Control of Glycoprotein Synthesis

LECTIN-RESISTANT MUTANT CONTAINING ONLY ONE OF TWO DISTINCT N-ACETYLGLUCOSAMINYLTRANSFERASE ACTIVITIES PRESENT IN WILD TYPE CHINESE HAMSTER OVARY CELLS*

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SAROJA NARASIMHAN,† PAMELA STANLEY,§ AND HARRY SCHACHTER‡

From the †Research Institute, Hospital for Sick Children, Toronto, and Department of Biochemistry, University of Toronto, and §Department of Medical Genetics, University of Toronto, Toronto, Canada

A Chinese hamster ovary cell line (Pha+) selected for resistance to a phytohemagglutinin from Phaseolus vulgaris has previously been shown to lack an N-acetylglucosaminyltransferase (GlcNAc-transferase) activity, present in wild type Chinese hamster ovary cells, that catalyzes the transfer of GlcNAc from UDP-GlcNAc to a glycopeptide prepared from human IgG with the structure

\[
\text{Man} \quad \text{Fuc} \\
\text{Man-GlcNAc-GlcNAc-Asn-peptide} \\
\text{Man}
\]

(Stanley et al. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3323-3327). We now show that wild type cells, but not Pha+ cells, can also effect the transfer of GlcNAc to

\[
\text{Man} \\
\text{Man-GlcNAc} \\
\text{Man}
\]

and Manα1-3Manβ1-4GlcNAc. Further, both Pha+ and wild type cells possess GlcNAc-transferase activity for the transfer of GlcNAc to acceptors with the structure

\[
\text{GlcNAc-Man} \\
\text{Man-GlcNAc-R} \\
\text{Man}
\]

where R is either -H or -FucGlcNAc-Asn-peptide. Wild type cells therefore exhibit at least two GlcNAc-transferase activities, GlcNAc-transferase I acting on structures with 1 or 2 terminal mannose residues, and GlcNAc-transferase II acting on structures with 1 terminal mannose residue and 1 terminal GlcNAc residue. Pha+ cells lack GlcNAc-transferase I activity but possess full GlcNAc-transferase II activity. Preliminary data suggest that both transferases effect the synthesis of GlcNAcβ1-2Man linkages. The acceptors for GlcNAc-transferases I and II compete for common enzyme active sites in extracts of wild type cells suggesting that the two transferases may share a catalytic subunit.

A line of Chinese hamster ovary cells (Pha+) selected for resistance to a phytohemagglutinin from Phaseolus vulgaris has been shown to lack an N-acetylglucosaminyltransferase (GlcNAc-transferase) activity, present in wild type Chinese hamster ovary cells, which transfers GlcNAc from UDP-GlcNAc to an acceptor prepared from human IgG1 glycopeptide (1); this acceptor had the structure Manα1-3[Manα1-6]Manβ1-4GlcNAcβ1-4(Fuc-βGlcNAc)-Asn-peptide (1, 2). The mutant Pha+ cells were, however, found to possess significant activity (~30% to 50% of wild type cell extracts) for the transfer of GlcNAc to an apparently similar acceptor prepared by sequential glycosidase treatment of α1-acid glycoprotein (1). Similar results have been obtained for Chinese hamster ovary cells selected for resistance to the toxin from Ricinus communis (2, 3), the agglutinin from wheat germ (3), and an agglutinin from Lens culinaris (3).

It appeared likely that the α1-acid glycoprotein preparation contained more than one acceptor for GlcNAc transfer and that wild type cells possessed at least two GlcNAc-transferase activities only one of which was deleted in Pha+ cells. In this paper, we examine the abilities of both wild type and Pha+ cells to transfer GlcNAc to a series of glycopeptides and oligosaccharides. The results show the presence of at least two GlcNAc-transferase activities in wild type cells, GlcNAc-transferase I acting on acceptors with 1 or 2 terminal mannose residues and GlcNAc-transferase II acting on acceptors with 1 terminal mannose residue and 1 terminal GlcNAc residue. GlcNAc-transferase I is deleted in Pha+ cells while GlcNAc-transferase II is present in both wild type and mutant cells. Some of this data has appeared in a preliminary communication (4).

1 The abbreviations used are: IgG, immunoglobulin G; GlcNAc-transferase, N-acetylglucosaminyltransferase; AGP, α1-acid glycoprotein; AGP: SA, Gal, GlcNAc, cellopodiase, β-galactosidase, βN-acetylglucosaminidase-treated AGP; Con A, concanavalin A; glycopeptide structures GS, GN, MS, MG, GnGn, MGn, and MM are defined in Fig. 1. Gal, GlcNAc, and Man always refer to the D-pyranose configuration and Fuc to the L-pyranose configuration.

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ROLE OF N-ACYTGLUCOSAMINYLTRANSFERASES IN LECTIN RESISTANCE

Experimental Procedures

Materials

Nonradioactive UDP-GlcNAc was purchased from Sigma and used to dilute UDP-N-acetyl-D-3H-glucosamine (56.5 mCi/mmol, New England Nuclear) to a specific activity of 4 × 10^6 cpm/μmol. Bio-Gel P-4 (minus 400 mesh), Bio-Gel P-10 (100 to 200 mesh), and AG 50W-X2 (H+) (200 to 400 mesh) were obtained from Bio-Rad Laboratories. Concanavalin A-Sepharose 4B, Sephadex G-25 (fine), and quater-P-4 (minus 400 mesh), Bio-Gel P-10 (100 to 200 mesh), and AC 50W-X2 (H+) (200 to 400 mesh) were obtained from Bio-Rad Laboratories.

Hydrolysis of N-Acetyl-n-galactosaminidase and β-N-acetylglucosaminidase (Fraction DE maclia. Methyl-cu-n-glucopyranoside was obtained from Pfanstiehl Laboratories. Human α, α-acid glycoprotein (AGP) was sequentially degraded with sialidase, β-galactosidase, and β-N-acetylglucosaminidase to yield the product AGP (SA, Gal, GlcNAc).

Preparation of Glycopeptides from Immunoglobulin G

Protein was determined by the procedure of Lowry et al. (12) using bovine serum albumin as standard; the levels of Triton X-100 present in the cell extracts did not interfere with these assays. Glycopeptides were detected in column effluents by absorbance at 230 nm and hexose analysis (13). Glycosidase activities were monitored by measuring the release of galactose (14) and N-acetylglucosamine (15) following digestion of glycopeptides. Buffers for high voltage paper electrophoresis were pH 9.0, 1% sodium tetraborate; pH 6.6, pyridineant./acetic acid/water (100:4:896); pH 3.6, pyridineant./acetic acid/water (1:10:89). Carbohydrate analysis of glycopeptides and oligosaccharides was performed by gas-liquid chromatography following methanolysis (0.5 M methanolic HCl at 80°C for 20 h) and trifluoroacetylation according to the method of Zanetta et al. (16); this method yields low values for the glucosamine content of glycopeptides (by about 1 residue) due to the resistance of the asparagine-N-acetylglucosaminyl linkage to methanolysis under the above conditions. Amino acid analysis of glycopeptides was carried out on a Beckman model 121 analyzer following hydrolysis with 5.7 N HCl at 110°C in evacuated tubes for 24 h.

Preparation of Glycopeptides from Immunoglobulin G

Frozen serum from a patient (NeI) with multiple myeloma was obtained from Dr. W. Pruzanski, Wellesley Hospital, Toronto. Immunoglobulin G (IgG) was purified (17) by passage through a column of QAE-Sephadex A-50 equilibrated with ethylenediamineacetate buffer (pH 2.7) according to a method used by others for purifying ovalbumin glycopeptides (10, 11). The column was eluted with the sodium acetate buffer and a glycopeptide peak emerged at the void volume. High voltage paper electrophoresis (pH 6.5) of this fraction indicated a single acidic band by both ninhydrin and periodate-benzidine (28) stains; the neutral glycopeptide and several other contaminating peptides adsorbed to the AG 50 column. The acidic glycopeptide (56 μmol) was concentrated and desalted by passage through a Bio-Gel P-10 column (2.5 × 90 cm) eluted with water.

Carbohydrate analysis of this preparation (Preparation I, Table I) showed that it contained a single sialic acid residue per mol. The carbohydrate composition of Preparation I, sequential glycosidase digestion of Preparation I (see below), and previous work by Kornfeld's group (19, 20) on the structure of human IgG oligoaccharides indicated that the major glycopeptide species in Preparation I had the structure GS shown in Fig. 1. Preparations II, III, IV, and V were then obtained by enzymatic digestion.
Role of N-Acetylglucosaminyltransferases in Lectin Resistance

prepared by sequential glycosidase degradation of Preparation I as described below and as outlined in Fig. 2. An aliquot of Preparation I (21 pmol) was digested with 0.3 unit of \textit{C. perfringens} \(\beta\)-galactosidase and 0.3 unit of \textit{C. perfringens} \(\beta\)-N-acetylglucosaminidase in 6.0 ml of 0.05 M potassium phosphate (pH 6.0) at 37° for 20 h under a layer of toluene. This treatment released 21 pmol of galactose (14) and 18 pmol of \(N\)-acetylgalactosamine (15). The incomplete release of GlcNAc, even after repeated glycosidase treatment, was observed in two separate preparations. The digest was concentrated and purified by gel filtration on Bio-Gel P-10 (2.5 x 90 cm) eluted with water. The carbohydrate composition of this preparation (Preparation II, Table I) and the amounts of galactose and GlcNAc released during digestion indicated that the major structure in this preparation was MS (Fig. 1) although the presence of about 14% GnG (Fig. 1) appeared likely due to incomplete GlcNAc release.

Preparation III-An aliquot (10 pmol) of Preparation II (10 pmol) was heated at 60° for 45 min to destroy residual glycosidase activities and was subsequently digested with 0.02 unit of sialidase in 1.0 ml of 0.1 M potassium acetate (pH 4.5) over chloroform at 37° for 24 h; a further 0.02 unit of sialidase was added and incubation continued for another 24 h. The total sialic acid content of this digest (21) was then equal to the total sialic acid content (22) indicating complete release of sialic acid. The degraded glycopeptide was isolated by gel filtration of the digest on Bio-Gel P-10 (2.5 x 90 cm) eluted with water. Carbohydrate analysis of this preparation (Preparation III, Table I) suggested that the major glycopeptide was MG (Fig. 1) although about 14% GnG (Fig. 1) was probably also present due to incomplete removal of GlcNAc in the previous step.

Preparation IV–Aliquots (5 pmol) of Preparation III were digested with either 0.4 unit of jack bean \(\beta\)-galactosidase in 0.5 ml of 0.05 M sodium citrate (pH 4.0) or 0.04 unit of a preparation of \textit{C. perfringens} \(\beta\)-galactosidase relatively free of \(\beta\)-N-acetylglucosaminidase in 0.6 ml of 0.05 M potassium phosphate (pH 6.0), at 37° for 20 h under a layer of toluene. The jack bean enzyme released about 50% of the bound galactose in Preparation III while the \textit{C. perfringens} enzyme released 100% of the bound galactose. However, the \textit{C. perfringens} preparation contained approximately 1.4 units of \(\beta\)-N-acetylglucosaminidase to release 1.4 units of GlcNAc. Both digests were subjected to gel filtration on Bio-Gel P-10 columns (2.5 x 90 cm) followed by gel filtration on Bio-Gel P-4 (1.5 x 25 cm) eluted with water. Carbohydrate analysis of the preparation using jack bean \(\beta\)-galactosidase (Preparation IV-1, Table I) and the release of galactose described above suggested that Preparation IV-1 contained approximately an equimolar mixture of MG and MGn (Fig. 1) with about 14% of a mixture of GnGn and GnG (Fig. 1) as indicated in the tables and figures. Incubations were at 37° for 1 h. Each reaction was terminated by the addition of 0.010 ml of 2% sodium tetraborate, 0.25 M EDTA followed by guest on March 24, 2020http://www.jbc.org/Downloaded from

Fractionation of Preparation IV-1 with Con A-Sepharose

Four columns of Con A-Sepharose (1.4 x 9 cm) were washed with 1% bovine serum albumin (30 ml) and equilibrated with 0.01 M Tris pH 7.0 containing 0.1 M NaCl. Preparation IV-1 (0.67 pmol per column) was loaded on the columns followed by elution first with 100 ml of buffer and then with 100 ml of 0.1 M methyl a-D-glucopyranoside in buffer. The buffer wash fractions and the methyl glucoside eluates were each concentrated and subjected to gel filtration on columns of Bio-Gel P-4 (1.5 x 25 cm) to remove salts and methyl glucoside from the glycopeptides. This resulted in 1.3 pmol of material eluting in the buffer wash (Preparation IV-1a) and 1.2 pmol of material adsorbed to Con A-Sepharose (Preparation IV-1b). Carbohydrate analyses (Table I) indicated that Preparation IV-1b was richer in MGn than Preparation IV-1 although there was probably still appreciable contamination with GnGn, MG, and GnG.

Preparation of Cell Extracts

Cells in logarithmic growth were harvested, washed, counted, and protein extracted with Triton X-100 as previously described (1). The protein concentrations of these cell extracts were usually between 10 and 14 mg/ml.

N-Acetylglucosaminyltransferase Assays

The standard incubation mixture contained 0.05 to 0.14 mg of enzyme protein, glycosyl acceptor (as indicated in the tables and figures), 2.6 pmol of (2-N-morpholino)ethanesulfonate buffer, pH 6.3, 0.5 pmol of MnCl\(_2\), and 25 nmol of UDP-N-acetyl-\(\beta\)-D-\textit{N-Acetylglucosaminidase (4 x 10\(^{-4}\) cm/ml) in a final volume of 0.04 ml. Incubations were at 37° for 6 h. Each reaction was terminated by the addition of 0.010 ml of 2% sodium tetrabrate, 0.25 M EDTA followed by high voltage paper electrophoresis in 1% sodium tetrabrate (23) and radioactivity, dried, and counted in a scintillation counter (24). All incubations were performed at a minimum of two protein concentrations both in the absence and presence of exogenous acceptor; the rate of product formation was proportional to enzyme protein concentration. Incorporation in the absence of acceptor was usually 0.5 to 1.0 nmol/h/mg and this value
was subtracted from that in the presence of acceptor in calculating enzyme velocities. Under the above assay conditions neither wild type nor lectin-resistant cell extracts showed detectable β-N-acetylglucosaminidase activity (1).

**Product Identification**

The standard N-acetylglucosaminyltransferase incubation (see above), containing wild type cell extract and either 1 mg of AGP (2S-AG, GlcNAc) or 0.2 μmol of Preparation VI-1 or 0.05 μmol of Preparation VII/0.040 ml, was scaled up 2- to 10-fold and incubated at 37°C for 3 to 5 h. The incubation containing glycoprotein was dialyzed extensively at 4°C against 0.1 M NaCl followed by water to remove salts and radioactive low molecular weight compounds. The resulting radioactive glycoprotein product was either hydrolyzed with 4 M HCl at 100°C for 4.5 h or treated with C. perfringens β-N-acetylglucosaminidase in 0.08 M potassium phosphate buffer (pH 5.0) at 37°C for 24 h under tunicamycin; these digests were analyzed by high voltage paper electrophoresis (pH 3.0) and descending paper chromatography in butanol/pyridine/water (45:25:40) for release of radioactive glucosamine and N-acetylglucosamine, respectively.

The incubations containing glycopeptide acceptors were subjected to high voltage paper electrophoresis (pH 3.6); radioactive glycopeptide product and free radioactive GlcNAc remained near the origin in this system while radioactive UDP-GlcNAc and GlcNAc-1-phosphate moved toward the anode. The origins were then washed by descending chromatography with 80% ethanol to remove any free radioactive GlcNAc that may have formed during the incubation. The radioactive glycopeptide products were eluted from the papers with water and subjected to gel filtration in water on columns of Bio-Gel P-4 (1.5 x 25 cm). A single radioactive glycopeptide peak was eluted from each of these columns (recovers were over 95%). These glycopeptides were acetylated by incubation with 1.5 μmol of acetic anhydride in 0.05 ml of 0.5% NaHCO₃ at room temperature for 1 h (24). Incubations were then flash-evaporated followed by gel filtration on Bio-Gel P-4 (1.5 x 25 cm) eluted with water.

The radioactive acetylated glycopeptides were analyzed with Con A/Sephaphore by the procedure of Ogata et al. (25). Con A/Sepharose columns (0.7 x 5 cm) were washed with 10 ml of 1% borvne serum albumin and then thoroughly equilibrated with at least 30 ml of 0.01 M Tris/HCl (pH 7.3) containing 0.1 M NaCl. Radioactive glycopeptides (2000 to 2500 cpm) were applied to these columns followed by 20 ml of 0.01 M Tris/HCl (pH 7.3) containing 0.1 M NaCl and then by 20 ml of 0.1 M methyl α-D-glucopyranoside in the same buffer. Fractions (1 ml) from the columns were added by 0.5-ml aliquots to 10 ml of Aquasol (New England Nuclear) and counting in a liquid scintillation spectrometer.

**RESULTS**

**Presence of Two GlcNAc-transferase Activities in Wild Type Cells** – Table IIA shows the results of GlcNAc-transferase assays of wild type and Pha₃cells extracts using as glycosyl acceptors Preparations VI, IV-1, IV-1b, and IV-2, prepared from IgG. Preparation VI contained primarily the structure MM (Fig. 1) and behaved in transferase assays as previously reported (1), i.e. Pha₃cells showed no activity with this acceptor while wild type cells had an activity of 4 to 5 nmol/h/mg of protein. In contrast, Preparations IV-1, IV-1b, and IV-2 all served as excellent glycosyl acceptors for both wild type and Pha₃cells and showing GlcNAc-transferase activities of 16 to 22 nmol/h/mg of protein. It appears from this data that wild type cells contain at least two GlcNAc-transferase activities, GlcNAc-transferase I acting on the structure MM and GlcNAc-transferase II acting on a structure present in Preparations IV-1, IV-1b, and IV-2. GlcNAc-transferase I is deleted in Pha₃cells while GlcNAc-transferase II is present at high levels in both wild type and Pha₃cells.

**Acceptor Specificity of GlcNAc-transferase I** – GlcNAc-transferase I activity was identified by its presence in wild type cells and its absence in Pha₃cells. Active acceptors for this enzyme were Preparation VI (Table IIA), the trisaccharide Manα1-3Manβ1-4GlcNAc (Table IID), and the branched tetrasaccharide Manα1-3[Manα1-6]Manβ1-4GlcNAc (Table IID); low activity was also shown with Preparations II and III (Table IIC) and with some, but not all, Manα1-2-terminal compounds (Table IIE). The high activity with the tri- and tetrasaccharides (Table IIA) indicated that neither the amino acid nor fucose contents of MM (Fig. 1) were essential for acceptor activity. However, the trisaccharide showed a higher Km (4.2 mm) and a higher Vmax (13 nmol/h/mg) than the glycopeptide MM (Km ,0.24 mm; Vmax, 5.0 nmol/h/mg) indicating that structures other than the trisaccharide sequence Manα1-3Manβ1-4GlcNAc were capable of influencing the activity of GlcNAc-transferase I.

GlcNAc-transferase I was unable to act on Preparations I and V (Table IIB) indicating that structures GS, GnGn, and GnG (Fig. 1) were ineffective acceptors. The low activity shown with Preparations II and III (Table IIC) was probably due to the α-Man-terminal compounds MS and MG (Fig. 1). The lack of activity with Preparation IV-1a (Table IIC), which appeared to contain the same structures as Preparation III (Table I), has not been explained; it should be pointed out, however, that Preparations III and IV-1a were not identical.

### Table II

**Substrate specificity of N-acetylglucosaminyltransferases**

| Glycosyl acceptor | Acceptor concentration | Wild type cells | Pha₃cells |
|------------------|------------------------|-----------------|-----------|
| Prep. VI (MM, 100%) | 1.3 | 3.7 | <0.2 |
| Prep. IV-1 (MGn, 43%; MG, 43%) | 2.5 | 17.6 | 18.1 |
| Prep. I (GS, 100%) | 2.5 | 0.6 | <0.2 |
| Prep. V (GnGn, 60%; GnG, 40%) | 2.5 | <0.2 | <0.2 |
| Prep. II (MS, 86%; GnS, 14%) | 2.5 | 1.7 | <0.2 |
| Prep. III (MG, 86%; GnG, 14%) | 2.5 | 2.2 | 0.5 |
| Prep. IV-1a (MG, 86%; GnG, 14%) | 1.3 | <0.2 | <0.2 |
| Prep. V (GnGn, 60%; GnG, 40%) | 2.5 | 0.6 | <0.2 |
| Gangliosidosis urine decasaccharide after two glycosidase treatments | 2.5 | 16.2 | 5.4 |
| Gangliosidosis urine decasaccharide after three glycosidase treatments: Manα1-3[Manα1-6Manβ1-4GlcNAc] | 2.5 | 2.6 | <0.2 |
| Manα1-2Manα1-3Manβ1-4GlcNAc | 3.8 | 4.1 | <0.2 |
| E. Manα1-2Manα1-3Manβ1-4GlcNAc | 2.5 | <0.2 | <0.2 |
| Manα1-2Manα1-3Manβ1-4GlcNAc | 5.0 | 0.5 | 0.3 |
| Manα1-2Manα1-3Manβ1-4GlcNAc | 2.0 | 2.1 | <0.2 |
| Manα1-2Manα1-3Manβ1-4GlcNAc | 5.0 | 6.9 | 1.8 |
| Ovalbumin glycopeptide | 2.5 | 1.8 | <0.2 |
Role of N-Acetylglucosaminyltransferases in Lectin Resistance

Fig. 1. Structures of the glycopeptides present in Preparations I to VI derived from human IgG (Table I) and the nomenclature used to designate these structures. Every structure is named according to the sugar residues at the nonreducing ends of the left and right branches, respectively, of the oligosaccharide chain. S, sialic acid; G, galactose; Gn, N-acetylglucosamine; M, mannose.

since Preparation III contained material which was both adherent and nonadherent to Con A/Sepharose while Preparation IV-la contained only material nonadherent to Con A/Sepharose (Fig. 2). It is not known whether GlcNAc-transferase I can act on MGn (Fig. 1) since all preparations enriched in this glycopeptide (Preps. IV-1, IV-1b, and IV-2) contained other α-Man-terminal glycopeptides which may have been responsible for the slightly higher activities seen with wild type cells relative to Pha-hc cell extracts (Table IIA).

It is not known whether GlcNAc-transferase I can attach a GlcNAc residue to both the Manα-3- and Manα-6- termini of MM. Unbranched Manα-6Manβ-terminal compounds were not available to test this point although it was shown that Manα-6GlcNAc was not an acceptor. However, according to the data of Baenziger and Kornfeld (20), incomplete glycosylation of human immunoglobulin oligosaccharides is restricted to the Manα-6- branch and, therefore, structures MS and MG (Fig. 1) should both have had Manα-6- termini; since MS and MG appeared to be substrates for GlcNAc-transferase I (Table IIC), it must tentatively be inferred that GlcNAc-transferase I can probably transfer GlcNAc to Manα-6- termini as well as to Manα-3- termini. The situation for Manα-2-terminal compounds was ambiguous since some compounds of this type appeared to be acceptors (Table IIE) while others were not (legend to Table II).

Acceptor Specificity of GlcNAc-transferase II - The existence of GlcNAc-transferase II was first suspected because the acceptor AGP(SA,Gal,GlcNAc) showed transferase activity with both wild type and Pha-hc cells (1) although the activity with the mutant cells was about 30 to 50% the activity of wild type cells. It was then observed that an oligosaccharide could be prepared by treating gangliosidosis urine decasaccharide

Fig. 2. Scheme for the preparation of IgG glycopeptides by sequential glycosidase digestion of the crude glycopeptide fraction (see text for details and Fig. 1 for structural nomenclature).
Ration V (a mixture of GnGn and GnG) was inactive with both acceptor; this has in fact been verified by showing that Prepa-

glucosaminyltransferase II, which acted on the structure MGn (Fig. 1); AGP(-SA, Gal,GlcNAc) and gangliosidosis urine decasaccharide after
imaging such that after two glycosidase treatments (Table IIA), I and V (Table IIB), and II, III, and IV-la (Table

to be an acceptor; this has in fact been verified by showing that Prepara-

Mycock and Mahalik, J. Biol. Chem. 248, 1100 (1973). The concentration of myoglobin in the sample was 1.2 mg/ml.

GlcNAc-transferase II attaches GlcNAc to this terminus. However, it is not known whether GlcNAc-transferase II can also attach GlcNAc to a Man6- terminus.

Product Identification—Both GlcNAc-transferase I and II required acceptors with terminal α-mannosyl residues (Table

The calculated velocities for the situation in which MM and MGn compete for common enzyme active sites were derived from the expression:

\[ v = \frac{V_1(A_1/K_1) + V_2(A_2/K_2)}{1 + A_1/K_1 + A_2/K_2} \]

where \( V_1 \) and \( V_2 \) are the maximum velocities for MM and MGn, respectively, \( K_1 \) and \( K_2 \) are the respective Michaelis constants, and \( A_1 \) and \( A_2 \) are the respective acceptor concentrations. Values of 5.0 nmol/h/mg and 0.24 nmol/hr/mg were derived for \( V_1 \) and \( K_1 \), respectively, by applying \( 1/v \) versus 1/S plots to incubations using Preparation VI alone. Values for \( V_2 \) (20 nmol/hr/mg) and \( K_2 \) (0.37 mM) were derived from incubations using Preparation IV-2 alone assuming competition between MM and MGn.

These values are the sums of the values obtained for Prepara-
tion VI and IV-2 alone.
ases therefore attach GlcNAc in β linkage to terminal α-mannosyl residues.

The nature of the linkages synthesized has been investigated with Con A/Sepharose chromatography as described by Ogata et al. (25). These workers suggested that oligosaccharides and glycopeptides adsorbed to Con A/Sepharose columns if they contained at least 2 α-mannosyl residues that were either at the nonreducing terminus or substituted in their carbon 2 positions; any other substitutions would prevent binding to Con A/Sepharose. Fig. 3 and 4 show the results of Con A/Sepharose analysis of the radioactive glycopeptide products of GlcNAc-transferase I and II, respectively. Both transferases produced products which adhered almost completely to Con A/Sepharose; however, while methyl α-glucopyranoside eluted the product of GlcNAc-transferase I as a sharp peak (Fig. 3), the product of GlcNAc-transferase II adhered strongly to the column and was eluted with relative difficulty by the glucoside (Fig. 4). These data suggested that both transferases catalyzed the synthesis of GlcNAcβ1-2Man linkages.

**Competition Studies** — Table III shows the results of mixed substrate experiments in which wild type cell extracts were assayed with Preparations VI and IV-2 separately and then together in the same tube. It is evident that the enzyme rates in the mixed substrate tubes were not additive as would be expected if GlcNAc-transferase I and II were different and independent enzymes. Rather, the results were compatible with the situation in which the two acceptors competed for common enzyme active sites. Although this type of experiment is not conclusive, the data suggest that GlcNAc-transferases I and II share a common catalytic subunit which is presumably identical for the enzyme active sites. These studies were motivated by the finding that a line of lectin-resistant (Pha") Chinese hamster ovary cells lacked completely an N-acetylglucosaminyltransferase activity assayed with a glycopeptide derivative of IgG as acceptor (1, 2) but showed appreciable transferase activity when the acceptor was prepared from α1-acid glycoprotein (1). This apparent discrepancy has now been resolved by the demonstration that wild type cells contain two transferases, GlcNAc-transferases I and II, only one of which (GlcNAc-transferase I) is deleted in Pha" cells. GlcNAc-transferase I transfers GlcNAc to Manα1-3[Manα1-6]Manβ1-4GlcNAc-R (where -R is -H or -[Fuc]-GlcNAc-Asn-peptide) to form GlcNAcβ1-2Manα1-3[Manα1-6]Manβ1-4GlcNAc-R. We have not shown that GlcNAc-transferase I is specific for the Manα1-3-terminus since there is low activity with some Manα1-2-terminal compounds (this may be a different transferase) and with the branched structures MG and MS (Fig. 1) which probably have Manα1-6- termini (19, 20); unbranched Manα1-6Manβ-terminal compounds were not available for testing as acceptors.

The only effective acceptor for GlcNAc-transferase II was found to be the asymmetric branched compound GlcNAcβ1-2Manα1-3[Manα1-6]Manβ1-4GlcNAc-R (where -R has the same assignment as above); the product of this reaction was believed to be GlcNAcβ1-2Manα1-3[GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAc-R. The basis for assigning the Manα1-6-terminus as the acceptor for GlcNAc in this reaction was the finding by Kornfeld’s group (19, 20) that human immunoglobulins yielded, on pronase digestion, the asymmetric structure G8 (Fig. 1) in which the sialic acid-containing arm was attached to Manα1-3-terminus; since we have not done this type of structural analysis on our Preparation I and since bovine IgG has been shown (26, 27) to carry an incompletely glycosylated oligosaccharide arm on the Manα1-5-branch rather than on the Manα1-6-branch claimed for human immunoglobulins (19, 20), the precise nature of the acceptor for GlcNAc-transferase II has not as yet been established. Further, it is not known whether GlcNAc-transferase II is specific for either the Manα1-5- or Manα1-6-termini of branched oligosaccharides or whether it can act on either arm; it is to be noted that GlcNAc-transferase II cannot act on unbranched Manα1-3-terminal compounds (Table III).

Fig. 5 summarizes the reactions suggested by the data. The assignment of linkages synthesized by GlcNAc-transferases I and II as GlcNAcβ1-2Man was based on the specificity of Con A/Sepharose columns (25). Since the products of both transferases adhered to the Con A/Sepharose, substitution of the terminal mannose residues at carbon atoms other than carbon 2 appeared highly unlikely. The difficulty observed in eluting the product of GlcNAc-transferase II (Fig. 4) suggested that the structure GnGn (Fig. 1) adhered more strongly to the column than the structure MGn (Fig. 1); this is in agreement with the finding of Kornfeld and Ferris (28) that GnGn was in fact a more effective competitive inhibitor of Con A binding to guinea pig erythrocytes than either MM or MGn.

Table I illustrates one of the major problems encountered in
Role of N-Acetylglucosaminyltransferases in Lectin Resistance

3933

this work, i.e. the difficulty of preparing homogeneous glycopeptides. Part of the problem was due to the inability of some glycosidase to remove completely certain sugar residues, e.g. the incomplete action of β-N-acetylglucosaminidase on GnS glycosidases to remove completely certain sugar residues, e.g. peptides. Part of the problem was due to the inability of some adherence is not known and is presently under investigation. Fortunately, the lack of completely homogeneous glycopeptides did not prevent the assignment of reasonable acceptor specificities to GlcNAc-transferases I and II. However, it is clear from our data and that of others, e.g. the work of Toyoshima et al. (29) on the sequential glycosidase degradation of porcine thyroglobulin glycopeptide B and of Kornfeld and Ferris (28) on similar degradations of human immunoglobulin glycopeptides, that incomplete release of sugars and nonhomogeneous glycopeptide preparations may be common problems that must be quantitatively assessed if valid conclusions are to be drawn.

Fig. 5 summarizes the above findings and recent work by Wilson et al. (7). The ManαGlcnAcα core of Asn-GlcNAc type glycopeptides (30) is presumably assembled as a dolichol-pyrophosphate-oligosaccharide and subsequently transferred to polypeptide (31). Elongation of this core commences when GlcNAc-transferase I attaches a single GlcNAcβ1-2 residue to either the Manα1-3- or Manα1-6- terminus of the core. This event triggers the addition by GlcNAc-transferase II of a second GlcNAcβ1-2 residue to the terminal mannose residue and by a fucosyltransferase (7) of a fucose residue to the most internal GlcNAc residue. GlcNAc-transferase I therefore controls the entire elongation process; when this enzyme is absent, as is the case in the Pha6 Chinese hamster ovary line (1-3), there is no addition of either GlcNAc or fucose to the ManαGlcnAcα core and, consequently, no addition of galactose to GlcNAc (32), nor of sialic acid to galactose (32), even though all other glycosyltransferases required for these reactions are present. This explains the resistance of the Pha6 cell line to the cytotoxic action of four different lectins, namely, the agglutinins from Phaseolus vulgaris, Ricinus communis, wheat germ, and Lens culinaris (1-3).

Both GlcNAc-transferase I and II are also present in rat liver and are equivalent to the UDP-GlcNAc:glycoprotein N-acetylglucosaminyltransferase previously described in the Golgi apparatus of this tissue (23, 33); the latter enzyme has also been described in goat colostrum (34), pig liver (33), and human, rat, and pig serum (35-37). Substrate specificity studies with the N-acetylglucosaminyltransferase activity from goat colostrum (34) showed no transfer of GlcNAc to a variety of low molecular weight compounds; of particular interest was the absence of activity with mannose, methyl α and β mannopyranosides, p-nitrophenyl-α- and β-mannopyranoside and αL2-linked α-mannosetriose. These acceptors have not been tested with Chinese hamster ovary cell extracts. We have, however, shown that low molecular weight acceptors can be prepared for this enzyme activity and the use of these well defined acceptors allowed us to differentiate at least two enzyme activities whereas previous work with a more complex glycoprotein acceptor had only defined a single enzyme.

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S Narasimhan, P Stanley and H Schachter

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