Enzymatic Basis for the Structural Changes of Asparagine-linked Sugar Chains of Membrane Glycoproteins of Baby Hamster Kidney Cells Induced by Polyma Transformation*

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Preferable studies indicated that enrichment of the GlcNAcβ1→6Manα1→ group with concomitant decrease of the GlcNAcβ1→4Manα1→ group occurs in the complex-type asparagine-linked sugar chains of the membrane glycoproteins of baby hamster kidney cells transformed by polyoma virus. The enzymatic basis of the chemical change is reported in this paper. By using oligosaccharides isolated from the urine of patients with a variety of exoglycosidase deficiencies, β-N-acetylglucosaminyltransferases in the cell homogenate were successfully assayed separately. Both baby hamster kidney cells and their polyoma transforms contain β-N-acetylglucosaminyltransferases I, II, IV, V, and VI, but not β-N-acetylglucosaminyltransferase III. The β-N-acetylglucosamine residue added by each β-N-acetylglucosaminyltransferase (GnT) is shown below.

Comparative studies of the specific activities of the five β-N-acetylglucosaminyltransferases in the two cell lines revealed that the value of β-N-acetylglucosaminyltransferase V in the polyoma transformant was twice of that in the normal cells, while those of the other four transferases in the two cell lines were not significantly different. Therefore the increase in β-N-acetylglucosaminyltransferase V may be the direct cause of the changes found in the sugar chains of surface glycoproteins in baby hamster kidney cells transformed by polyoma virus.

Several differences have been found in the structures of sugar chains of glycoproteins produced in normal and malignant cells. One of them is a characteristic increase in the molecular weight of the asparagine-linked sugar chains which has been widely observed in the plasma membrane glycoproteins of transformed and malignant cells from a variety of mammals (1); malignant cells in culture were examined in addition to those derived from solid tumors. By studying the behavior of the glycopeptides obtained from baby hamster kidney (BHK)1 cells and their polyoma transformants (Py-BHK cells) on a concanavalin A-Sepharose column, we suggested that the increase in size of the sugar chains of glycoproteins in these transformed cells is probably induced by an increased number of outer chain moieties of complex-type asparagine-linked sugar chains (2). Supporting evidence for this assumption has been reported from several research groups including ourselves (3-5).

The recent establishment of controlled hydrazinolysis (6), a chemical method for the release of asparagine-linked sugar chains of glycoproteins, has enabled us to analyze quantitative and qualitative differences in plasma membrane glycoproteins of cells (7, 8) as well as purified glycoproteins. Comparative studies of the asparagine-linked sugar chains of membrane glycoproteins of BHK and Py-BHK cells using this method have revealed that the transformation induces specific increases of the outer chains linked at the C-6 position of the Manα1→6 residue of the trimannosyl cores of the complex-type asparagine-linked sugar chains (9). The basis for this phenotypic change could arise from an altered β-N-acetylglucosaminyltransferase activity originating from altered genetic control. However, it could also arise from changes in the membrane components since such transferases are embedded in the Golgi membrane. In order to elucidate which of these possibilities is correct, we have analyzed the activities of a series of β-N-acetylglucosaminyltransferases responsible for the formation of the outer chain moieties of complex-type sugar chains in BHK cells and Py-BHK cells.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Thus far, six β-N-acetylglucosaminyltransferases, shown in Fig. 1 as GnT I to VI, are considered to be responsible for the

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1 The abbreviations used are: BHK, baby hamster kidney; XylNAc, N-acetylxylosaminol. Subscript OT is used to indicate NaBH₄-reduced oligosaccharides. In the same way, subscript OH is used to indicate NaBH₃-reduced oligosaccharides. All sugars mentioned in this paper were of the D-configuration except for fucose which has the L-configuration.

2 Portions of this paper (including "Experimental Procedures" and Tables I-III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1021, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
addition of different β-N-acetylglucosamine residues to the trimannosyl core. GnT VI catalyzes the addition of the β-N-acetylglucosamine residue which is not linked directly to the core but to the galactosyl residue of the Galβ1→4GlcNAc outer chains to initiate formation of the N-acetyllactosamine repeating structures. Since the purpose of this study was to determine the concentrations of all six β-N-acetylglycosaminyltransferases, we had to devise special means for the accurate determination of the activity of each transferase in cell homogenates. Like many other glycosyltransferases, the six β-N-acetylglycosaminyltransferases have been shown to have fairly strict specificities toward the acceptor sugar chains: GnT I (10, 11), II (11), III (12), IV (13), V (14), and VI (15). Therefore, it was considered possible that the activity of each enzyme might be determined in a cell homogenate, if an appropriate sugar chain was chosen as an acceptor.

So we have thoroughly investigated the radioactive products obtained by incubation of the oligosaccharides listed in Table I with UDP-N-acetyl[U-14C]glucosamine and homogenates of BHK cells or of Py-BHK cells as sources of enzymes. Except for Mz. GN, F-GN, GN-Mz. GN, and GN2-Mz. GN, all oligosaccharides listed in Table I can be converted to either one or two radioactive oligosaccharides by such incubations. This evidence indicated that GnT III might be deficient in both BHK cells and Py-BHK cells, because Gm1-GN served as a good acceptor for this enzyme in homogenates of hen oviduct.3 This inference was further confirmed by the complete absence of the bisecting N-acetylglucosamine residue in all radioactive products described below. Absence of the enzyme in BHK cells and in Py-BHK cells is also reflected in the structures of asparagine-linked sugar chains of glycoproteins from both cells, which completely lack the bisecting N-acetylglucosaminyl residue (9).

Structural Analyses of the Radioactive Products from Mz. GN, Mz. GN, and Mz. GN—Only one radioactive product was obtained from Mz. GN and Mz. GN (Fig. 2, A and B, respectively). From the differences in mobilities between acceptors in studies with Py-BHK cell homogenates. The black arrows at the top of the figure indicate the elution positions of glucose oligomers (numbers indicate the glucose units), and the white arrows indicate those of authentic oligosaccharides as follows: 1, Manβ1→3Manβ1→4GlcNAc; 2, Manβ1→4XylNAcC; 3, N-acetylgalactosamine. A, B, and C, the radioactive products obtained from Mz. GN, Mz. GN, and Mz. GN, respectively (the black triangles indicate the elution positions of acceptor oligosaccharides); D, the radioactive peaks in A, B, and C reduced with NaBH4 and then incubated with diplococcal β-N-acetylgalactosaminidase; E, the radioactive peak b in C incubated with jack bean α-mannosidase; F, the radioactive peak in E reduced with NaBH4 and incubated with diplococcal β-N-acetylgalactosaminidase (dotted line, radioactivities determined on an aliquot of each fraction by liquid scintillation spectrometer, solid line, obtained by monitoring effluent with a radioanalyzer); G, the dotted line (tritium labeled) peak in F subjected to periodate oxidation.

was recovered in a peak which corresponded to N-acetylgalactosamine (Fig. 2D). This result indicated that all radioactive N-acetylgalactosamine residues added to the two acceptor oligosaccharides occur as the GlcNacβ1→2Man group because β-N-acetylgalactosaminidase can only cleave this linkage among the five β-N-acetylgalactosaminyl linkages connected directly to the trimannosyl core shown in Fig. 1 (16).

Fig. 2. Bio-Gel P-4 column chromatography of the radioactive products from Mz. GN, Mz. GN, and Mz. GN and their enzymatic and chemical degradation products. Only data obtained from experiments where BHK cell homogenates were used as sources of enzymes are presented; the same results were also found in studies with Py-BHK cell homogenates. The black arrows at the top of the figure indicate the elution positions of glucose oligomers (numbers indicate the glucose units), and the white arrows indicate those of authentic oligosaccharides as follows: 1, Manβ1→3Manβ1→4GlcNAc; 2, Manβ1→4XylNAcC; 3, N-acetylgalactosamine. A, B, and C, the radioactive products obtained from Mz. GN, Mz. GN, and Mz. GN, respectively (the black triangles indicate the elution positions of acceptor oligosaccharides); D, the radioactive peaks in A, B, and C reduced with NaBH4 and then incubated with diplococcal β-N-acetylgalactosaminidase; E, the radioactive peak b in C incubated with jack bean α-mannosidase; F, the radioactive peak in E reduced with NaBH4 and incubated with diplococcal β-N-acetylgalactosaminidase (dotted line, radioactivities determined on an aliquot of each fraction by liquid scintillation spectrometer, solid line, obtained by monitoring effluent with a radioanalyzer); G, the dotted line (tritium labeled) peak in F subjected to periodate oxidation.

3 K. Yamashita, T. Ohkura, and A. Kobata, unpublished result.

4 Although diplococcal β-N-acetylgalactosaminidase cannot cleave the GlcNAcβ1→4Man linkage in GlcNAcβ1→4Manα1→6GlcNAcβ1→4(GlcNAcβ1→4GlcNAcβ1→2)Manα1→3)Manβ1→4GlcNAc. As reported previously (16), the enzyme readily cleaves the linkage in GlcNAcβ1→2Manα1→6GlcNAcβ1→4(GlcNAcβ1→4)Manα1→3)Manβ1→4GlcNAc. Therefore, the oligosaccharide must be reduced beforehand in order to use the substrate specificity of diplococcal β-N-acetylgalactosaminidase for the linkage determination of β-N-acetylglucosaminyl residues in the outer chain moiety.

Fig. 1. A structure showing the possible β-N-acetylglucosamine residues that can be attached to the trimannosyl cores of the complex-type asparagine-linked sugar chains. β-N-Acetylglucosaminyltransferases (abbreviated in this paper as GnT) responsible for the addition of each β-N-acetylglucosamine residue will be called by the number indicated in this figure.
active oligosaccharide with the same mobility as the radioactive peak in Fig. 2A by the α-mannosidase digestion (data not shown). This result showed that the two nonreducing terminal α-mannosyl residues of the Manα1→6(Manα1→3)Manα1→6 group were not substituted by the newly added β-N-acetylglucosamine residue. Therefore, the structure of the radioactive product from Mα2-GN should be that shown in Table II.

When the radioactive peak b in Fig. 2C was incubated with jack bean α-mannosidase, one mannose was removed (Fig. 2E). In order to determine which of the two α-mannosyl residues was removed by enzymatic digestion, the radioactive component in Fig. 2E was reduced with NaBH₄ and then digested with diplococcal β-N-acetylhexosaminidase. As shown in Fig. 2F, two radioactive products were obtained by this treatment. The peak with the same mobility as N-acetylglucosamine contained only ¹⁴C radioactivity, while the peak with a mobility of 4.2 glucose units contained only ³H radioactivity. This result showed that the radioactive component should be a 1:1:N-acetylglucosaminyl derivative. This result is in agreement with the reports of Hartzog and Schachter (11) and of Oppenheimer et al. (17) but is different from the report of Mendicino et al. (18) which indicated that Mα3-GN-Asn can be an acceptor of both GlcNAcT I and GlcNAcT II. Therefore, the activity of GlcNAcT II alone could be determined by using GN-Mα3-GN. When the pentasaccharide was used as an acceptor, a single radioactive product corresponding to peak a in Fig. 2C was detected (data not shown).

Structures of the Radioactive Products from GNα1-Mα1-GN and GNα2-Mα1-GN—Although a single radioactive peak was obtained from GNα2-Mα1-GN when BHK and Py-BHK cell homogenates were used as sources of enzyme, the elution patterns of the two reaction products were slightly different (Fig. 3, A and B, respectively). The mobilities of the products on the Bio-Gel P-4 column indicated that an N-acetylglucosamine residue was added to the hexasaccharide acceptor. All the radioactive N-acetylglucosamine residues should have the β-anomeric configuration because all radioactivities in the peaks were released as N-acetylglucosamine by incubation with jack bean β-N-acetylhexosaminidase (data not shown). When the radioactive peaks in Fig. 3, A and B, were reduced with NaBH₄ and subjected to paper chromatography, both of them were separated into two radioactive components (Fig. 4, A and B). The mobilities of the two radioactive components a and b were the same as authentic GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcαHex and GlcNAcβ1→4(GlcNAcβ1→2)Manα1→6Manα2→6Manα→4GlcNAcαHex, respectively (Fig. 4, A and B). Incubation with diplococcal β-N-acetylhexosaminidase released one radioactive N-acetylglucosamine from component a and two nonradioactive N-acetylglucosamines from component b of the BHK product (Fig. 3, C and D, respectively) and of the Py-BHK product (data not shown). As reported in the previous paper (16), the enzyme converts GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcαHex to GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6(GlNAcβ1→2Manα1→3)Manβ1→4GlcNAcαHex and GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3(GlcNAcβ1→}
by measuring the radioactivities in components fore, the relative activities of GnT V and IV can be determined acceptors: G2. GN2. Ms. GN (Fig. 4C), GN, released as N-acetylglucosamine by incubation with jack bean galactosidase as the enzyme source. The result showed that only [14C]N-acetylglucosamine and [3H]galactitol, which were identified by paper chromatography using butanol:1-ethanol:water (4:1:1 by volume) as a solvent and paper electrophoresis with borate buffer (19). Although the linkage between the N-acetylglucosamine and the galactose of the disaccharide fragment was not confirmed, the structures of the asparagine-linked sugar chains of the surface glycoproteins of BHK and Py-BHK cells (9) and the substrate specificity of the endo-β-galactosidase (26) suggested that the radioactive β-N-acetylglucosamine residues were added at the C-3 position of one of the terminal β-galactosyl residues of the three oligosaccharide acceptors by the action of GnT VI.

Optimal Conditions for the Assay of Each β-N-Acetylglucosaminyltransferase—The results described so far indicated that the concentrations of GnT I, II, IV, V, and VI in BHK cells and in Py-BHK cells can be determined by using appropriate oligosaccharides listed in Table I. Furthermore, important evidence concerning the substrate specificities of GnTs has been revealed by this study. That the N-acetylglucosamine residue cannot be transferred to M2. GN-F. GN indicated that GnT II in BHK and Py-BHK cells cannot use a linear sugar chain, unless a fucose residue inhibits GnT II. That GN-M2. GN cannot be an acceptor of any β-N-acetylglucosaminyltransferases in these cells indicated that GnT IV and GnT V cannot use GlcNAcβ1→2Manα1→3Manβ1→4GlcNAc as their acceptors, respectively. Therefore, the oligosaccharide substrate of these transferases must contain a trimannosyl core.

Optimal conditions for the assay of each enzyme were studied in more detail. All five β-N-acetylglucosaminyltransferases showed rather broad pH optima ranging from pH 6.0 to 7.0. Therefore, pH 7.0 was used for the assay of all enzymes to suppress the action of lysosomal glycosidases as much as possible. GnT V did not need added Mn2+ as reported by Cummings et al. (14), but all other enzymes required this cation. Therefore, this cation was added in a final concentration of 20 mM to all reaction mixtures, except for GN2. M2. GN which was incubated with and without Mn2+ to determine the activity of GnT IV + GnT V only.

Preliminary experiments revealed that the amounts of radioactive N-acetylglucosamine transferred to oligosaccharides after 16-h incubation were the same when using the concentration of UDP-GlcNAc was higher than 2.5 mM. We also found that almost no donor nucleotide remained after 16-h incubation when used in a concentration of 0.5 mM. Therefore, we chose to use UDP-GlcNAc (2.5 mM), so that sufficient amounts of the donor remained when the reaction was over.

The kinetic properties of each β-N-acetylglucosaminyltransferase were studied by using oligosaccharides in Table I as substrates. Although only the data obtained by using GN2. M2. GN and G2. GN2. M2. GN are shown in Fig. 6, the reaction rates were proportional to protein concentration and were

![Fig. 5. Bio-Gel P-4 column chromatography of the radioactive products from G2. GN2. M2. GN, G2. GN2. M2. GN, and G2. GN2. M2. GN and their enzymatic degradation products. The black arrows are the same as in Fig. 2, and the white arrow indicates the elution position of GlcNAcβ1→3Gal. A, C, and E, the radioactive products obtained from G2. GN2. M2. GN, G2. GN2. M2. GN, and G2. GN2. M2. GN, respectively (the black triangles indicate the elution positions of acceptor oligosaccharides); B, D, and F, the radioactive peaks in A, C, and E incubated with diplococcal β-galactosidase (solid line) and then with endo-β-galactosidase (dotted line), respectively.](image-url)
Kinetic data of GnT IV, V, and VI. A and C, effect of incubation time on the reaction rate determined with BHK or Py-BHK cell homogenate (150 μg of protein), respectively. The conditions of incubation are the same as described under "Experimental Procedures" except that the time of incubation was varied as indicated. B and D, effect of enzyme concentration on the reaction rate determined with BHK or Py-BHK cell homogenate, respectively. The conditions of incubation are the same as described under "Experimental Procedures" except that protein concentration was varied as indicated.

Comparison of the Concentrations of Five N-Acetylglucosaminyltransferases in BHK Cells and Py-BHK Cells—The activities of the five β-N-acetylglucosaminyltransferases in the homogenates of BHK and Py-BHK cells were determined using the optimal conditions described above and the results are summarized in Table III. Because peak a in Fig. 2C contains two N-acetyl[U-14C]glucosamine residues, the activity of GnT I was calculated on the basis of the radioactivity in peak b plus half of that in peak a in Fig. 2C, and the activity of GnT II was estimated on the basis of half the radioactivity in peak a. The data indicated that GnT I and GnT II activities are one order higher than other β-N-acetylglucosaminyltransferases in both cell homogenates. As is evident from the Py-BHK/BHK ratio of each enzyme in the two homogenates, both cells have the same concentrations of GnTs I, II, IV, and VI. In contrast, the activity of GnT V is twice that in Py-BHK cells irrespective of the different oligosaccharide acceptors.

The results indicated that the increase in concentration of GnT V may be the direct cause of the structural change in the asparagine-linked sugar chains of BHK cells by malignant transformation. In Fig. 7, a biosynthetic network responsible for the formation of a series of complex-type asparagine-linked sugar chains is shown. Because of the acceleration of the two pathways indicated by the bold arrows, Py-BHK cells synthesize more tri- and tetra-antennary sugar chains with the GlcNAcβ1→6Manα1→ group. Van den Eijnden and Schiphorst (20) recently reported that GnT VI purified from Novikoff tumor cell ascites fluid works most favorably on Galβ1→4GlcNAcβ1→6Man group. If it is a general characteristic of GnT VI, increments of the highly branched complex-type asparagine-linked sugar chains with the Galβ1→4GlcNAcβ1→6Man group may enhance the formation of sugar chains with N-acetyllactosamine repeating structures in their outer chain moiety. This may explain why tri- and tetraantennary complex-type sugar chains with N-acetyllactosamine repeating structures of the membrane bound glycoproteins of Py-BHK cells showed a higher turnover rate than those of BHK cells as reported in the previous paper (9).

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REFERENCES
Supplemental Material to

ENZYMIC BASIS FOR THE STRUCTURAL CHANGES OF ASPARAGINE-LINKED SUGAR CHAINS OF MAMMARY GLANDULOIN OF RATS

The details of 'Experimental Procedures' are presented.

EXPERIMENTAL PROCEDURES

Cell Homogenate—Rat mammary gland cells (106 cells/100 ml) and their polyoma transformant (106 cells/100 ml) were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 ng/ml) and streptomycin (100 μg/ml).

Chemicals and Enzymes—Structures and abbreviations of the oligosaccharides used in this study are summarized in Table 1. α-N- and β-N-acetyl-D-glucosamine were isolated from the urine of hamsters injected with [α-3H]glucosamine (485 pmol/kg) and [β-3H]glucosamine (525 pmol/kg), respectively. The α-N- and β-N-acetyl-D-glucosamine were obtained by acid hydrolysis of the urine of the hamsters injected with [α-3H]glucosamine (485 pmol/kg) and [β-3H]glucosamine (525 pmol/kg), respectively. The α-N- and β-N-acetyl-D-glucosamine were isolated from the urine of hamsters injected with [α-3H]glucosamine (485 pmol/kg) and [β-3H]glucosamine (525 pmol/kg), respectively. The α-N- and β-N-acetyl-D-glucosamine were isolated from the urine of hamsters injected with [α-3H]glucosamine (485 pmol/kg) and [β-3H]glucosamine (525 pmol/kg), respectively. The α-N- and β-N-acetyl-D-glucosamine were isolated from the urine of hamsters injected with [α-3H]glucosamine (485 pmol/kg) and [β-3H]glucosamine (525 pmol/kg), respectively. The α-N- and β-N-acetyl-D-glucosamine were isolated from the urine of hamsters injected with [α-3H]glucosamine (485 pmol/kg) and [β-3H]glucosamine (525 pmol/kg), respectively.
### Table II. Proposed structures of oligosaccharides synthesized from each acceptor by cell homogenates and assignment of α-N-acetylglucosaminyltransferases responsible for their formation. X represents Man3→GlcNAc.

<table>
<thead>
<tr>
<th>Acceptors</th>
<th>Products</th>
<th>GTn</th>
<th>Acceptors</th>
<th>Products</th>
<th>GTn</th>
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<tbody>
<tr>
<td>N₃-GlcNAc</td>
<td>(Man)⁺→N₄-GlcNAc</td>
<td>I</td>
<td>N₄-GlcNAc</td>
<td>(Man)⁺→N₄-GlcNAc</td>
<td>V</td>
</tr>
<tr>
<td>N₃-GlcNAc</td>
<td>N₄-GlcNAc</td>
<td>I</td>
<td>N₄-GlcNAc</td>
<td>N₄-GlcNAc</td>
<td>R</td>
</tr>
<tr>
<td>N₃-GlcNAc</td>
<td>(Man)⁺→N₄-GlcNAc</td>
<td>I</td>
<td>N₄-GlcNAc</td>
<td>(Man)⁺→N₄-GlcNAc</td>
<td>R</td>
</tr>
<tr>
<td>N₄-GlcNAc</td>
<td>(Man)⁺→N₄-GlcNAc</td>
<td>I</td>
<td>N₄-GlcNAc</td>
<td>(Man)⁺→N₄-GlcNAc</td>
<td>R</td>
</tr>
<tr>
<td>N₄-GlcNAc</td>
<td>(Man)⁺→N₄-GlcNAc</td>
<td>I</td>
<td>N₄-GlcNAc</td>
<td>(Man)⁺→N₄-GlcNAc</td>
<td>R</td>
</tr>
</tbody>
</table>

### Table III. Relative activities of α-N-acetylglucosaminyltransferases I to VI in the cell homogenates of RhB and Py-RhB.

<table>
<thead>
<tr>
<th>Acceptors</th>
<th>GTn</th>
<th>RhB Cells</th>
<th>Py-RhB Cells</th>
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<tr>
<td>N₃-GlcNAc</td>
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<td>4.68±0.82</td>
<td>6.75±4.45</td>
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<td>39.8±39.8</td>
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<td>41.1±41.1</td>
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<td>N₃-GlcNAc</td>
<td>I</td>
<td>30.5±30.5</td>
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<tr>
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<td>IV</td>
<td>1.33±1.33</td>
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<td>1.16±1.16</td>
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<td>2.57±2.57</td>
<td>2.60±2.60</td>
</tr>
</tbody>
</table>

The numbers in the parentheses are the mean values of two experiments.

A. The calculation was based on the value of radioactivity in the peak g plus half of the radioactivity in the peak h in Fig. 2C.

B. The calculation was based on half of the radioactivity in the peak g in Fig. 2C. Since the radioactive peak g was formed by the concerted action of GT I and GT II, the number is not so reliable as others.

C. Incubation was performed without N₃-GlcNAc.