Purification and Characterization of UDP-GlcNAc: GlcNAcβ1-6(GlcNAcβ1–2)Manα1-R [GlcNAc to Man]–β1, 4-N-acetylg glucosaminyltransferase VI from Hen Oviduct*

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A new β1,4-N-acetylg glucosaminyltransferase (GnT) responsible for the formation of branched N-linked complex-type sugar chains has been purified 64,000-fold in 16% yield from a homogenate of hen oviduct by column chromatography procedures using Q-Sepharose FF, Ni2+-chelating Sepharose FF, and UDP-hexanolamine-agarose. This enzyme catalyzes the transfer of GlcNAc from UDP-GlcNAc to tetraantennary oligosaccharide and produces pentaantennary oligosaccharide with the β1–4-linked GlcNAc residue on the Manα1–6 arm. It requires a divalent cation such as Mn2+ and has an apparent molecular weight of 72,000 under nonreducing conditions. The enzyme does not act on biantennary oligosaccharide (GnT I and II product), and β,6-N-acetylg glucosaminylation of the Manα1–6 arm (GnT V product) is essential for its activity. This clearly distinguishes it from GnT IV, which is known to generate a pentaantennary glycan, which is a pentaantennary glycan with a bisecting GlcNAc residue, were found in hen ovomucoid (2, 3) and in the fish egg glycoprotein known as hyosophorin (4). GnTs I–V have been purified and the corresponding genes have been cloned (5–19). Only GnT VI has not been purified, and its gene structure remains unknown.

This study reports the purification of GnT VI from hen oviduct, which has been previously shown to have high activity (20). By successive column chromatographies using Q-Sepharose FF, Ni2+-chelating Sepharose FF, and UDP-hexanolamine-agarose with a newly developed assay method (21) wherein pyridylaminated agalactotetraantennary oligosaccharide ([(2,4),(2,6)]-PA) (see Fig. 2) was used as an acceptor substrate and the reaction product was pyridylaminated agalacto-pentaantennary oligosaccharide ([(2,4),(2,4,6)]-PA) (see Fig. 2), this enzyme was purified 64,000-fold from a homogenate of hen oviduct. Using several acceptor compounds, the purified enzyme was shown to have an absolute requirement of GlcNAcβ1–2(GlcNAcβ1–6)Manα1–6 substrate. The GnT VI enzyme is distinct from GnT IV, which generates a β1–4-linked GlcNAc residue only on the Manα1–3 arm (13).

EXPERIMENTAL PROCEDURES

Materials—All materials were obtained from the following suppliers: UDP-GlcNAc, UDP-hexanolamine-agarose (ligand concentration of 2.4 μmol/ml), and GlcNAc from Sigma; Q-Sepharose FF and chelating Sepharose FF from Amersham Pharmacia Biotech (Uppsala, Sweden); Tris, HEPES, MES, and MOPS from Nacalai Tesque (Kyoto, Japan); Triton X-100, (p-aminophenyl)methanesulfon fluoride hydrochloride, glycine, DTT, and metal chlorides from Wako (Osaka, Japan); [(2,4,6)]-PA from Takara Co. (Kyoto); and hen oviduct from Benchoyo (Osaka).

Determination of GnT VI Activity—GnT VI activity was assayed as described previously (21) with minor modifications. The standard incubation mixture contained the following components in a total volume of 50 μl: 150 mM HEPES (pH 8.0), 100 mM GlcNAc, 30 mM MnCl₂, 0.5% Triton X-100, 2 mg/ml bovine serum albumin, 24 or 120 pmol of substrate ([(2,4),(2,6)]-PA), and 2 μl of enzyme fraction. After incubation at 37 °C for 4 h, 40 μl of water was added, and the enzyme reaction was stopped by boiling for 1 min. After centrifugation at 13,000 rpm for 5 min, 10 μl of the supernatant from the reaction mixture was applied to a TSK-Gel ODS-80TM column (4.6 × 75 mm; Tosoh, Tokyo, Japan). Elution was performed at 55 °C with 20 mM ammonium acetate (pH 4.0) at a flow rate of 1.6 ml/min. Fluorescence was monitored with excitation and emission wavelengths of 320 and 400 nm, respectively. The specific activity of the enzyme is expressed as picomoles of product formed per h/mg of protein. The protein concentration was determined with a BCA kit using bovine serum albumin as the standard. The activities of GnTs III–V were measured according to the method of Nishikawa et al. (22). Preparation of Oligosaccharides—The structures of all oligosaccharides used in this assay are shown in Fig. 2. [(2,4),(2,6)]-PA was prepared from human α1-acid glycoprotein essentially as described previ-
FIG. 1. GlcNAc-transferases (GnTs I–VI) involved in antenna formation on the cores of N-linked complex-type sugar chains.

FIG. 2. Structures and abbreviations for pyridylaminated sugar chains used in this study. The numbers in brackets show the positions of the two α-Man residues to which nonreducing terminal GlcNAc residues are linked. The first set of parentheses indicate GlcNAc residues linked to the α-Man residue (Man-4) that is linked to the β-Man residue by 1–3 linkage, and the second set of parentheses indicate GlcNAc residues linked to the α-Man residue (Man-4) that is linked to the β-Man residue by 1–6 linkage. For example, (2,2),(2,6)-PA indicates the structure in which two GlcNAc residues are linked to Man-4 by β1–2 and β1–4 linkages and two GlcNAc residues are linked to Man-4 by β1–2 and β1–6 linkages.

RESULTS

Purification of GnT VI—The activity of GnT VI was assayed using the fluorescently labeled agalactotetraantennary sugar chain as an acceptor substrate basically according to the original method of Taguchi et al. (21). Since bovine serum albumin (2 mg/ml) was found to be effective in preserving enzyme activity at 37 °C, especially for the highly purified enzyme fraction (Steps 5 and 6), it was routinely included in the standard assay mixture.

Like other glycosyltransferases, GnT VI activity was found in the microsomal fraction. At least 80% of the GnT VI activity was associated with the microsomal fraction. GnT VI activity was successfully solubilized from the microsomal fraction by extraction with Triton X-100. Substantial amounts of proteins were separated from GnT VI by Q-Sepharose FF and Ni2+-chelating Sepharose FF chromatographies (Fig. 3, A and B). After the Ni2+-chelating Sepharose FF column chromatography (Step 4), the enzyme was more stable than in Steps 2 and 3. As opposed to results with GnTs III and IV (11, 13), no GnT VI activity was eluted after application to a Cu2+-chelating Sepharose FF column. Following the above two column chromatography steps, the GnT VI active fraction still contained GnT III and β1,4-galactosyltransferase activities. Cation exchange, dye affinity, gel filtration, and lectin column chromatographies did not step forward to be effective for further purification of the enzyme fraction after Step 4. The use of an affinity column (Steps 5 and 6) (Fig. 3C) packed with an analog of the common donor substrate for GnTs (UDP-hexanolamine) as a ligand was effective. This affinity column has been proven to be very effective for purification of GnTs and was first used for the purification of GnT I by Oppenheimer and Hill (5). The activity of GnT III was

that had been equilibrated with Buffer C. Fractions of 40 ml were collected through this column chromatography. The column was washed with Buffer C until the protein concentration was reduced to 1.0 mg/ml. Elution was then carried out with a linear gradient established between 1000 ml of Buffer C and 1000 ml of 0.8 M NaCl/Buffer C. The fractions containing GnT VI activity were combined.

Ni2+-chelating Sepharose FF Column Chromatography (Step 4)—The pool of fractions from Step 3 was applied directly to a column of Ni2+-chelating Sepharose FF (2.5 × 10 cm) that had been equilibrated with Buffer D. Ni2+-chelating Sepharose FF resin was layered on the chelating Sepharose FF resin without metal ions (2.5 × 5 cm) to avoid any possible leakage of Ni2+. Fractions of 11 ml were collected through this column chromatography. All of the GnT VI activity was retained by the column. After washing the column with Buffer D until the protein concentration was reduced to 0.2 mg/ml, GnT VI activity was eluted with a linear gradient established between 300 ml of Buffer D and 300 ml of 0.1 M glycine/Buffer D. The fractions containing GnT VI activity were combined, and the buffer in this fraction was replaced by Buffer E by means of an Amicon Diaflow Ultrafiltrater using a YM-30 membrane (Amicon, Inc., Beverly, MA).

UDP-hexanolamine-Agarose Affinity Column Chromatography (Step 5)—After the above step, the column was siliconized with Sigma-Aldrich (Sigma), and siliconized tubes (Assist, Tokyo) were used for fractionation. The UDP-hexanolamine-agarose column (1.5 × 15 cm) had been equilibrated with 0.05 M NaCl/Buffer E, and the concentrated enzyme fraction from Step 4 was applied to the column, followed by washing with 100 ml of Buffer E. GnT VI activity was eluted with Buffer F. At the loading and washing steps, fractions of 5 ml were collected. At the elution step, fractions of 1.4 ml were collected, and those containing GnT VI activity were pooled.

UDP-hexanolamine-Agarose Affinity Column Chromatography (Step 6)—An equal volume of Buffer G was added to the pool of fractions from Step 5, and this solution was rechromatographed by the same procedure as described for Step 5. This purified GnT VI fraction was used for the enzyme characterization.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (23) using 10% gels. Molecular markers (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) were used for size standards. Proteins on the gels were stained with a silver staining kit (DAIICHII 2D silver stain II, Daiichi Pure Chemicals, Tokyo).
not bound to this column under the conditions described under "Experimental Procedures." The majority of GnT VI activity was bound to this affinity column and was eluted by the buffer lacking MnCl₂. Since the eluted fraction (after Step 5) still contained several proteins as revealed by SDS-polyacrylamide gel electrophoresis (data not shown), this fraction was rechromatographed (Step 6) under the same conditions as described for Step 5. The addition of DTT was crucial in preserving GnT VI activity after Steps 5 and 6, whereas it was without effect in Step 4. The enzyme fraction of Step 6 showed a single broad band with a molecular weight of 72,000 on SDS-polyacrylamide gel electrophoresis under nonreducing conditions (Fig. 4) and of 60,000 under reducing conditions (data not shown), suggesting that the enzyme was a glycoprotein. This band was also correlated with GnT VI activity by elution with UDP (5 mM) or NaCl (0.4 M) from a UDP-hexanolamine-agarose column, suggesting that this band was GnT VI (data not shown). No GnT III–V activities were detected in this fraction when [2,2]-PA was used as an acceptor substrate. All experiments for enzyme characterization described below were performed using the chromatographic Step 6 material. Table I summarizes the purification of GnT VI, which was purified 64,000-fold in 16% yield.

**pH Optimum**—GnT VI possessed activity over a relatively broad pH range with an optimum at pH 7.75.

**Effect of MnCl₂ Concentration on GnT VI Activity**—GnT VI activity was Mn²⁺-dependent and was high around 5 mM. When the MnCl₂ concentration was increased, the activity gradually decreased.

**Effect of Divalent Cations on GnT VI Activity**—The effects of divalent cations on GnT VI activity were examined. The activity was maximal with Mn²⁺. Co²⁺, Mg²⁺, and Ni²⁺ could partially substitute for Mn²⁺, whereas Ca²⁺, Zn²⁺, Fe²⁺, and Cu²⁺ showed no significant effect (Table II).

**Acceptor Substrate Specificity**—The acceptor substrate specificity of GnT VI was examined using complex-type sugar chains. Purified GnT VI was revealed to select its substrate clearly (Table III). It could transfer GlcNAc to [(2,4),(2,6)]-PA and [2,(2,6)]-PA (GnT V product), whereas no activity was observed when [2,2]-PA or [(2,4),2]-PA (GnT IV product) was used as a substrate.

**DISCUSSION**

Six different GnTs (I–VI) are known to be mainly involved in antenna formation on the cores of N-linked complex-type sugar chains (Fig. 1). In addition, Raju and Stanley (24) recently identified two other distinct GnT activities designated GnTs VII and VIII from Chinese hamster ovary mutant cells. GnT VI activity is defined as that catalyzing the transfer of GlcNAc to the Man₁₋₆ arm and forms a GlcNAc β₁₋₄Man₁₋₆ linkage. Only GnT VI has not been purified in the GnTs that act on α-Man residues. The substrate specificity of GnT VI has not been clearly determined, although it has been suggested, by using hen oviduct microsomes as an enzyme source, that GnT VI acts after assembly of GlcNAcβ₁₋₂Man₀₁₋₃, GlcNAcβ₁₋₂Man₀₁₋₆, and GlcNAcβ₁₋₆Man₀₁₋₆ antennae by the action of GnTs I, II, and V, respectively (20). The tissue distribution of this enzyme seems to be highly restricted. So far, only avian oviduct (20) and fish ovary (25) have been shown to express this enzyme activity. The transferrin synthesized by the human hepatocarcinoma cell line HepG2 was reported to have a pentantennary glycan chain (26), which is the product of GnT VI activity.

In this study, we have purified GnT VI from hen oviduct 64,000-fold with a newly developed assay method (21) wherein [(2,4),(2,6)]-PA (Fig. 2) was used as an acceptor substrate, and the reaction product was [(2,4),(2,4,6)]-PA (GnT IV product) was used as a substrate.
TABLE I

| Step                | Total protein | Total activity | Specific activity | Yield | Purification |
|---------------------|---------------|----------------|-------------------|-------|--------------|
|                     | mg            | nmol/h         | pmol/h/mg protein | %    | -fold        |
| 1. Homogenate       | 11,800        | 350            | 29.7              | 100   | 1            |
| 2. Triton extract   | 950           | 250            | 263               | 71    | 8.9          |
| 3. Q-Sepharose FF   | 445           | 184            | 413               | 53    | 14           |
| 4. Ni²⁺-chelating Sepharose FF | 100 | 125 | 36            | 30    | 12          |
| 5. UDP-hexanolamine-agarose (1st) | 0.1 | 106 | 1,060,000 | 30    | 36,000       |
| 6. UDP-hexanolamine-agarose (2nd) | 0.03 | 57.0 | 1,900,000 | 16    | 64,000       |

TABLE II

| Addition | Activity |
|----------|----------|
| MnCl₂    | 100      |
| CoCl₂    | 80       |
| MgCl₂    | 60       |
| NiCl₂    | 27       |
| CuCl₂    | 8.5      |
| ZnCl₂    | ND       |
| FeCl₂    | ND       |
| CuCl₂    | ND       |
| None     | ND       |
| EDTA     | ND       |

TABLE III

| Acceptor | Activity |
|----------|----------|
| [2,2]-PA | ND       |
| [2,4,2]-PA | ND     |
| [2,6,2]-PA | 340    |
| (2,4,2,6)-PA | 100 |

Purification of GnT VI from hen oviduct

GnT VI activity was assayed as described under “Experimental Procedures” at substrate levels of 12 μM. GnT VI activity in the presence of [(2,4),(2,6)]-PA as an acceptor is taken as 100%. 100% enzyme activity corresponds to 4.7 nmol/h/ml. The structures of the acceptor sugar chains are shown in Fig. 2. ND, not detected.

Addition Activity %
MnCl₂ 100
CoCl₂ 80
MgCl₂ 60
NiCl₂ 27
CuCl₂ 8.5
ZnCl₂ ND
FeCl₂ ND
CuCl₂ ND
None ND
EDTA ND

Substrate specificity of GnT VI

GnT VI activity was assayed as described under “Experimental Procedures” at substrate levels of 12 μM. GnT VI activity in the presence of [(2,4),(2,6)]-PA as an acceptor is taken as 100%. 100% enzyme activity corresponds to 4.7 nmol/h/ml. The structures of the acceptor sugar chains are shown in Fig. 2. ND, not detected.

Addition Activity %
MnCl₂ 100
CoCl₂ 80
MgCl₂ 60
NiCl₂ 27
CuCl₂ 8.5
ZnCl₂ ND
FeCl₂ ND
CuCl₂ ND
None ND
EDTA ND

this GnT VI gene is now in progress based on the peptide sequences obtained from the purified enzyme. The specificity of this purified GnT VI is summarized as follows (see Table III). (a) no GnT III and IV activities were observed, both of which can act on [2,2]-PA and produce GlcNAcβ1-4 linkages on the β-linked Man residue and the Manα1–3 residue, respectively (11, 13). (b) GnT VI activity was observed when [2,4,2]-PA and [2,6,2]-PA were used as acceptor substrates. (c) GnT VI activity was not detected when [(2,4,2)-PA was used as a substrate. From these observations, it is concluded that β1,6-N-acetylgalactosaminyltransferase to the Manα1–6 arm (GnT V product) is essential for its activity. This defines GnT VI activity as UDP-GlcNAc:GlcNAcβ1-6GlcNAcβ1-2Manα1-R [GlcNAc to Man]-β1,4-N-acetylgalactosaminyltransferase. This characteristic clearly distinguishes this enzyme from GnT IV, which is known to generate a β1,4-linked GlcNAc residue only on the Manα1–3 arm (13). In addition, it should be pointed out that this purified GnT VI does not have GnT VI activity, which is defined as that making GlcNAcβ1-2[GlcNAcβ1-4]Manα1−6 linkage without the requirement of a GlcNAcβ1-6Manα1-6 structure (27), since no product was detected when [2,2]-PA was used as a substrate. In conjunction with previous observations, the biosynthetic pathways leading to a bisected pentaantennary glycan chain are depicted in Fig. 5.

The substrate specificity of purified GnT VI agrees with the results reported by Brochhausen et al. (20) in a study using hen oviduct microsomes as the enzyme source and GlcNAcβ1-6GlcNAcβ1-2Manα1-6Manα1-[CH₂]₇COOCH₃ as an acceptor substrate. Some differences from their results in terms of optimal conditions for activity, e.g. the effect of Mn²⁺ concentration and the effects of divalent cations, are to be noted. Differences are also noted for the optimal conditions of enzyme from hen oviduct microsomes (21) and this purified GnT VI using the same substrate, [(2,4),(2,6)]-PA. Such discrepancies could be due to the presence of another (or other) GnT VI(s) in hen microsomes.

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Purification and Characterization of GnT VI

32602

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