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Aglycone structure influences α-fucosyltransferase III activity using *N*-acetyllactosamine glycoside acceptors

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We showed previously that Chinese hamster ovary cells took up and utilized a variety of *N*-acetylglucosaminides as primers of oligosaccharide biosynthesis (Ding *et al.*, 1999, *J. Carbohydr. Chem.*, 18 : 471–475). In this study, a library of *N*-acetylglucosaminides was enzymatically galactosylated *in vitro* to yield type 2 chain *N*-acetyllactosaminides bearing a variety of aglycones. Those disaccharides are potential acceptors for fucosyltransferases. As an extension of the previous study, we tested the type 2 chain disaccharyl glycosides (Galβ1,4-GlcNAcβ-R) for their aglycone-dependent acceptor specificity for α-L-fucosyltransferase III (Fuc-TIII). The enzyme activity significantly depended on the aglycone structures, suggesting that, in addition to the polar groups on the sugar moiety, the hydrophobic aglycone can substantially contribute to recognition in this reaction.

Keywords: **substrate specificity, fucosyltransferase, glycosides, *N*-acetyllactosamine, *N*-acetylglucosaminides**

Abbreviations: **Fuc-T, fucosyltransferase; MU, 4-methylumbelliferyl; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; Lac, lactose; LacNAc, Galβ(1–4)GlcNAc; GlcNAc, *N*-acetyl-D-glucosamine.**

Introduction

Artificial glycosides have been used to study cellular glycosylation by mimicking endogenous acceptors, such as glycosaminoglycans [1–4], glycoproteins [5,6] and glycolipids [7]. These glycosides, also called primers, resemble biosynthetic intermediates and serve as substrates for oligosaccharide assembly, thereby diverting the synthesis from endogenous glycoconjugates. The efficiency of glycoside utilization by cells depends on many factors, such as their membrane permeability, stability, affinity for glycosyltransferases, cellular toxicity, and intracellular entry into selected organelles. Among these possible determinants, major factors contributing to the fate of those primers in cells are the structure of the aglycone and its linkage to the sugar moiety. Flitz *et al.* reported that the priming activity strongly depended on aglycone structure [8]. Recently, we developed an efficient strategy for synthesizing a library of *β*-*N*-acetylglucosaminides (GlcNAc*β*-R) with various aglycones [9]. In the previous study, the library of GlcNAc*β*-R had been shown to serve as primers in CHO cells to carry poly-*N*-acetyllactosamines with or without terminal sialic acid, where the amounts of product and the structure of oligosaccharide in the products were strongly influenced by the aglycone structure.

In view of GlcNAc-related cell surface carbohydrate molecules in glycoproteins and glycolipids, α(1,3/4)-fucosylated *N*-acetyllactosamines are of great interest, since Lewis-type and sialylated Lewis-type carbohydrate related structures have been found to play a role in various biological phenomena including selectin-dependent cell-adhesion [10,11]. Thus, molecules containing the sialyl Lewis x (sLe^x) or sialyl Lewis a (sLe^a) determinants can be efficiently employed for inhibition of cellular interactions and, consequently, are potential tools for therapeutic treatment in inflammation and in cancer metastasis. The biosynthesis of fucose-containing glycoconjugates relies on critical fucosyltransferases (Fuc-T). Each member of the fucosyltransferase

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family has different preferences for specific linkages and types of oligosaccharide acceptors [12,13]. Among them, Fuc-TIII has a relatively broad substrate specificity and produces all Lewis-type determinants [14]. Once the primers penetrate the cells and reach the Golgi, the main factors affecting production of elongated sugar chains on the primers are the kinetic parameters of the particular glycosyltransferase for a substrate. For the biosynthesis of Lewis–related oligosaccharides, the key and indispensable step is fucosylation of pre-formed type 1 or type 2 oligosaccharides. In this study, we have attempted to clarify the relationship between the aglycone structure and acceptor activity toward Fuc-TIII using a library of type 2 β(1,4)-galactosylated β-GlcNAc-R compounds.

Materials and methods

Materials

Bovine milk β1-4-galactosyltransferase, *p*-nitrophenyl β-*N*-acetylglucosamine (GlcNAcβ-pNP), silica gel (15–40 μm), lacto-*N*-tetraose (LNT, Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-neo-tetraose (LNnT, Galβ1-4GlcNAcβ1-3Galβ1-4Glc), and 4-methylumbelliferyl lactose (Lacβ-MU, **2**) were purchased from Sigma, St. Louis, MO. Labeling of LNT and LNnT with 2-aminobenzamide and the purification were carried out as described previously [4]. UDP-Galactose was from Fluka, Milwaukee, WI. GDP-Fucose was purchased from Calbiochem, San Diego, CA. GDP-[^{3}H]fucose (5.2 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. Gal(β1,3)GlcNAc-*O*-$(CH_2)_8COOCH_3$ (**3**) [25], Gal(β1,4)GlcNAc-*O*-$(CH_2)_8COOCH_3$ (**4**) [27], and a library of β-GlcNAc-R [9] were prepared as previously described. Fucose metabolism-deficient CHO cells stably transfected with Fuc-TIII plus the polyoma T antigen, CHO-FTIII, was kindly provided by J.B. Lowe, University of Michigan Medical School, MI. C18 silica gels (Bonded C18 reversed phase silica gel and UNIBOUND C18 SPICE Tube) were from Analtech, Inc., Newark, DE.

Preparation of CHO-FTIII extracts as a Fuc-TIII source

The CHO-FTIII cells from confluent monolayers in 162 cm^2 flask were washed with PBS and then collected after trypsinization. The cells were lysed with 700 μl of 1% Triton X-100 in PBS containing proteinase inhibitors, then mixed briefly. The solution was kept on ice for 30 min, then sonicated five times for 10 sec. The cell lysate was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and stored at −80°C before use. The protein concentration was estimated to be 10 μg/μl by MicroBCA, Pierce, using bovine serum albumin as standard.

Galactosylation of GlcNAcβ-R by bovine milk β1-4-galactosyltransferase

To prepare acceptors for Fuc-TIII from a library of GlcNAcβ-R, enzymatic galactosylation of *N*-acetylglucosaminides employing bovine milk β1-4-galactosyltransferase was carried out. The standard reactions were performed in a final volume of 50 μl containing 125 mM MES (pH 6.7), 1 mM GlcNAcβ-R, 6 mU enzyme, 3 mM UDP-Gal, and 10 mM $MnCl_2$ at 37°C for 24 h. The reaction products were collected by a C18 spin column as described below. The combined eluates were dried using SpeedVac concentrator. The products were further purified by a silica gel spin column, as described previously [4]. The reaction was monitored by thin-layer chromatography (TLC) using a solvent of chloroform–methanol–water (60 : 35 : 6). The spots on TLC plates were visualized under UV lamp or with sulfuric acid.

Fucosyltransferase Assays

Fucosyltransferase assays were performed in duplicate in a volume of 20 μl and contained 50 mM sodium cacodylate (pH 6.4), 5 mM ATP, 20 mM $MnCl_2$, 10 mM L-fucose, 0.1% Triton X-100, 3 μM GDP-[^{3}H]Fuc (0.1 μCi/10 μl), 3 mM GDP-Fuc, and 10 μg of cell extract protein. Acceptor glycosides were added to a final concentration of 1 mM. Control assays without acceptors were performed using the same conditions. Reactions were incubated at 37°C for 1 h. Assays were terminated by addition of 200 μl of 20 mM $NaHCO_3$ containing 5 mM EDTA. After heating at 100°C for 5 min and centrifugation at 14,000 × *g* for 5 min, the supernatant was applied to a C18 spin column, which was preactivated with methanol and then equilibrated with 20 mM $NaHCO_3$ containing 5 mM EDTA. After centrifugation at 2,000 × *g* for 1 min, the spin column was washed three times with 400 μl of 20 mM $NaHCO_3$ containing 5 mM EDTA. The products were then eluted with three 400 μl portions of 50% methanol. The incorporation of [^{3}H]Fuc was determined by liquid scintillation counting of the eluate.

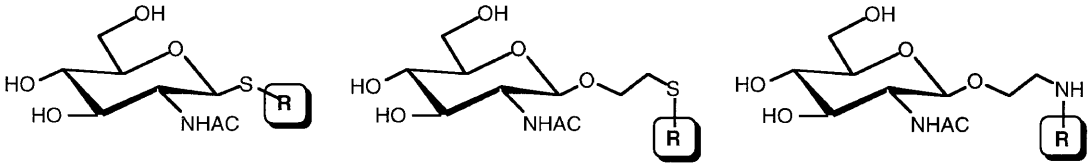
Kinetic studies

Apparent kinetic parameters for acceptor substrate were determined under the above standard assay conditions. The acceptor concentration was varied up to 50 mM (five to seven different concentrations). The radiolabeled products were isolated as above using a C18 spin column and eluates were counted for radioactivity.

Results

A library of GlcNAcβ-R

As shown in Figure 1, three classes of intermediates, which differ in the types of linkage or spacer arm lengths were used to synthesize a GlcNAcβ-R library with different hydrophobic aglycone structures. The synthesis of the structures and their



Alkylthio GlcNAc **Alkylthioethyl GlcNAc** **Acylaminoethyl GlcNAc**

7 R = A 11 R = E
8 R = B 12 R = F
9 R = C 13 R = G
10 R = D

14 R = A
15 R = B

16 R = H 21 R = M
17 R = I 22 R = N
18 R = J 23 R = P
19 R = K 24 R = Q
20 R = L

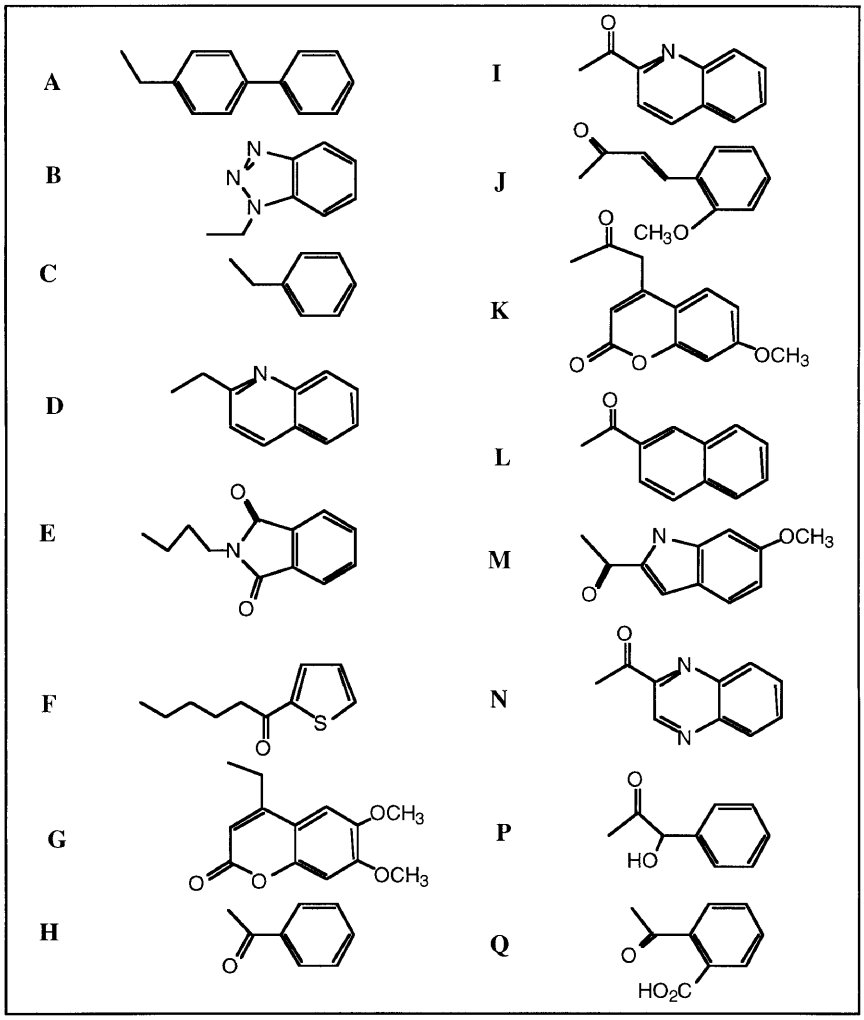


Figure 1. Structures of GlcNAcβ-R. Three intermediates having different glycosidic linkages or spacer arms were conjugated to various hydrophobic aglycones as indicated. The identification and confirmation of the structures was reported previously [9].

confirmation by NMR and mass spectrometry were previously reported [9].

Preparation of galactosylated disaccharides from the GlcNAcβ-R library

Type 2 substrates, Galβ(1–4)GlcNAcβ-R, were prepared from the GlcNAcβ-R library by enzymatic galactosylation. In addition to the GlcNAcβ-R library, GlcNAcβ-pNP and GlcNAcβ-benzyl were also converted to their corresponding LacNAc glycosides. After the β1,4-galactosyltransferase reaction, there was no detectable starting material on thin-layer chromatograms developed with chloroform–methanol–water (60 : 35 : 6). The reaction mixture was then applied to a spin column packed with a C18 resin to isolate the glycosides. The purified β(1–4) galactosylated glucosaminides were reconstituted in dimethylsulfoxide at 50 mM and used in the following assays.

Type 1 preference of Fuc-TIII over type 2 oligosaccharides

Since the Fuc-TIII has been reported to show different substrate specificities in the literature [15–19], we first confirmed the ability of Fuc-TIII to utilize both type 1 (Galβ(1–3)GlcNAc) and type 2 (Galβ(1–4)GlcNAc) chains, leading to the Le^a and Le^x structure, respectively. Besides using type 1 and type 2 disaccharides, having the aglycone methoxycarbonyloctyl ($-(CH_2)_8-COOCH_3$), type 1 lacto-*N*-tetraose (LNT) and type 2 lacto-*N*-neotetraose (LNnT) were labeled with the fluorescence dye 2-aminobenzamide (2AB) for this study. The 2AB labeling enables purification of the product by reversed-phase chromatography after the transferase reaction, and it also eliminates the possibility that fucose is transferred to the reducing-end glucose residue. This had been reported previously on this compound as an acceptor in a Fuc-TIII-catalyzed reaction [19]. The methoxycarbonyloctyl glycosides were tested at 1 mM, while the 2AB-labeled tetrasaccharides were used at 0.1 mM. In each experimental condition, the activities were compared to that of LacNAcβ-pNP as shown in Table 1. All the glycosides tested served as substrates for Fuc-TIII, although Fuc-TIII showed a significant preference for type 1 glycosides over type 2 glycosides using both tetrasaccharides and disaccharides. The activities of both type 2 glycosides were 6–8% of the corresponding type 1 glycosides. In spite of its type 2 structure, LacNAcβ-pNP showed 25–34% as much activity as type 1 methoxycarbonyloctyl glycosides. To our knowledge, very few studies have examined the importance of aglycone specificity for Fuc-TIII reactions. In general, reducing oligosaccharides and some methoxycarbonyloctyl glycosides have been used for these assays, leading to the conclusion that a β-anomeric configuration of GlcNAc residue in the substrate is preferred by Fuc-TIII [19]. The enzyme has been known to act on both glycoproteins and glycolipids, suggesting a broad aglycone specificity. But the aglycone preference in an acceptor for Fuc-TIII is not clear. The results from the data here suggested that the aglycone could affect significantly the substrate recognition. We therefore screened the library of galactosylated GlcNAcβ-R compounds with different aglycone structures.

Aglycone specificity of fucosyltransferase III

Each β(1–4)galactosylated β-*N*-acetylglucosaminide was tested as a substrate for Fuc-TIII. Since CHO cells have been reported to lack any Fuc-T activities [20], CHO cells were stably transfected with Fuc-TIII (CHO-FTIII) plus the polyoma T antigen, and lysates were assayed for enzyme activity. The Fuc-TIII reaction was linear up to 4 h under the conditions used (data not shown). The activity of Fuc-TIII-catalyzed fucose transfer to the acceptors having a variety of aglycones is shown in Figure 2. Clearly, Fuc-TIII was active with several type 2 glycosides with different aglycones, but the activity significantly depended on the structure of the aglycone. Among the glycosides tested, glycosides **8**, **15**, **24** possessed high activity, comparable to that of LacNAcβ-pNP, whereas the activity of the other glycosides was widely variable.

Kinetics of Fuc-TIII for a panel of type 2 glycosides

The apparent K_m and V_{max} values of Fuc-TIII for a panel of acceptors are listed in Table 2. Only the K_m for the type 1 methoxycarbonyloctyl glycoside was in sub mM range among those measured, while the K_m value for the other type 2

Table 1. Acceptor specificity of Fuc-TIII toward type 1 and type 2 acceptors

Substrates	*Relative activity (%)* [a]	*Relative activity (%)* [b]
LacNAcβ-pNP, **1**	100	ND
Galβ(1–3)GlcNAcβ(1,3)Galβ(1,4)Glc-2AB, **5**	298	ND
Galβ(1–4)GlcNAcβ(1,3)Galβ(1,4)Glc-2AB, **6**	23	ND
LacNAcβ-pNP, **1**	ND	100
Galβ(1–3)GlcNAcβ-*O*-$(CH_2)_8$-$COOCH_3$, **3**	ND	391
Galβ(1–4)GlcNAcβ-*O*-$(CH_2)_8$-$COOCH_3$, **4**	ND	22

ND: Not determined.
[a] Relative rates, measured at 100 μM acceptor concentration, are expressed with respect to 1.
[b] Relative rates, measured at 1 mM acceptor concentration, are expressed with respect to 1.

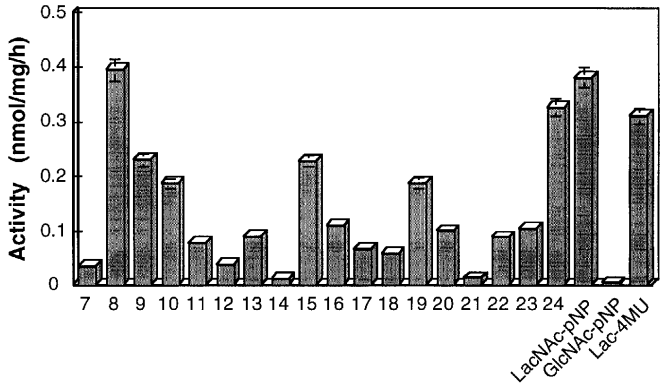


Figure 2. Fuc-TIII activity with a library of LacNAc disaccharide substrate acceptors. Fucosyltransferase assays were performed three times in duplicate as described in the Methods. Acceptor glycosides were added to a final concentration of 1 mM. Control assays without acceptors were performed using the same conditions. Reactions were incubated at 37°C for 1 h. Error bars indicate standard deviations, which were typical of all the analyses.

glycosides ranged from 5 to 50 mM comparable to the substrate preference as shown in Figure 2. On the contrary, the V_{max} values are in a very similar range for all the glycosides. Some of the type 2 glycosides used in this study had a higher V_{max} than that of type 1 methoxycarbonyloctyl glycoside. These data reveal that the aglycone of the acceptor significantly affects the substrate specificity of Fuc-TIII.

Discussion

We previously reported an efficient strategy for preparing a library of GlcNAcβ-R in which different glycosidic linkages and variable spacer arms were linked to a panel of hydrophobic aglycones. These glycosides were readily taken up by CHO cells and used for oligosaccharide synthesis [9]. This versatile synthetic strategy can be used to prepare a diverse variety of glycosides, which then can be applied to screen a large number of candidate molecules for a desired property. Incubating CHO cells with glycosides in the presence of [^{3}H]galactose generated a range of glycoside products from a single LacNAc to molecules with several LacNAc repeats with or without sialic acids [9]. Also the amount of the products recovered from the culture medium was dependent on the aglycone structure. Interestingly, the recovery of [^{3}H]galactose-labeled materials from the incubation with GlcNAcβ-*S*-benzyl was 80% greater than that of GlcNAcβ-*O*-benzyl, suggesting that the β-hexosaminidase-resistant thio-glycosidic linkage has significant advantages as a primer. The differences in the types of products made are probably due to substrate specificity or kinetics of the glycoside for a series of sequential or alternative glycosyltransferases.

In this report, we studied the contribution of the aglycone to the specificity of Fuc-TIII-catalyzed fucosylation of a library of type 2 LacNAc molecules. In glycoproteins. β-*N*-acetylglucosamine residues are usually followed by β-galactose except in the chitobiose core. Among LacNAc-based oligosaccharides, Lewis-type-related structures are of great interest, since various Fuc-Ts can be involved in their biosynthesis [11,21,22]. The addition of fucose to make sLe^a or sLe^x determinants is considered to be a final step of this biosynthetic pathway. Substrate specificities of rat liver Galβ(1–4)GlcNAc α2,6-sialyltransferase (α2,6ST) and Galβ(1–3/4)GlcNAc α2,3-sialyltransferase (α2,3ST) have been studied by Wlasichuk, et al [23]. Neither sialyltransferase can use any α(1–3/4)-fucosylated type 1 or type 2 glycoside substrate. The apparent K_m values for the transfer of sialic acid to a type 2 methoxycarbonyloctyl glycoside using α2,6ST and α2,3ST have been reported to be 0.4 mM and 0.9 mM, respectively. In this study, the apparent K_m values for the Fuc transfer to the type 1 and type 2 methoxycarbonyloctyl glycoside by the Fuc-TIII were estimated to be 70 µM and 15 mM, respectively. If ST and Fuc-TIII are colocalized in the same Golgi compartment, these kinetic data would argue that sialylation of type 2 chains would occur preferentially over fucosylation. While with type 1 chains, the kinetic data suggest that fucosylation would occur first, preventing sialylation and the production of sLe^a structures. Information on kinetics and localization of the enzymes within the Golgi could be very important for designing primers that generate a desired oligosaccharide structure.

In our previous study, prerequisite galactosylation of GlcNAcβ-R was investigated in CHO cells using [^{3}H]Gal-metabolic labeling. Glycoside **9**, GlcNAcβ-*S*-benzyl, was the most effective primer for synthesizing a LacNAc structure,

Table 2. Acceptor specificity of Fuc-TIII toward type 1 and type 2 acceptors

Substrates	K_m *(mM)*	V_{max} *(pmol/mg/min)*
Lacβ-MU, **2**	5.5	24
Galβ(1–3)GlcNAcβ-*O*-$(CH_2)_8$-$COOCH_3$, **3**	0.072	20
Galβ(1–4)GlcNAcβ-*O*-$(CH_2)_8$-$COOCH_3$, **4**	15	6.1
8	16	40
10	22	31
12	50	17
20	38	20

while the benzotriazole compounds, *i.e.* **8** and **15**, were moderate for LacNAc production. Thus, taken together with results presented here, the glycosides (**8**, **9**, **15** and **24**) should be suitable for the production of Lewis-related structures on GlcNAcβ-R in cells with Fuc-TIII.

Data in Table 2 suggest that the linkage of the disaccharide, *i.e.* type 1 or type 2, makes a greater difference in substrate binding than in catalysis. Aglycone structure significantly affected both substrate binding and catalysis. Hindsgaul *et al.* proposed previously [24] hydrogen bonding interactions between acceptor substrate and the enzyme during the glycosyltransferase-catalyzed reactions. In addition, critical key polar groups in the disaccharide acceptors have been described for α(1–3)fucosyltransferase as hydroxyls at C-6 of Gal and C-3 or C-4 of GlcNAc [19,25]. Besides those key polar hydroxyl groups on the disaccharide, the aglycone portion also could provide hydrogen bonding through nitrogen or oxygen. In compounds, **8** and **15**, benzotriazole contains three nitrogen atoms capable of hydrogen bondings, suggesting an additional enhancing effect of polar atoms in the hydrophobic aglycones.

Effective disaccharide primers have been reported for the synthesis of oligosaccharide chains that mimic glycoproteins [26] and glycolipids [7]. Using those disaccharide primers may prove to be more potent and be specific for the synthesis of the desired oligosaccharides, diverting the assembly of sugar chains from a particular class of endogenous glycoconjugates. The aglycone preference studied here provides direct insights into utilization of the disaccharides as *in vivo* primers. The compounds capable of mimicking endogenous glycans may prove to be advantageous modifiers of biological responses by inhibiting a specific glycosyltransferase or controlling the cellular/molecular interactions involving carbohydrates.

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