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Glycopeptide-preferring Polypeptide GalNAc Transferase 10 (ppGalNAc T10), Involved in Mucin-type *O*-Glycosylation, Has a Unique GalNAc-*O*-Ser/Thr-binding Site in Its Catalytic Domain Not Found in ppGalNAc T1 or T2*[S]

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Mucin-type *O*-glycosylation is initiated by a large family of UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferases (ppGalNAc Ts) that transfer GalNAc from UDP-GalNAc to the Ser and Thr residues of polypeptide acceptors. Some members of the family prefer previously glycosylated peptides (ppGalNAc T7 and T10), whereas others are inhibited by neighboring glycosylation (ppGalNAc T1 and T2). Characterizing their peptide and glycopeptide substrate specificity is critical for understanding the biological role and significance of each isoform. Utilizing a series of random peptide and glycopeptide substrates, we have obtained the peptide and glycopeptide specificities of ppGalNAc T10 for comparison with ppGalNAc T1 and T2. For the glycopeptide substrates, ppGalNAc T10 exhibited a single large preference for Ser/Thr-*O*-GalNAc at the +1 (C-terminal) position relative to the Ser or Thr acceptor site. ppGalNAc T1 and T2 revealed no significant enhancements suggesting Ser/Thr-*O*-GalNAc was inhibitory at most positions for these isoforms. Against random peptide substrates, ppGalNAc T10 revealed no significant hydrophobic or hydrophilic residue enhancements, in contrast to what has been reported previously for ppGalNAc T1 and T2. Our results reveal that these transferases have unique peptide and glycopeptide preferences demonstrating their substrate diversity and their likely roles ranging from initiating transferases to filling-in transferases.

Mucin-type *O*-glycosylation is a common post-translational modification of secreted and membrane-associated proteins. *O*-Glycan biosynthesis is initiated by the transfer of GalNAc from UDP-GalNAc to the hydroxyl groups of serine or threonine residues in a polypeptide, catalyzed by a family of polypeptide *N*-α-acetylgalactosaminyltransferases (ppGalNAc Ts).[5] To date, 16 mammalian members have been reported in the literature (1–16) with a total of at least 20 members currently present in the human genome data base. Multiple members of the ppGalNAc T family have also been identified in *Drosophila* (9, 10, 14), *Caenorhabditis elegans* (3, 8), and single and multicellular organisms (17–20). Several members show close sequence orthologues across species suggesting that the ppGalNAc Ts are responsible for biologically significant functions that have been conserved during evolution. For example, in *Drosophila* four isoforms have close sequence orthologues to the mammalian transferases. Of the two that have been recently compared, nearly identical peptide substrate specificities have been observed between the fly and mammals, suggesting common but presently unknown functions preserved across these diverse species (21).

Recently, several ppGalNAc T isoforms have been shown to be important for normal development or cellular processes. For example, inactive mutations in the fly PGANT35A (the T11 orthologue in mammals) are lethal because of the disruption of the tracheal tube structures (9, 10, 22), whereas mutations in PGANT3 alter epithelial cell adhesion in the *Drosophila* wing blade resulting in wing blistering (23). In humans, mutations in ppGalNAc T3 are associated with familial tumoral calcinosis, the result of the abnormal processing and secretion of the phosphaturic factor FGF23 (24, 25). Human ppGalNAc T14 has been suggested to modulate apoptotic signaling in tumor cells by its glycosylation of the proapoptotic receptors DLR4 and DLR5 (26), and very recently the specific *O*-glycosylation of the

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[5] The abbreviations used are: ppGalNAc T, UDP-α-GalNAc:polypeptide *N*-α-acetylgalactosaminyltransferase; UDP-GalNAz, uridine diphosphate *N*-α-azidoacetylgalactosamine; GP-I, GP-II, P-VI, and P-VII random (glyco)-peptide substrates; ELISA, enzyme-linked immunosorbent assay; PTH, phenylthiohydantoin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

TGFB-II receptor (ActR-II) by the GalNTL1 has been shown to modulate its signaling in development (16).

Historically, the major targets of the ppGalNAc Ts have been thought to be heavily *O*-glycosylated mucin domains of membrane and secreted glycoproteins. Such domains typically contain 15–30% Ser or Thr, which are highly (>50%) substituted by GalNAc. One question in the field is as follows. How is this high degree of peptide core glycosylation achieved and is it related to the large number of ppGalNAc isoforms, some of which may even have specific mucin domain preferences? Interestingly, some members of the ppGalNAc T family are known to prefer substrates that have been previously modified with *O*-linked GalNAc on nearby Ser/Thr residues, hence having so-called glycopeptide or filling-in activities, *i.e.* ppGalNAc T7 and T10 (8, 27–29). Others simply possess altered preferences against glycopeptide substrates, *i.e.* ppGalNAc T2 and T4 (30–33), or may be inhibited by neighboring glycosylation, *i.e.* ppGalNAc T1 and T2 (29, 34, 35). These latter transferases have been called early or initiating transferases, preferring nonglycosylated over-glycosylated substrates. Presently, little is known about which factors dictate the different peptide/glycopeptide specificities among the ppGalNAc Ts.

The ppGalNAc Ts consist of an N-terminal catalytic domain tethered by a short linker to a C-terminal ricin-like lectin domain containing three recognizable carbohydrate-binding sites (36). Because ppGalNAc T7 and T10 prefer to transfer GalNAc to glycopeptide acceptors, it has been widely assumed that their C-terminal lectin domains would play significant roles in this activity, as has been demonstrated for other family members (27, 28, 32). Recently, Kubota *et al.* (37) solved the crystal structure of ppGalNAc T10 in complex with Ser-GalNAc specifically bound to its lectin domain. In this work (37), the authors further demonstrated that a T10 lectin domain mutant indeed had altered specificity against GalNAc-containing glycopeptide substrates when the acceptor Ser/Thr site was distal from the pre-existing glycopeptide GalNAc site. However, it was also observed that the lectin mutant still possessed relatively unaltered glycopeptide activity when the acceptor Ser/Thr site was directly N-terminal of a pre-existing glycopeptide GalNAc site. Kubota *et al.* (37) therefore concluded that for ppGalNAc T10, both its lectin and indeed its catalytic domain must contain distinct peptide GalNAc recognition sites. In support of this, Raman *et al.* (33) have shown that the complete removal of the ppGalNAc T10 lectin domain only slightly alters its specificity against distal glycopeptide substrates while showing no difference in its ability to glycosylate residues directly N-terminal of an existing site of glycosylation. Thus, it seems that the catalytic domain of ppGalNAc T10 may have specific requirements for a peptide *O*-linked GalNAc in at least the +1 position (toward the C terminus) of residues being glycosylated. As no systematic determination of the glycopeptide binding properties of the ppGalNAc Ts catalytic domain has been performed, it is unknown whether additional GalNAc peptide-binding sites exist in T10 or, for that matter, any of the other ppGalNAc Ts.

We have recently reported the use of oriented random peptide substrates, GAGA$(X)_n$T$(X)_n$AGAGK (where *X* indicates randomized amino acid positions and $n = 3$ and 5) for determining the peptide substrate specificities of mammalian ppGalNAc T1, T2, and their fly orthologues (21, 38). In the present work, we extend this approach to the determination of the catalytic domain glycopeptide (Ser/Thr-*O*-GalNAc) substrate preferences for ppGalNAc T1, T2, and T10 employing two $n = 4$ oriented random glycopeptide libraries (Table 1). The first library has Ser-*O*-GalNAc and other amino acids in the random "*X*" regions (GP-I), and the second library has a central Thr-*O*-GalNAc with nonglycosylated Ser and other amino acid residues in the random *X* regions (GP-II). From these two complementary random glycopeptides, and the use of the azido-labeled UDP-GalNAc analogue UDP-GalNAz, we have obtained the first systematic determination of the glycopeptide preferences of the catalytic domains of the ppGalNAc T1, T2, and T10. We observe that ppGalNAc T10 has a large and pronounced preference for Ser/Thr-*O*-GalNAc only at the +1 position from the acceptor site, whereas T1 and T2 have significantly reduced and variable preferences for Ser/Thr-*O*-GalNAc. We have also obtained preferences of ppGalNAc T10 against the $n = 5$ random peptide substrates for comparison with ppGalNAc T1 and T2 (21). Interestingly, ppGalNAc T10 displays few significant enhancements and specifically lacks the Pro residue enhancements observed for ppGalNAc T1 and T2. These findings further demonstrate the vast substrate diversity of the catalytic domains of the ppGalNAc T family of transferases.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—Soluble recombinant bovine ppGalNAc T1 was a gift of Ake Elhammer (Kalamazoo, MI). Soluble bovine ppGalNAc T1, human ppGalNAc T2, and human ppGalNAc T10 have been characterized previously (33, 38). UDP-GalNAz, alkyne-biotin reagent (supplemental Fig. S4*C*), and tris-(benzyltriazolylmethyl)amine were synthesized as described (39, 40). The optimal T2 peptide substrate (38), GAGAPGPTPGPAGAGK, was synthesized by Biotechnology Resources, Center for Biologics Evaluation and Research (National Institutes of Health). Random peptide substrates, P-VI and P-VII, GAGA*XXXXX*(T)*XXXXX*AGAGK, where *X* indicates Gly, Ala, Pro, Val, Leu, Tyr, Glu, Gln, Arg, and His (P-VI) or *X* indicates Gly, Ala, Pro, Ile, Met, Phe, Asp, Asn, Arg, and Lys (P-VII), were custom-synthesized by QCB Inc. (Hopkinton, MA). Random glycopeptides (GP-I), GAGA*XXXX*-(T)*XXXX*AGAG, where *X* = Gly, Ala, Pro, Val, Ile, Phe, Tyr, Glu, Asp, Asn, Arg, Lys, His, and Ser-*O*-GalNAc and (GP-II), GAGA*XXXX*(Thr-*O*-GalNAc)*XXXX*AGAG, where *X* = Gly, Ala, Pro, Val, Ile, Phe, Tyr, Glu, Asp, Asn, Arg, Lys, His, and Ser were custom-synthesized by Bachem Americas (King of Prussia, PA). All peptide substrates were adjusted to pH ~7.5 with dilute NaOH and/or HCl and lyophilized multiple times from water prior to use. UDP-[^{3}H]GalNAc and UDP-[^{14}C]GalNAc were purchased from ARC Inc. (St. Louis, MO). GalNAc-binding immobilized lectins SJA (*Sophoro japonica*), SBA (*Glycine max*), and HPA (*Helix pomatia*) were obtained from EY Laboratories (San Mateo, CA); immobilized VVA (*Vicia villosa*) was acquired from Vector Laboratories (Burlingame, CA). Sephadex G-10 and Dowex (1X8) ion-exchange resin (100–200 mesh) were supplied from GE Healthcare and Organics, respec-

tively. Liquid scintillation counting was performed on a Beckman model LS5801.

ppGalNAc T10 Glycosylation of Random Peptide (P-VI and P-VII)—The glycosylation of random peptides P-VI and P-VII was performed as described previously (21, 38). Reactions consisted of 2–4 mM UDP-GalNAc (^{3}H- or ^{14}C-labeled, 0.01 μCi/μl), ~0.24 mg/ml ppGalNAc T10, and 22–45 mM random peptide substrates P-VI or P-VII, with final volumes of 53–200 μl. Based on radiolabel incorporation, less than 2% of either peptide was glycosylated after overnight incubation at 37 °C. Isolation of the glycosylated peptide was performed via mixed bed lectin column chromatography as described previously in Gerken *et al.* (21). GalNAc-eluted fractions were pooled based on ^{3}H/^{14}C content, lyophilized, and chromatographed on Sephadex G-10. Glycopeptide fractions were pooled and sequenced as described (21). (See comments in the supplemental material on the presence of *N*-deacetylated UDP-[^{3}H]GalNAc label.)

UDP-GalNAz Glycosylation of GP-I by ppGalNAc T1, T2, and T10—Glycosylation reactions consisted of 0.1 mM EDTA, 100 mM HEPES, pH 7.8, 10 mM $MnCl_2$, protease inhibitor mixtures P8340 and P8849 (1:100 dilution), 0.1–1.5 mM UDP-GalNAz, trace UDP-[^{3}H]GalNAc (0.01 μCi/μl), 0.071–0.25 mg/ml transferases, and 18–24 mM random glycopeptide (GP-I). Reactions were incubated overnight at 37 °C and stopped by the addition of a 2× volume of 250 mM EDTA. UDP, nonhydrolyzed UDP-[^{3}H]GalNAc, and GalNAz were removed by passage over Dowex 1X8 anion-exchange columns. The eluant was lyophilized and chromatographed on a Sephadex G-10 column (0.7 × 113 cm) in 50 mM acetic acid buffer, pH 4.5, with NH_4OH. Fractions were monitored for absorbance at 220 and 280 nm and incorporation of radioactivity by scintillation counting. Based on radiolabel incorporation of [^{3}H]GalNAc, rough estimates of GP-I glycosylation by GalNAz ranged from ~2 to 4%, assuming equal transfer rates. Tracer radiolabeled glycopeptide fractions were pooled based on absorbance and ^{3}H content and lyophilized.

Biotin Labeling of GalNAz Glycopeptide by Copper(I)-catalyzed 1,4-Cycloaddition (41, 42)—Briefly, lyophilized glycopeptide G-10 fractions (0.045–0.11 μmol) were dissolved in 75 μl of 10 mM potassium phosphate buffer, pH 8. Alkyne-biotin (supplemental Fig. S4*C*) (1.3 mM), tris(2-carboxyethyl)phosphine hydrochloride (2 mM), and tris-(benzyltriazolylmethyl)-amine (1 mM) were then added to the peptide solution (final concentrations in 100 μl given in parentheses). After the mixture was vortexed for 60 s, the solution was made 2 mM $CuSO_4$ and incubated overnight at 37 °C. The reaction products were chromatographed on a Sephadex G-10 column in 50 mM ammonium bicarbonate buffer, pH 6.5, to reduce hydrolysis (see supplemental Methods and Results). Fractions were monitored for absorbance at 220 and 280 nm and for the ^{3}H radiolabel (Fig. 2*A*). Glycopeptide fractions were pooled as described above and lyophilized. The structure of the biotinylated GalNAz-GP-I product is given in supplemental Fig. S4*D*.

Isolation of Biotinylated GalNAz GP-I Product on Immobilized Monomeric Avidin—Immobilized monomeric avidin (Pierce) (1-ml gel-bed volume) was prepared for use following the supplier's recommendations. Following equilibration with phosphate-buffered saline (0.15 M NaCl, pH 7.0), the G-10 isolated biotinylated GP-I was applied to the column and allowed to incubate at room temperature for 30 min. The column was washed with phosphate-buffered saline, and bound biotinylated random glycopeptide was eluted with 2-mM D-biotin in phosphate-buffered saline. The biotin eluant was lyophilized and passed over Sephadex G-10 in 50 mM acetic acid buffer, pH 4.5; fractions were monitored for absorbance at 220 and 280 nm (see Fig. 2*B*). Biotinylated glycopeptide was detected by a standard ELISA approach (43) using a 96-well HB plate (Fisher). Nonspecific binding was blocked with 3% bovine serum albumin, and the biotinylated glycopeptide was detected after incubation with streptavidin-alkaline phosphatase (Pierce) and *p*-nitrophenyl phosphate (Aldrich). Absorbance at 405 nm was monitored on a VERSAMAX Microplate Reader (Sunnyvale, CA). Fractions containing the biotinylated product were pooled and lyophilized for Edman sequencing.

Glycosylation of Random Glycopeptide GP-II by ppGalNAc T1, T2, and T10—The glycosylation of random glycopeptide GP-II was performed for ppGalNAc T10 initially as described previously for P-VI and P-VII using 2 mM ^{3}H-labeled UDP-GalNAc (0.01 μCi/μl) and ~10 mM random glycopeptide substrate GP-II. Because of low ^{3}H incorporation under the above conditions, glycosylations with ppGalNAc T1 and T2 were performed in the absence of unlabeled UDP-GalNAc using 2.5–5 μl of ^{3}H-labeled UDP-GalNAc (1 μCi/μl) giving 0.125–0.250 μM UDP-GalNAc. Enzyme concentrations consisted of 0.36 mg/ml ppGalNAc T10 and ~0.14 mg/ml ppGalNAc T1 or T2. After separation on a Sephadex G-10 column, fractions were pooled based on radioactivity and lyophilized, and portions were Edman sequenced. Random glycopeptide GP-II was ~1.8% glycosylated by ppGalNAc T10 and less than ~0.01% glycosylated by ppGalNAc T1 and T2 based on radiolabel incorporation. [^{3}H]GalNAc incorporation at each random position was determined by scintillation counting of the collected Edman sequencer PTH product (see below).

Edman Amino Acid Sequencing—Pulsed liquid phase Edman amino acid sequencing was performed on an Applied Biosystems Procise 494 protein sequencer (Applied Biosystems, Foster City, CA) at a C18 PTH column temperature at 55 °C. Data analysis was performed based on methods developed earlier (38, 44). Automatic integration errors were corrected by manual integration via cutting and weighing of the peaks. For the analysis of ^{3}H content, the post-PTH conversion step at each cycle was re-directed to a fraction collector and submitted to scintillation counting. At least three independent experiments were performed for ppGalNAc T10 with P-VI and P-VII, and the data were averaged. Two independent experiments were performed for GP-I for each transferase, and the data were averaged accordingly. For GP-II, at least two different experiments were performed for each transferase.

NMR and Mass Spectrometry Methods—Proton NMR spectra (see Fig. S3) of the optimal T2 peptide before and after glycosylation by UDP-GalNAz were obtained at the Cleveland Center for Structural Biology using the 900-MHz Bruker Ultrastabilized™ spectrometer (Billerica, MA) in D_2O. The ^{1}H

chemical shifts were referenced taking the HDO resonance as 4.7 ppm. The MALDI-TOF experiments (see Fig. S4) were performed on SCIEX prTOF™ 2000 mass spectrometer (PerkinElmer Life Sciences) with 2,5-dihydroxybenzoic acid utilized as the matrix.

RESULTS

Several members of the ppGalNAc transferase family, *i.e.* ppGalNAc T7 and T10 (4, 8, 29), possess so-called glycopeptide activity, preferring previously glycosylated peptides, although others appear to be inhibited by neighboring glycosylation, *i.e.* ppGalNAc T1 and T2 (29, 34, 35). Quantifying the role of peptide sequence and neighboring glycosylation is critical to understanding the biological significance of the different ppGalNAc T isoforms. In this study, both the peptide and glycopeptide substrate specificities of ppGalNAc T10 have been determined utilizing a series of random peptide and glycopeptide substrates for comparison with ppGalNAc T1 and T2.

TABLE 1
ppGalNAc transferase random substrates utilized in this work
PVI, PVII, GP-I, and GP-II random (glyco)peptide substrates.

Peptide	Sequence	No. of unique sequences
	GAGA*XXXXX*T*XXXXX*AGAGK	
P-VI	X = G, A, P, V, L, Y, E, Q, R, H	10×10^9
P-VII	X = G, A, P, I, M, F, D, N, R, K	10×10^9
	GAGA*XXXX*T*XXXX*AGAG	
GP-I	X = G, A, P, V, I, F, Y, E, D, N, R, K, H, and Ser-*O*-α-GalNAc	1.47×10^9
	GAGA*XXXX*(Thr-*O*-α-GalNAc)*XXXX*AGAG	
GP-II	X = G, A, P, V, I, F, Y, E, D, N, R, K, H, S	1.47×10^9

Determination of ppGalNAc T10 Peptide Substrate Preferences—Random peptide substrates P-VI and P-VII (Table 1) were used to determine the peptide preferences for ppGalNAc T10 as described previously for ppGalNAc T1 and T2 (21, 38). As ppGalNAc T10 has comparatively reduced activity against nonglycosylated substrates, the enzyme and substrate concentrations were increased ~5–10-fold from our previous studies with ppGalNAc T1 and T2 (21, 38). After isolation of the glycopeptide product by mixed bed lectin chromatography (supplemental Fig. S1) and subsequent Edman sequencing, the amino acid enhancement values were obtained as displayed in Fig. 1. In these plots, values greater than 1 indicate an elevated preference for a given residue by the transferase, whereas values less than 1 indicate a decreased preference. Strikingly, ppGalNAc T10 displays no highly significant preferences, having most of its enhancement values close to 1. Interestingly, the large 2–5-fold enhancements observed for Pro at the −1, +1, and +3 positions seen in ppGalNAc T1 and T2[6] (21, 38) are not observed for ppGalNAc T10 (see supplemental Fig. S2 for comparison). Instead, T10 only shows modest Pro enhancements (~1.5 or less) at the −1, +1, and +2 positions. Ala is also modestly increased at the +2 position, whereas slight enhancements for Gly and Arg are observed at random positions N-ter-

[6] These Pro enhancements are also observed for ppGalNAc T5 and T12 (T. Gerken, unpublished data).

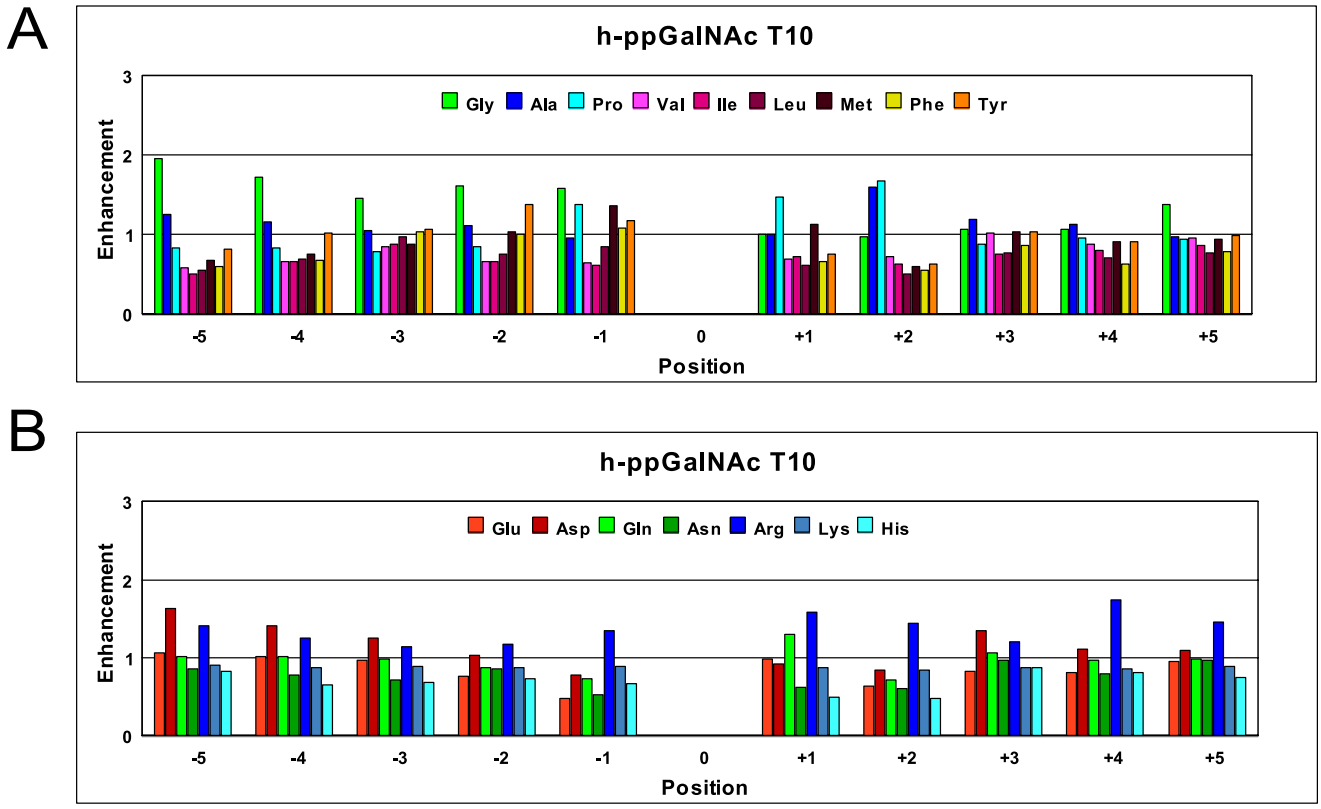


FIGURE 1. **Amino acid residue enhancement factors for ppGalNAc T10 obtained from random peptide substrates P-VI and P-VII (Table 1).** *A,* enhancement factors for the hydrophobic amino acid residues Gly, Ala, Pro, Val, Ile, Leu, Met, Phe, and Tyr *versus* the site of glycosylation (position 0). *B,* enhancement factors for the hydrophilic amino acid residues Glu, Asp, Gln, Asn, Arg, Lys, and His *versus* the site of glycosylation (position 0). The values represent the average of three or more determinations as described under "Experimental Procedures."

minal and C-terminal, respectively, of the central Thr glycosylation site. These findings suggest that specificity for ppGalNAc T10 is largely independent of the nature of the nonglycosylated peptide residues neighboring the acceptor site in contrast to ppGalNAc T1 and T2.[6]

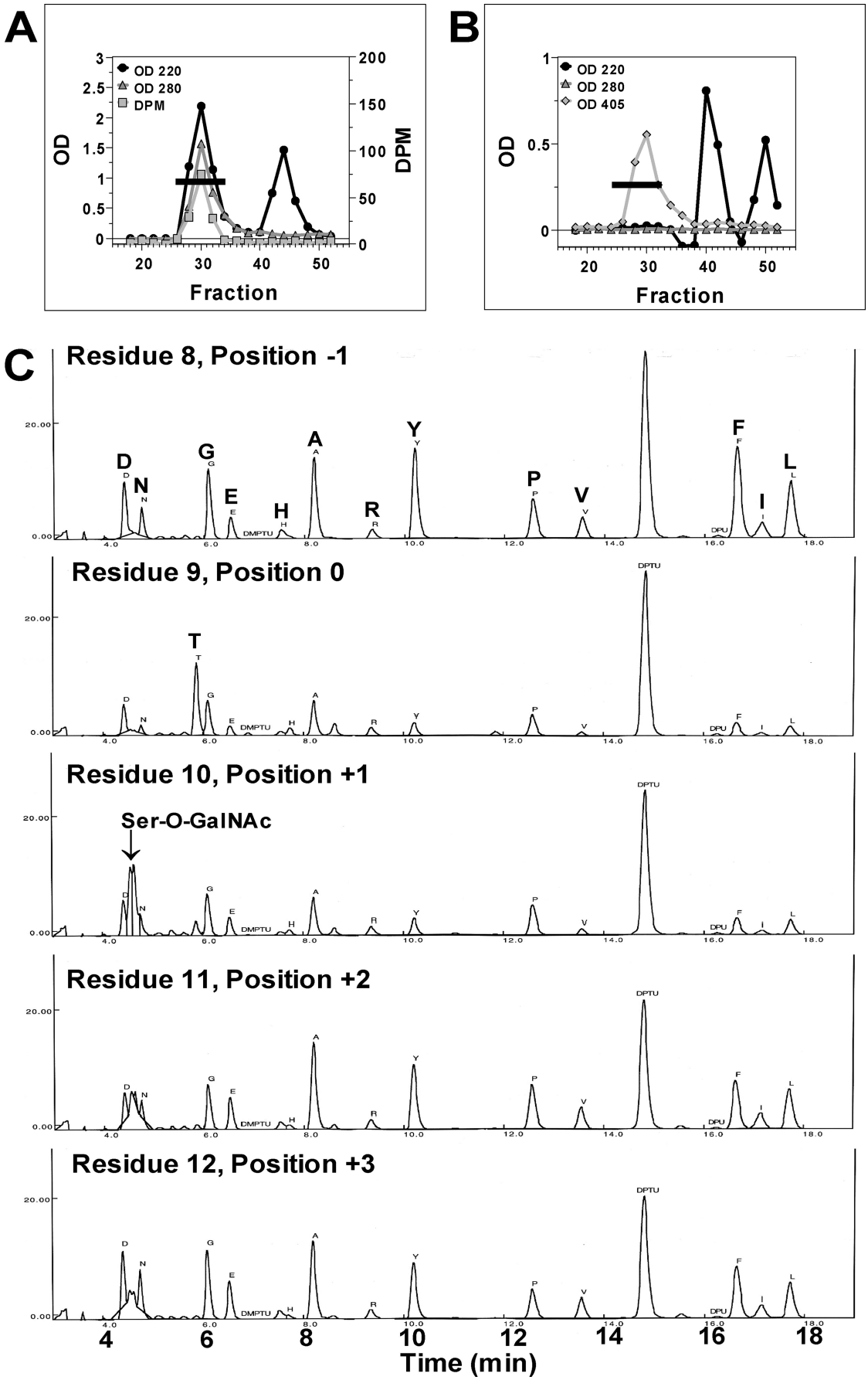
ppGalNAc T1, T2, and T10 Studies with Random Glycopeptide GP-I—To address the role of neighboring GalNAc glycosylation, a random peptide substrate GP-I was designed, where Ser-*O*-GalNAc residues were included in the randomized amino acid *X* regions (Table 1). Rather than synthesizing two or more GP-I substrates with different and overlapping random residues (21, 38), only a single GP-I substrate was synthesized containing 14 unique randomized residues, including Ser-*O*-GalNAc although lacking Thr, Ser, Trp, Cys, Met, Lys, and Gln (Table 1). Lectin column chromatography is unsuitable for isolating the ppGalNAc T GP-I product. Therefore, the azido-labeled UDP-GalNAc analogue, UDP-GalNAz, was used to introduce a chemically reactive species for ppGalNAc T product isolation, taking advantage of the fact that the ppGalNAc Ts can utilize UDP-GalNAz (29, 45). Multiple approaches for conjugating biotin labels to azido sugars have been described, including the phenyl phosphine Staudinger ligation (45–47) and the alkyne cycloaddition reaction (48). The major drawbacks with the Staudinger ligation is that the phenyl phosphine biotin reagent has limited solubility in water; it is susceptible to air oxidation, and the reaction is not highly efficient (45–47). Nevertheless, a number of initial attempts with this approach were made that proved unsuccessful (data not shown). Therefore, the copper(I)-catalyzed cycloaddition approach was implemented, where the alkyne-biotin reagent was found to be highly soluble and the reaction significantly more efficient (41, 49). As discussed in the supplemental Methods and Results, the peptide GalNAz-biotin cycloaddition product was found to be partially unstable to acidic conditions. Therefore, appropriate buffers were used for its isolation (see supplemental Methods and Results).

Isolation and Edman Sequencing of GalNAz-biotinylated GP-I—The Sephadex G-10 chromatography of the ppGalNAc T10 GP-I-GalNAz-biotin cycloaddition product before and after its isolation on monoavidin is shown in Fig. 2*A* and *B*, respectively. The biotinylated GP-I fraction was pooled from the final G-10 column run as indicated in Fig. 2*B* and submitted to Edman sequencing. Upon Edman sequencing, the PTH-Ser-*O*-GalNAc derivative chromatographs as a doublet, between PTH-Asp and PTH-Asn (44), thereby allowing its quantification along with the remaining random amino acid residues. Representative Edman sequencing chromatograms of the biotinylated GP-I substrate after glycosylation by ppGalNAc T10 are shown in Fig. 2*C*, where it is evident that the PTH-Ser-*O*-GalNAc doublet is significantly increased at the +1 position (residue 10). Enhancement values thus obtained for GP-I with ppGalNAc T10 are given in Fig. 3*A*, whereas enhancement values for ppGalNAc T1 and T2 are given in Fig. 3, *B* and *C*. For clarity, the enhancement values for Ser-*O*-GalNAc alone are given for each transferase in the panels in Fig. 3*D*.

Except for a few outliers, the nonglycosylated residue enhancements shown in Fig. 3, *A–C,* for ppGalNAc T10, T1, and T2 on GP-I are generally very similar to those enhancements obtained for these transferases on the nonglycosylated random substrates (see Fig. 1 and supplemental Fig. S2 and the comparison plots in supplemental Figs. S5, S6, and S7). For example, the plot of the nonglycosylated residue GP-I enhancements *versus* those obtained previously for ppGalNAc T1 and T2 (21, 38) give r^2 correlations of 0.57 and 0.73, respectively (see supplemental Figs. S5 and S6). Thus, the previously observed (21, 38) ppGalNAc T1 and T2 Pro enhancements at the −1, +1, and +3 positions are also readily observed with GP-I. Even the unique ppGalNAc T1, +3 Tyr enhancement is seen in GP-I. Interestingly, for ppGalNAc T1, Val is considerably more enhanced at the −1 site in GP-I compared with the nonglycosylated peptide substrates (21, 38), although no highly significant differences in enhancement values are observed for ppGalNAc T2 between GP-I and our previous results (21, 38). However, principally because the ppGalNAc T10 enhancement values cluster close to one, the comparison plot for T10 shows no mathematical correlation between the values for GP-I and those for PV-I and PV-II (supplemental Fig. S7). Interestingly, ppGalNAc T10 shows an elevated Phe and Tyr enhancement at the −1 position (and at several other positions) in GP-I that are not observed in random peptides P-VI and P-VII. We therefore conclude, despite some differences, that the glycosylated GP-I substrate provides reliable and reproducible enhancement values reasonably consistent with the enhancement values obtained previously from the nonglycosylated substrates for all three transferases.

In contrast to the nonglycosylated residue enhancements, ppGalNAc T10 shows a very large Ser-*O*-GalNAc enhancement (~9-fold) at the +1 position, with slight preferences at the +2 and +4 positions (Fig. 3*D*). Interestingly, no strong Ser-*O*-GalNAc enhancements were observed for ppGalNAc T1 and T2, all being less than 1, except at the +2 and −4 positions for T1 and T2, respectively. It is noteworthy that the +1 Ser-*O*-GalNAc enhancement observed for ppGalNAc T10 is the largest that we have observed to date for any residue type. Thus, it appears that the substrate specificity of ppGalNAc T10 is dominated almost entirely by a very high preference for a GalNAc-glycosylated residue at the +1 position, whereas ppGalNAc T1 and T2 do not seem to have any significant preferences for neighboring GalNAc glycosylated residues, and instead have preferences that are dominated by the nature and pattern of neighboring nonglycosylated residues. As discussed below, these results suggest that all of these ppGalNAc T preferences must be the result of specific interactions of the acceptor substrate with the catalytic domain of ppGalNAc Ts and not with direct interactions with its lectin domain.

ppGalNAc T1, T2, and T10 Studies on Random Glycopeptide GP-II—To confirm the Ser-*O*-GalNAc preferences obtained from the GP-I studies above and to assess the role of a single Thr-*O*-GalNAc residue on targeting the site of ppGalNAc T glycosylation, we next utilized random glycopeptide GP-II (Table 1) as an acceptor substrate. GP-II has been previously used as a T-synthase substrate (50) and is similar to GP-I, except that it contains a central Thr-*O*-Gal-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C
Residue 8, Position -1
Residue 9, Position 0
Residue 10, Position +1
Ser-O-GalNAc
Residue 11, Position +2
Residue 12, Position +3
DPTU
DMPTU
DPU
Time (min)

NAc residue that is flanked by randomized residues that also contain nonglycosylated Ser (50). Here we monitored the incorporation of [^{3}H]GalNAc by ppGalNAc T10, T1, and T2 into the Ser residues at each random position of the GP-II substrate by scintillation counting of each Edman cycle. A plot of the results for ppGalNAc T10, T1, and T2 are given in Fig. 4, *A*–*C*. As shown in Fig. 4*A*, ppGalNAc T10 incorporates a peak of radiolabeled GalNAc almost entirely at the −1 position relative to the central Thr-*O*-GalNAc of GP-II. This result is entirely consistent with our above findings with GP-I (Fig. 3*D*), which demonstrated that ppGalNAc T10 highly prefers a Ser-*O*-GalNAc residue at the +1 position relative to the acceptor Thr residue (*i.e.* position 0 of GP-I). Indeed, it can be shown that the inverse of the radiolabeled GalNAc incorporation patterns obtained for GP-II should parallel the Ser-*O*-GalNAc enrichment values obtained for GP-I at all positions (*i.e.* the GP-II ^{3}H pattern should mirror the enhancements of GP-I). Therefore, in the *right-hand panels* of Fig. 4 (*D*–*F*) we have also plotted the transformed (inverse) GP-II ^{3}H incorporation data next to the GP-I enhancement values for all three transferases. From these plots (Fig. 4, *A* and *D*), it is clear that ppGalNAc T10 almost exclusively recognizes a Ser/Thr-*O*-GalNAc-glycosylated residue directly C-terminal of an acceptor Ser or Thr residue.

In contrast to ppGalNAc T10, the [^{3}H]GalNAc incorporation patterns for ppGalNAc T1 and T2 on GP-II are less concordant with the Ser-*O*-GalNAc enhancement values obtained for these transferased with GP-I (see Fig. 4, *E* and *F*). For example, ppGalNAc T1 shows peak [^{3}H]GalNAc incorporation at the −4 position of GP-II (corresponding to +4 position on GP-I) (Fig. 4, *B* and *E*), whereas on GP-I the highest Ser-*O*-GalNAc preference is found at the +2 position with the +4 position having the second highest preference for this transferase. For ppGalNAc T2, [^{3}H]GalNAc incorporation into GP-II appears to be spread across more residues, with peak incorporation at the −4, −2, −1, and +4 positions (corresponding to positions −4, +1, +2, and +4 on GP-I) (Fig. 4*F*), and for GP-I the highest preference was found at the −4 position for this transferase. Nevertheless, the patterns N-terminal of the site of glycosylation for ppGalNAc T2 appear to be similar for both GP-I and GP-II (Fig. 4*F*).

The differences in glycosylation patterns observed for ppGalNAc T1 and T2 with GP-I and GP-II may stem from several factors. These would include their different acceptor residues (*i.e.* Thr *versus* Ser), the differences in having single *versus* multiple acceptor (and glycosylated) sites, and finally the fact that these transferases do not prefer neighboring glycosylated residues. In addition, each uses a different donor, UDP-GalNAc or UDP-GalNAz. Therefore, depending on which factors dominate the substrate binding interactions, the observed preference patterns could be expected to vary between GP-I and GP-II. Because Ser residues are much poorer acceptors compared with Thr, it is possible that with the multiple Ser acceptor GP-II, the specificities of the transferases would be more likely to be modulated by the binding of the single Thr-*O*-GalNAc residue in the transferase peptide-binding cleft. In contrast, for the single Thr acceptor substrate, GP-I, it is possible that the binding of the single acceptor Thr residue at the catalytic site of GalNAc transfer would dominate over the binding of neighboring GalNAc- glycosylated residues. However, in the case where a transferase possesses a very strong GalNAc-binding site, such as ppGalNAc T10, both glycopeptide substrates GP-I and GP-II would be expected to behave similarly, as indeed is observed.

DISCUSSION

Essential to understanding the biological functions of the individual isoforms of the ppGalNAc T family of *O*-glycan, initiating transferases is the characterization of their peptide acceptor substrate preferences. Severely complicating this is the varied and relatively unknown effects of prior substrate glycosylation on transferase substrate specificity. For example, two members of this family have near-absolute preferences for α-GalNAc-modified substrates, *i.e.* ppGalNAc T7 and T10 (8, 27, 28, 33, 37), whereas others display altered specificity upon substrate glycosylation, *i.e.* ppGalNAc T2 and T4 (30–33). For others, prior glycosylation may also inhibit substrate glycosylation as shown for ppGalNAc T1 and T2 (29, 34, 35). Presently, the role of prior glycosylation is very poorly understood. Only until recently has a systematic and quantitative determination begun of the acceptor peptide substrate preferences of the ppGalNAc Ts (21, 38). In this study, we extend these studies to the investigation of the peptide and glycopeptide acceptor substrate specificities of the glycopeptide-favoring transferase, ppGalNAc T10, using a series of previously described oriented random peptides and a new series of novel glycopeptide substrates.

ppGalNAc T10 Nonglycosylated Peptide Substrate Preferences—Although ppGalNAc T10 is thought to be a glycopeptide requiring transferase, variable reports of the activity of ppGalNAc T10 against nonglycosylated substrates have been reported ranging from no activity to moderate activity (8, 28, 33, 37).[7] Therefore, the specificity of ppGalNAc T10

[7] Note, however, that the activity of ppGalNAc T10 against the Muc 1a peptide, AHGVTSAPDTR, as reported by Cheng *et al.* (28), has not been found in our hands.

FIGURE 2. **Isolation of the ppGalNAc T10 GP-I-GalNAz-biotin cycloaddition product by Sephadex G-10 before and after isolation on monomeric avidin (*A* and *B*), and selected Edman amino acid sequencing chromatograms (*C*).** *A,* Sephadex G-10 gel filtration chromatography of the GalNAz-biotinylated GP-I reaction product. Fractions (*dark bar*) were pooled for isolation on monomeric avidin, as described under "Experimental Procedures." *B,* Sephadex G-10 gel filtration chromatography of the ppGalNAc T10 GalNAz-biotinylated GP-I product after elution from monomeric avidin. Fractions (*dark bar*) representing the biotinylated GP-I (ELISA, OD_{405}) were pooled for Edman sequencing. *A* and *B*, *circles* represent the absorbance at 220 nm; *triangles* represent the absorbance at 280 nm; *squares* represent the [^{3}H]GalNAc; and *diamonds* represent the biotin by ELISA absorbance at 405 nm. *C,* Edman sequencing chromatograms (residues 8–10) of the ppGalNAc T10 GalNAz-biotinylated GP-I product pooled in *B*. Amino acid residues are assigned by their single-letter abbreviations in cycles (residues) 8 and 9, and Ser-*O*-GalNAc is assigned in residue 10. Note that the presence of free Thr in cycle 9 is because of the partial elimination of GalNAz-alkyne-biotin as described in supplemental Methods and Results.

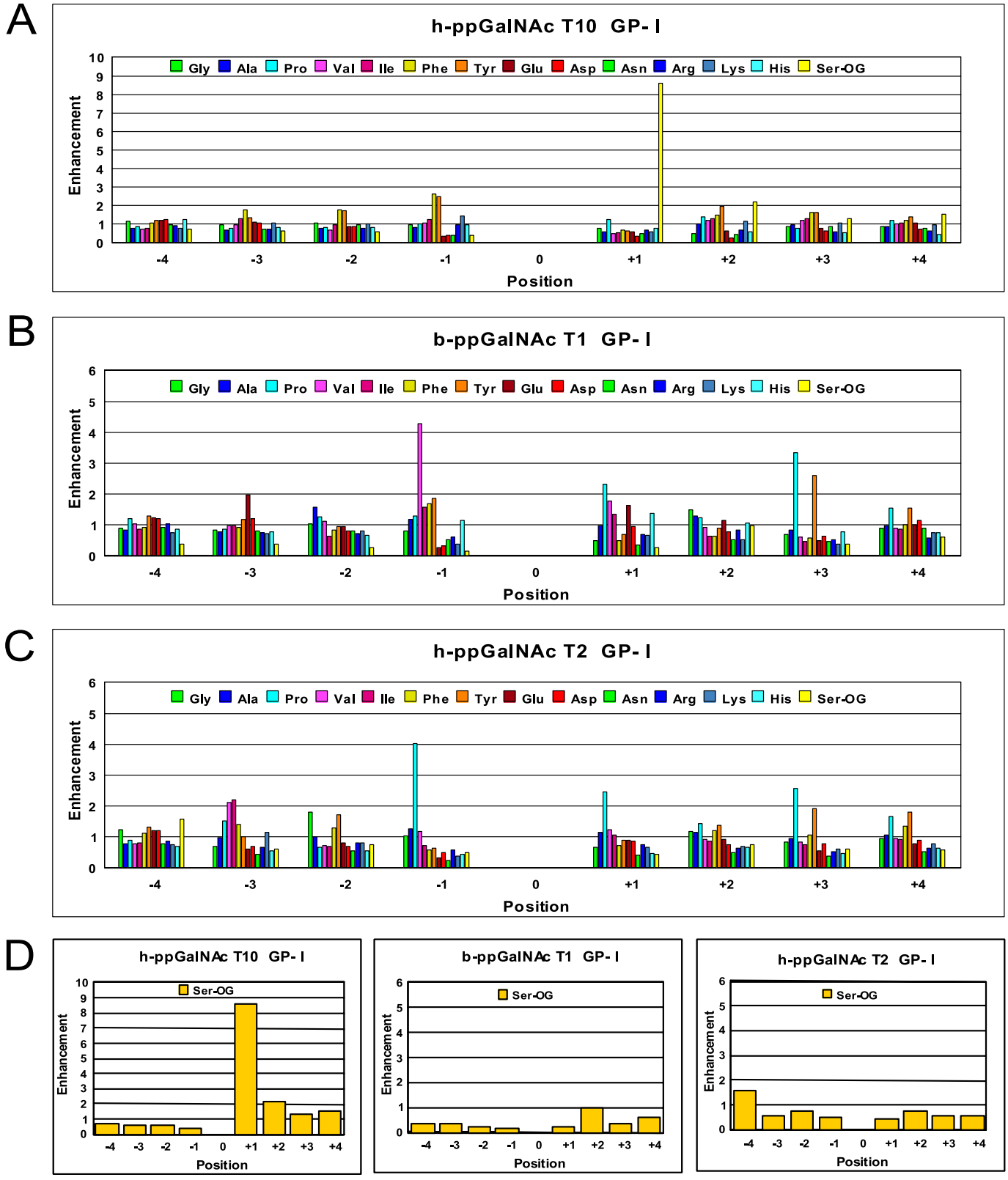

FIGURE 3. **Amino acid residue enhancement factors for ppGalNAc T10, T1, and T2 obtained from random peptide substrate GP-I, utilizing UDP-GalNAz.** *A–C*, enhancement factors for the hydrophobic and hydrophilic amino acid residues for ppGalNAc T10, T1, and T2, respectively. *D*, plot of Ser-*O*-GalNAc enhancement factors for ppGalNAc T10, T1, and T2. Values represent the average of two determinations as described under "Experimental Procedures."

against random peptide substrates P-VI and P-VII was performed as reported previously for ppGalNAc T1 and T2 (21, 38). As shown in supplemental Fig. S1, ppGalNAc T10 was indeed capable of transferring GalNAc to these peptide substrates, although its UDP-GalNAc hydrolyzing activity was significantly greater (supplemental Fig. S1, *A* and *D*). Inter-

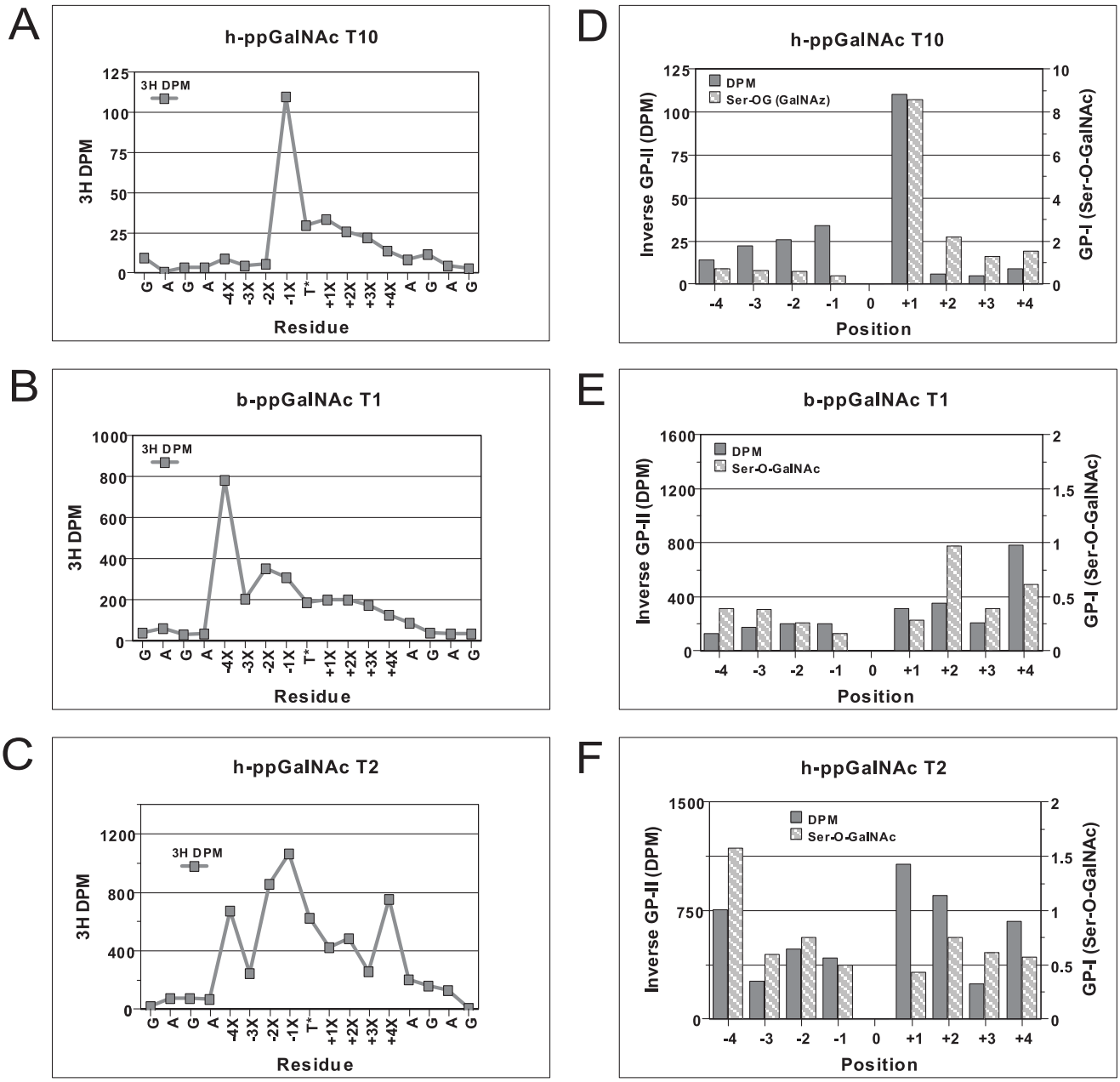

FIGURE 4. **[^{3}H]GalNAc incorporation patterns for ppGalNAc T10, T1, and T2 on the random peptide substrate GP-II (Table 1) and comparison with the enhancement values obtained for GP-I (Fig. 3).** *A–C*, plots of ^{3}H content *versus* GP-II residue for ppGalNAc T10, T1, and T2, respectively. Disintegrations/min values were corrected for cycle lag during Edman sequencing. Note that some [^{3}H]GalNAc incorporation is observed at the Thr-*O*-GalNAc position of GP-II because of a small fraction of unglycosylated Thr at this position (data not shown). *D–F*, plots of the transformed (inverse) GP-II ^{3}H incorporation data (*dark gray bars*) and GP-I enhancement values (*light bars*) for ppGalNAc T10, T1, and T2, respectively. With the transformation of the GP-II data, the site of glycosylation effectively shifts the 0 position of the peptide sequence, therefore allowing the direct comparison of the GP-II and GP-I data. The *vertical scales* of the GP-II plots in *D–F* were arbitrarily adjusted to approximately match the GP-I enhancement data. Note also that for clarity, the GP-II disintegration/min values at the 0 position were omitted in *D–F*.

estingly, as shown in Fig. 1, ppGalNAc T10 revealed no significant hydrophobic or hydrophilic residue enhancements, in contrast to what was observed for ppGalNAc T1 and T2 (and others ppGalNAc Ts studied to date) (see supplemental Fig. S2). Strikingly, the 2–5-fold enhancements for Pro at the −1, +1, and +3 positions observed for ppGalNAc T1 and T2 (and other ppGalNAc Ts)[6] are absent with ppGalNAc T10. Only very weak Pro enhancements are observed at the −1, +1, and +2 positions with ppGalNAc T10. These findings suggest that ppGalNAc T10 does not possess any previously undiscovered highly specific nonglycosylated peptide motifs, and that its specificity is largely independent of the nature of the neighboring nonglycosylated residues. Thus, ppGalNAc T10 may tolerate a wide range of residue types in its peptide-binding site, although it does not glycosylate these substrates very efficiently as demonstrated by its high UDP-GalNAc hydrolyzing activity and its low transfer to peptide activity.

ppGalNAc T10 Glycosylated Peptide Substrate Preferences—As ppGalNAc T10 has been reported to possess high glycopep-

tide activity (8, 28, 33, 37), we designed a series of two complementary random glycopeptide substrates, GP-I and GP-II (Table 1), to quantify its glycopeptide specificity. In GP-I, a single acceptor Thr residue is flanked by random residues that include Ser-*O*-GalNAc, whereas in GP-II multiple acceptor Ser residues flank a single central Thr-*O*-GalNAc residue. UDP-GalNAz was utilized as the sugar donor in the GP-I studies followed by its biotinylation via copper(I)-catalyzed 1,4 cycloaddition "click chemistry" (see under "Experimental Procedure"). By Edman amino acid sequencing of the biotinylated glycopeptide, the enrichment factors for Ser-*O*-GalNAc (and other nonglycosylated residues) were obtained. GP-II incubations were performed with UDP-[^{3}H]GalNAc, and [^{3}H]GalNAc incorporation was determined at each random position by Edman sequencing. We found for both GP-I and GP-II, that ppGalNAc T10 exhibited a single very large preference for Ser/Thr-*O*-GalNAc at the +1 (C-terminal) position relative to the Ser or Thr acceptor site (Figs. 3 and 4). As discussed below, this finding is consistent with previous reports on ppGalNAc T10. In contrast, our studies with ppGalNAc T1 and T2 revealed no significant enhancements for Ser/Thr-*O*-GalNAc utilizing these glycopeptide substrates (Figs. 3 and 4). Indeed, except for 1 or 2 positions, Ser-*O*-GalNAc appears to be inhibitory for ppGalNAc T1 and T2, once more in keeping with previous findings for these transferases (29, 34, 35).

In addition to obtaining neighboring Ser-*O*-GalNAc enhancement values, our studies on GP-I have also yielded enhancement values for several nonglycosylated residues. Generally, for ppGalNAc T1, T2, and T10, these enhancement values are very similar to the values observed from the random peptide substrates with only a few outliers (compare Fig. 3 with Fig. 1 and supplemental Figs. S2 and S5–S7) (21, 38). Interestingly, the largest differences seem to occur with specific hydrophobic residues at the −1 site, with Val and both Phe and Tyr showing elevated enhancements in GP-I with ppGalNAc T1 and T10, respectively. The differences between the enhancement values from GP-I and the random peptide substrates are not fully understood, but they may be due to the use of UDP-GalNAz instead of UDP-GalNAc. Nevertheless, the high similarity of the nonglycosylated residue enhancements between GP-I and the nonglycosylated substrates further supports the notion that these transferases recognize each substrate position relatively independent of its neighbor, as suggested earlier (38, 51).

Our findings that ppGalNAc T10 has a +1 Ser/Thr-*O*-GalNAc preference is consistent with previously published data for this transferase, where the sites of identified glycosylation are typically at Ser/Thr residues N-terminal of a previous site of glycosylation (8, 29, 33, 37). As the lectin domain mutants of ppGalNAc T10 show the same +1 glycopeptide preference (33, 37), it is clear that this activity is not directed by substrate binding to the lectin domain. Preliminary molecular docking studies of GalNAc glycopeptides onto the catalytic domain of ppGalNAc T10 suggest the possibility of a +1 GalNAc-binding site in the catalytic domain of ppGalNAc T10.[8] Thus, it is clear that ppGalNAc T10 possesses a single +1 Ser/Thr-GalNAc-binding site in its catalytic domain. Interestingly, the crystal structure of ppGalNAc T10 co-crystallized in the presence Ser-*O*-GalNAc shows GalNAc bound to the β subdomain of its lectin domain and not to its catalytic domain (37). This may suggest that efficient binding of Ser/Thr-*O*-GalNAc-containing glycopeptide substrates at the catalytic domain may require longer lengths of flanking amino acid residues. Alternatively, the lectin domain may indeed have a higher affinity for Ser/Thr GalNAc glycopeptides than the catalytic domain suggesting that the lectin domain could further modulate the overall activity or specificity of ppGalNAc T10 against certain glycopeptide substrates. This is supported by the data of Pratt *et al.* (29) where the rate of glycosylation of Thr-6 in the EA2 glycopeptide, PTTDST(Thr-*O*-GalNAc)PAPTTKK, by ppGalNAc T10 is significantly enhanced (~10-fold) by the prior glycosylation of Thr-11 or Thr-12, which are 5 and 6 residues C-terminal from the site of glycosylation. Likewise, Kubota *et al.* (37) have shown that the lectin domain of ppGalNAc T10 is required for the glycosylation of Thr-4 (with subsequent glycosylation of Ser-3) in the IgA-hinge glycopeptide, VPSTPPTPSP(Ser-*O*-GalNAc)-TPPTPSPS, where the Ser-*O*-GalNAc is +7 residues from the initial site of glycosylation. Thus, ppGalNAc T10 appears to have a GalNAc glycopeptide-binding site in its lectin domain that significantly accelerates the rate of glycosylation without significantly altering its specificity. Further studies on longer random glycopeptide substrates are required to further address these additional potential peptide-GalNAc-binding sites.

In contrast to ppGalNAc T10, our results for ppGalNAc T1 (Figs. 3 and 4) suggest that this transferase highly disfavors the presence of GalNAc-glycosylated residues at all positions except perhaps +2. This is consistent with the findings of Pratt *et al.* (29) that showed the unglycosylated EA2 peptide as the best substrate for this transferase. In the case of ppGalNAc T2, our results suggest that a glycosylated residue at the −4 position may slightly enhance glycosylation (Figs. 3 and 4). Again, this is observed in the data of Pratt *et al.* (29) for EA2, where the prior glycosylation of Thr-3 results in an ~30% increase in rate of glycosylation compared with the nonglycosylated EA2 peptide (assuming Thr-7 is the site of glycosylation in both substrates). Interestingly, these workers also observed rate enhancements for ppGalNAc T2 against EA2 glycopeptides that were previously glycosylated at positions −/+ 5 residues from the site of glycosylation, Thr-7. Therefore, similar to ppGalNAc T10, ppGalNAc T2 may also possess additional remote glycopeptide-binding sites in its catalytic or lectin domain.

In conclusion, by using a series of random peptide and glycopeptide substrates, we have successfully completed the most thorough determination to date of the peptide and glycopeptide substrate preferences of the catalytic domains of three important members of the ppGalNAc T family, T1, T2, and T10. Our results reveal that these transferases have unique peptide and glycopeptide preferences demonstrating their substrate diversity and their likely roles ranging from initiating transferases to filling-in transferases. It is anticipated that from the transferase-specific preferences that methods for the prediction of specific sites of glycosylation will become possible,

[8] C. L. Perrine and T. A. Gerken, work in progress.

leading to the identification and confirmation of transferase-specific protein targets of biological significance.

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