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# Distinct Orders of GalNAc Incorporation into a Peptide with Consecutive Threonines

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**Mucin O-glycosylation is initiated by a transfer of *N*-acetyl-D-galactosamine (GalNAc) to Ser and Thr residues in polypeptides with a family of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (pp-GalNAc-Ts). In this paper, four human pp-GalNAc-Ts (pp-GalNAc-T1, T2, T3, and T4) were tested for their preferential orders of GalNAc incorporation into FITC-PTTTPITTTTK, a portion of the tandem repeat of human MUC2. The products were separated by reverse-phase HPLC and characterized by MALDI-TOF MS and peptide sequencing. pp-GalNAc-T1 showed preference for acceptor sites, but the order of the incorporation into these sites seemed to be random. In contrast, the GalNAc incorporation by pp-GalNAc-T2, T3, or T4 was not only site-specific but also according to the specific orders. Furthermore, pp-GalNAc-T2, T3, or T4 had distinct maximum numbers of GalNAc incorporations into this peptide.** 

***Key Words:*** **mucin; MUC2; UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase (pp-GalNAc-T); O-glycosylation; biosynthesis; cellular recognition; lectin domain.**

O-Glycosylation of polypeptides is initiated by the attachment of *N*-acetyl-D-galactosamine (GalNAc) to Thr and Ser residues. This reaction is known to be catalyzed by a family of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (pp-GalNAc-Ts). To date, 9 human pp-GalNAc-Ts (pp-GalNAc-T1, T2, T3, T4, T5, T6, T7, T8, and T9) have been reported (1–8). pp-GalNAc-Ts of other vertebrates and nematodes have also been isolated and identified (9–17). The expression of these enzymes does not seem to be mutually exclusive in a single cell. Thus, there may be a competition and coordination between different pp-GalNAc-Ts in a cell. In fact, some pp-GalNAc-Ts such as human pp-GalNAc-T4 and T7 and rat pp-galNAc-T9 have been shown to have a preference toward peptide partially modified by GalNAc incorporations by other pp-GalNAc-Ts (3, 6, 16). We previously observed that there were only two "pathways" in the incorporation of GalNAc residue into a peptide, FITC-PTTTPITTTTK, representing a portion of the tandem repeat of MUC2, when microsome fractions of human colon carcinoma LS174T cells, which contain at least 4 distinct pp-GalNAc-Ts, were used as the source of pp-GalNAc-Ts (18; Takeuchi *et al.*, submitted; Kato *et al.*, in press). The result was remarkable showing that these two pathways were chosen out of 13,700 possibilities by the mixture of at least 4 pp-GalNAc-Ts expressed in these cells (18; Takeuchi *et al.*, submitted; Kato *et al.*, in press). Therefore, it was hypothesized that each threonine residue within a peptide is glycosylated by a distinct pp-GalNAc-T according to a predetermined scenario. To prove or disprove this hypothesis, all biosynthetic intermediates should be isolated and examined as acceptor substrates for each pp-GalNAc-T. As the initial step, we show in the present report that the pathways of GalNAc incorporations into FITC-PTTTPITTTTK by pp-GalNAc-T1, T2, T3, and T4 are distinct from each other.

Abbreviations used: FITC, fluorescein isothiocyanate; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; PTH, phenylthiohydantoin; pp-GalNAc-T, UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferases; TFA, trifluoroacetic acid; UDP-GalNAc, UDP-*N*-acetylgalactosaminide.

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## MATERIALS AND METHODS

*Synthesis of acceptor substrates.* Oligopeptide used as the acceptor substrate, PTTTPITTTTK, was synthesized with a Model 9020 peptide synthesizer (Milligen, Burlington, MA) with a lysine residue attached at the C-terminus. This peptide was labeled with fluorescein isothiocyanate at its N-terminal amino acid at pH 7.5 adjusted

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with 100 mM Hepes buffer. Under this condition, $\epsilon$-amino group is not supposed to be modified.

*GalNAc incorporation into peptides.* Incubations for the determination of the pathway of GalNAc-incorporation were performed as previously described (Kato *et al.*, in press) with slight modifications. Briefly, the enzyme reaction mixture consisted of 50 mM Hepes buffer (pH 7.5), 5 mM 2-mercaptoethanol, 20 mM $MnCl_2$, 2 mM PMSF, protease inhibitors (1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, 0.5 $\mu$g/ml pepstatin A), 1 mM UDP-GalNAc (Sigma, St. Louis, MO), 5 $\mu$M synthetic oligopeptide, and pp-GalNAc-Ts. pp-GalNAc-T1 (0.236 $\mu$g), T2 (1.10 $\mu$g), T3 (1.37 $\mu$g), or T4 (2.80 $\mu$g) was applied with various incubation periods. The amounts of the enzymes were chosen to adjust the activity at 0.2 unit. One unit was defined as the amount that can catalyze 1 nmol of GalNAc attachment to 5 $\mu$M PTTTPITTTTK peptide in 30 min in the 50 $\mu$l of reaction mixture. Reactions were performed at 37°C for up to 72 h and terminated by adding 1/5 volume of 0.5 M EDTA (pH 8.0).

*Characterization of glycosylated peptides.* The glycosylated peptides in the reaction mixtures were separated by reverse-phase HPLC (JASCO, Tokyo, Japan). A Cosmosil ($5C_{18}$-AR, 10 × 250 mm, Nacalai Tesque, Kyoto, Japan) was used. The column was eluted with a linear gradient of 0 to 50% of solvent B (0.05% TFA/2-propanol) in solvent A (0.05% TFA/$H_2O$) at a flow rate of 2 ml/min for 30 min. Eluates were monitored by fluorescence intensity at 520 nm (ex: 492 nm).

In MALDI-TOF MS analysis, concentrated glycopeptides corresponding to the peaks after HPLC were applied on a stainless steel plate and mixed with a 10 mg/ml solution of $\alpha$-cyano-4-hydroxycinnamic acid dissolved in 0.1% TFA–50% ethanol in water. All mass spectra were obtained on a Voyager Elite in the linear mode with the delayed extraction setting. Acceleration voltage was set at 20,000, grid voltage at 93.5, and guide wire voltage at 0.05%. Laser intensity was chosen so that optimum resolution could be obtained. A major signal peak was obtained in each case. Minor peaks in the mass chart at mass numbers corresponded to peptides with smaller numbers of GalNAc residues did not seem to result from contaminating (glyco-)peptides for the following reasons: (a) The relative intensity of these associated peaks depended on the laser intensity. (b) GalNAc-peptides with different numbers of GalNAc usually eluted at different positions on reverse-phase HPLC. Relative molecular mass numbers were determined using angiotensin I as a standard.

Pulsed liquid Edman degradation amino acid sequencing of glycopeptides was performed on the Applied Biosystems 492 Procise protein sequencing system (Perkin Elmer, Norwalk, CT). With this system, a phenylthiohydantoin (PTH) derivative of GalNAc-attached Thr was identified as a pair of peaks eluted near the positions of PTH-Ser and PTH-Thr. Amino acid sequencing of a fully glycosylated peptide (PT*T*T*PLK: * represents a GalNAc residue) confirmed these eluting positions (data not shown) (19).

## RESULTS

### *Unique Combination of Glycosylation Products after Incubation of FITC-PTTTPITTTTK with pp-GalNAc-Ts*

We have reported previously that all Thr residues in the peptide PTTTPITTTTK containing three and four consecutive Thr residues were glycosylated by microsome fractions of the colon carcinoma cell line LS174T through two distinct pathways (18; Kato *et al.*, in press). The cell line was shown to express at least four pp-GalNAc-Ts, T1, T2, T3, and T4. Hypothetically, each of these two pathways could be generated by one of these pp-GalNAc-Ts. Alternatively, each step of these pathways might be driven by a distinct pp-GalNAc-T. To understand the regulation of mucin glycosylation, all glycosylation products obtained by incubations of pp-GalNAc-T1, T2, T3, or T4 with FITC-PTTTPITTTTK were separated and characterized.

As shown in Fig. 1, the elution profiles of the glycosylation products with each pp-GalNAc-T were unique. The number of GalNAc residues attached to the peptide was determined by MALDI-TOF MS analyses and

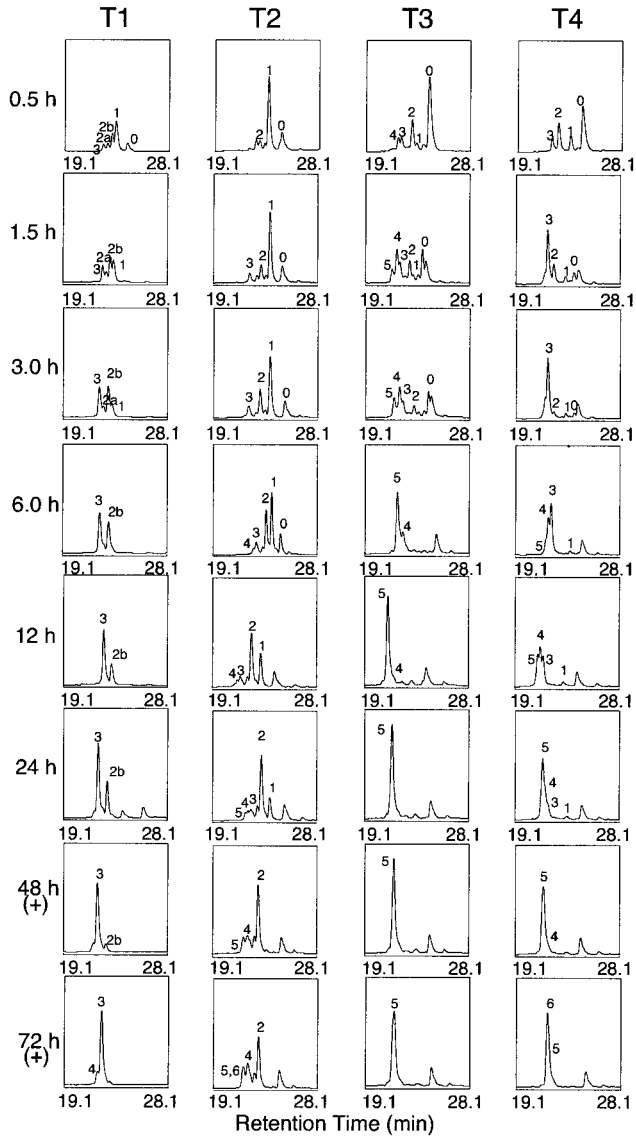


**FIG. 1.** Elution profiles of glycosylated FITC-PTTTPITTTTK peptides by pp-GalNAc-T1, T2, T3, and T4. FITC-PTTTPITTTTK peptides were incubated with each pp-GalNAc-T (T1, T2, T3, or T4) up to 72 h. Each pp-GalNAc-T could not transfer the maximum number of GalNAc to FITC-PTTTPITTTTK peptide. A symbol (+) indicates addition of same amount of UDP-GalNAc and each pp-GalNAc-T in reaction mixtures. The number above each peak indicates the number of GalNAc with FITC-PTTTPITTTTK peptides. The number of attached GalNAc was confirmed by the MALDI-TOF MS analyses.

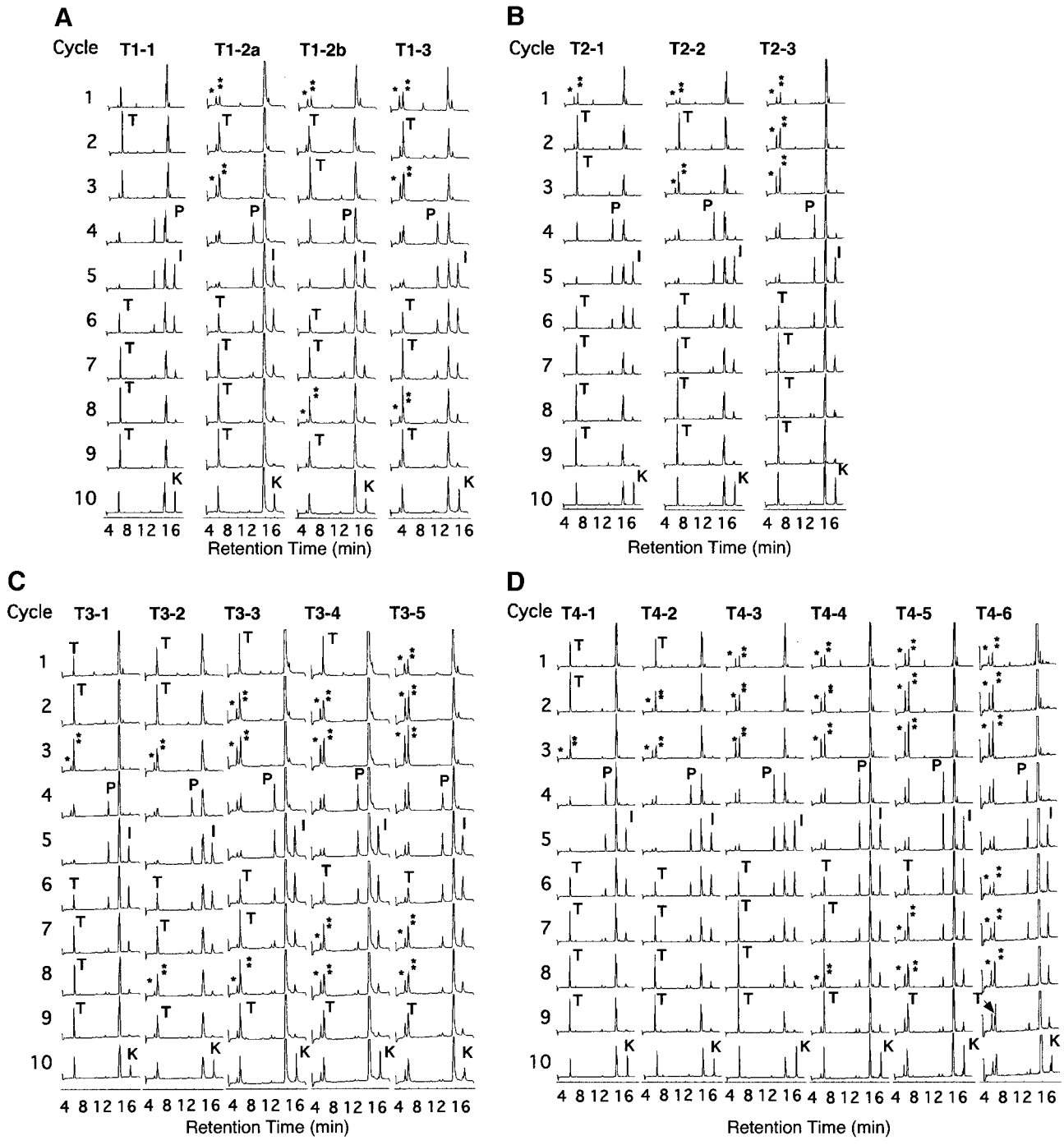


**FIG. 2.** Profiles of amino acid sequencing chromatograms of the FITC-PTTTPITTTTK peptide and its derivatives when pp-GalNAc-T1 (A), T2 (B), T3 (C), and T4 (D) were used. Glycopeptides generated by pp-GalNAc-T1 (A), T2 (B), T3 (C), and T4 (D) were further analyzed to determine the positions where the GalNAc were attached. Maximum numbers of the GalNAc that attached to FITC-PTTTPITTTTK peptide were three, six, five, and six respectively. The peptide with one GalNAc residue generated by pp-GalNAc-T1 seemed to be a mixture of two products that have GalNAc at Thr-2 or Thr-4. The peptide with four, five, or six GalNAc generated by pp-GalNAc-T2 could not be determined by amino acid sequencing because of their minute quantity. Asterisks indicate putative peaks derived from α-GalNAc-threonine. Other amino acids are indicated by one-letter abbreviations.

indicated in each panel. The major final product after prolonged incubation with pp-GalNAc-T1 was the peptide with 3 GalNAc residues (Fig. 1). At least two species of glycopeptides with two GalNAc residues, 2a and 2b, were observed by the reverse-phase HPLC separation when products with pp-GalNAc-T1 were analyzed. pp-GalNAc-T2, T3, or T4 also seemed to generate single major final products. The numbers of

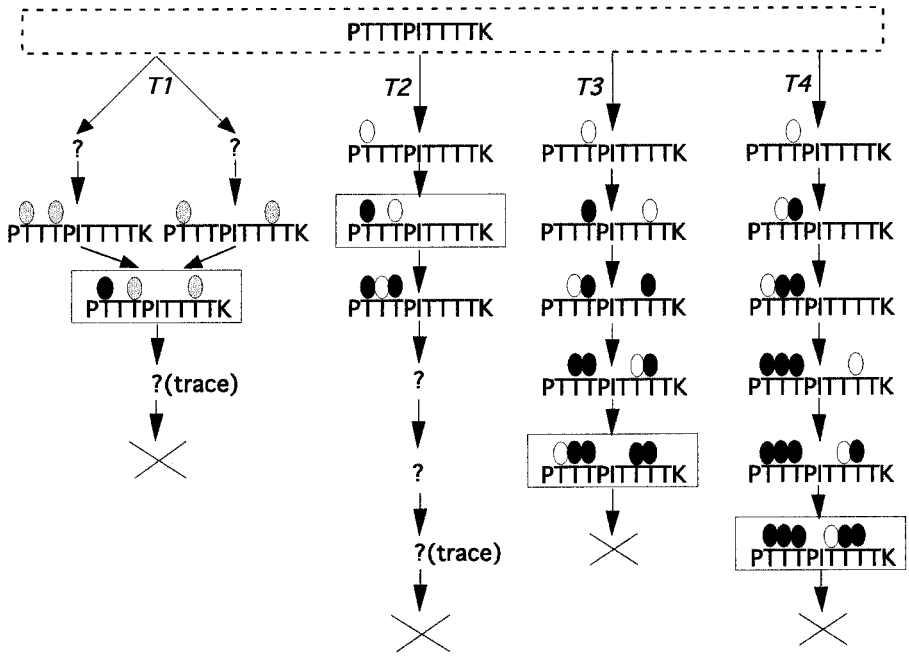


**FIG. 3.** Schematic representation of the pathways of GalNAc incorporation into FITC-PTTTPITTTTK peptide incubated with UDP-GalNAc and recombinant pp-GalNAc-Ts. Closed circles and open circles indicate GalNAc residue attached to the peptide. Open circles represent GalNAc residues likely to be transferred in the reaction immediately prior to the formation of the structure. In the case of pp-GalNAc-T1 the order of GalNAc incorporations is not clear and the residues are shown by shaded circles. Boxed intermediates were the major glycopeptides generated by pp-GalNAc-Ts after 72 h reaction time period. Initial glycosylation products using pp-GalNAc-T1 seemed to be a mixture of at least two distinct glycopeptides. However, they could not be separated using reverse-phase HPLC. From the incubation mixtures with pp-GalNAc-T1, two distinct products with two GalNAc residues, FITC-PT*TT*PITTTTK and FITC-PT*TTPITTT*TK, were identified. The glycosylation product with the largest number of GalNAc residues generated by pp-GalNAc-T1 was FITC-PT*TT*PITTT*TK. pp-GalNAc-T2, T3, and T3 generated unique glycosylation products that can be attributed to single pathways. Both pp-GalNAc-T3 and T4 generated the products PTTT*PITTTTK and PT*T*T*PITT*T*TK, although the other intermediate products generated were different for each enzyme.

GalNAc residues in these major final products for pp-GalNAc-T2, T3, and T4 were 2, 5, and 6 respectively. Maximum numbers of GalNAc residues transferred to this peptide after 72 h incubation period with pp-GalNAc-T2, T3, and T4 were 6, 5, and 6 respectively (Fig. 1).

### *Preferential Pathways of O-GalNAc Attachment to FITC-PTTTPITTTTK*

Each peak in Fig. 1 was pooled and its glycosylation sites were determined by a peptide sequencer. As shown in Fig. 2, a PTH-Thr conjugated with GalNAc residues results in two peaks of diastereomers (asterisks). A PTH-Pro was not detected in cycle 1 because FITC-Pro should be released by TFA prior to the PITC modification in the standard Edman degradation. The sequence of glycopeptide with one GalNAc residue generated by pp-GalNAc-T1 was not clearly defined probably because it was a mixture of at least two species (Fig. 2A). Thus, apparently there were multiple pathways in GalNAc incorporation by pp-GalNAc-T1 though the final product was FITC-PT*TT*PITTT*TK (Fig. 2A). On the contrary, each glycosylation product with a given number of GalNAc residue after incubation with pp-GalNAc-T2, T3, or T4 consisted of only a single glycoform (Figs. 2B–2D). Furthermore, the order of appearance of glycoforms was unique to each pp-GalNAc-T. In other words, it appears that each pp-GalNAc-T2, T3, and T4 exhibits a preferential pathway of GalNAc incorporation into the FITC-PTTTPITTTTK peptide sequence. The predicted glycosylation pathways for each pp-GalNAc-T are shown in Fig. 3.

## DISCUSSION

O-Glycosylation of mucins has recently been shown not to be a random process but strictly governed by specific pp-GalNAc-T isozymes apparently working in a coordinated fashion. One remarkable finding that supports this hypothesis is our recent result that there are only two pathways in the glycosylation of a portion of the MUC2 tandem repeat PTTTPITTTTK, containing consecutive Thr residues, when microsome fractions of LS174T cells are used as an enzyme source (Kato *et al.*, in press). At least four pp-GalNAc-Ts were found to be expressed in this cell line and the functions

of these and potentially other isoforms must be reconciled with such an apparent strict regulation. In the present paper, specificity of pp-GalNAc-T1, T2, T3, and T4 toward FITC-PTTTPITTTTK was studied. The results indicated that pp-GalNAc-T1 transferred GalNAc residues to the preferential positions through a few pathways, whereas pp-GalNAc-T2, T3, and T4 transferred GalNAc residues to the peptide through distinct pathways.

Although substrate specificity and kinetic properties of pp-GalNAc-Ts toward various peptides were previously investigated, the present study is the first to determine the order of GalNAc incorporations by pp-GalNAc-Ts into a stretch of consecutive Thr residues. Interestingly, the acceptor site specificity of GalNAc-T1, which did not have a preferential order, seemed to be distinct from pp-GalNAc-T2, T3, or T4.

It has been predicted that pp-GalNAc-Ts, as far as those used in the present work, contain ricin-like lectin domains. Mouse pp-GalNAc-T1 did not seem to require this domain for its activity with peptide substrates (12), but the lectin-like domain was shown to be essential for the GalNAc-glycopeptide substrate specificity of human pp-GalNAc-T4 while the same enzyme activity with peptide substrates was independent of the lectin domain (20). It is likely that human pp-GalNAc-T2 and T3 also have functional lectin domains that recognize GalNAc residues incorporated into peptides and that these participate in directing glycosylation of clustered acceptor sites, although evidence of this is not available at present. Attachment of GalNAc residues to the peptide may change the conformation of this peptide, so that the next GalNAc might have a greater accessibility. In this context it is clear that the three isoforms showing GalNAc-glycopeptide specificity appear to exhibit different acceptor specificities with respect to peptide sequence and glycoform of glycopeptide (6, 16, 21). Furthermore, it was found that rat pp-GalNAc-T7 and T9 had preferential order in GalNAc attachment toward glycosylated human MUC5AC peptides (16, 21). These pp-GalNAc-Ts might also use ricin-like domain for their specific interactions with glycosylated peptides. pp-GalNAc-T1, on the other hand, seems to recognize a unique peptide sequence, regardless of the prior GalNAc attachment.

We previously used a peptide, FITC-PTTTPLK, mimicking the tandem repeat portion of MUC2 to examine how GalNAc was incorporated into three consecutive Thr residues by pp-GalNAc-T1, T2, T3, and T4 (19; Takeuchi *et al.*, submitted). These four enzymes showed different orders of GalNAc incorporation into this peptide. The position and the order of the GalNAc attachment to the three consecutive Thr motif with surrounding Pro residues (PTTTP) were almost the same as the orders shown in the present work with FITC-PTTTPITTTTK.

Recently, we also reported that FITC-PTTTPITTTTK peptide was glycosylated up to 7 potential O-glycosylation sites (18; Kato *et al.*, in press) when microsome fractions of LS174T cells were used as the enzyme source. LS174T cells contained at least pp-GalNAc-T1, T2, T3, and T4. However, pp-GalNAc-T1, T2, T3, and T4 alone (present paper) or mixtures (unpublished) were not able to form peptide with seven GalNAc residues. Other pp-GalNAc-Ts (5–8) should play important roles in the incorporation of the seventh GalNAc residue. Involvement of enzymes other than pp-GalNAc-T1, T2, T3, or T4 in the GalNAc incorporation by the LS174T cell microsome fractions was also suggested by the fact that the pathways were distinct from any pathway with one of these isozymes.

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