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Asparagine-linked carbohydrate chains of inducible rat parotid proline-rich glycoprotein contain terminal β-linked *N*-acetylgalactosamine

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Rats treated with daily injection of DL-isoproterenol for 10 consecutive days (25 mg kg^{-1} body weight) showed marked induction of a proline-rich glycoprotein (GPRP) of 220 kDa. Proteinase K digestion of GPRP produced a homogeneous glycopeptide with an average chemical composition as follows (residues per mol): Pro_4, Glx_3, Asx_2, Gly_1, His_1, Thr_1, Arg_1, $GlcNAc_5$, $GalNac_1$, Man_3, Gal_{2-3}, and Fuc_1. The structural analysis of the asparagine-linked carbohydrate unit was performed by methylation, periodate oxidation and enzymatic degradation. Methylation studies indicated that the three mannosyl residues were substituted at 1,2-, 1,2,4-, and 1,3,6-positions. Fucose, *N*-acetylgalactosamine, 1.5 residues of galactose and 0.35 residues of *N*-acetylglucosamine were terminally located and one galactose residue was 1,4-substituted. Approximately four of the 5 *N*-acetylglucosamine residues were substituted at 1,4-position and approximately 1 residue of *N*-acetylglucosamine was substituted at 1,4,6-positions. Periodate oxidation studies and exoglycosidase results were consistent with the methylation data. Based on the results of Smith degradation, methylation and sequential exoglycosidase digestions a triantennary oligosaccharide structure having terminal *N*-acetylgalactosamine in one of the branches is proposed for the major Asn-linked carbohydrate moiety of GPRP.

***Keywords*: Parotid, salivary, proline-rich protein, inducible, glycoprotein, asparagine-linked carbohydrate, *N*-acetylgalactosamine, isoproterenol**

Introduction

The proline-rich proteins (PRPs), characterized by a predominance of the amino acids proline, glycine and glutamine/glutamic acid, are members of a multigene family of proteins present in the saliva and salivary glands of several mammalian species including rats, rabbits, mice subhuman primates and humans (reviewed in [1] and [2]). Based on their charge and chemical composition, these proteins can be divided into acidic, basic and glycosylated PRPs. Several important functions attributed to PRPs include masticatory lubrication [3, 4], maintaining the integrity of the tooth surface by regulating the concentration of ionic calcium in saliva at its physiological concentration [5], formation of acquired enamel pellicle by heterotypic complexing with other salivary proteins [6, 7], and formation of dental plaque by mediating attachment of microorganisms to the tooth surface [8]. Acidic PRPs have been strongly implicated in the microbial adherence of oral pathogens *Porphyromonas gingivalis* [8], *Streptococcus mutans* [9, 10], *Streptococcus gordonii* [11], and *Actinomyces viscosus* [12] to the tooth surface or hydroxyapatite. The role of human salivary glycosylated PRP in lubrication and mechanical cleansing of the oral tissue has been suggested [3]. Deglycosylation of the human salivary glycosylated PRP (GPRP) resulted in marked decrease in the lubricating ability relative to the intact molecule [4]. The GPRPs also serve as receptors for several strains of streptococci [13, 14] and *Fusobacterium nucleatum* [15].

Whereas PRPs make up approximately 70% of the protein content of the human parotid saliva, these proteins are present only in small amounts in normal rat saliva or parotid glands but are induced by β-adrenergic agonists to constitute 50% of the total glandular protein [16, 17]. In a family of more than 10 PRPs induced in the rat parotid glands, there is a glycoprotein with an apparent molecular weight of 220 000 [18, 19]. Another inducible glycosylated PRP with an apparent molecular weight of 158 000 (GP-158) has been isolated from submandibular gland extract of isoproterenol-treated rats [20]. Recently the cDNAs for two glycoproteins, the 158 kDa submandibular gland

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glycoprotein (GP-158) and the parotid gland glycoprotein have been cloned [21]. Both the submandibular and parotid glycoproteins contain an identical protein backbone but differ in their glycosylation patterns [21]. Rat parotid glycosylated proline-rich protein (GPRP) exhibits several features clearly distinct from submandibular gland GP-158. As compared to rat submandibular gland GP-158, rat parotid GPRP lacks sialic acid and contains *N*-acetylgalactosamine.

Since glycosylated PRPs both in the parotid and the submandibular glands of isoproterenol-treated rats are products of a single copy gene and are generated through differential glycosylation, the carbohydrate structural analysis of GPRPs will be very useful to study the biosynthesis and post-translational modifications of glycoproteins in general and salivary glycoproteins in particular. The present communication describes the carbohydrate structure of the asparagine-linked glycans of rat parotid GPRP. The data presented in these studies could also form the basis for studies on functional aspects of the carbohydrate sidechains such as bacterial binding and lubrication.

Materials and methods

Materials

N-acetylneuraminic acid, α-amino-β-guanido-propionic acid HCl, D-(−)-arabinose, sodium metaperiodate, ethylene glycol, sodium borohydride, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), urea, tris (hydroxymethyl) aminomethane hydrochloride, manganese chloride ($MnCl_2 \times 4H_2O$), sodium azide, and acetic anhydride were all obtained from Sigma Chemical Co. (St Louis, MO). Calcium chloride dihydrate, ammonium bicarbonate and D(+)glucose were purchased from Fisher Scientific (Pittsburgh, PA). Proteinase K was obtained from Calbiochem Corporation (San Diego, CA). Petroleum ether, sodium acetate trihydrate, dimethyl-sulfoxide, methanol, chloroform and pyridine were from J.T. Baker Chemical Co. (Phillipsburg, NJ). The sodium diluent for amino acid hydrolysate analysis was a product of Beckmann (San Ramon, CA). NaB^3H_4 was obtained from NEN Research Products (DuPont), (Boston, MA). Sephadex G-50 and G-25 were purchased from Pharmacia, Inc. (Piscataway, NJ). BioGel P-4, Bio-Gel P-2, AG1-X2 and AG50W-X4 were purchased from Bio-Rad Laboratories (Richmond, CA). Sodium hydride and methyl iodide were products of Aldrich Chemical Co., Inc. (Milwaukee, WI). Anhydrous hydrazine was obtained from Pierce (Rockford, IL). Dura-Bond (DB) capillary gas chromatography columns DB-1, DB-17 and DB-225 were products of J&W Scientific Co. (Folsom, CA). Fluorophore 8-aminonapthalene-1,3,6-trisulfonic acid (ANTS) was purchased from Glyko Inc. (Novato, CA).

Endopeptidase peptide-*N*-glycosidase F (*Flavobacterium meningosepticum*) (PNGase F) was purchased from Boehringer Mannheim Biochemica. Exoglycosidases used for sequential deglycosylation were β-galactosidase (*Aspergillus niger*, Grade V), α-mannosidase (Jack Beans) and β-*N*-acetylhexosaminidase (*A. niger*), purchased from Sigma Chemical Co. (St Louis, MO). These enzymes were tested for their specificity toward p-nitrophenyl-D-glycopyranoside substrates as described previously [22] and were found to be free from cross-contamination with other glycosidases.

GPRP Purification

The high molecular weight inducible glycosylated proline-rich protein (GPRP) was isolated from aqueous extracts of parotid glands from isoproterenol treated animals by sequential chromatography on columns of DEAE-Sepharose-CL 6B, Sephadex G-100 and FPLC on Superose-12 column [23]. The glycoprotein employed in these studies was homogeneous by polyacrylamide gel electrophoresis.

Preparation and isolation of GPRP glycopeptide

The purified GPRP (15 mg) was digested with 0.5% (by weight) proteinase K in 0.1 M ammonium bicarbonate, pH 8.0. Two more additions of proteinase K (0.25% each) were made at 2 h and 4 h intervals. The proteinase K peptides were separated by gel filtration on a column of Sephadex G-50 (superfine, 200 × 1.5 cm), eluted with 0.002 N acetic acid. The fractions were monitored for peptides by absorbance at 230 nm and for carbohydrates by the phenol-sulfuric acid method [24]. The fractions containing glycopeptide were lyophilized and further purified by gel filtration on a column (1.5 × 100 cm) of Sephadex G-25, and subsequently by reverse phase HPLC chromatography on a Beckmann (C_{18}) ultrasphere ODS reverse-phase column (250 × 4.6 mm). A Beckmann Model 421 liquid chromatograph equipped with gradient elution capability and a model 165 variable wavelength detector was employed for high-performance liquid chromatography. Elution of peptides was achieved by the use of a linear gradient from 0.1% TFA (solvent A) to 0.1% TFA in acetonitrile (solvent B) at a constant flow rate of 1 ml min^{-1}.

Preparation of oligosaccharide from GPRP

To liberate the asparagine-linked oligosaccharides by hydrazinolysis, 5 mg of the glycoprotein was heated with 5 ml of anhydrous hydrazine in a sealed tube at 100 °C for 16 h as described by Takasaki *et al.* [25]. After repeated evaporations with toluene to remove hydrazine, the oligosaccharides were separated by gel filtration of Sephadex G-50 column (1.5 × 100 cm). The lyophilized oligosaccharide was dissolved in 1 ml saturated sodium bicarbonate and *N*-acetylated with 5 × 10 μl additions of acetic anhydride at 10 min intervals. After desalting on a Biogel P-2 column (1 × 30 cm), the oligosaccharides were reduced with $NaB^3H_4/NaBH_4$ and purified by chromatography on Biogel P-2 column. A sample of ≈25 μmol of the intact GPRP (5 mg) yielded ≈1 μmol of oligosaccharide.

The asparagine-linked oligosaccharides were also released from GPRP by enzymatic cleavage with peptide-*N*-glycosidase F (PNGase F). Prior to enzymatic digestion 1 mg of GPRP was denatured by boiling for 5 min in 25 μl of 10 mM sodium phosphate buffer, pH 7.4, containing 0.1% SDS, 0.05 M 2-mercaptoethanol and 0.01 M EDTA. Na_2, cooled to room temperature, mixed with 5 μl (1 U) of PNGase and incubated at 37 °C overnight. The mixture was fractionated by FPLC on a Superdex-75 column, eluted with 0.5% ammonium bicarbonate. Fractions were monitored for protein by absorbance at 280 nm and for carbohydrate by the phenol-sulfuric acid method [24].

Fluorophore-labeling and polyacrylamide gel electrophoresis of oligosaccharides

The asparagine-linked oligosaccharides released from GPRP or glycopeptide were labeled with 8-aminonapthalene-1,3,6-trisulfonic acid (ANTS) as described by Jackson [26]. Briefly, 50–100 μg of dried enzymatically released oligosaccharides were mixed with 5 μl of 0.15 M ANTS solution (0.15 M in acetic acid:water (3:17, by vol)) and 5 μl of 1 M sodium cyanoborohydride in DMSO and incubated for 16 h at 37 °C. Reaction mixture was desalted on FPLC desalting column and lyophilized.

Polyacrylamide gel electrophoresis separation of ANTS-derivatized oligosaccharides was performed on 20% polyacrylamide gels using the SDS-PAGE method of Laemmli [27], but omitting use of SDS throughout. After completion of the run, the electrofluorograms were viewed using a UV transilluminator box and photographed using a Polaroid 553 film.

Analytical methods

SDS-PAGE was performed in Mini-Protean-II apparatus (Bio-Rad) according to the method of Laemmli using a 10% resolving gel [27]. Molecular weights of intact GPRP and deglycosylated protein were estimated from the log molecular weight versus relative mobility of prestained protein markers (Bio-Rad) myosin (200 kDa), β-galactosidase (118 kDa), BSA (68 kDa), ovalbumin (47 kDa), carbonic anhydrase (31.5 kDa), and lysozyme (18.9 kDa). The molecular mass of the glycopeptide before and after enzymatic deglycosylation was determined by matrix assisted laser desorption mass spectrometry using Perseptive Biosystems Voyager Biospectrometry workstation. Amino acid analyses were performed after hydrolysis of the sample in 6 N HCl at 110 °C for 24 h in evacuated sealed glass bulbs. Samples for hexosamine analyses were hydrolyzed in 4 N HCl at 100 °C for 4 h. Samples were analyzed on a Beckmann System Gold amino acid analyzer using α-amino-β-guanidinopropionic acid as an internal standard. The total carbohydrate content of the samples was estimated by the phenol-sulfuric acid procedure [24]. Individual neutral sugars and acetylhexosamines were estimated by gas-liquid chromatography as alditol acetates. The samples were hydrolyzed with 2 N HCl for 2 h at 100 °C, reduced with sodium borohydride and acetylated. The samples were analyzed by GLC using a Varian Gas Chromatograph Series 3400 on a column packed with 3% OV-225 at 170 °C isothermally for 5 min followed by an increase in temperature of 5 °C min^{-1} to 190 °C and held at that temperature for 20 min, followed by another linear increase in temperature of 5 °C min^{-1} to 210 °C and held at that temperature for 25 min. Arabitol was used as an internal standard. Sialic acid was determined according to the thiobarbituric acid procedure of Warren [28].

Periodate oxidation and Smith degradation

GPRP glycopeptide (1.0 μmol) was oxidized with 2 ml of 0.08 M $NaIO_4$ in 0.05 M acetate buffer, pH 4.5 at 4 °C for 60 h, in the dark [29]. Excess periodate was destroyed by adding 50 μl of ethylene glycol. The reaction mixture was adjusted to pH 8.0 with 1 N NaOH. A 2 M sodium borohydride solution was added to a final concentration of 0.2 M and reduction continued for 24 h at 4 °C. Excess borohydride was destroyed by the addition of a few drops of glacial acetic acid. The reaction mixture was desalted on a column (1.5 × 100 cm) of Sephadex G-25 (fine). Glycopeptide fractions were pooled, lyophilized and dissolved in 1 ml water. Appropriate aliquots were taken for amino acid, hexosamine and neutral sugar analyses. The remaining sample was subjected to partial acid hydrolysis in 0.05 N H_2SO_4 for 90 min at 80 °C. The products of partial acid hydrolysis of periodate oxidized-reduced glycopeptide were fractionated on a column (1.5 × 100 cm) of Sephadex G-25.

Inter-sugar linkage analysis of the glycopeptide fraction: methylation and analysis by gas chromatography-mass spectrometry (GC-MS)

Partially-methylated alditol acetates (PMAAs) were prepared by methylation of the glycopeptide fraction according to the method of Anumula and Taylor [30]. Approximately 100 μg neutral sugar equivalents of the lyophilized glycopeptide fraction were taken for the permethylation procedure. The PMAAs were analyzed on a Finnegan 4500 gas chromatography-mass spectrometry (GC/MS) system operating in the electron impact mode. Separation of partially methylated hexitol and hexosaminitol acetates was achieved on a DB-1 capillary column (30 m long, 0.25 mm i.d., 0.25 μm phase thickness). The carrier gas was helium (19 psi, 38 cm s^{-1}). Injections were performed in the splitless mode. The column program consisted of an initial isocratic hold at 80 °C (2 min), a linear gradient to 150 °C (10 °C min^{-1}) and a final linear gradient to 280 °C (2 °C min^{-1}) maintained for 10 min. The temperatures of the ion source, injector and transfer lines were 180 °C, 250 °C, and 290 °C, respectively. A scan time of 1.5 s per scan was used and the INCOS Data System was used to manage the data.

The sugar derivatives were identified by comparison of their retention times relative to those of PMAA standard sugars available in the laboratory. Methylated reference compounds from the thiostatin glycopeptides: (a) were as described previously [31]. The following oligosaccharides used for the preparation of the additional methylated reference compounds were purchased from Sigma: (b) 2-acetamido-2-deoxy-4-*O*-(4-*O*-β-D-galactopyranosyl-β-D-galactopyranosyl)-D-glucopyranose; (c) methyl-2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside; (d) 2-acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-glucopyranoside; (e) benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; (f) methyl-β-*O*-α-D-mannopyranosyl-α-D-mannopyranoside; (g) methyl-3-*O*-(2-acetamido-2-deoxy-4-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside; and (h) 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucopyranose. Each of the above sugars was dissolved separately as a 1 mg ml^{-1} solution in HPLC grade water, and 200 µl aliquots (200 µg) taken for permethylation as described above.

PMAA samples also were gas chromatographed on a Varian Series 3400 instrument equipped with a flame ionization detector and connected to a Varian 4290 integrator. The PMAA samples were separated on a DB-17 capillary column (15 m long, 0.519 mm i.d., 1.0 µm phase thickness). The nitrogen carrier flow rate was 60 ml min^{-1} and the hydrogen and air flow rates were 40 ml min^{-1} each. Injections were made in the split-less mode. The program consisted of an initial isocratic step at 140 °C for 10 minutes, a linear gradient to 200 °C (2 °C min^{-1}) held for 5 min followed by a gradient up to 225 °C (5 °C min^{-1}) and maintained at that temperature for 5 min.

Enzymatic hydrolysis of GPRP-oligosaccharide

Radiolabeled oligosaccharide (100–200 nmol, 1×10^5 cpm) in 200 µl of 0.05 M citrate/phosphate buffer, pH 4.8, was treated with β-*N*-acetylhexosaminidase (0.5–1.0 U), β-galactosidase (0.2–0.5 U) and α-mannosidase (0.2–0.5 U) either individually or as a mixture. The incubation was continued for 2 days at 37 °C in an atmosphere of N_2. The sample was layered with toluene by adding a drop of solvent to avoid any bacterial growth. The products of the enzymatic digest of radiolabeled oligosaccharides were fractionated on a column (1.5×150 cm) of Biogel P-4, packed in and eluted with 0.005 N acetic acid. In order to locate the released sugars the column was calibrated using radiolabeled *N*-acetylglucosaminitol. Oligosaccharide containing fractions were pooled, lyophilized and dissolved in a known volume of water. Appropriate aliquots were taken for hexosamine and neutral sugar analyses. Fractions containing released sugars were passed through anion exchangers AG-1 and AG-50, eluted with water, reduced with $NaBH_4$ and acetylated. The sugars released by enzymes were analyzed by GLC.

Results

Preparation of glycopeptides and oligosaccharides

No oligosaccharides were released from the purified GPRP following β-elimination using conditions described for the release of O-linked oligosaccharides from rat submandibular gland mucin [32]. Deglycosylation of the purified GPRP with PNGase F produced a single homogeneous band of apparent molecular weight of 150 000. PNGase-treated protein did not stain with periodate-Schiff's reagent (not shown), suggesting that all the oligosaccharides were *N*-linked to the protein backbone. The oligosaccharides released from the glycoprotein were derivatized with ANTS and analyzed by polyacrylamide gel electrophoresis. Derivatized oligosaccharides revealed a major band and a minor band with relative mobilities similar to $(Glc)_{12}$ and $(Glc)_{11}$ of the maltooligosaccharide series derived from starch, respectively (data not shown).

The glycopeptides were prepared from GPRP by proteinase K digestion followed by the fractionation of the peptides and glycopeptides by gel filtration on Sephadex G-50 (superfine). Four major peptide containing peaks were obtained (Figure 1). Compositional analyses revealed that pool B contained most of the carbohydrate (83%) present in GPRP and no significant difference was observed in the carbohydrate composition of pools A and B. The oligosaccharides released from both pool A and pool B fractions after derivitization with ANTS showed a similar patterns on polyacrylamide gel electrophoresis, a major band and a minor band with relative mobilities similar to $(Glc)_{12}$ and $(Glc)_{11}$. Glycopeptide present in pool B was further purified by gel filtration on Sephadex G-25 and subsequently by HPLC on C_{18} reverse phase column (Figure 2, inset).

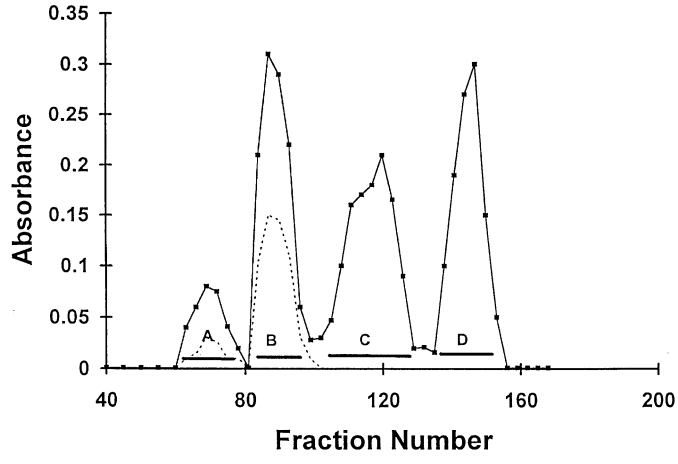

Figure 1. Sephadex G-50 elution of Proteinase K digested GPRP. The proteinase K digest was applied to and then eluted from a Sephadex G-50 column (200 × 1.5 cm) equilibrated with 0.002 N acetic acid. Two-ml fractions were collected. A_{230} nm (■) was recorded and 50 µl aliquots were assayed for hexose (A_{490} nm, - - -) by phenol sulfuric acid method.

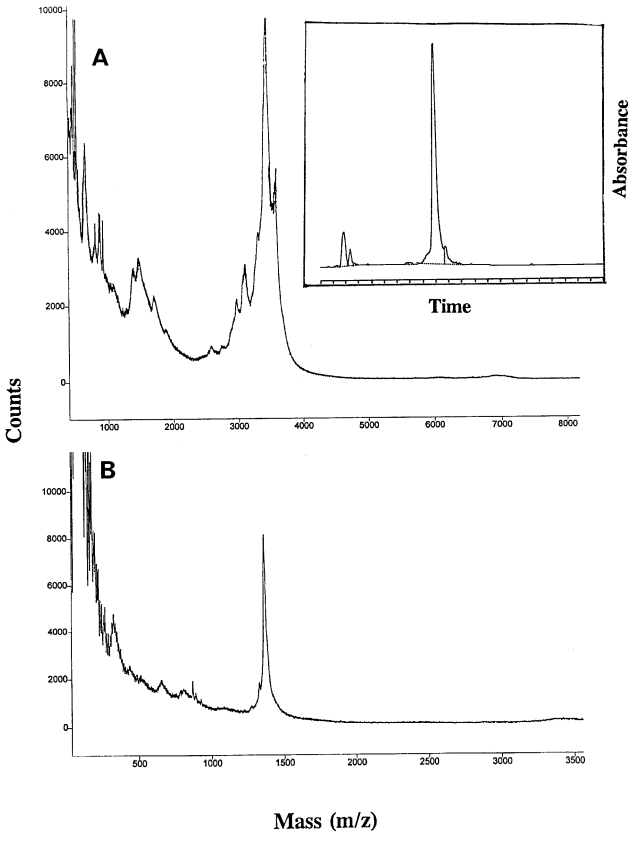

Figure 2. Matrix-assisted laser desorption mass spectrum of the glycopeptide prepared from GPRP by proteinase K digestion (upper panel) and the deglycosylated peptide generated by digestion of glycopeptide with PNGase F (lower panel). Analytical reverse-phase HPLC of the purified glycopeptide used in these studies is shown in inset.

Physico-chemical characterization of asparaginyl glycopeptide and oligosaccharide

Proteinase K digestion of GPRP produced a glycopeptide with an average chemical composition as follows (residues/mol): Pro_4, Glx_3, Asx_2, Gly_1, His_1, Thr_1, Arg_1, $GlcNAc_5$, $GalNAc_1$, Man_3, Gal_{2-3}, and Fuc_1. Minor heterogenicity was revealed in the purified glycopeptide by matrix-assisted laser desorption mass spectral analysis (Figure 2A). Molecular weight of 3694 ± 36 for the major purified glycopeptide is consistent with the expected molecular mass based on compositional analysis. Additional peaks observed may represent sugar moieties containing fucose (m/z 3829 ± 23) or lacking terminal galactose (m/z 3535 ± 25). Deglycosylation of glycopeptide following treatment with PNGase F generated a single peptide with a molecular mass of 1462 ± 20, which is consistent with the calculated mass of 1470 (Figure 2B). The carbohydrate composition of the oligosaccharide was also similar to that of glycopeptide. Based on 3 residues of mannose, the molar composition of the oligosaccharide is fucose (0.8), mannose (3.0), galactose (2.7), *N*-acetylglucosamine (5.1) and *N*-acetylgalactosamine (1.0).

Methylation analysis

In order to determine the glycosidic linkages the intact glycopeptide was partially methylated, hydrolyzed, reduced and acetylated. The partially methylated alditols and hexosaminitol acetates were identified by their retention times relative to 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol by comparison with those of authentic standards (Figure 3 and Table 1). Confirmatory evidence was obtained by GLC/MS analysis. Fragment patterns were compared with those reported by Jannson *et al.* [33] for partially methylated alditol acetate standards of neutral sugars and by Stellner *et al.* [34] for partially methylated acetylated hexosaminitol standards and also with known methylated sugar standards. Table 1 shows the methylated sugar derivatives identified and the relative number of residues of various methylated derivatives obtained from GPRP glycopeptide. Identical ratios were obtained for partially methylated GPRP oligosaccharide (data not shown).

Smith degradation

The treatment of the GPRP glycopeptide with eight- to 10-fold excess of $NaIO_4$ at 4 °C for 60 h caused almost complete destruction of fucose, galactose and *N*-acetylgalactosamine, 1 of the 3 residues of mannose and 0.2 residues out of the 4.9 residues of *N*-acetylglucosamine (OR-1, Table 2). During mild acid hydrolysis of periodate-oxidized, borohydride-reduced glycopeptide 1 residue of *N*-acetylglucosamine was lost, leaving 2 residues of mannose and 4 residues of *N*-acetylglucosamine per 2 mol of Asx (ORH-1, Table 2). A second cycle of the periodate oxidation of the remaining glycopeptide resulted in destruction of 1.2 additional residues of *N*-acetylglucosamine, whereas both mannose residues remained intact.

Treatment of GPRP oligosaccharide with exoglycosidases

The sequence of peripheral monosaccharides in oligosaccharide, and their anomeric configurations, were determined by individual and sequential treatment of the radiolabeled oligosaccharide with specific glycosidases. The oligosaccharides after each glycosidase treatment were recovered by gel filtration on Bio-Gel P4 column, and their carbohydrate composition determined (Table 3). The released sugars were also analyzed by GLC following desalting of the excluded fractions from Biogel P-4 column by mixed ion-exchangers, reduction and acetylation. Treatment of oligosaccharide with β-*N*-acetylhexosaminidase from *A. niger* resulted in the liberation of 0.4 mol of *N*-acetylglucosamine and 0.2 mol of *N*-acetylgalactosamine per mol of oligosaccharide. Approximately one-half of the galactose residues was released by treatment with β-galactosidase alone, and almost 100% of galactose residues were released from oligosaccharide when treated with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase.

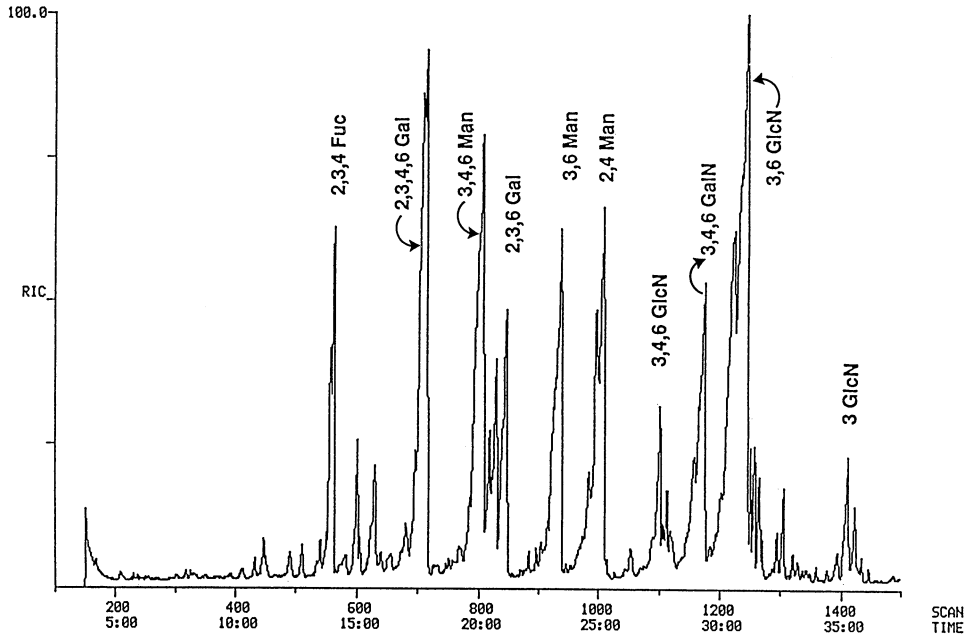

Figure 3. Gas-liquid chromatography of partially methylated alditol acetates (PMMAs) obtained from GPRP glycopeptide. A DB-1 capillary column (30 m long, 0.25 mm i.d., 0.25 μm phase thickness) was used to resolve the PMMAs on a Finnegan 4500 GC-MS system operating in the electron impact mode as described in methods section.

Table 1. Linkage analysis, relative retention time and molar ratios of partially methylated alditol acetates obtained from the methylated glycopeptide of rat parotid gland proline-rich glycoprotein

Methylated sugar	*Position substituted*	*Relative retention time*[a]		*Molar ratio*[b]
		GPRP	*Reference compounds*	
Fucose				
2,3,4-tri-*O*-methyl	1-*O*-	0.698	0.694[c]	0.60
Galactose				
2,3,4,6-tetra-*O*-methyl	1-*O*-	0.872	0.873[d]	1.50
2,3,6-tri-*O*-methyl	1,4-di-*O*-	1.008	1.005[d]	1.01
2,3,4-tri-*O*-methyl	1,6-di-*O*-	1.051	1.049[c]	0.13
Mannose				
3,4,6-tri-*O*-methyl	1,2-di-*O*-	1.000	1.000[g]	1.00
3,6-di-*O*-methyl	1,2,4-tri-*O*-	1.158	1.158[c]	1.01
2,4-di-*O*-methyl	1,3,6-tri-*O*-	1.250	1.252[e]	1.00
2-Deoxy-2-*N*-methylacetamidoglucose				
3,4,6-tri-*O*-methyl	1-*O*-	1.360	1.361[f]	0.35
3,6-di-*O*-methyl	1,4-di-*O*-	1.511	1.511[d]	3.75
3-*O*-methyl	1,4,6,-tri-*O*-	1.611	1.613[c]	0.54
2-Deoxy-2-*N*-methylacetamidogalactose				
3,4,6-tri-*O*-methyl	1-*O*-	1.420	1.421[g]	1.06

[a] With respect to 3,4,6-tri-*O*-methyl-D-mannitol.
[b] Calculated assuming 2,4-di-*O*-methyl-D-mannitol as 1.0.
[c–g] Reference compounds used were: c, thiostatin glycopeptide; d, 2-acetamido-2-deoxy-4-*O*-(4-*O*-*β*-D-galactopyranosyl-*β*-D-galactopyranosyl)-D-glucopyranose; e, methyl 2-*O*-*α*-D-mannopyranosyl-*α*-D-mannopyranoside; f, 2-acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-*β*-D-glucopyranosyl)-D-glucopyranose; g, benzyl-2-acetamido-2-deoxy-*α*-D-galactopyranoside.

Table 2. Smith degradation of glycopeptide of rat parotid proline-rich glycoprotein.

Sugar[a]	*Native*	*OR-1*[b]	*ORH-1*[b]	*ORH-2*[b]
Fucose	0.8	0	0	0
Mannose	3.0	2.4	2.3	1.9
Galactose	2.6	0.3	0	0
N-Acetylglucosamine	4.9	4.7	3.7	2.5
N-Acetylgalactosamine	1.0	0.1	0.1	0

[a] Number of residues of sugar based on two residues of Asx in glycopeptide.
[b] OR, periodate-oxidized, borohydride-reduced; ORH, periodate-oxidized, borohydride-reduced, acid hydrolyzed; numbers refer to cycle of Smith degradation.

Treatment with a mixture of β-*N*-acetylhexosaminidase and β-galactosidase also resulted in the release of 3 of the 5 residues of *N*-acetylglucosamine and the complete removal of *N*-acetylgalactosamine. Subsequent treatment with α-mannosidase released 40–45% of the mannose residues.

Discussion

Unlike the constitutive nature of the PRPs in human saliva, the salivary glands of mice, rat and hamsters normally contain low levels of PRPs. Chronic treatment of these animals with β-agonist isoproterenol results in a marked induction of PRPs in both parotid and submandibular glands [16–20]. The proteins induced in rat parotid glands include a glycosylated proline-rich protein of apparent molecular weight of 220 000 [17, 19, 20]. The glycoprotein isolated from rat parotid glands is substantially larger in molecular weight than glycosylated PRPs isolated from rat submandibular glands [20], human saliva [35–37], subhuman primate *Macaca fascicularis* [38] and rabbit parotid saliva [39]. The amino acid composition of parotid GPRP follows a pattern similar to that reported for GPRPs from various sources [20, 36, 38, 40]. Although the amino acid composition of rat parotid GPRP [23] is similar to that reported from submandibular gland glycoprotein GP-158, subtle differences are observed in the carbohydrate composition of rat parotid gland GPRP and rat submandibular gland GP-158. Rat parotid GPRP contains 2.52 mol percent of *N*-acetylgalactosamine but no sialic acid was detected in rat parotid GPRP. On the contrary, rat submandibular gland GP-158 contains no *N*-acetylgalactosamine and a much higher mol percent of sialic acid [20]. The carbohydrate composition of parotid GPRP also differs from the carbohydrate composition of glycosylated PRPs from human saliva, subhuman primate saliva and rabbit saliva.

The compositional analysis of the oligosaccharide prepared by hydrazinolysis confirms that *N*-acetylgalactosamine is an integral part of the asparagine-linked sugar moiety. Although *N*-acetylgalactosamine is commonly present as linkage monosaccharide in O-glycosidic linked carbohydrate units, its presence in the *N*-glycosidic carbohydrate has also been previously reported [41–47]. Rabbit PRP also contains *N*-acetylgalactosamine but the nature of the glycosidic linkage has not been determined [39].

Based on the results of methylation, periodate oxidation and enzymatic hydrolysis, the following structure (Figure 4) is proposed for the carbohydrate units of GPRP. The major *N*-linked carbohydrate structure of rat parotid GPRP consists triantennary glycan, with β-*N*-acetylgalactosamine in a terminal position. In the proposed structure *N*-acetylgalactosamine is shown on any one of the galactose residues, since its presence on either of the three galactose residues cannot be ruled out based on the present data. As discussed below, the proposed structure shows some microheterogeneity with respect to part of the oligosaccharide chains lacking terminal Gal. This is consistent with the presence of at least two bands on PAGE following release of oligosaccharides with PNGase.

Table 3. Treatment of GPRP-oligosaccharide with individual or mixture of glycosidases

Glycosidase	*Molar ratio*[a]				
	Fuc	*Man*	*Gal*	*GlcNAc*	*GalNAc*
(1) Control	0.80	3.00	2.70	5.10	1.00
(2) β-*N*-Acetylhexosaminidase	0.80	3.00	2.70	4.70	0.76
(3) β-Galactosidase	0.75	3.00	1.25	4.9	0.96
(4) β-*N*-Acetylhexosaminidase + β-Galactosidase	0.80	3.00	0	1.70	0
(5) β-*N*-Hexosaminidase + β-Galactosidase + α-Mannosidase	0.35	1.35	0	1.00	0

[a] Calculated on the basis of 3 mannose residues except for the treatment with mannosidase where the molar ratio is relative proportion of sugars.

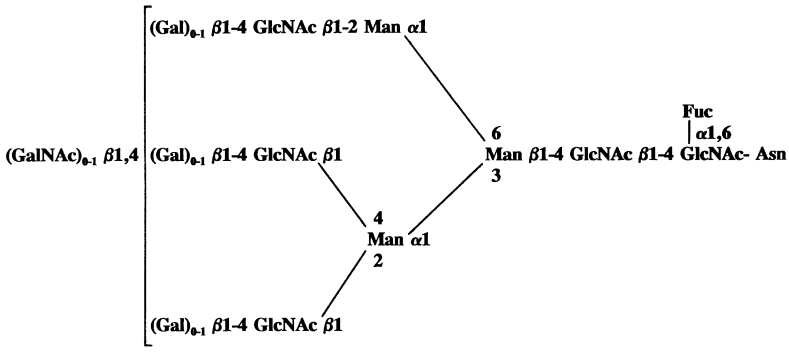

Figure 4. Proposed structure of the asparagine-linked carbohydrate units of GPRP. The presence of *N*-acetylgalactosamine on either of the three galactose residues cannot be ruled out based on the present data. The proposed structure also shows some microheterogeneity with respect to part of the oligosaccharide chains lacking terminal Gal.

The nonreducing terminal location of fucose, *N*-acetylgalactosamine, 1.5 residues of galactose and 0.35 residues of *N*-acetylglucosamine was established by methylation analysis. The destruction of galactose, fucose, *N*-acetylgalactosamine and 1 of the 3 residues of mannose is in agreement with the methylation data. Since only trace amounts of *N*-acetylglucosamine residues were destroyed by periodate oxidation, most of the *N*-acetylglucosamine residues must have been substituted at 0–3 or 0–4 or both. Methylation data confirmed that these sugars were substituted either at 0–4 or at both 0–4 and 0–6 positions. The presence of 3.75 residues of 1,4-di-*O*-substituted and 0.54 residues of 1,4,6-tri-*O*-substituted *N*-acetylglucosamines suggest that most of the glucosamine residues are internal residues. The presence of 0.35 residues of terminal *N*-acetylglucosamine and 1.5 residues of terminal galactose suggests that one of the non-reducing branches may be lacking terminal galactose. This is further confirmed by release of 0.4 residues of GlcNAc following treatment with *N*-acetylhexosaminidase, as well as destruction of 0.2 residues of GlcNAc during the first cycle of Smith degradation (OR-1, Table 2). Further, methylation data showed that one residue of galactose was substituted at position 4.

On methylation analysis of GPRP glycopeptide three mannose residues were detected as 3,4,6-tri-*O*-methylmannitol, 2,4-di-*O*-methylmannitol and 3,6-di-*O*-methylmannitol in a ratio of about 1:1:1, suggesting a triantennary carbohydrate chain structure. The branching structure of the three mannose residues was further confirmed by Smith degradation. Periodate oxidation resulted in the destruction of 1 of 3 mannosyl residues, indicating that this mannosyl residue was linked by 1,2 linkage identified by methylation analysis. When the periodate oxidized-reduced glycopeptide was subjected to mild acid hydrolysis, one residue of *N*-acetylglucosamine was lost. This indicates that *N*-acetylglucosamine was linked to mannose by 1,2 linkage.

Although the non-reducing terminal location of *N*-acetylgalactosamine was established by both methylation analysis and Smith degradation, the treatment of the oligosaccharide with β-*N*-acetylhexosaminidase from *A. niger* resulted in the liberation of only 0.2 mol of *N*-acetylgalactosamine per mol of oligosaccharide. The reason for lack of cleavage of GalNAc with β-*N*-acetylhexosaminidase alone is not understood at present. In view of the fact that terminal GalNAc has frequently been observed to be sulfated [48], we checked for the presence of sulfate and did not detect any sulfate present in oligosaccharide isolated from GPRP. Furthermore, all GalNAc residues were released following simultaneous treatment of oligosaccharide with β-galactosidase and β-*N*-acetylhexosaminidase, suggesting that the structure may be causing some steric hindrance to cleavage with β-*N*-acetylhexosaminidase alone. Oligosaccharide treated with β-galactosidase alone still contained 1.25 residues of galactose, confirming that at least one galactosyl residue is substituted at its terminal position with *N*-acetylhexosamine. Although from the proposed structure one would expect a ratio of Fuc:Man:GlaNAc of 1:1:2 following treatment with α-mannosidase (Table 3 last line), only 40–45% of mannose residues were released. This discrepancy may be because of the partial cleavage with α-mannosidase but could not be confirmed because of the limited availability of material.

There are several regulatory points in the biosynthesis and tissue-specific expression of glycoproteins. It has been demonstrated that different tissues can generate distinct glycoforms of otherwise identical proteins based on the differential expression of glycosyltransferases [49]. Two distinct glycoforms of carbonic anhydrase have been identified in bovine submandibular and parotid glands [50]. These glycoforms differ from each other in that the majority of Asn-linked oligosaccharides in the submandibular form terminate with GalNAc-4-SO_4 but the terminal GalNAc in parotid glands is not sulfated [50]. It has been demonstrated that differential expression of glycosyltransferases results in the distinct glycoforms of carbonic anhydrase in bovine parotid and submandibular glands [50]. GalNAc-4-sulfotransferase is not expressed in bovine parotid glands, therefore carbonic anhydrase synthesized in bovine parotid

gland terminates with β1,4-linked GalNAc which is not sulfated. Because of the unique, inducible character of rat salivary PRPs, these glycoproteins provide a useful model with which to explore basic questions about the expression and regulation of glycosyltransferases. Influence of β-adrenergic stimulation on glycosylation of *N*-asparagine linked glycoproteins in rat parotid glands has been reported [51], and an increased expression of glycosyltransferases has been observed as a consequence of chronic injection of isoproterenol [52]. Because of the unique inducible nature of glycosylated proline-rich glycoproteins and different glycoforms of the same protein in submandibular and parotid glands structural information of these glycoproteins will be helpful in understanding the mechanisms of tissue-specific expression and posttranslational modification which operate in response of β-adrenergic stimulation.

The most abundant carbohydrate chains of human salivary GPRP are biantennary oligosaccharide substituted with two fucose residues on one of the antenna [35]. It has been suggested that binding to *Fusobacterium nucleatum* is mediated through unsubstituted lactosamine sequence in the biantennary carbohydrate structure [35]. The data obtained in these studies shows various salient and unique features of rat parotid GPRP, like the presence of triantennary structure with *N*-acetylgalactosamine in the terminal position. Binding properties of rat parotid glycoprotein to bacteria may provide additional information about structure-function relationship of glycosylated salivary glycoproteins.

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