Isolation of Galactoprotein a from Hamster Embryo Fibroblasts and Characterization of the Carbohydrate Unit†

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ABSTRACT: A major cell surface labeled glycoprotein of hamster embryo fibroblasts, with a subunit molecular weight of 230000, which is deleted on viral transformation [Gahmberg, C. G., & Hakomori, S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3329–3333; Hynes, R. O. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3170–3174], was quantitatively extracted and purified on an insolubilized *Ricinus communis* lectin–poly(acrylhydrazido)agarose column. A glycopeptide with a molecular weight of 2000 was isolated from the glycoprotein after exhaustive digestion with protease of *Streptomyces griseus*. The carbohydrate structure of the isolated glycopeptide was determined, as seen in Figure 7, by carbohydrate analysis, stepwise degradation by exoglycosidases followed by hydrolysis with endo-β-*N*-acetylglucosaminidase of *Diplococcus pneumoniae*, methylation analysis with gas chromatography–mass spectrometry, and direct probe mass spectrometry of the methylated core oligosaccharide, released by endo-β-*N*-acetylglucosaminidase.

The cell surface glycoproteins of hamster embryonic fibroblasts and their changes associated with viral transformation have attracted much attention since we introduced the surface-labeling procedure with galactose oxidase and NaB^3H_4 (Gahmberg & Hakomori, 1973a). One of the prominent transformation-dependent changes of surface galactoproteins was the deletion of a high molecular, protease-sensitive glycoprotein with a subunit molecular weight of 230000, which was termed "galactoprotein a"[1] (hereafter abbreviated as Gap a) (Gahmberg & Hakomori, 1973b, 1974, 1975; Gahmberg et al., 1974). A glycoprotein similar to or identical with Gap a was described in various laboratories during 1973 to 1974 and was termed LETS (large external transformation sensitive protein) (Hynes, 1973, 1976), Zeta (Wickus et al., 1974), SF210 (Vaheri & Ruoslahti, 1974), CSP (Yamada & Weston, 1974), and Fibronectin (Kuusela et al., 1976); for a review see Hynes (1976). The Gap a increased at cell contact, at the G_1 phase of the cell cycle (Hynes & Bye, 1974; Gahmberg et al., 1974; Gahmberg & Hakomori, 1974), and interacted with concanavalin A, *Ricinus communis* lectin (Gahmberg & Hakomori, 1975), collagen (Pearlstein, 1976; Engvall & Ruoslahti, 1977), and possibly fibrinogen and fibrin (Mosesson et al., 1975). The 230000 molecular weight subunits may be involved in a disulfide-linked intercellular matrix (Hynes & Destree, 1977; Chen et al., 1978). The physical properties and amino acid and carbohydrate composition of the isolated cell surface glycoprotein have been recently reported (Carter & Hakomori, 1977; Yamada et al., 1977) although the structure of this glycoprotein is not known. Gap a released from the cell surface into culture medium is immunologically cross-reactive to a well-known serum component, cold insoluble globulin (abbreviated as CIG; Morrison et al., 1948). The cold insoluble globulin is distinctively different from cell-bound Gap a or LETS) in molecular weight, solubility, and aggregative property. Nevertheless, most of the chemical studies have been made on the released form of the glycoprotein or CIG because of their availability (Mosesson & Umfleet, 1970; Mosher, 1975).

This paper describes the isolation of cell surface Gap a, a glycopeptide derived from isolated Gap a, and the structure of the carbohydrate moiety of the glycopeptide through degradation with exo- and endoglycosidases, methylation analysis, and direct probe mass spectrometry of an oligosaccharide fragment.

Materials and Methods

Cells and Cell Culture. Hamster embryo fibroblasts were prepared by teasing and trypsinizing 13- to 14-day-old Syrian hamster embryos, followed by culturing adherent cells in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Cells were frozen after two passages and utilized as stock cells for all cell cultures.

Surface Labeling. Galactose oxidase–NaB^3H_4 surface labeling was carried out essentially as previously described (Gahmberg & Hakomori, 1973a). Cells on glass roller bottles were washed three times with salt/P_i buffer and then incubated with galactose oxidase (Kabi, Aktebalaget, Fack, Stockholm) in salt/P_i buffer (3 units/mL) for 2 h at 37 °C. The adherent oxidized cells were washed three times with salt/P_i buffer, scraped with a rubber policeman, and then centrifuged at 800*g* for 10 min. The cell pellet was suspended in four volumes of salt/P_i buffer to which was added 2.5 mCi of NaB^3H_4 (New England Nuclear; 8.2 Ci/mmol)/mL of cell suspension. After a 30-min reduction, the label was chased with 1 mM $NaBH_4$ for 15 min, followed by four washings with salt/P_i buffer.

Metabolic Labeling of Cells. Subconfluent cultures of hamster embryo fibroblasts were metabolically labeled with tritiated glucose (New England Nuclear; 34.0 Ci/mmol) by culturing in Dulbecco's modified Eagle's medium with 10% fetal calf serum for approximately 72 h (2 μCi/mL of media). Cells were harvested by rubber policeman after washing three times with salt/P_i buffer.

Polyacrylamide Gel Electrophoresis. Linear polyacrylamide gradient (5–14%) slab gels containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$) were prepared following the basic stacking $NaDodSO_4$ gel procedure of Laemmli (1970). Cell

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[1] Abbreviations used: ATP, adenosine triphosphate; CIG, cold insoluble globulin; EDTA, ethylenediaminetetraacetate; Gap a, galactoprotein a; GC, gas chromatography; GC–MS, combination equipment of gas chromatography and mass spectrometry; LETS, large external transformation sensitive protein; MS, mass spectrometry; RCA, *Ricinus communis* lectin; RCA–PAHA, *Ricinus communis* lectin bound to poly(acrylhydrazido)agarose; salt/P_i buffer, 10 mM sodium phosphate buffer, pH 7.2, containing 0.9% NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$; $NaDodSO_4$, sodium dodecyl sulfate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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samples (75–150 μg of protein) were dissolved in the sample buffer containing 2% $NaDodSO_4$ and 5% 2-mercaptoethanol and heated in a boiling-water bath for 5 min. Slab gels were stained with Coomassie blue R-250. Fluorography of slab gels followed the procedure of Bonner & Laskey (1974). Protein was determined utilizing the fluorescamine assay (Udenfriend et al., 1972).

Preparation of Hamster Skeletal Muscle Actin and Myosin. Hamster skeletal muscle actin was prepared as described by Mommaerts (1951). Hamster skeletal muscle myosin was prepared as described by Yagi (1974).

Preparation of Ricinus communis Lectin–Poly(acrylhydrazido)agarose. The *Ricinus communis* lectin purified from castor beans (Nicolson et al., 1974) was covalently attached to glutaraldehyde-activated poly(acrylhydrazido)-agarose (RCA–PAHA) (Wilchek & Miron, 1974), followed by reduction with $NaBH_4$ (Miron et al., 1976).

Sequential Extraction of Cell Surface Glycoproteins and Purification by Lectin Affinity Chromatography. Extraction and purification of Gap a followed the basic conditions as described by Carter & Hakomori (1977). Galactose oxidase–NaB^3H_4 or metabolically labeled cells were suspended in five volumes of ice-cold 20 mM Tris-HCl buffer, pH 7.6, containing 0.34 M sucrose, 1 mM ATP, 1 mM EDTA, and 0.2 mM TLCK. After 60 min on ice the cell suspension was centrifuged at 800*g* for 10 min (low speed), and the supernatant was removed and recentrifuged at 35000*g* for 20 min (high speed). The high-speed pellet was added back to the low-speed pellet and reextracted as above. The first and second high-speed supernatants were pooled and labeled sucrose–ATP–EDTA extract. The above procedure was repeated on the cell pellet utilizing two 60-min extraction cycles of ice-cold 20 mM Tris-HCl buffer, pH 7.6, containing 25 mM KCl, 5 mM $MgCl_2$, 5 mM, $CaCl_2$, 1.0% Empigen BB (Albright and Wilson Ltd., England), and 0.2 mM TLCK. The two combined high-speed supernatants were labeled Empigen BB extract. The remaining cell pellet was suspended in five volumes of 50 mM Tris buffer, pH 7.6, containing 8 M urea and 0.2 mM TLCK. The suspension was heated in a boiling-water bath for 5 min and then centrifuged at 35000*g* for 20 min. Lower urea concentrations or extraction at 4 °C resulted in reduced solubilization of radioactivity. The supernatant was removed and labeled urea extract.

The labeled glycoproteins in each of these extracts were respectively purified on an RCA–PAHA column. (1) The sucrose–ATP–EDTA extract was applied to a 25-mL bed-volume RCA–PAHA column containing 3 mg of RCA/mL of resin which had been equilibrated with 20 mM Tris-HCl buffer, pH 7.6, washed with the same buffer until no further radioactivity was eluted, and then eluted with the same buffer containing 0.5 M galactose. The peak of radioactivity eluted with galactose was extensively dialyzed against water and lyophilized. (2) The Empigen BB extract was chromatographed on an RCA–PAHA column in the same way as above except all buffers contained 0.5% Empigen BB. (3) The 8 M urea extract was first diluted to 2 M urea with 50 mM Tris-HCl buffer, pH 7.6, containing 1% Empigen BB, and then chromatographed on an RCA–PAHA column in 2 M urea and 0.5% Empigen BB. The glycoproteins in the Empigen BB and urea extract absorbed on an RCA–PAHA column and eluted with galactose were extensively dialyzed against water, 80% acetone, and water (Rice, 1974), lyophilized, and weighted.

The partially purified lectin receptors obtained from the sucrose–ATP–EDTA extract and Empigen BB extract are to be described elsewhere. Preparations of Gap a were obtained from the urea extract and were subject to further analysis.

Preparation of Glycopeptide by Pronase Digestion. Gap a isolated from hamster embryonic fibroblasts which were metabolically labeled with [^{3}H]glucose or cell surface labeled by the galactose oxidase–NaB^3H_4 method were subject to pronase (protease of *Streptomyces greseus*) digestion, followed by molecular sieve chromatography.

High-Voltage Paper Electrophoresis. Paper electrophoresis was perfomed using pyridine–acetic acid–H_2O (3:1:387 by volume), pH 5.4, on Whatman No. 1 paper as described by Tai et al. (1975a) at 53 V/cm for either 4.25 or 2.5 h. Preliminary electrophoresis runs were made for 60 min utilizing aspartic acid as a high-mobility ion, to insure that no other high-mobility glycopeptides might be lost during extended electrophoresis. Radioactive glycopeptides were detected with a Packard Model 720 radiochromatogram scanner or by cutting the chromatograms into strips, wetting, and counting in a liquid scintillation counter.

Carbohydrate Composition. The carbohydrate content of glycoprotein and glycopeptides was determined by gas–liquid chromatography (GC) of the sugars released by hydrolysis in 90% acetic acid containing 0.5 N H_2SO_4, under a nitrogen atmosphere, at 80 °C, in an oven, for 8 h. These hydrolysis conditions are modifications of previously described procedures (Yang & Hakomori, 1971; Ito et al., 1977) and shall be described in detail elsewhere (Powell & Fukuda, unpublished results). Subsequent reduction and acetylation were as previously described (Yang & Hakomori, 1971). [^{14}C]Inositol was included as an internal standard throughout the analysis. The alditol acetate derivatives were injected on a 6-ft column of 3% OV-225 using helium as a carrier gas. The temperature was maintained at 160 °C for 10 min and then programmed to 210 °C at 1 °C min. A flame ionization detector was used for quantitation of the released alditol acetate derivatives by comparison to alditol acetate derivatives of standard monosaccharides. All release sugars were confirmed by gas chromatography–mass spectrometry (GC–MS).

All released sugars were confirmed to be of cellular origin by detection of radioactivity in the separated alditol acetate sugar derivatives obtained from [^{3}H]glucose metabolically labeled Gap a. The flame of the flame ionization detector was extinguished, and derivatives were collected in a capillary pipet cooled with dry ice as previously described (Gahmberg & Hakomori, 1973a). The derivatives were eluted from the capillary tubes with scintillation fluid and counted. Retention time of radioactive derivatives was based on the elution time relative to the [^{14}C]inositol derivative.

Sialic acid was quantitated with the fluormetric assay described by Hammond & Papermaster (1976) after hydrolysis with 0.05 N H_2SO_4 or 0.01 N HCl at 80 °C for 1 h.

Methylation Analysis. Glycopeptides and oligosaccharides were methylated as described by Hakomori (1964) and were isolated by chromatography on LH-20 in methanol–acetone (1:1). The purified methylated glycopeptide and oligosaccharide were hydrolyzed, reduced, and acetylated as described above for the unmethylated glycopeptide except that the hydrolysis was extended to 14 h for the permethylated glycopeptide. [^{14}C]Inositol was included as an internal standard. The partially O-methylated hexitol acetates and partially O-methylated 2-deoxy-2-(*N*-methylacetamido)hexitol acetates were separated and identified by GC–MS. Derivatives were separated on a 6-ft column of 3% OV-225 on Supelcoport (Supelco Co., Bellefonte, PA) programmed from 160 to 230 °C at 1 °C/min and confirmed by separation on a 6-ft column of 1.5% OV-17 on Gas-Chrom Q programmed from 140 to 230 °C/min. Glycopeptide isolated from bovine IgG was analyzed as described above and served as a source for

standard partially methylated sugar derivatives.

Due to the small quantities of Gap a glycopeptide analyzed (~10–25 μg), noncarbohydrate reagent contamination interfered with quantitation of some of the partially methylated derivatives on the basis of peak area from the gas chromatograph. The reagent contamination occurred during the process of hydrolysis and derivatization of partially O-methylated sugar acetates. Uncontaminated peaks were readily identified and quantitated from the reconstructed gas chromatograph. However, derivative peaks which cochromatographed with reagent contamination were not quantifiable but could be reliably identified on the basis of retention time and by scanning the entire GC elution profile for selected mass numbers (m/e).

Mass spectrometry was performed under the following conditions on a Finnigan Model 3300 (with an all-glass separator) with a Finnigan 6110MS data system: mass range, 40–325 amu; electron energy, 70 V; ion energy, 3.9 V; extractor, +9.8 V; lens, −40 V; emission, 0.5 mA; electron multiplier, 2100 V; and sensitivity, 10^{-7} A/V.

Direct Probe Mass Spectrometry of Oligosaccharides without Hydrolysis. Oligosaccharides (10–20 μg) were dissolved in 100 μL of 0.01 M NaOH containing 2.5 mCi of NaB^3H_4 (Amersham, IL; 5–10 Ci/mM) and incubated on ice for 6 h, followed by reduction in 120 μL of $NaBH_4$ (2 mg/mL) for 3 h at room temperature. The solution was made acidic with acetic acid, followed by addition of methanol–acetic acid (100:1), and evaporated under a stream of N_2. The methanol–acetic acid was added and evaporated four more times. The residue was dissolved in H_2O, lyophilized and then dried in vacuo over P_2O_5 at room temperature, and then methylated as described by Hakomori (1964). The methylation reaction mixture was dissolved in chloroform and extracted with H_2O. The chloroform layer was chromatographed on LH-20 in acetone, and the radioactive peak was collected and partitioned between chloroform and H_2O again. The chloroform layer was dried and redissolved in acetone. Aliquots were dried in direct probe capillary tubes and subject to direct probe mass spectrometry under the following conditions: electron energy, 30 V; ion energy programmed from 4.7 V; extractor, +2.7 V; lens, −20 V; emission, 0.5 mA; electron multiplier, 2200 V; and sensitivity 10^{-7} A/V.

Sequential Digestion with Exoglycosidases. Metabolically labeled glycopeptide was subject to sequential removal of carbohydrate by treatment with mild acid or glycosidases. After each hydrolysis the reaction was stopped, and the hydrolysate was analyzed by descending paper chromatography in ethyl acetate–pyridine–H_2O (12:5:4) for 18 h on Whatman 3 MM to identify released monosaccharides. The chromatogram was dried, cut in 1-cm strips, wetted, and counted in scintillation fluid. Alternatively, the hydrolysate was subject to the next hydrolysis procedure instead of paper chromatography. For example, glycopeptide was dissolved in 0.01 N HCl and hydrolyzed for 60 min at 80 °C in a nitrogen atmosphere and then frozen and lyophilized. The residue was dissolved in 25 μL of 50 mM sodium citrate, pH 4.25, containing 0.18 units of Jack Bean β-galactosidase (Li & Li, 1972) and incubated at 37 °C for 24 h in a toluene atmosphere. The sample was placed in a boiling-water bath for 1 min and then lyophilized. *N*-Acetyl-β-D-glucosaminidase of beef kidney (Boehring Mannheim), 0.04 units, dissolved in 25 μL of 10 mM sodium citrate buffer, pH 4.25, was added to the lyophilized residue and digested for 24 h at 37 °C. The sample was then analyzed by paper chromatography.

Digestion with Endo-β-N-acetylglucosaminidase H and D. Isolated galactose oxidase–NaB^3H_4 labeled glycopeptide was dissolved in 50 μL of 100 mM sodium citrate buffer, pH 5.2, containing 2.5 m units of endo-β-*N*-acetylglucosaminidase H (Tarentino & Maley, 1974) from *Streptomyces griseus* (Seikagaku Fine Biochemicals, Tokyo, Japan) and incubated for 18 h at 37 °C in a toluene atmosphere. The products were analyzed by high-voltage paper electrophoresis as described above.

Isolated [^{3}H]glucose metabolically labeled glycopeptide was digested with endo-β-*N*-acetylglucosaminidase D (Muramatsu, 1971) from *Diplococus pneumoniae* (Seikagaku Fine Biochemical, Tokyo, Japan) both before and after digestion with exoglycosidases. Glycopeptide was digested in 30 μL of 150 mM phosphate–citrate buffer, pH 6.5, containing 0.17 units/mL of endo-β-*N*-acetylglucosaminidase D for 24 h at 37 °C in a toluene atmosphere. The digestion products were analyzed by descending paper chromatography as described above and/or by molecular sieve chromatography on Bio-Gel P-2.

Standard Oligosaccharides. Glycopeptide was prepared from bovine IgG (Sigma Chemical Co.) by pronase digestion and molecular sieve chromatography basically as described in the legend in Figure 2. The bovine IgG core tetrasaccharide (Tai et al., 1975b) was prepared by sequential digestion with exoglycosidases and endo-β-acetylglucosaminidase D basically as described in the legend in Figure 5 for Gap a.

Fetuin glycopeptide (Spiro, 1973) ($NANA_3Gal_3Man_3GlcNAc_5Fuc_1Asx$) labeled with [^{14}C]acetic anhydride was a gift from Minoru Fukuda of this laboratory. Ovalbumin core oligosaccharide ($Man_5GlcNAc$) was prepared from ovalbumin glycopeptide fraction V (Tai et al., 1975a) by digestion with endo-β-*N*-acetylglucosaminidase D (by Michiko Fukuda, Fred Hutchinson Cancer Research Center, Seattle, WA) Human IgM core glycopeptide ($Man_3GlcNAc_2Fuc_1Asx$) was a gift from A. L. Tarentino, State of New York, Department of Health, Albany, NY, through Minoru Fukuda (Tarentino et al., 1975). Human IgM core tetrasaccharide ($Man_3GlcNAc_1$) was prepared from the glycopeptide by digestion with endo-β-*N*-acetylglucosaminidase D. Stachyose was purchased from Pfanstiehl Laboratories, Waukegan, IL.

Results

Isolation and Homogeneity of Galactoprotein a. Hamster embryonic fibroblasts were metabolically labeled with [^{3}H]glucose for 3 days to ensure uniform labeling of all sugar residues associated with glycoproteins (Shen & Ginsburg, 1967). Alternately, cell surface glycoproteins were oxidized with galactose oxidase, followed by reduction with NaB^3H_4. The labeled, harvested cells were then subject to selective, sequential extractions as previously described (Carter & Hakomori, 1977), first with Tris buffer containing sucrose, EDTA, and ATP, followed by extraction with the zwitterionic surfactant, Empigen BB. Finally, the cell residue was extracted with 8 M urea. The first two extractions remove most of the membrane and cytoplasm including Gap a found in the cytoplasm which may be incompletely glycosylated. The first two extracts were applied to an insolubilized *Ricinus communis* lectin affinity column. Elution with galactose afforded partially purified cell surface glycoproteins which are to be described elsewhere. The empigen-extracted residue was found to contain all of the cell surface labeled Gap a along with at least two proteins showing the same electrophoretic mobility as skeletal muscle myosin and actin (Figure 1, column 2). The residue was then extracted with 8 M urea and found to contain, minimally, 75% of the Gap a, most of the actin, and no myosin.

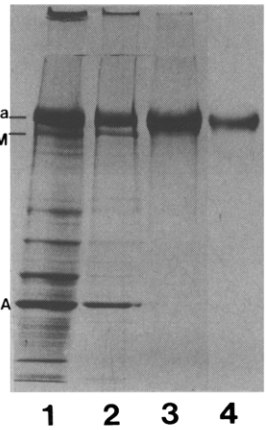

FIGURE 1: Electrophoretic pattern of galactoprotein a, isolated from [^{3}H]glucose metabolically labeled hamster embryo fibroblasts on pH 7.6 polyacrylamide gradient (5–14%) slab gels in the presence of 0.1% $NaDodSO_4$. Cells were metabolically labeled with [^{3}H]glucose, followed by successive extraction with sucrose–ATP–EDTA and with Empigen BB detergent. Gap a was finally extracted with 8 M urea from the detergent-insoluble residue and was purified from the diluted urea extract by affinity chromatography on insolubilized *Ricinus communis* lectin column. (1) Protein stain of hamster embryo fibroblast cells. (2) Protein stain of the Empigen BB extracted cell residue. (3) Protein stain of affinity purified Gap a. (4) Fluorograph of gel 3. (A) Migration of hamster skeletal muscle actin. (M) Migration of hamster skeletal muscle myosin. Direction of migration is from top (–) to bottom (+).

Application of the diluted urea extract to an insolubilized *Ricinus communis* lectin column, followed by washing and elution with galactose, afforded a preparation of Gap a in high yield. Routinely, the purified glycoprotein was found to contain variable quantities of the actin-like component.[2] This actin-like component was not labeled with radioactive carbohydrate precursors and therefore is not a glycoprotein and does not affect subsequent carbohydrate analysis. Extraction of the purified, lyophilized Gap a with chloroform–methanol (2:1) failed to solubilize any glycolipid material which might affect carbohydrate studies. No obvious radioactivity was detected at the top of the gel (Figure 1, column 4), which would indicate the presence of labeled mucin that would not be detected by protein stain. All the radioactivity detected by fluorography was associated with the Gap a band (Figure 1).

Carbohydrate Composition of Isolated Galactoprotein a. The isolated, metabolically labeled Gap a was subject to carbohydrate analysis by gas–liquid chromatography (GC) of alditol acetate derivatives after hydrolysis. The identity of each detected sugar residue was confirmed by GC–MS. Gap a was found to contain 5.5% carbohydrate by weight consisting of fucose, mannose, galactose, glucosamine, sialic acid, and galactosamine (Table I). The analysis detected small quantities of glucose in addition to the above sugar residues. However, monitoring of the radioactivity of each sugar derivative separated on GC established that glucose was not radiolabeled and therefore a minor contaminant of noncellular origin. Despite the extended period of incubation with radioactive sugar precursors, the radioactive monosaccharides detected in Gap a were of variable specific activity. As a result, detection of radioactive monosaccharides was useful in qualitative detection and in insuring that the residues were of cellular origin. All quantitative analyses were calculated on the basis of mass utilizing a flame ionization detector of a GC or the reconstructed gas chromatograph of a GC–MS.

[2] A possible association of actin and other cytoskeletal proteins with a specific component of plasma membrane has been studied and will be described elsewhere.

Table I: Carbohydrate Composition of Galactoprotein a and a Major Glycopeptide and the Core Glycopeptide Isolated from Galactoprotein a[a]

residue	glycoprotein (mol of sugar/mol of Gap a)[b]	glycopeptide (mol of sugar/mol of Gap a)[c]	glycopeptide (mol of sugar/mol of asparagine)	core glycopeptide (mol of sugar/3 mol of mannose)
fucose	5.9	4.0	0.6 (1)[f]	0.9
mannose	16.8	14.8	2.3 (3)	3.0
galactose	11.5	12.0	1.9 (2)	0.3
glucosamine	22.4	22.3	3.5 (4)	1.4
galactosamine	5.2	trace	trace	trace
sialic acid[d]	2.0	2.1	0.3 (0 or 1)	ND
aspartic acid[e]			1.00	

[a] The carbohydrate content was determined by gas–liquid chromatography and mass spectrometry of the alditol acetate derivatives of sugars released from known dry weight of glycoprotein after hydrolysis. A subunit molecular weight of 230 000 was utilized for calculations. Glycoproteins and glycopeptides were isolated from cells labeled metabolically with [^{3}H]glucose, and the cellular origin of sugars was confirmed by radioactivity detection. [b] Values based on the average of three determinations. [c] Values based on recovery of radioactive glycopeptide from Gap a of known specific activity. [d] Sialic acid was determined by fluorometric assay (Hammond & Papermaster, 1976). [e] Amino acid analysis performed by AAA Laboratory, Seattle, after 24-h 6 N HCl hydrolysis at 110 °C; serine increased by 10% and threonine increased by 5% to compensate for destruction by acid. Relative AA composition based on 1 mol of aspartic acid: aspartic acid (1.00), proline (0.85), threonine (0.64), alanine (0.47), glycine (0.39), glutamic acid (0.21), other amino acids (trace). [f] Values in parentheses were rounded up. This was done on the assumption that more than 1 mol of aspartic acid was present per mole of glycopeptide either as trace contaminants or in the incompletely digested peptide portion.

Variable quantities of labeled GalNAc were detected in a ratio to GlcNAc of approximately 1:7. GalNAc isolated from metabolically labeled Gap a possessed the same specific activity as GlcNAc. However, GalNAc from Gap a labeled by the galactose oxidase–NaB^3H_4 procedure possessed less than 5% of the specific activity of galactose. This suggests that the GalNAc is not in a terminal nonreducing position.

Reductive Alkaline Hydrolysis of Galactoprotein a. Alkaline borohydride treatment of galactose oxidase–NaB^3H_4 labeled Gap a (1 M $NaBH_4$ in 0.05 M KOH at 45 °C for 15 h) as described by Carlson (1968), followed by chromatography on Bio-Gel P-2, resolved a small quantity of radioactive material (~10% of total radioactivity) with heterogeneous low molecular weight (results not shown). However, GC analysis of the released material and the digested glycoprotein failed to detect any preferential release of carbohydrate. In particular, the GalNAc was not released from the glycoprotein. This suggests that the carbohydrate units may not be attached through an O-glycosidic linkage.

Pronase Digestion of Galactoprotein a. Pronase digestion of metabolically labeled Gap a (5.3 mg), followed by chromatography on Bio-Gel P-10, afforded two radioactive peaks (Figure 2A). Peak II contained 50% of the applied radioactivity, 100% of the detectable free amino groups, and only trace quantities of carbohydrate (mannose, glucose, and galactose) as determined by GC analysis. Peak I had a relative molecular weight of 2000 and was pooled and subject to a second pronase digestion and molecular sieve purification (Figure 2B).

A sample of Gap a (3.9 mg), labeled by the galactose oxidase–NaB^3H_4 method and subject to pronase digestion and

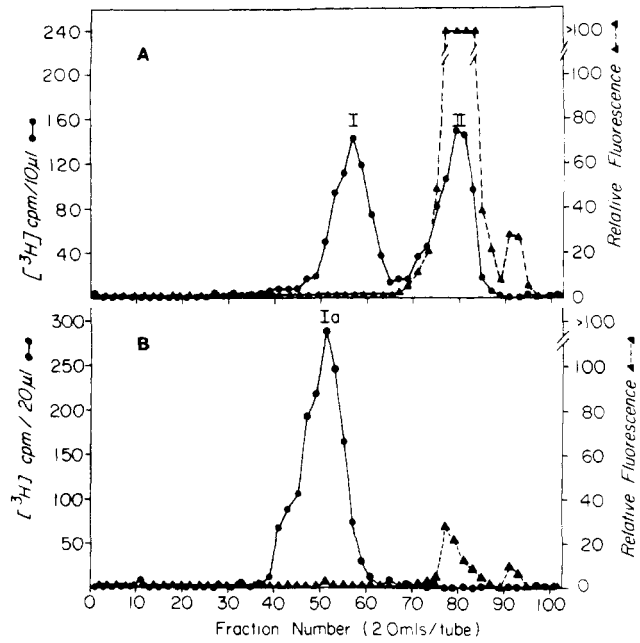

FIGURE 2: Gel filtration chromatography of [^{3}H]glucose metabolically labeled galactoprotein a after pronase digestion. (A) Isolated, metabolically labeled Gap a (5.3 mg) was suspended in 2.0 mL of 100 mM sodium borate buffer, pH 8.0, containing 5 mM $CaCl_2$. Pronase (86 400 PUK/g) was dissolved in the borate–$CaCl_2$ buffer (2 mg/mL) and 100 µg was added to the Gap a suspension. The digestion mixture was incubated in a toluene atmosphere at 40 °C. Additions (50 µg) of pronase were made at 24, 48, and 72 h of incubation. After 4-days incubation, the digestion mixture was centrifuged and the small pellet washed with 0.1 M pyridine acetate buffer, pH 5.0. This washed pellet contained 0.4% of the total radioactivity and was discarded. The supernatant and pellet wash were pooled and applied to a Bio-Gel P-10 column (200–400 mesh, 1.6 × 90 cm) equilibrated and developed in 0.1 M pyridine acetate buffer, pH 5.0; 2-mL fractions were collected. (B) Peak I (pooled tubes 46–65) of Figure 2A was subjected to a second Pronase digestion under the conditions described above and chromatographed on Bio-Gel P-6 (200–400 mesh, 116 × 1.5 cm) in pyridine acetate buffer. Peak Ia (pooled tubes 39–62) was subject to further analysis. (▲) Relative fluorescence by fluorescamine assay (Udenfriend et al., 1972). The columns were calibrated utilizing the following standards with indicated elution positions for graph A: fetuin glycopeptide (3000 g/mol eluted in fraction 45), IgM core glycopeptide (1200 g/mol eluted in fraction 65), stachyose (648 g/mol eluted in fraction 77), and galactose (180 g/mol eluted in fraction 83).

chromatography as described above, afforded a single major radioactive peak with a relative molecular weight of 2000 (results not shown). In addition, 4% of the applied radioactivity was recovered in the void volume. Analysis of this high molecular weight radioactive material detected small quantities of Gal, GlcNAc, and GalNAc in the approximate ratio of 3:2:1. This high molecular weight radioactive material was not detected in the metabolically labeled fractions and may represent minor contamination of Gap a by an unknown heteroglycan (see Discussion). Too little material was available for further characterization.

Homogeneity of Glycopeptide Isolated from Galactoprotein a. As seen in Figure 3A, high-voltage paper electrophoresis of labeled glycopeptide resolved three radioactive peaks. Essentially identical results were obtained with metabolically labeled or galactose oxidase–NaB^3H_4 labeled glycopeptide. Removal of sialic acid by mild acid hydrolysis resulted in the disappearance of a minor high-mobility peak. Subsequent blockage of peptide carboxyl groups by amidation with water-soluble carbodiimide and ethanolamine resulted in only one major electrophoretic form of the glycopeptide (Figure 3D). This result suggests that the observed electrophoretic

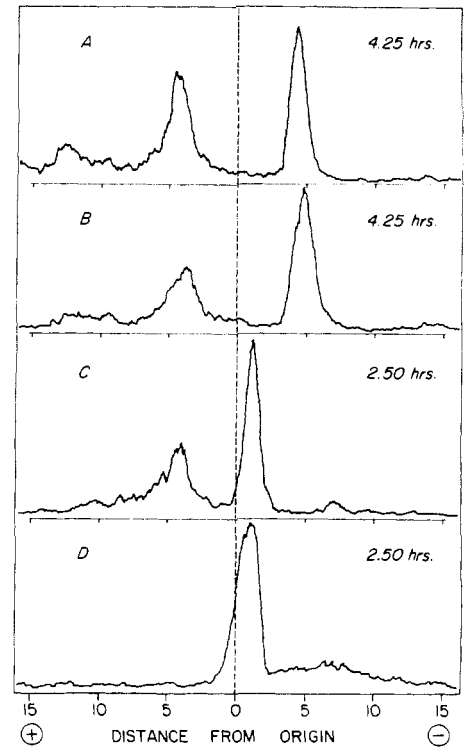

FIGURE 3: High-voltage paper electrophoresis of galactoprotein a glycopeptide, asialoglycopeptide, and amidated asialoglycopeptide at pH 5.4. (A) Gap a glycopeptide was isolated from galactose oxidase–NaB^3H_4 labeled Gap a by Pronase digestion and subject to high-voltage paper electrophoresis. (B) Glycopeptide was subject to mild acid hydrolysis (0.01 N HCl; 80 °C; 60 min) to remove sialic acid prior to electrophoresis. (C) Repeat of (B) at shorter electrophoresis time. (D) Asialolglycopeptide was subject to quantitative modification of carboxyl groups by reaction with ethanolamine in the presence of water-soluble carbodiimide [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride] as described by Hoare & Koshland (1967) prior to electrophoresis. Ordinate: tritium radioactivity. Abscissa: distance of migration from origin at 0.

heterogeneity of the glycopeptide is due to heterogeneity in the sialic acid content and acidic amino acid content of the glycopeptide and not in the neutral carbohydrate content.

Reaction of the glycopeptide with sodium nitrite, followed by mild hydrolysis with acetic acid as described by Bayard & Fournet (1976), failed to cleave the glycopeptide as detected by chromatography on Bio-Gel P-4 (results not shown), suggesting that all the amino groups of the hexosamine are acetylated.

Carbohydrate Composition of the Isolated Glycopeptide. As can be seen in Table I, all of the carbohydrate detected in Gap a is recovered in a homogeneous, asparagine-linked glycopeptide. The glycopeptide possesses a carbohydrate composition (normalized to 1 mol of aspartic acid) of $fucose_1$, $mannose_3$, $galactose_2$, $glucosamine_4$, and $sialic\ acid_{0-1}$. This minimal composition is compatible with the estimated relative molecular weight of 2000 (Figure 2). The small quantities of GalNAc present in the original Gap a preparation were absent from the glycopeptide.

Sequential Degradation of the Glycopeptide with Exoglycosidases and Endoglycosidases. Enzymatic digestion of the metabolically labeled glycopeptide Ia with β-galactosidase released substantial quantities of galactose present in glycopeptide Ia (Figure 4). However, approximately 10–20% of terminal galactose must be sialylated based on the content of sialic acid (Table I) and methylation analysis (Table II). It is evident from the relative radioactive peak areas of Figure 4A that the released monosaccharides exhibit different specific

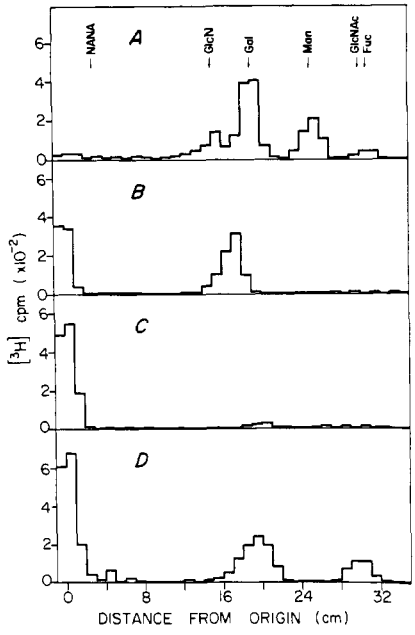

FIGURE 4: Sequential degradation of galactoprotein a glycopeptide with exoglycosidases, followed by paper chromatography. Glycopeptide, metabolically labeled with [^{3}H]glucose, was subject to milk acid hydrolysis to remove sialic acid and/or sequential digestion with exoglycosidases. Released monosaccharides were qualitatively identified by paper chromatography of standard sugars and the digestion mixture (see Materials and Methods for digestion and chromatography conditions). (A) Radioactive monosaccharide composition of glycopeptide resulting from full hydrolysis in 1.0 N HCl for 4 h at 100 °C in a nitrogen atmosphere. (B) Chromatography following digestion with β-galactosidase. (C) Chromatography following digestion with β-*N*-acetylglucosaminidase. (D) Chromatography following sequential mild acid hydrolysis of sialic acid, β-galactosidase digestion, and β-*N*-acetylglucosaminidase digestion. In (A), arrows indicate migration positions of standard sugars. Origin is at 0 distance of migration.

activities (see Table I for quantitative values). As a result, radioactivity was used only for qualitative detection. Treatment of the glycopeptide with β-*N*-acetylglucosaminidase had no effect on bound GlcNAc. These results suggest, qualitatively, that Gal is the major, terminal nonreducing sugar residue, which is compatible with the fact that Gap a is strongly labeled externally with the galactose oxidase–NaB^3H_4 method. GlcNAc was released after sequential removal of sialic acid and galactose, followed by β-*N*-acetylglucosaminidase digestion (Figure 4). The remaining undigested core glycopeptide was isolated by chromatography on Bio-Gel P-2 (Figure 5A). The composition of the core glycopeptide is seen in Table I as $Man_3GlcNAc_{1-2}Fuc_1$. The low recovery of GlcNAc in the core glycopeptide probably results from incomplete hydrolysis of the asparagine-linked *N*-acetylglucosamine. Hydrolysis conditions capable of giving quantitative release of reducing terminal GlcNAc result in extensive destruction of peripheral sugar residues. Comparing compositions of the core glycopeptide with the intact glycopeptide (Table I) indicates that sequential hydrolysis of nonreducing terminal sialic acid, galactose, and *N*-acetylglucosamine removed 0–1 mol of sialic acid, 2 mol of galactose, and 2 mol of *N*-acetylglucosamine per 3 mol of total mannose.

The intact glycopeptide was insensitive to digestion by endo-β-*N*-acetylglucosaminidase H, confirming that the structure was not of the high mannose type (see Materials and Methods for experimental details). However, after removal of the terminal sialic acid, Gal, and GlcNAc, the core glycopeptide was readily digested by endo-β-*N*-acetylglucosaminidase D releasing an oligosaccharide which coeluted with

Table II: Partially Methylated Neutral and Amino Sugars Detected in the Hydrolysates of Permethylated Core Tetrasaccharide and Glycopeptide Ia Derived from Galactoprotein a and Comparison with Bovine IgG Glycopeptides

partially O-methylated hexitol and hexosaminitol acetates	core tetrasaccharide		glycopeptide	
	Gap a	IgG	Gap a	IgG
fucitol				
2,3,4-tri-*O*-Me	–[a]	–	+	+ (0.6)
galactitol				
2,3,4,6-tetra-*O*-Me	–	–	+ (1.8)[b]	+ (0.8)
2,4,6-tri-*O*-Me[c]	–	–	+[f]	–
2,3,4-tri-*O*-Me	–	–	–	+ (0.4)[f]
mannitol				
2,3,4,6-tetra-*O*-Me	+	+	*d*	*d*
3,4,6-tri-*O*-Me	–	–	+ (2.1)	+ (2.3)
2,4-di-*O*-Me	+	+	+ (1.0)	+ (1.0)
2-deoxy-(2-*N*-acetyl)-2-acetamido glucitol				
3,4,6-tri-*O*-Me	–	–	–	+ (0.5)[f]
3,6-di-*O*-Me	+	+	+ (1.2)[e]	+ (1.3)[e]
3-mono-*O*-Me	–	–	+[e]	+[e]

[a] Sugars, qualitatively identified on the basis of relative retention time on GC columns, OV 17 and OV 225, and on characteristic mass ions, are indicated as (+). Absence of sugar is indicated as (–). Due to the small quantities of Gap a glycopeptide (10–25 μg) and oligosaccharide available for analysis, exact quantification of some peaks was impossible due to partial overlap of the sugar peak by nonsugar contaminant peaks. [b] In parentheses is shown the molar ratio of the peaks obtained from the total mass ion intensity of GC–MS calculated on 2,4-di-*O*-methylmannitol as 1.0. [c] Not separated from 2,3,6-tri-*O*-methylmannitol and 2,3,6-tri-*O*-methylglucitol. [d] A peak corresponding to 2,3,4,6-tetra-*O*-methylglucitol, which is indistinguishable from 2,3,4,6-tetra-*O*-methylmannitol, was detectable due to glucose contamination. [e] Lower yield of amino sugars as compared to neutral sugars was observed due to incomplete hydrolysis. [f] The presence of these partially methylated derivatives reflects the heterogeneity in terminal nonreducing sialic acid, galactose, and glucosamine, present in bovine IgG (Tai et al., 1975b).

bovine IgG core tetrasaccharides (Tai et al., 1975b), $Man_3GlcNAc$ (Figure 5).

Direct Probe Mass Spectrometry and Methylation Analysis of the Core Oligosaccharide. The mass spectra from direct probe analysis of the core oligosaccharide released by a combination of exoglycosidases and endo-β-*N*-acetylglucosaminidase D digestion of Gap a glycopeptide is seen in Figure 6A. The presence of mass ions *m/e* 219 and 187 (219 – 32) indicates the presence of terminal hexose. *m/e* 276 is indicative of terminal, reduced *N*-acetylhexosamine. The mass ion *m/e* 875 (919 – 45 – 1) was the highest ion detected and corresponds to a molecular weight of 919 for the oligosaccharide. No *m/e* 189 or 157 (189 – 32) was detected for terminal fucose, suggesting that the fucose detected in the core glycopeptide (Table I) is not present in the core oligosaccharide released by endo-β-*N*-acetylglucosaminidase D and therefore must be bound to the asparagine-linked *N*-acetylglucosamine residue. These results, combined with the composition of the core glycopeptide, suggest that the core oligosaccharide is a tetrasaccharide composed of $Man_3GlcNAc_1$.

Methylation analysis of the isolated core oligosaccharide (Table II) was performed following methylation, hydrolysis, reduction, and acetylation. The resulting partially methylated sugars were identified by their retention times and characteristic mass ions. The detection of 2,4-dimethylmannose and the known cleavage requirements for endo-β-*N*-acetylglucosaminidase D (Tai et al., 1975a) further suggest that the tetrasaccharide is a branched structure with two terminals, nonreducing mannose residues, similar to bovine IgG core tetrasaccharide.

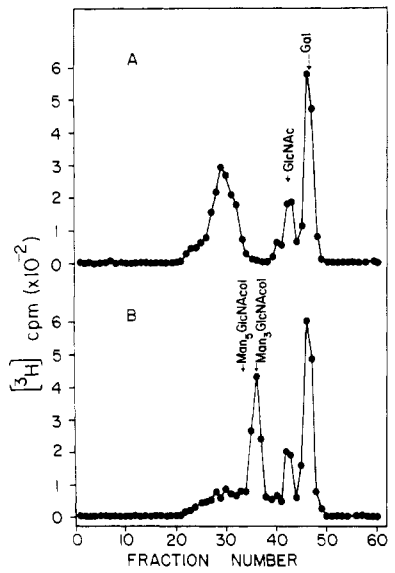

FIGURE 5: Digestion of galactoprotein a glycopeptide by endo-β-*N*-acetylglucosaminidase D after sequential removal of terminal monosaccharides. (A) Galactoprotein a glycopeptide, metabolically labeled with [^{3}H]glucose, was subject to sequential treatment with mild acid, exo-β-galactosidase, and exo-β-*N*-acetylglucosaminidase (see Materials and Methods). The resulting "core glycopeptide" was separated from the released terminal sugars by chromatography on Bio-Gel P-2 (200–400 mesh, 115 × 0.8 cm), equilibrated, and developed in 0.1 M pyridine acetate buffer, pH 5.0, with 1-mL fractions collected. The released radioactive monosaccharides were identified by GC–MS of their alditol acetate derivatives. Migration of standard monosaccharides are indicated. (B) The core glycopeptide could be further degraded by digestion with endo-β-*N*-acetylglucosaminidase D and analyzed by molecular sieve chromatography as described above. The elution positions of standard oligosaccharides $Man_3GlcNAcol_1$ (from human IgM) and $Man_5GlcNAcol_1$ (from ovalbumin) are indicated. The released core oligosaccharide had the same elution volume as $Man_3GlcNAcol$.

For comparative purposes, the mass spectra and structure of the core tetrasaccharide released from bovine IgG glycopeptide is presented in Figure 6B. The mass spectrum for Gap a core tetrasaccharide is consistent with spectra obtained from the IgG core tetrasaccharide. The peaks at *m/e* 668 and 627 of the two mass scans exhibit noticeable differences in their relative ion intensities. These differences probably do not reflect structural differences in the two tetrasaccharides since the corresponding precursor and product mass ions, 745 and 408, have similar ion intensities. It should be emphasized that approximately five times more core tetrasaccharide from IgG than from Gap a was analyzed by direct probe. These quantity differences may account for the small differences in the spectra, particularly in the high molecular weight ion range.

Methylation Analysis of Gap a Glycopeptide. The isolated intact glycopeptide was also subject to methylation followed by hydrolysis, reduction, and acetylation. The major proportion of galactose was detected as a 2,3,4,6-tetra-*O*-methyl derivative indicating a terminal position (Table II). A small quantity of 2,4,6-tri-*O*-methylgalactitol was also detected and may result from the small quantities of terminal sialic acid linked to the 3 position of a penultimate galactose. Fucose was recovered as a 2,3,4-tri-*O*-methyl sugar, indicating a terminal linkage position. *N*-Acetylglucosamine was detected as a 2,3-di-*O*-methyl and a 3-*O*-methyl derivative, indicative of internal linkages with a possible branch point at the monomethyl derivative. Similarly, mannose was recovered as both a 3,4,6-tri-*O*-methyl and a 2,4-di-*O*-methyl derivative, which are characteristic of internal linkages with a branch point at the dimethyl derivative. Methylation analysis data for bovine IgG glycopeptide is presented for comparative purposes.

Proposed Structure of the Glycopeptide. A proposed structure for the major asparagine-linked glycopeptide of galactoprotein a from hamster embryo fibroblasts is presented in Figure 7. The terminal nonreducing carbohydrate sequence and linkages were determined by degradation with specific exoglycosidases and methylation analysis. The tetrasaccharide within the dashed rectangle was isolated after digestion with endo-β-*N*-acetylglucosaminidase D and characterized by direct probe mass spectrometry and methylation analysis. The designation of linkages to the anomeric carbons of sugars in the core tetrasaccharide was based on the cleavage specificity of endo-β-*N*-acetylglucosaminidase D (Tai et al., 1975a).

Discussion

In spite of various biological interests currently described for Gap a (see the introduction), the cell-bound glycoprotein has only recently been isolated in high yield (Carter & Hakomori, 1977) and its molecular structure has not been well characterized.

The major difficulty for isolation and characterization of cellular Gap a is the poor yield and unusual solubility and aggregative property of the glycoprotein. Recently we have described a method for isolation of various cell surface glycoproteins, involving sequential and selective extraction with Tris buffers containing (1) EDTA and ATP, (2) Empigen BB, a zwitterionic detergent, and (3) 8 M urea (Carter & Hakomori, 1977). Almost all proteins and glycoproteins were extracted from cells by steps 1 and 2 alone, leaving essentially all Gap a, some cytoskeletal proteins, and the nuclei unextracted. The last step of extraction with 8 M urea solubilizes Gap a,[3] and the glycoprotein was purified from the urea extract by affinity chromatogaphy on insolubilized *Ricinus communis* lectin columns. As previously described, the purified Gap a exhibits a disulfide-dependent subunit interaction and after reduction migrates on polyacrylamide gels containing $NaDodSO_4$ with a relative molecular weight of 230 000 (Carter & Hakomori, 1977). An extensive disulfide-dependent cross-linking of LETS protein was also reported (Hynes & Destree, 1977). We have prepared Gap a starting from surface-labeled or metabolically labeled cells in culture. The overall yield of the purified glycoprotein was about 12.9 mg from 2 to 3 × 10^9 cells, which was about 25 mL of packed hamster embryo fibroblasts. A total of 11.0 mg of the isolated glycoprotein was utilized in these carbohydrate studies.

Structure analysis of glycopeptide has been greatly advanced recently by improved methylation analysis and specific degradation by endo- and exoglycosidases. However, past studies have required milligram quantities of glycopeptides (e.g., Tai et al., 1975a; Kawasaki & Ashwell, 1976; Ito et al., 1977; Kornfeld, 1978). Cell surface glycoproteins of cultured cells such as Gap a are extremely limited in quantity; therefore their carbohydrate structures have not been studied so far. In this study we have evaluated the carbohydrate structure of the limited amounts of Gap a available by utilizing the following methods: (1) enzymatic degradation was studied with the glycopeptide metabolically labeled with [^{3}H]glucose, (2) methylation analysis was performed using 10–25 μg of the glycopeptide through computer-programmed scanning of GC elution profile for selected mass numbers, and (3) direct probe

[3] Quantitative solubilization of cell-bound Gap a was obtained with the final 8 M urea extraction in most cases. Occasionally, however, reduction with dithiothreitol in urea was required for complete solubilization. The reasons for this variability in disulfide cross-linking and its effect on Gap a solubility are, as yet, unexplained. Possibly, the passage number of the cultures or the length of time the cells are maintained at confluence may affect the extent of disulfide cross-linking. The role of disulfide bonds in the attachment and function of LETS protein has been recently published (Ali & Hynes, 1978a, b; Chen et al., 1978).

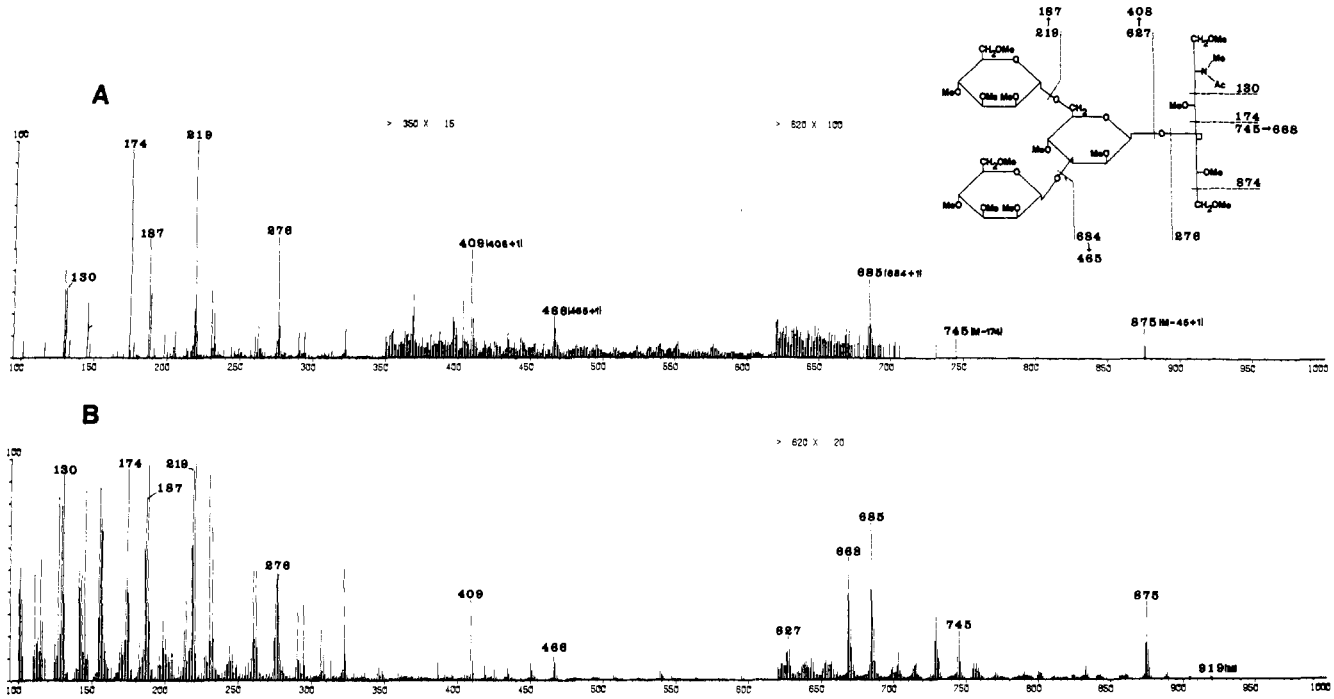

FIGURE 6: Direct probe mass spectra of permethylated core oligosaccharide liberated from the galactoprotein a core glycopeptide by endo-β-*N*-acetylglucosaminidase D and comparison to IgG core tetrasaccharide structure. (A) Galactoprotein a core oligosaccharide was isolated by sequential treatment of glycoprotein with mild acid, β-galactosidase, β-*N*-acetylglucosaminidase, and endo-β-*N*-acetylglucosaminidase D, followed by chromatography on Bio-Gel P-2 (see Figure 5). The isolated oligosaccharide was reduced with NaB^3H_4 and then $NaBH_4$, permethylated, and purified by LH-20 chromatography and by partitioning between chloroform and water. The chloroform layer was dried under N_2 and the residue dissolved in acetone. The sample was analyzed by direct probe mass spectrometry without hydrolysis. (B) Glycopeptide from bovine IgG was isolated by Pronase digestion of IgG essentially as described in Figure 2. Subsequent sequential digestion of the isolated glycopeptide as described above released a tetrasaccharide with a previously established structure (Tai et al., 1975b). The IgG core tetrasaccharide was permethylated and analyzed by direct probe mass spectrometry as described above. Ordinate: relative ion intensity. Abscissa: *m/e*.

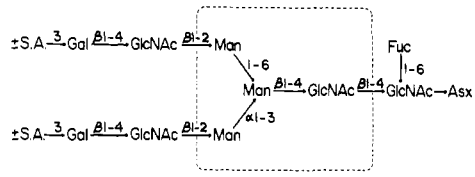

FIGURE 7: Proposed structure of the major asparagine-linked glycopeptide of galactoprotein a from hamster embryonic fibroblasts.

mass spectrometry of permethylated core oligosaccharide which was liberated by enzymatic degradation was used. Essentially all of the carbohydrate detected in Gap a was recovered in a glycopeptide with the relative molecular weight of 2000 after pronase digestion. Each Gap a subunit, molecular weight 230 000, contains 5 or 6 asparagine-linked, complex-type structure as shown in Figure 7 in which no unusual or unique carbohydrate structure was detected. This structure is similar to that proposed for the released form of Gap a isolated from conditioned media of hamster embryo fibroblasts (Fukuda & Hakomori, 1979). Heterogeneity of the glycopeptide was mainly due to variation in the amino acid and sialic acid content. It is noteworthy, however, that only a small portion (10–15%) of the terminal galactosyl residues of the glycopeptide were sialylated; therefore it is well labeled with galactose oxidase–NaB^3H_4. It may be of interest to compare the carbohydrate structure of Gap a obtained from sparse and confluent cell cultures, particularly the peripheral sequence of the carbohydrate moiety. In contrast to Gap a of hamster fibroblasts, the same glycoprotein of mouse 3T3 cells was not labeled by galactose oxidase–NaB^3H_4, but it was labeled only after sialidase treatment (Itaya & Hakomori, 1976). Therefore, the degree of sialylation in the peripheral carbohydrate structure may differ according to the cell type and species. Indeed Yamada et al. (1977) have reported that the similar if not identical glycoprotein from chicken fibroblasts (termed CSP) contains more than twice as much sialic acid as found in hamster fibroblast Gap a. CSP was also reported to contain glucose and xylose but not galactosamine.

The presence of galactosamine in intact Gap a was noteworthy since galactosamine was virtually absent in the major glycopeptide, fraction Ia (Figure 2). Small quantities of GalNAc were detectable in almost all the high molecular weight fractions of the molecule sieve elution profile of pronase-digested Gap a, and the highest ratio of GalNAc to other sugars was detected in the void volume of the molecular sieve column. The GalNAc was not released from the glycoprotein by alkaline borohydride treatment. These results suggest that the GalNAc is not involved in a usual O-glycosydically linked oligosaccharide chain as found in most mucin-type glycopeptides (for a review see Kornfeld & Kornfeld, 1976). The possibility that the GalNAc is involved in the core structure of the glycopeptide as described for follitropin, lutropin, and thyrotropin glycoproteins (Hara et al., 1978) is also unlikely due to the very low quantities present in the glycopeptide Ia. More reasonably, the GalNAc may be a component of an uncharacterized base-stable heteroglycan that is associated with or copurified with Gap a, which forms a heterogeneous population of GalNAc-containing heteroglycans upon pronase digestion. Recently, a base-stable linear heteroglycan was isolated from the pronase digestion product of cell surface glycoprotein of human erythrocyte membrane (Finne et al., 1978). Kornfeld (1978) has detected nonstoichiometric quantities of GalNAc present in glycopeptide fractions isolated from calf thymus membranes.

The complex-type asparagine-linked oligosaccharide as shown in Figure 7 is very similar or identical to the proposed structure of the carbohydrate moiety of human IgG, IgE, IgA, and bovine IgG (for review see Kornfeld & Kornfeld, 1976). Some minor differences were detected however. Sialic acid present in Gap a glycopeptide appears to be linked to the 3

position of galactose, not the 6 position as in the human immunoglobulin as listed above. The microheterogeneity of sialic acid in Gap a appears to be limited to the linkage of sialosyl residues but not to the subterminal structure which is exclusively composed of Gal. Unusual sequences of carbohydrate such as Galβ1→3Galβ1→4GlcNAc or the highly branched structures as described by Kornfeld (1978) for glycopeptides of calf thymus plasma membrane were not detectable. Fibronectin isolated from human plasma (Wrann, 1978) has also been reported to contain sialic acid linked either to the 4 or 6 position of the penultimate galactose. Wrann also did not report any terminal fucose in the human plasma fibronectin, thus suggesting possible specific differences in the linkage and content of terminal nonreducing sialic acid and fucose residues of hamster and human glycoproteins. The similarity of the carbohydrate moieties of Gap a and many serum glycoproteins makes it unlikely that the carbohydrate unit is important for specific cell interaction or in recognition of other cellular proteins. Recently, Olden et al. (1978) have suggested that the carbohydrates may stabilize the glycoprotein against proteolytic degradation.

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