated platelets from 242 units of blood (≈121 L) yielded 1.18 mg of macroglycopeptide.

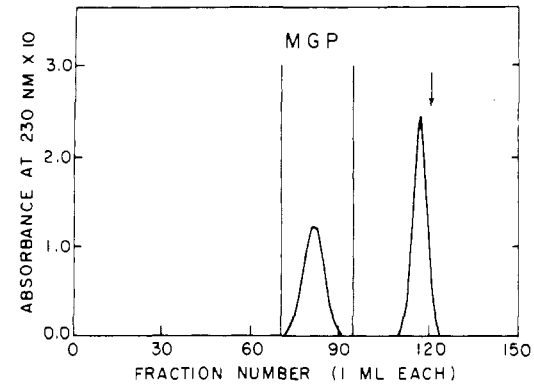

FIGURE 1: Denaturing gel filtration chromatography of 154 units of macroglycopeptide. A column of Sepharose 4BCL, 0.9 × 217 cm (138 mL), was equilibrated and developed in 6 M guanidine hydrochloride and 0.01 M potassium phosphate (adjusted to pH 6.4 with phosphoric acid) and operated at about 20 °C by gravity flow at about 6 cm^3/h. Elution points were determined by weighing each fraction. The arrow marks the point of elution of tritiated water. MGP indicates the fractions pooled as macroglycopeptide.

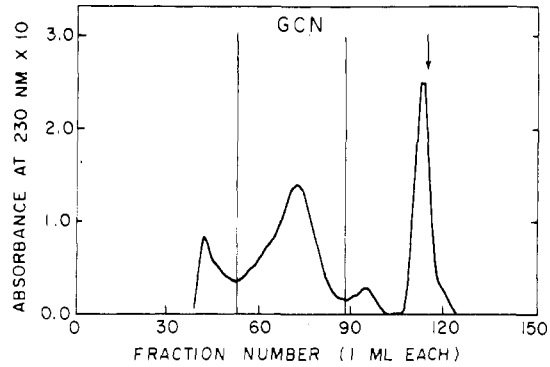

FIGURE 2: Denaturing gel filtration chromatography of 367 units of glycocalicin on Sephacryl S-300. The sample was applied to a 0.9 × 217 cm column of Sepharose 4BCL in 6 M guanidine hydrochloride and 0.01 M potassium phosphate (pH 6.4) at near 20 °C. The arrow marks the point of elution of tritiated water. Glycocalicin is found in pool GCN.

Purification of Glycocalicin. Supernatant prepared from isotonically lysed human platelets by centrifugation to remove cell debris and membranes showed, on SDS–PAGE, a number of Coomassie Blue stained bands but only one PAS-stained band centered about an M_r of 137 000. This glycocalicin band was purified by using procedures identical with those for macroglycopeptide. Figure 2 shows glycocalicin eluting from the final column used. Outdated platelets from 367 units of blood (≈183 L) provided 1.05 mg of glycocalicin.

Purity of Product. The purity of macroglycopeptide and glycocalicin was assessed by using radiolabeling of these peptides with iodinated Bolton–Hunter ester and reduction with sodium borotritide after periodate oxidation. After labeling was completed, samples were analyzed by reduced

Table I: Compositional Analyses of Glycocalicin (GCN) and Macroglycopeptide (MGP)

	Okumura et al. (1976)				present work						Judson et al. (1982),
	MGP		GCN		MGP			GCN			MGP
	mol %[a]	mol %[b]	mol %[a]	mol %[b]	mol %[a]	mol %[b]	res[c]	mol %[a]	mol %[b]	res[c]	mol %[a]
NANA	21.1		12.5		18.5		37.7	11.3		41.0	14.9
Man	0.9		1.2		0.8		2.9	1.0		6.6	4.2
Fuc	1.3		1.9		1.2		4.8	1.9		13.7	0.3
Gal	20.2		15.1		19.1		70.0	13.4		87.0	17.8
Glc	1.6		2.3		0.2		0.7	0.1		0.6	NS[e]
GlcNAc	10.9		7.2		12.0		35.2	6.5		33.9	10.8
GalNAc	10.0		5.9		11.4		33.5	5.4		27.8	10.9
Lys	2.1	6.3	3.3	6.3	2.1	5.8	9.9	2.7	4.4	22.2	1.4
His	0.6	1.8	1.1	2.1	0.5	1.5	2.4	1.3	2.1	10.0	2.9
Arg	trace	trace	1.1	2.3	0.4	1.2	1.6	1.6	2.6	10.6	1.1
Asp	0.9	2.7	4.7	8.8	1.1	3.0	5.8	5.3	8.8	48.7	1.3
Thr	7.9	23.1	7.6	14.0	7.2	19.6	42.7	5.8	9.6	60.4	7.2
Ser	4.8	14.1	4.9	8.9	5.3	14.3	36.3	5.3	8.8	64.4	4.6
Glu	3.6	10.7	5.8	10.5	4.0	10.8	18.5	8.0	13.2	65.1	3.2
Pro	6.8	20.1	6.8	12.6	6.3	17.2	39.0	6.0	9.9	65.4	6.1
Gly	0.7	2.0	2.7	5.1	1.7	4.6	17.7	7.0	11.5	128.9	1.0
Ala	1.6	4.6	2.3	4.4	1.8	4.8	14.9	1.5	2.5	22.4	1.4
half-Cys	ND[d]	ND	ND	ND	0.5	1.3	2.7	1.3	2.1	12.9	ND[d]
Val	0.6	1.8	2.5	4.6	1.0	2.7	6.1	3.2	5.3	34.2	1.3
Met	trace	trace	0.8	1.4	0.7	1.8	3.0	0.3	0.5	2.6	0.1
Ile	1.0	3.0	1.3	2.3	1.0	2.7	5.3	1.7	2.9	16.3	1.4
Leu	2.3	6.7	6.7	12.9	1.8	4.9	9.5	6.2	10.3	58.2	2.7
Tyr	trace	trace	1.2	2.1	0.4	1.1	1.5	1.2	1.9	7.5	1.8
Phe	1.1	3.1	1.6	3.0	1.0	2.8	4.2	2.0	3.4	14.6	2.9

[a] Mole percentages including carbohydrate constituents. [b] Calculated as mole percentages of amino acids only. [c] Residues per molecule. [d] ND = none detected. [e] NS = not stated.

SDS–PAGE followed by slicing and counting. Surprisingly, neither macroglycopeptide nor glycocalicin was detectably labeled with the Bolton–Hunter reagent, presumably reflecting steric inaccessibility of the relatively few lysine residues in these heavily substituted glycopeptides. But since no other radiolabeled components were detected in either sample (except a large peak which runs with the bromophenol blue tracking dye), it appears that all nonglycopeptide contaminants have been removed. Control peptides examined under these conditions were readily labeled by Bolton–Hunter reagent. Tritiated macroglycopeptide showed a single peak of M_r 108 000, and tritiated glycocalicin showed a single peak of M_r 118 000 (Figure 3).

Compositional Analysis of Macroglycopeptide and Glycocalicin. Table I compares the compositions of macroglycopeptide and glycocalicin determined in this study with those by Okumura et al. (1976) of the peptides purified by a different chromatographic procedure and with the composition of the major chymotryptic glycopeptide of the platelet surface (Judson et al., 1982).

Molecular Weights of Macroglycopeptide and Glycocalicin. Partial specific volumes of macroglycopeptide and glycocalicin were calculated from their compositional analyses and are, respectively, 0.64 and 0.66. The density of the solvent used, 6 M guanidine hydrochloride and 0.01 M potassium phosphate (pH 6.4), was found to be 1.15017 g cm^{-3} at 20 °C. Molecular weights for the two species were obtained by using these values and the meniscus-depletion sedimentation equilibrium data. The weight-average molecular weights determined were 59 700 ± 200 for macroglycopeptide and 105 600 for glycocalicin. The complete plots of ln (net fringe displacement) against r^2 appeared to be linear within experimental error since, when the data fitted to a parabola in r^2 [$\ln \delta = a_0 + a_1 r^2 + a_2 (r^2)^2$], the coefficient of the $(r^2)^2$ term was not statistically different from zero.

Availability of molecular weights allowed the compositions of macroglycopeptide and glycocalicin to be recalculated in terms of residues per molecule as shown in Table I.

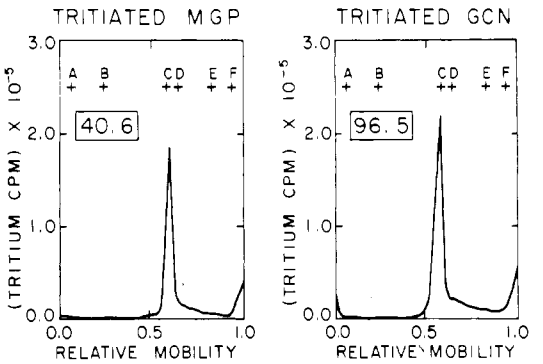

FIGURE 3: Profiles of counts per slice plotted against relative mobility for macroglycopeptide (MGP) and glycocalicin (GCN) from Sepharose 4BCL chromatography in 6 M guanidine hydrochloride after 7.5% reduced Laemmli SDS–PAGE. The inset in each panel indicates the total namomoles of amino acids applied to that particular gel. Separate gels to which twice as much sample material was applied showed no visible bands when stained with Coomassie Blue. Standard marker proteins (molecular weights in parentheses) are indicated as follows: (A) myosin heavy chain (220 000); (B) β-galactosidase (116 000); (C) bovine serum albumin (68 000); (D) ovalbumin (45 000); (E) chymotrypsinogen A (25 800); (F) ribonuclease A (13 700).

Discussion

Pepper & Jamieson (1970) have purified macroglycopeptide by concentration of trypsin and Pronase digests of external membrane proteins of intact human platelets by rotary evaporation, desalting on Sephadex G-25 at pH 8.5, gel filtration chromatography on Sephadex G-200 at pH 8.9, and ion-exchange chromatography on DEAE-Sephadex A-50 at pH 2.5. Judson et al. (1982) purified the major glycopeptide from chymotryptic digests of intact platelets by concentration using ultrafiltration and gel filtration chromatography on Sephadex G-200 pH 8.9. Glycocalicin was purified by Okumura et al. (1976) from a suspension of human platelets

prepared by sonication at pH 7.4. Their procedure involved precipitation with ammonium sulfate (at pH 7.4), pH adjustment from 7.4 to 5.4 with 0.2 N HCl, gel filtration chromatography on Sephadex G-200 at pH 6.8, hydroxylapatite chromatography at pH 6.8, and precipitation from 60% saturated ammonium sulfate adjusted to pH 3 with 0.2 N sulfuric acid.

Because of the presence of potentially acid-labile sialic acid residues (Codington et al., 1976), it seemed desirable to develop a chromatographic scheme which would avoid acidic pH values. Similarly, since variability in hydroxylapatite preparation is a recognized source of nonreproducibility (Bernardi, 1971), a purification scheme without this material seemed desirable. A purification scheme which met these two objectives was developed, and analyses by SDS–PAGE using sensitive radiolabeling methods of the macroglycopeptide and glycocalicin products showed their freedom from significant contamination.

Determinations of the size of the glycopeptides gave somewhat surprising results. It is known (Leach et al., 1980) that polyacrylamide gel electrophoretic behavior of SDS–glycopeptide complexes yields abnormally high molecular weight estimates. Several empirical treatments of SDS–PAGE data for glycopeptides failed to correct reliably for their aberrant behavior (Leach et al., 1980). Thus, our values and other values for the molecular weights of the purified glycopeptides obtained by SDS–PAGE were expected to be high. However, when sedimentation equilibrium centrifugation in 6 M guanidine hydrochloride was used to determine the weight-average molecular weights of macroglycopeptide and glycocalicin, the values obtained, 59 700 and 105 600, respectively, were unexpectedly low. Determination of the weight-average molecular weights of either of these two moieties by any primary physical method has not previously been reported.

Pepper & Jamieson (1970) have indirectly calculated a molecular weight for macroglycopeptide. They estimated the Stokes radius of macroglycopeptide to be 100 Å by using a Sephadex G-200 column calibrated with human fibrinogen and three other proteins. The sedimentation constant, $s^{1\%}_{20,w}$, was determined to be 3.2 S from sucrose density gradient centrifugation. The partial specific volume calculated from their compositional analysis was 0.688 $cm^3\ g^{-1}$. From these data, Pepper & Jamieson (1970) calculated a molecular weight of 120 000 for macroglycopeptide. The value for the Stokes radius and thus the molecular weight of the macroglycopeptide given by Pepper & Jamieson (1970), however, seem likely to be in error since Nozaki et al. (1976) have reported that the Stokes radius of fibrinogen is 108 Å when determined hydrodynamically, but apparently only 71 Å when determined from gel chromatography. Its retardation on calibrated gel filtration columns was attributed to the end-on insertion of this highly asymmetric molecule into the gel pores. Nozaki et al. presented data indicating that such an anomalous Stokes radius determination by gel chromatography is a general problem with highly asymmetric molecules. If an "apparent" Stokes radius for fibrinogen of 71 Å is used for calibration, then the Pepper and Jamieson data yield a Stokes radius for macroglycopeptide of near 70 Å.

As indicated in Table I, macroglycopeptide contains a total of 63.2 mol% (77.8 wt %) of carbohydrate. Galactose and NANA are the predominant monosaccharides (and constituents) and are present in approximately equimolar amounts, while the amino sugars are also nearly equimolar but at half the level of galactose and NANA. The presence of glucose in such a minor quantity, particularly in our preparation, and the lack of any precedent for its inclusion within glycoproteins after normal processing of N-linked oligosaccharides argue for its being a contaminant. The most plentiful amino acids are threonine (7.2 mol %), proline (6.3 mol %), serine (5.3 mol %), and glutamic acid/glutamine (4.0 mol %). Our results are in excellent agreement with the carbohydrate analysis of macroglycopeptide performed by Okumura et al. (1976) and, except for the NANA content, with that reported for the chymotryptic glycopeptide purified by Judson et al. (1982). All three reports find an identical pattern of the four most abundant amino acids, although the mole percentages differ. Judson et al. agree with the present work that arginine, methionine, and tyrosine are minor but measurable constituents, but with Okumura et al. that cysteine is absent.

Judson et al. (1982) subjected the chymotryptic glycopeptide derived from the platelet surface to β-elimination and isolated a single O-linked oligosaccharide accounting for more than 80% of the released material. They showed this to be an O-linked sialohexasaccharide composed of 2 mol each of NANA and galactose and of 1 mol each of GlcNAc and GalNAc, for which they proposed two alternative structures. Because of incomplete characterization of all the released material and of the carbohydrate persisting on the peptide chain, they were unable to exclude the presence of N-linked oligosaccharides. Our findings are consistent with the presence of approximately 35 such heterosaccharides per mole of macroglycopeptide each containing 2 mol of galactose, one each of GlcNAc and GalNAc, but only one of NANA. The smaller quantities of mannose and fucose may suggest the presence of at least a single more complex heterosaccharide side chain or a partial modification of the O-linked structures.

Glycocalicin is composed of 39.60 mol % (56.5 wt %) of carbohydrate with the same sugar components present in the same relative proportions as in macroglycopeptide. Glucose is presumed to be a contaminant. The most abundant amino acids were glutamic acid/glutamine (8.0 mol %), glycine (7.0 mol %), leucine (6.2 mol %), proline (6.0 mol %), and threonine (5.8 mol %). In contrast, the amino acid analysis of glycocalicin as performed by Okumura et al. (1976) and shown in Table I reported threonine (7.6 mol %), proline (6.8 mol %), leucine (6.7 mol %), glutamic acid/glutamine (5.8 mol %), and serine (4.9 mol %) as the most plentiful amino acids. The carbohydrate analysis of glycocalicin reported by Okumura et al. (1976) agrees with the relative prevalence of the sugar constituents but not with their molar percentages as determined in this work. These differences presumably are because the glycocalicin purified by Okumura et al. contained tenacious contaminants which are removed by our chromatographic procedure. The carbohydrate analysis of pure glycocalicin suggests that the bulk of the carbohydrate remains in the major fragment, macroglycopeptide, on further proteolysis with trypsin. The heterosaccharide containing two galactose residues, one GlcNAc, one GalNAc, and one NANA appears to be the predominant component, with, again, perhaps 35 such heterosaccharides per mole of glycocalicin. The explanation for the smaller amount of GalNAc found in glycocalicin is not known. The slightly larger amounts of fucose and mannose found in glycocalicin may suggest that a portion of a second heterosaccharide type is largely lost on hydrolysis to macroglycopeptide.

In the absence of protein sequence determination or peptide map comparison, it is not possible to say that macroglycopeptide is structurally related to glycocalicin. However, the derivation of macroglycopeptide from glycocalicin by pro-

teolysis, initially suggested by Okumura et al. (1976) from studies of direct proteolysis of glycocalicin, is entirely consistent with the close similarities in carbohydrate and amino acid composition given in Table I.

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