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Purification and Properties of the Glycoprotein Processing *N*-Acetylglucosaminyltransferase II from Plants†

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ABSTRACT: The presence of an *N*-acetylglucosaminyltransferase (GlcNAc-transferase) capable of adding a GlcNAc residue to $GlcNAcMan_3GlcNAc$ was demonstrated in mung bean seedlings. This enzyme was purified about 3400-fold by using (diethylaminoethyl)cellulose and phosphocellulose chromatographies and chromatography on Concanavalin A–Sepharose. The transferase was assayed by following the change in the migration of the [^{3}H]mannose-labeled GlcNAcβ1,2Manα1,3(Manα1,6)Manβ1,4GlcNAc on Bio-Gel P-4, or by incorporation of [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc into a neutral product, $(GlcNAc)_2Man_3GlcNAc$. Thus, the purified enzyme catalyzed the addition of a GlcNAc to that mannose linked in α1,6 linkage to the β-linked mannose. GlcNAcβ1,2Manα1,3(Manα1,6)Manβ1,4GlcNAc was an excellent acceptor while Manα1,6(Manα1,3)Manβ1,4GlcNAc, Manα1,6(Manα1,3)Manα1,6-(Manα1,3)Manβ1,4GlcNAc, and Manα1,6(Manα1,3)Manα1,6[GlcNAcManα1,3]Manβ1,4GlcNAc were not acceptors. Methylation analysis and enzymatic digestions showed that both terminal GlcNAc residues on $(GlcNAc)_2Man_3GlcNAc$ were attached to the mannoses in β1,2 linkages. The GlcNAc transferase had an almost absolute requirement for divalent cation, with Mn^{2+} being best at 2–3 mM. Mn^{2+} could not be replaced by Mg^{2+} or Ca^{2+}, but Cd^{2+} showed some activity. The enzyme was also markedly stimulated by the presence of detergent and showed optimum activity at 0.15% Triton X-100. The K_m for UDP-GlcNAc was found to be 18 μM and that for $GlcNAcMan_3GlcNAc$ about 16 μM.

The N-linked or asparagine-linked glycoproteins occur in plants as well as in animals (Kornfeld & Kornfeld, 1985; Lehle & Tanner, 1983), and the plant glycoproteins may contain either high-mannose or modified oligosaccharide chains (Takahashi et al., 1986). Some of these modified or complex oligosaccharides of plants have a galactose-β1,4GlcNAcβ1,2-sequence attached to each of the α-linked mannoses of the trimannose core, in close analogy to the complex structures of animal cells (Ishihara et al., 1979; Kobata, 1984). However,

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the modified chains of plants differ from animal cell glycoproteins in that they lack sialic acid and frequently have a β1,2-linked xylose on the β-linked mannose (Sturm et al., 1986). Xylose has also been found in animal cell glycoproteins (Van Kuik et al., 1985), but its presence is unusual.

Many of the reactions that involve the formation of the lipid-linked oligosaccharide precursor, i.e., $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryldolichol,[1] have been well studied in animal cells (Struck & Lennarz, 1980; Snyder, 1984), and a number of the enzymes that participate in the modification of the oligosaccharide after transfer to protein have been purified and characterized (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985). Thus, the three glucose residues are removed by two endoplasmic reticulum bound glucosidases: glucosidase I removes the outermost α1,2-linked glucose (Turco et al., 1977; Elting et al., 1980; Ugalde et al., 1980; Hettkamp et al., 1984) and then glucosidase II removes the next two α1,3-linked glucoses (Burns & Touster, 1982; Reitman et al., 1982; Spiro et al., 1979). The resulting high-mannose structure [i.e., $Man_9(GlcNAc)_2$] is then a substrate for the endoplasmic reticulum α-mannosidase (Bischoff & Kornfeld, 1983), which removes one or two α1,2-linked mannoses. Several Golgi α1,2-mannosidases (referred to as mannosidase IA and mannosidase IB) can also trim up to four mannose residues to give a $Man_5(GlcNAc)_2$–protein (Opheim & Touster, 1978; Forsee & Schutzbach, 1981; Tabas & Kornfeld, 1979). A GlcNAc-transferase I then adds a GlcNAc residue to the mannose linked α1,3 to the β-linked mannose (Harpaz & Schachter, 1980; Oppenheimer & Hill, 1981), and this addition is apparently a signal for another Golgi mannosidase, called mannosidase II, to remove the α1,3- and α1,6-linked mannoses from the 6 arm of the $GlcNAcMan_5(GlcNAc)_2$–protein (Tabas & Kornfeld, 1978; Tulsiani et al., 1982). The resulting GlcNAc-$Man_3(GlcNAc)_2$–protein is the substrate for a second GlcNAc transferase (GlcNAc-transferase II) that adds a GlcNAc to the 6-linked mannose to give $(GlcNAc)_2Man_3(GlcNAc)_2$–protein (Harpaz & Schachter, 1980; Oppenheimer et al., 1981; Medicino et al., 1981). Then other sugars of the complex chains, i.e., galactose, sialic acid, fucose, etc., may be added to give a variety of structures.

Much less is known about the biosynthesis of the N-linked glycoproteins of plants (Elbein, 1979). Several laboratories have shown the presence of or biosynthesis of the $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryldolichol in various plants (Staneloni et al., 1981; Lehle, 1981; Hori et al., 1982). In addition, in vivo studies and pulse–chase studies suggested that processing of the oligosaccharide chains of the plant glycoproteins was occurring (Hori & Elbein, 1983; Vitale & Chrispeels, 1984).

Recently, glucosidase I (Szumilo et al., 1986a) and mannosidase I (Szumilo et al., 1986b) were purified from mung bean seedling and shown to have properties quite similar to those of the corresponding enzymes from animal cells. In addition, evidence has been obtained for the presence of mannosidase II in mung bean seedlings (Szumilo et al., 1986c), as well as for the GlcNAc-transferase (Davies & Delmer, 1981; Chrispeels, 1985).

In this paper, we demonstrate the presence of a second GlcNAc-transferase in mung bean seedlings. This enzyme was purified about 3400-fold from the microsomal fraction, and its properties were examined. The enzyme was distinct from GlcNAc-transferase I and showed specificity requirements analogous to GlcNAc-transferase II of animal cells. Thus, the best acceptor for GlcNAc was the GlcNAcβ1,2Manα1,3(Manα1,6)Manβ1,4GlcNAc oligosaccharide. On the basis of enzyme digestion studies and methylation analysis, both the GlcNAc on the substrate, GlcNAcManα1,3(Manα1,6)ManβGlcNAc, and GlcNAc on the product, GlcNAcManα1,6(GlcNAcManα1,3)-ManβGlcNAc, were linked in β1,2 linkages. Thus, plants are able to process at least some of the N-linked oligosaccharides to the $(GlcNAc)_2Man_3$- stage and beyond.

Experimental Procedures

Materials. UDP-[^{3}H]GlcNAc (20 Ci/mmol) was from New England Nuclear Co., and [2-^{3}H]mannose (25 Ci/mmol) was from American Radiolabel Co. UDP-GlcNAc, UDP-galactose, UDP-xylose, UDP-glucose, concanavalin A–Sepharose, dithiothreitol, DE-52, and ovalbumin were from Sigma Chemical Co. Pronase was from Calbiochem, and endo-β-*N*-acetylglucosaminidase H was from Miles Laboratories. Swainsonine was isolated from *Astragalus emoryanus* as previously described (Szumilo & Elbein, 1985).

Preparation of [^{3}H]Mannose-Labeled GlcNAcMan$_3$GlcNAc Substrate. The starting material for the preparation of $GlcNAcMan_3GlcNAc$ was the [^{3}H]mannose-labeled $Man_9GlcNAc$. This oligosaccharide was prepared by raising influenza virus in [2-^{3}H]mannose in the presence of the mannosidase I inhibitor, deoxymannojirimycin, as previously described (Szumilo et al., 1986b). The viral glycoproteins were digested with Pronase and then with endoglucosaminidase H to produce $Man_9GlcNAc$. The purified $Man_9GlcNAc$ was incubated with a partially purified mannosidase I in MES buffer, pH 6.0, in the presence of Ca^{2+} and swainsonine. The resulting [^{3}H]$Man_5GlcNAc$ was isolated by gel filtration and purified on calibrated columns of Bio-Gel P-4. $Man_5GlcNAc$ was incubated with unlabeled UDP-GlcNAc plus the plant GlcNAc-transferase I in the presence of swainsonine to produce $GlcNAcMan_5GlcNAc$ (Szumilo et al., 1986c). After purification, this substrate was incubated with a partially purified plant mannosidase II to give the radioactive $GlcNAcMan_3GlcNAc$.

Preparation of Unlabeled Oligosaccharide Substrates. Glycopeptides were prepared from ovalbumin by exhaustive digestion with Pronase. The Man_5 glycopeptide was purified by gel filtration on Sephadex G-25 and ion-exchange chromatography on Dowex 50-X-2 as previously described (Szumilo et al., 1986c). The Man_5 glycopeptide was digested with endoglucosaminidase H to obtain $Man_5GlcNAc$. This oligosaccharide was treated sequentially with GlcNAc-transferase I to obtain GlcNAc-$Man_5GlcNAc$ and then with mannosidase II to obtain $GlcNAcMan_3GlcNAc$ as described above.

Preparation of the Microsomal Fraction from Mung Bean Seedlings. Mung beans were soaked overnight in running tap water and were placed on moist cotton in the dark for germination. The seedlings were collected after 3 or 4 days and kept on ice until used. Three kilograms of seedlings was blended in 1.5 L of extraction buffer [50 mM Tris-HCl, pH

[1] Abbreviations: $Glc_3Man_9(GlcNAc)_2$, Glcα1,2Glcα1,3Glcα1,3-Manα1,2Manα1,2Manα1,3[Manα1,2Manα1,6(Manα1,2Manα1,3)-Manα1,6]Manβ1,4GlcNAcβ1,4GlcNAc; $Man_9(GlcNAc)_2$, Manα1,2Manα1,2Manα1,3[Manα1,2Manα1,6(Manα1,2Manα1,3)-Manα1,6]Manβ1,4GlcNAcβ1,4GlcNAc; $Man_5GlcNAc$, Manα1,6-(Manα1,3)Manα1,6(Manα1,3)Manβ1,4-GlcNAc; $Man_3GlcNAc$, Manα1,6(Manα1,3)Manβ1,4GlcNAc; $GlcNAcMan_3GlcNAc$, GlcNAcβ1,2Manα1,3(Manα1,6)Manβ1,4GlcNAc; $(GlcNAc)_2Man_3$-GlcNAc, GlcNAcβ1,2Manα1,6(GlcNAcβ1,2Manα1,3)-Manβ1,4GlcNAc; GlcNAc-transferase, *N*-acetylglucosaminyltransferase; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane.

7.2, containing 0.25 M sucrose, 0.5 μM dithiothreitol, 1 mM EDTA, and 0.25% poly(vinylpyrrolidone)]. The blending involved three 15-s bursts in a Waring blender at maximum speed. The resulting suspension was passed through six layers of cheesecloth, and the filtrate was centrifuged at 3000*g* for 30 min to remove whole cells and large debris. The supernatant liquid from this centrifugation was centrifuged at 100000*g* for 45 min to isolate the microsomes. The microsomal GlcNAc-transferase II was solubilized with Triton X-100 and purified as described under Results.

Assay of GlcNAc-transferase II. Two different assay methods were used to measure transferase activity: a gel filtration assay and an ion-exchange method. At the initial stages of purification, the gel filtration assay was used. In this case, incubation mixtures contained 60 mM MES buffer, pH 6.5, 3000 cpm of [^{3}H]mannose-labeled GlcNAcMan$_3$GlcNAc, 2.5 μmol of unlabeled UDP-GlcNAc, 10 μmol of $MnCl_2$, 2 μmol of AMP, 0.1% sodium azide, and various amounts of enzyme, all in a final volume of 0.2 mL. In some experiments, various components were omitted to determine whether they were necessary for activity. Incubations were done for various times as indicated in the figures. At the end of the incubation, 2 mL of $CHCl_3$ and 0.3 mL of H_2O were added, and the mixtures were shaken vigorously to extract most of the Triton X-100. The mixtures were centrifuged, and the $CHCl_3$ was removed with a Pasteur pipet. To the aqueous phase was added 5 volumes of absolute ethanol, and the mixture was kept in the freezer for 2 h to precipitate the protein. The precipitate was removed by centrifugation, and the supernatant liquid was dried under a stream of nitrogen. The residue was dissolved in 1 mL of 0.1% acetic acid and chromatographed on a Bio-Gel P-4 column (1.5 × 150 cm).

With more purified enzyme fractions, the GlcNAc-transferase II activity was measured according to an ion-exchange method. In this case, incubation mixtures contained 60 mM MES buffer, pH 6.5, 5 mM $MnCl_2$, 0.1% Triton X-100, 6–30 μM unlabeled GlcNAcMan$_3$GlcNAc, 25 μM UDP-[^{3}H]GlcNAc, and various amounts of enzyme, all in a final volume of 0.1 mL. Incubations were for various times. At the end of the incubation, 1 mL of cold water was added and the mixture was passed through a column of Dowex 1-X-8 (100–200 mesh; contained in a Pasteur pipet). Unreacted UDP-[^{3}H]GlcNAc was retained on the column whereas [^{3}H]GlcNAc transferred to GlcNAcMan$_3$GlcNAc passed through the column. The columns were washed twice with 1-mL portions of water, and the washes were counted as a measure of activity. The radioactivity in the wash was checked periodically by gel filtration and was found to correspond to (GlcNAc)$_2$Man$_3$GlcNAc.

Characterization of (GlcNAc)$_2$Man$_3$GlcNAc Product. The product resulting from the transfer of GlcNAc to GlcNAcMan$_3$GlcNAc was initially isolated and characterized by chromatography on a long (1.5 × 200 cm) calibrated column of Bio-Gel P-4 (200–400 mesh). The column was standardized with a number of oligosaccharides including Man$_5$GlcNAc, GlcNAcMan$_3$GlcNAc, (GlcNAc)$_2$Man$_3$GlcNAc, and so on. The oligosaccharide was also tested for its susceptibility to a number of glycosidases, including jack bean β-*N*-acetylhexosaminidase and jack bean α-mannosidase. For the β-*N*-acetylhexosaminidase, the oligosaccharide (labeled or unlabeled in the GlcNAc) was placed in 50 mM citrate buffer, pH 5.0, with 42 milliunits of enzyme. Incubations were for 24 h under a toluene atmosphere. For α-mannosidase, oligosaccharides were placed in 50 mM citrate buffer, pH 4.5, containing 250 milliunits of enzyme and 1 mM $ZnCl_2$. Again incubations were for 24 h. In both cases, the release of labeled monosaccharides or a change in migration of the oligosaccharide was monitored by chromatography on the Bio-Gel P-4 columns. The [^{3}H]mannose-labeled GlcNAcMan$_3$GlcNAc and (GlcNAc)$_2$Man$_3$GlcNAc were subjected to methylation as described by Hakamori (1964), and the methylated mannose derivatives were identified by thin-layer chromatography.

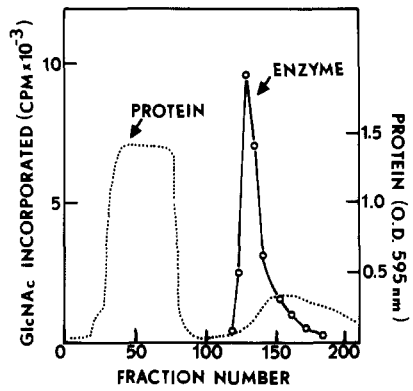

FIGURE 1: Purification of GlcNAc-transferase II on phosphocellulose. The enzyme from the first DEAE-cellulose column (96 mL and about 270 mg of protein) was applied to a 2.2 × 35 cm column of phosphocellulose that had previously been equilibrated with 10 mM Tris-HCl buffer, pH 7.2, containing 20% glycerol, 0.1% Triton X-100, and 0.5 μM dithiothreitol. After most of the unretained protein had been washed through the column, active enzyme was eluted with a gradient (200 mL each) of 0–1.5 M NaCl. Fractions were collected and assayed for protein and for GlcNAc-transferase II activity.

RESULTS

Purification of GlcNAc-transferase II

Solubilization of the Enzyme. The microsomal fraction obtained as described under Experimental Procedures was washed with 50 mM Tris-HCl buffer, pH 7.2, containing 20% glycerol and 0.5 μM dithiothreitol, and the particles were isolated by centrifugation at 100000*g* for 45 min. The washed pellet was suspended in solubilizing buffer (50 mM Tris-HCl, pH 7.2, 20% glycerol, 0.5 μM dithiothreitol, 1 mM NaCl, and 2% Triton X-100) and homogenized for 5 min in a Dounce homogenizer. The solution was then stirred for 45 min in an ice bath. The suspension was centrifuged for 1 h at 100000*g*, and the supernatant liquid, which contained the solubilized GlcNAc-transferase II, was removed and saved. The residue was reextracted with the solubilization buffer, and this supernatant liquid was combined with that from the first extraction.

Purification in DEAE-cellulose. The solubilized enzyme (about 100 mL containing 1500 mg of protein) was applied to a 2.3 × 30 cm column of Whatman DE-52 that had been equilibrated with the ion-exchange buffer (10 mM Tris-HCl, pH 7.2, 20% glycerol, 0.1% Triton X-100, and 0.5 μM dithiothreitol). The buffer also contained 100 mM NaCl in this case. The column was washed with 200 mL of this ion-exchange buffer, and the enzymatic activity emerged in the wash, whereas much of the protein bound to the column and required 0.3 M NaCl for elution (data not shown).

Cellulose Phosphate Chromatography. The most active fractions from the first DE-52 column (about 96 mL) were pooled, dialyzed overnight, and applied to a 2.2 × 35 cm column of cellulose phosphate as described in Figure 1. Most of the protein was not retained on this column and emerged in the wash. After most of the protein had washed through the column, the transferase activity was eluted with a gradient

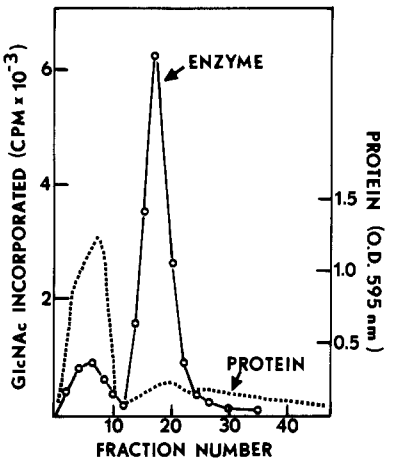

FIGURE 2: Fractionation of GlcNAc-transferase II on DEAE-cellulose. The concentrated and active enzyme from the phosphocellulose column was dialyzed against 10 mM Tris-HCl buffer, pH 7.2, containing 20 glycerol, 0.1% Triton X-100, and 0.5 μM dithiothreitol. This enzyme was applied to a 1.2 × 12 cm column of DEAE-cellulose, equilibrated with the same buffer. The column was washed well with buffer, and the GlcNAc-transferase II was eluted with a gradient (150 mL each) of 0–0.25 M NaCl. Fractions were tested for protein and for enzyme activity.

Table I: Purification Procedure for GlcNAc-transferase II[a]

	vol (mL)	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)
crude extract	2400	11520	31.1	0.0027	100
solubilized enzyme	85	1147	26.4	0.023	85
first DEAE-cellulose	112	258	16.3	0.063	52
cellulose phosphate	75	17	14.5	0.85	47
second DEAE-cellulose	40	5.2	13.0	2.5	42
concanavalin A–Sepharose	8	0.82	7.6	9.3	24

[a] One unit is defined as that amount of enzyme that catalyzes the transfer of 1 nmol of GlcNAc min^{-1} (mg of protein)$^{-1}$.

of NaCl (0–1.5 M). The enzyme emerged in a fairly sharp peak about 0.5 M NaCl. Active fractions were pooled and concentrated by Amicon filtration on a P-30 membrane.

Second DEAE-cellulose Chromatography. The enzyme preparation from the cellulose phosphate step was then applied to DEAE-cellulose. As presented in Figure 2, the column was eluted with a gradient of NaCl and the enzyme emerged in a fairly sharp and symmetrical peak about 0.075–0.1 M NaCl. Active fractions were pooled and concentrated on an Amicon filtration apparatus to about 8 mL.

Chromatography on Concanavalin A–Sepharose. The dialyzed fraction from the second DE-52 column was treated with concanavalin A–Sepharose as follows: the enzyme solution was made 0.5 M with respect to NaCl and 1 mM with respect to Mn^{2+}, Ca^{2+}, and Mg^{2+}. The enzyme solution was then mixed with 1 mL of washed concanavalin A–Sepharose. The mixture was incubated at 5 °C overnight with stirring, and then the gel was removed by centrifugation and washed 3 times with 10 mM Tris buffer, pH 7.2, containing 0.5 M NaCl and 1 mM each of Mn^{2+}, Ca^{2+}, and Mg^{2+}. The enzyme was eluted by suspending the gel in 2 mL 0.1 M methyl α-mannoside in the same buffer. Two elutions with methyl α-mannoside were done, and the two eluates were pooled. The pooled fraction was diluted with buffer to 20 mL and concentrated on the Amicon to 2 mL. This was repeated once again to remove most of the methyl α-mannoside.

With the use of these procedures, GlcNAc-transferase II was purified about 3400-fold with a recovery of about 24%. These data are summarized in Table I. Many of the experiments described in this paper were done with the second DE-52 fraction, which is purified about 1000-fold. This enzyme fraction was fairly stable to freezing and could be kept for several weeks at −20 °C, as long as 20% glycerol, 0.5 μM dithiothreitol and 0.1% Triton X-100 were in the buffer. The enzyme could also be stored for at least 1 week in ice.

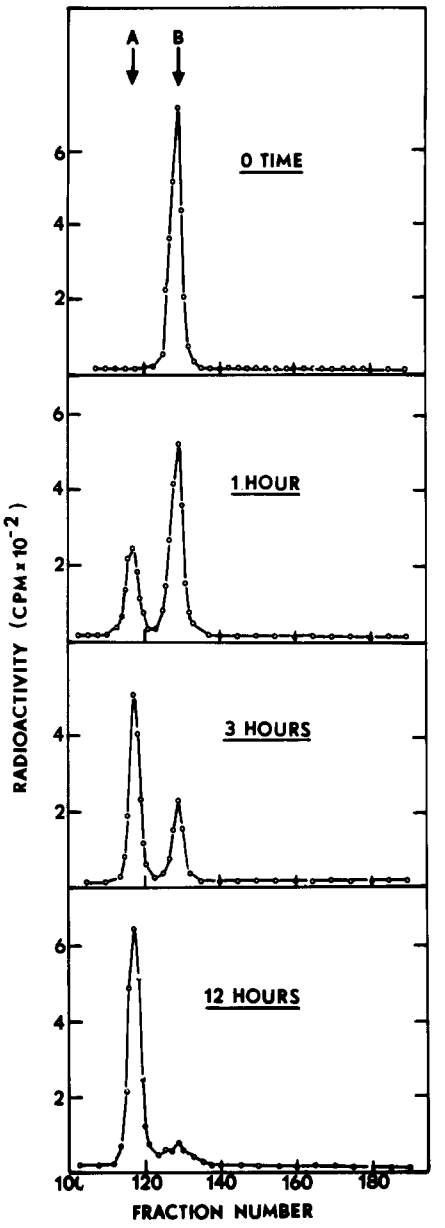

FIGURE 3: Gel filtration assay demonstrating dependence of reaction on time. Incubations with [^{3}H]mannose-labeled $GlcNAcMan_3GlcNAc$ and unlabeled UDP-GlcNAc were prepared as described in the text. In these incubations, 5 μg of DE-52 enzyme was used. Aliquots of the reaction mixture were removed at the times indicated and subjected to gel filtration on columns of Bio-Gel P-4. Aliquots of each faction were counted to determine their radioactive content. Standards shown by the arrows are as follows: A, $(GlcNAc)_2Man_3GlcNAc$; B, $GlcNAcMan_3GlcNAc$.

Properties of the Purified Enzyme

Requirements for Activity. The plant GlcNAc-transferase II could be detected by using a gel filtration assay or an ion-exchange method. In Figure 3, the time-dependent formation of $(GlcNAc)_2Man_3GlcNAc$ is shown by using the gel filtration assay. In this case, [^{3}H]mannose-labeled $GlcNAcMan_3GlcNAc$ was incubated with the DE-52 enzyme in the presence of unlabeled UDP-GlcNAc, and the reaction products were examined at various times. It can be seen that

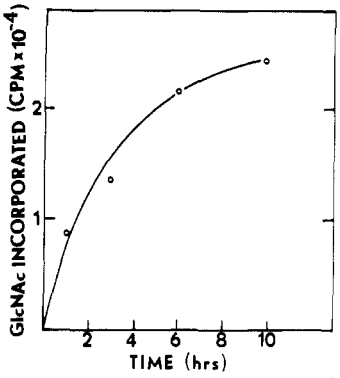

FIGURE 4: Time course of incorporation of [^{3}H]GlcNAc by GlcNAc-transferase II. Incubations containing unlabeled GlcNAcMan$_3$GlcNAc and UDP-[^{3}H]GlcNAc were prepared as described in the text and contained 4 μg of phosphocellulose enzyme. The incorporation of GlcNAc was determined by the ion-exchange method.

Table II: Requirements for GlcNAc-transferase II

omissions or additions	radioactivity in product
none (complete)[a]	4630
$-Mn^{2+}$	0
-GlcNAcMan$_3$GlcNAc	352
-UDP-GlcNAc, +UDP-Gal	74
-enzyme	0
+Triton X-100	7542

[a] Complete incubations were as described in the text and contained unlabeled GlcNAcMan$_3$GlcNAc, Mn^{2+}, UDP-[^{3}H]GlcNAc, and 1 μg of enzyme from the second DE-52 column. After 30 min of incubation, the formation of product was determined by the ion-exchange method.

a new, slower-moving peak appeared during the incubation, and the amount of radioactivity in this peak increased proportionately with time of incubation. This new peak was identified as (GlcNAc)$_2$Man$_3$GlcNAc (see below). The formation of (GlcNAc)$_2$Man$_3$GlcNAc could also be assayed by measuring the incorporation of [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc into GlcNAcMan$_3$GlcNAc. Figure 4 shows that this incorporation was fairly linear with time for at least several hours, using the ion-exchange assay method. The requirements for GlcNAc-transferase II activity with the 1000-fold purified enzyme and the ion-exchange assay are presented in Table II. Thus, the enzyme showed an absolute requirement for divalent cation and an almost absolute requirement for oligosaccharide acceptor and UDP-GlcNAc. Furthermore, the addition of Triton X-100 above the usual amount (0.1%) caused a considerable stimulation in activity. The small amount of neutral radioactivity observed in the absence of acceptor may represent some degradation of the substrate UDP-[^{3}H]GlcNAc to neutral GlcNAc.

Effect of pH and Metal Ions. The transfer of GlcNAc to GlcNAcMan$_3$GlcNAc showed a fairly sharp pH optimum of 6.5–7.0, while activity was greatly diminished at pH values of 5–6 or those above 7.5 [see Figure 8 in the supplementary material (see paragraph at end of paper regarding supplementary material)]. The incorporation of GlcNAc also showed an absolute dependence on the presence of a divalent cation as demonstrated in Figure 5. Thus, Mn^{2+} at 2–3 mM caused a great stimulation in activity, and this requirement for Mn^{2+} could not be replaced by Mg^{2+} or Ca^{2+}. In fact, only Cd^{2+} showed some ability to replace Mn^{2+}, and Figure 5 shows that activity with Cd^{2+} reached a peak about 0.5–1 mM. Other divalent cations, i.e., Co^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, and Ca^{2+}, were ineffective in stimulating at either 1 or 2.5 mM (data not shown).

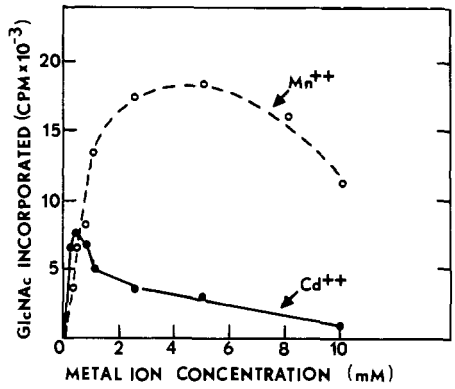

FIGURE 5: Effect of Mn^{2+}, Cd^{2+}, or EDTA on the activity of GlcNAc-transferase II. Incubations were as described in the text using the unlabeled GlcNAcMan$_3$GlcNAc and UDP-[^{3}H]GlcNAc, except that Mn^{2+} or Cd^{2+} were added at various concentrations as shown. In one case, various amounts of Mn^{2+} were added as well as 2.5 mM EDTA. Reactions were initiated by the addition of 3 μg of enzyme (second DEAE-cellulose), and incubations were for 60 min. Incorporation of GlcNAc was measured by the ion-exchange method.

Kinetics of GlcNAc-transferase II. By use of the purified enzyme, the effect of substrate concentration was examined. GlcNAc incorporation increased with increasing concentration of UDP-GlcNAc up to about 100 μM, and the K_m for UDP-GlcNAc was estimated to be about 18 μM. The effect of concentration of the other substrate, GlcNAcMan$_3$GlcNAc, was also examined. In this case, GlcNAc incorporation also increased with increasing amounts of oligosaccharide up to about 30 or 35 μM, but the incorporation was inhibited at higher oligosaccharide concentrations. This inhibition may be due to the presence of an inhibitory material, perhaps another oligosaccharide, in the preparation. Nevertheless, the K_m for GlcNAcMan$_3$GlcNAc was estimated to be 16.6 μM. These kinetic data are shown in Figures 9 and 10 (supplementary material).

Substrate Specificity of GlcNAc-transferase II. A number of oligosaccharides and glycoproteins were tested as GlcNAc acceptors by the purified enzyme. Essentially all of these were inactive, including Manα1,6(Manα1,3)Manα1,6(Manα1,3)-ManβGlcNAc, Manα1,6(Manα1,3)ManβGlcNAc, Manα1,2Manα1,3ManβGlcNAc, Manα1,3ManβGlcNAc, Manα1,6Manβ(GlcNAc)$_2$, *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl β-D-mannopyranoside, *p*-nitrophenyl β-D-maltoside, fetuin, asialofetuin, and ovomucoid. We also tested a number of sugar nucleotides to see whether they could replace UDP-[^{3}H]GlcNAc as sugar donor to GlcNAcMan$_3$GlcNAc. All of these were inactive, including UDP-[^{14}C]GalNAc, UDP-[^{14}C]galactose, UDP-[^{14}C]xylose, UDP-[^{3}H]glucose, and GDP-[^{14}C]fucose. Xylose and fucose were tested since the plant modified chains may contain these two sugars.

Effect of Detergent on Activity. The incorporation of GlcNAc to form (GlcNAc)$_2$Man$_3$GlcNAc was markedly stimulated by the addition of detergent above the usual 0.015%. Maximum activity occurred at about 0.1%, where activity was increased at least 3-fold (data not shown).

Characterization of the Product

The [^{3}H]mannose-labeled (GlcNAc)$_2$Man$_3$GlcNAc produced from unlabeled UDP-GlcNAc or the [^{3}H]GlcNAc-labeled (GlcNAc)$_2$Man$_3$GlcNAc produced from UDP-[^{3}H]-GlcNAc was isolated by chromatography on columns of Bio-Gel P-4, as shown in Figure 3. The radioactive peaks emerged from the column in the same area as (GlcNAc)$_2$Man$_3$GlcNAc and were clearly distinct from the starting substrate, GlcNAcMan$_3$GlcNAc.

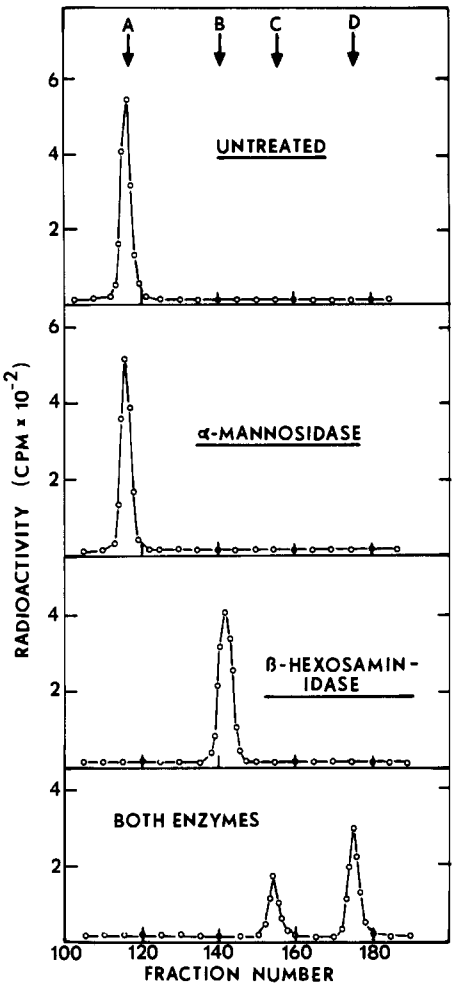

FIGURE 6: Characterization of reaction product on Bio-Gel P-4. The oligosaccharide product prepared from mannose-labeled $GlcNAcMan_3GlcNAc$ and unlabeled UDP-GlcNAc was chromatographed on Bio-Gel P-4 columns as shown in the top profile. This oligosaccharide was treated with jack bean α-mannosidase in the second profile, and the reaction mixture was examined on the Bio-Gel column. Another aliquot of the oligosaccharide was treated with β-hexosaminidase, and the reaction products were run on the Bio-Gel P-4 column (third profile). Finally, a third aliquot of the product was incubated with both α-mannosidase and β-hexosaminidase before being run on the column. Standards shown at the top are as follows: A, $(GlcNAc)_2Man_3GlcNAc$; B, $Man_3GlcNAc$; C, ManGlcNAc; D, mannose.

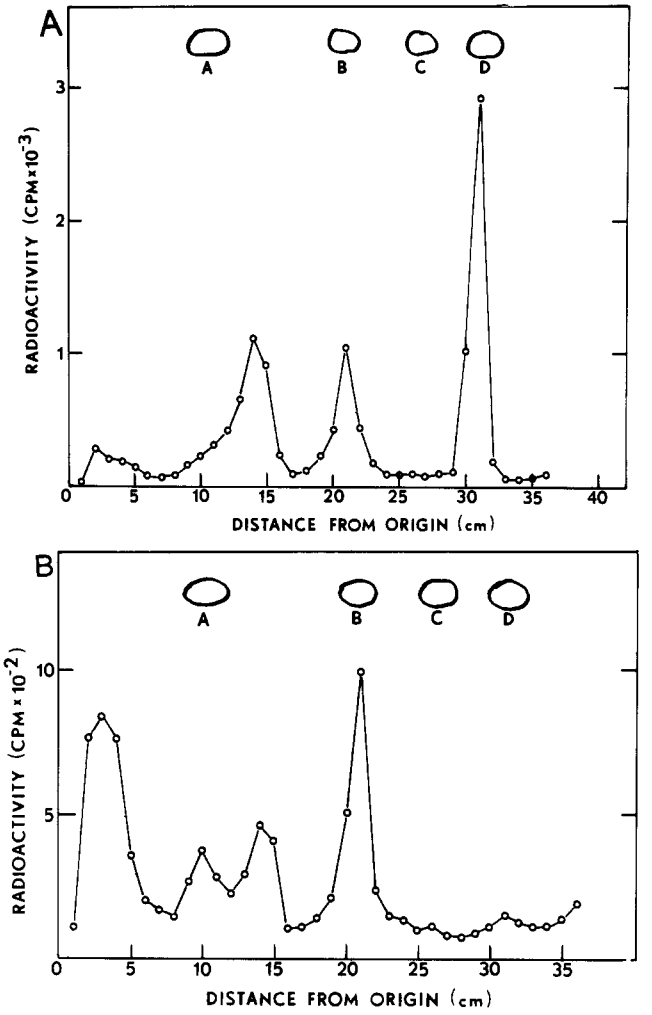

FIGURE 7: Methylation analysis of substrate, $GlcNAcMan_3GlcNAc$, and product, $(GlcNAc)_2Man_3GlcNAc$. The [^{3}H]mannose-labeled substrate and product were subjected to methylation as described, and the methylated oligosaccharides were isolated by gel filtration and hydrolyzed. The methylated sugars were identified by thin-layer chromatography in benzene/acetone/H_2O/ammonium hydroxide (50:200:1:1.35). Standards: A, 2,3-dimethylmannose; B, 3,4,6-trimethylmannose; C, 2,4,6-trimethylmannose; D, 2,3,4,6-tetramethylmannose. In profile A, the oligosaccharide substrate $GlcNAcMan_3GlcNAc$ is shown, while in profile B, the product $(GlcNAc)_2Man_3GlcNAc$ is presented.

The [^{3}H]mannose-labeled $(GlcNAc)_2Man_3GlcNAc$ was treated with a number of enzymes to aid in the characterization. As shown in Figure 6, after each treatment, the products were identified by chromatography on columns of Bio-Gel P-4. When the oligosaccharide was treated with jack bean α-mannosidase, there was no change in the elution pattern of the oligosaccharide and no radioactive mannose was released. On the other hand, treatment with β-hexosaminidase caused a considerable change in the migration of the oligosaccharide which now migrated near the $Man_3GlcNAc$ standard. Again no radioactive mannose was released. On the other hand, when the oligosaccharide was treated with both α-mannosidase and β-hexosaminidase, the original oligosaccharide was replaced by two new peaks that corresponded to free mannose and ManGlcNAc. The radioactivity in the free mannose was almost twice that in the ManGlcNAc. These data are consistent with a $(GlcNAc)_2Man_3GlcNAc$ structure. When the [^{3}H]GlcNAc-labeled product was treated with β-hexosaminidase, all of the radioactivity was released as free GlcNAc.

The oligosaccharide substrate, [^{3}H]mannose-labeled $GlcNAcMan_3GlcNAc$, and the oligosaccharide product, [^{3}H]mannose-labeled $(GlcNAc)_2Man_3GlcNAc$, were subjected to complete methylation in order to determine the glycosidic linkages of the terminal GlcNAc residues. After methylation, the oligosaccharides were isolated by gel filtration and hydrolyzed to release the sugars, and the methylated mannose derivatives were isolated and identified by thin-layer chromatography as shown in Figure 7. In Figure 7A, the methylated mannose derivatives released from the substrate, $GlcNAcMan_3GlcNAc$, are shown. Besides the peak of radioactivity at the origin, which probably represents unhydrolyzed oligosaccharide, three other radioactive peaks were observed. In order of migration from the origin, these peaks correspond to 2,4-dimethylmannose (i.e., 3,6-linked mannose), 3,4,6-trimethylmannose (i.e., 2-linked mannose), and 2,3,4,6-tetramethylmannose (i.e., terminal mannose). The amount of radioactivity in the 2-linked mannose was nearly the same as that in the terminal mannose, while the 3,6-linked mannose had only about 60% as much radioactivity. The product of the GlcNAc-transferase II reaction, i.e.,

$(GlcNAc)_2Man_3GlcNAc$, was also subjected to methylation analysis as shown in Figure 7B. In this case, the oligosaccharide was not completely hydrolyzed since a peak of radioactivity remained at the origin and a second small peak migrated slightly from the origin. However, the important features of this analysis are the loss of terminal mannose and the substantial increase in 2-linked mannose. These data indicate that both GlcNAc residues are linked to the mannose residues in β1,2 linkages.

Discussion

The results described in this paper demonstrate the presence of a second GlcNAc-transferase in plants that is distinct from the previously reported GlcNAc-transferase of mung beans (Davies & Delmer, 1981; Chrispeels, 1985; Szumilo et al., 1986c). We also report the 3000-fold purification of this enzyme starting with microsomes from *Phaseolus aureus*. On the basis of the specificity of this enzyme for the $GlcNAcMan_3GlcNAc$ acceptor, and the fact that methylation analysis of the product indicated that a GlcNAc had been added in β1,2 linkage to the 6-linked mannose, this enzyme appears to be analogous to the animal GlcNAc-transferase II (Schachter et al., 1983). This enzymatic activity has not been previously described in plants.

However, it is known that plants produce modified oligosaccharide structures that resemble the biantennary complex chains of animal cells, except for the absence of sialic acid in plants and the frequent presence of a β1,2-linked xylose. Thus, sycamore cells secrete the enzyme laccase, an N-linked glycoprotein that has a Galβ1,4-GlcNAcβ1,2 sequence attached to each of the α-linked mannose residues (Takahashi et al., 1986). Most of these oligosaccharides also have a β1,2-linked xylose on the β-mannose. On the basis of what is known about the processing reactions in animal cells, we would expect the formation of the laccase oligosaccharides to require the action of glucosidase I, glucosidase II, mannosidase I, GlcNAc-transferase I, mannosidase II and GlcNAc-transferase II (Hubbard & Ivatt, 1981).

During the purification of GlcNAc-transferase II, we were able to separate this activity from that of GlcNAc-transferase I. However, during this purification, we found no evidence for the presence of other GlcNAc-transferases that have been reported in animal systems. Thus, GlcNAc-transferase III, which adds a bisecting GlcNAc, has been shown to represent an important crossroad since addition of this GlcNAc prevents a number of other reactions and therefore can determine the final oligosaccharide structure (Schachter et al., 1983). While this bisecting GlcNAc apparantly is absent in plants, its place may be taken by the xylose that becomes linked in β1,2 linkage to the β-mannose. That is, it seems possible that this xylose serves as some sort of signal to direct the oligosaccharides down certain pathways. It will be necessary to isolate and purify the xylosyltransferase and determined its specificity before such regulation can be established.

While it seems likely, on the basis of the substrate specificity of GlcNAc-transferase II, that the second GlcNAc is added after the action of mannosidase II, it is not known at what stage the xylose is added nor when fucose is attached. It will be of considerable interest to determine the substrate specificities of these transferases in order to establish these points.

Supplementary Material Available

Figures 8–10 showing effect of incubation mixture pH on GlcNAc-transferase II, effect of UDP-GlcNAc concentration on GlcNAc-transferase II activity, and effect of $GlcNAcMan_3GlcNAc$ concentration on GlcNAc-transferase II activity (3 pages). Ordering information is given on any current masthead page.

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Isolation and Characterization of Four Heparin-Binding Cyanogen Bromide Peptides of Human Plasma Apolipoprotein B†

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ABSTRACT: Apolipoprotein B-100 (apoB-100) is the major protein constituent of human plasma low-density lipoproteins (LDL). On the basis of its amino acid sequence [Chen, S.-H., Yang, C.-Y., Chen, P.-F., Setzer, D., Tanimura, M., Li, W.-H., Gotto, A. M., Jr., & Chan, L. (1986) *J. Biol. Chem. 261*, 12918–12921], apo B-100 is one of the largest monomeric proteins known with a calculated molecular weight of 512 937. Heparin binds to the LDL surface by interacting with positively charged amino acid residues of apoB-100, forming soluble complexes in the absence of divalent metals and insoluble complexes in their presence. The purpose of this study was to isolate and characterize the heparin-binding domain(s) of apoB-100. Human plasma LDL were fragmented with cyanogen bromide (CNBr). After delipidation and reduction–carboxymethylation, the CNBr peptides were fractionated by sequential chromatography on DEAE-Sephacel, Mono S, and high reactive heparin (HRH) AffiGel-10; HRH was purified by chromatography of crude bovine lung heparin on LDL AffiGel-10. Heparin-binding peptides were further purified by reverse-phase high-performance liquid chromatography. Heparin-binding activity was monitored by a dot-blot assay with ^{125}I-HRH. The amino-terminal sequences of four CNBr heparin-binding peptides (CNBr-I–IV) were determined. CNBr-I–IV correspond to residues 2016–2151, 3109–3240, 3308–3394, and 3570–3719, respectively, of the amino acid sequence of apoB-100. Each CNBr peptide contains a domain(s) of basic amino acid residues which we suggest accounts for their heparin-binding activity. CNBr-I is located near the middle of the apoB-100 sequence whereas CNBr-II, -III, and -IV are clustered near the carboxyl-terminal end of the protein. The proposed heparin-binding domains of CNBr-II (residues 3150–3157) and -IV (residues 3670–3677) show structural homology to the reported heparin-binding region of human vitronectin; CNBr-III (residues 3361–3368) shows structural homology to the known receptor/heparin-binding region of apolipoprotein E (residues 144–151). We suggest that one or more of these heparin-binding domains possibly corresponds to the receptor-binding region(s) of LDL as heparin is known to displace LDL from its membrane receptor.

Plasma low-density lipoproteins (LDL)[1] are the major carriers of cholesterol in the circulation. LDL are isolated in the density range 1.019–1.063 g/mL and represent a distribution of particles differing in size, hydrated density, and lipid composition (Crouse et al., 1985; Fisher, 1983; Krauss & Burke, 1982; Shen et al., 1981). LDL contain one major protein termed apolipoprotein B-100 (Kane et al., 1980) with a calculated molecular weight of 512 937 based on the complete amino acid sequence reported by Chen et al. (1986). In normal man, very low density lipoproteins (VLDL) represent the major apoB-100-containing precursor lipoproteins of plasma LDL. LDL are removed from plasma by either receptor-dependent (Goldstein & Brown, 1984; Brown & Goldstein, 1986: Dietschy, 1984) or receptor-independent processes (Spady et al., 1986); the liver is the major tissue site for LDL clearance.

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[1] Abbreviations: apoB-100, apolipoprotein B-100; VLDL, very low density lipoprotein(s); LDL, low-density lipoprotein(s); GAG, glycosaminoglycans; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRH, high reactive heparin; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; CNBr, cyanogen bromide; PTH, phenylthiohydantoin; HBD, heparin-binding domain.

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