Carbohydrates and Activity of Natural and Recombinant Tissue Factor*†

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The effect of glycosylation on tissue factor (TF) activity was evaluated, and site-specific glycosylation of full-length recombinant TF (rTF) and that of natural TF from human placenta (pTF) were studied by liquid chromatography-tandem mass spectrometry. The amidolytic activity of the TF-factor VIIa (FVIIa) complex toward a fluorogenic substrate showed that the catalytic efficiency (Vmax) of the complex increased in the order rTF1–243 (Escherichia coli) < rTF1–263 (<S9 insect cells) < pTF for the glycosylated and deglycosylated forms. Substrate hydrolysis was unaltered by deglycosylation. In FXase, the Km of FX for rTF1–263-FVIIa remained unchanged after deglycosylation, whereas the kcat decreased slightly. A pronounced decrease, 4-fold, in kcat was observed for pTF-FVIIa upon deglycosylation, whereas the Km was minimally altered. The parameters of FX activation by both rTF1–263D-FVIIa and pTFD-FVIIa were identical and similar to those for rTF1–243-FVIIa. In conclusion, carbohydrates significantly influence the activity of TF proteins. Carbohydrate analysis revealed glycosylation on asparagines 11, 124, and 137 in both rTF1–263 and pTF. The carbohydrates of rTF1–263 contain high mannose, hybrid, and fucosylated glycans. Natural pTF contains no high mannose glycans but is modified with hybrid, highly fucosylated, and sialylated sugars.

Tissue factor (TF) is an integral membrane glycoprotein, which upon injury or stimulus is exposed on cellular surfaces to circulating blood (1). TF, the nonenzymotic cofactor, is involved in the extrinsic factor Xase (FXase) formation with FVIIa, which initiates coagulation by activating FIX and FX to the corresponding serine proteases FIXa and FXa, respectively. The TF-FVIIa complex assembly accelerates the generation of FXa by FVIIa by 7 orders of magnitude (2). Serine proteases FIXa and FXa form the intrinsic FXase with FVIIa and the prothrombinase complex with FVa, respectively. These membrane-bound complexes accelerate thrombin generation, which subsequently converts fibrinogen to fibrin and forms a stable clot (3).

TF is a 263-amino acid glycoprotein containing three domains: the extracellular domain (residues 1–219), the transmembrane domain (residues 220–242), and the cytoplasmic domain (residues 243–263) (4). The extracellular domain contributes to coagulation by binding FVIIa and thereby forming the extrinsic FXase (5). The interaction of TF with FVIIa is calcium-dependent, and the cleavage of both FIX and FX by the complex is membrane-dependent (6, 7). The transmembrane domain serves as an anchor to the cell membrane, whereas the cytoplasmic domain has been suggested to play a role in signal transduction (8, 9). Studies on the distribution of TF have found high activity levels in the brain, lung, heart, and placenta, and precise localization has been assigned to endothelial, monocytic, and vascular adventitia (10, 11). Although TF is expressed in many human tissues, the overall concentration of TF in vivo is extremely low. Surrogate rTFs from various expression systems have been widely used for functional studies; however, the structural and functional comparison of the natural protein with the recombinant forms has been lacking (12).

Posttranslational modifications of TF have also been partially described, and their potential role in activity of the protein has been analyzed (13, 14). Variations in posttranslational modifications of proteins have accentuated the fact that specific modifications, reflective of the production system, may play a role in the activity of proteins (15–17). The most pronounced diversity of posttranslational modification structures is found in Asn-linked (N-linked) and Ser/Thr (O-linked) glycosylation (18, 19). The carbohydrate moieties of some coagulation proteins have been described and shown to have influence on the activity of those proteins (20–24).

Although it is widely accepted that TF is the key initiator of blood coagulation, many controversies related to TF structure and function are continuously debated (25–29). Experimental data used for the understanding of TF structure and function are mostly acquired with rTF proteins. The cDNA deduced amino acid sequence of TF protein indicates that TF contains four potential sites for N-linked glycosylation based on the Asn-X-Ser/Thr motif (18). These sites include Asn-11, Asn-124, and Asn-137 in the extracellular domain and Asn-261 in the cytosolic domain. However, there is no consensus related to the influence of glycosylation on TF activities (12). Some reports...
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state that TF glycosylation has no influence on TF procoagulant activity (30–35). Other observations suggest that glycosylation may be important (36–38).

In the present study, we provide experimental data showing the role of carbohydrates in the function of natural TF. We describe the activity of TF proteins from three different sources, including rTF1–243 expressed in Escherichia coli, rTF1–263 expressed in Sf9 insect cells, and, most importantly, affinity-purified natural human placental TF (pTF). We show that the activity of the natural TF protein is different relative to its recombinant full-length counterpart. In a previous report (39), we have shown that the mobility, by SDS-PAGE, of the recombinant protein is different from that of natural TF purified from human placenta and human monocytes.

We have also focused on the comparative analysis of the carbohydrate composition of the recombinant and natural TF. We describe the carbohydrate moieties of full-length rTF1–263 and natural pTF. The sites of glycosylation and the composition of glycans at each site are identified and described. The study revealed that the composition of carbohydrates at each site varies between the recombinant and natural human pTF. The activity of these proteins before and after deglycosylation revealed that glycosylation does alter TF activity.

EXPERIMENTAL PROCEDURES

Proteins—rTF1–243 (E. coli) was a gift from Drs. S. Liu and R. Lundblad (Baxter Healthcare Corp., Duarte, CA). rTF1–263 expressed in Sf9 insect cells, was a gift from Dr. R. Jenny (Haematologic Technologies, Inc.). pTF (Thromborel S) was a gift from Dr. D. Barrow (Behring Diagnostics Inc.). rFVIIa was provided as a gift by Dr. U. Hedner (Novo Nordisk). The concentration of rFVIIa was calculated using a molecular mass of 50,000 Da and an extinction coefficient (ε) of 1.39 (40). Human FX was isolated from fresh frozen plasma using a method described elsewhere (39). Peptide-N-glycosidase F (PNGase F), native protein deglycosylation kit, proteomics grade trypsin, horseradish peroxidase-labeled goat anti-mouse Ig, and Glu-C enzyme were purchased from Sigma.

Materials—Synthetic phospholipids 1,2-dioleoyl-sn-glycero-3-phospho-L-serine and 1,2-dioleoyl-sn-glycero-3-phosphocholine were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Spectrozyme FXa was obtained from American Diagnostica Inc. (Greenwich, CT). Fluorogenic substrate 6-(p-FPR)amino-1-naphthalenethiol-sulfonamide was synthesized in house (41). Sinapinic acid, DL-dithiothreitol, and iodoacetamide were purchased from Sigma. CHAPS, water, and acetonitrile were purchased from Fisher. Luminex microspheres were purchased from Luminex (Austin, TX). R-Phycoerythrin-streptavidin was purchased from Invitrogen. Formic acid was purchased from VWR (West Chester, PA).

Purification and Quantitation of TF—Human pTF was purified from the commercial thromboplastin, Thromborel S®. by a procedure described previously (39). The contents of 100 vials of Thromborel S were resuspended in Tris-buffered saline, pH 7.4, containing 0.2% Triton X-100 and affinity-purified on Sepharose-coupled anti-TF-5 mAb (2.5 mg of mAb/ml of resin). Proteins were quantitated by fluorescence-linked immunosay (FLI) by capture on anti-TF-5 mAb-coupled beads, probed with biotinylated anti-TF-48 mAb, and detected with R-phycoerythrin-streptavidin (39). Each point in the FLI measurement is a mean of two experiments ± 1 S.D. The concentration of TF was confirmed by absorbance at 280 nm using an ε value of 1.5 for rTF1–263 and 1.4 for rTF1–243 and binding stoichiometry to rFVIIa (42, 43). The efficiency of the oligosaccharide removal was assessed by immunoblotting. For carbohydrate analysis, enzymatic deglycosylation of rTF1–263 was performed under non-denaturing conditions using PNGase F. One μl (1 unit) of the enzyme was added to 10 μg of TF protein, followed by a 4-h incubation at 37 °C. The efficiency of the glycosidase reaction buffer, followed by a 4-h incubation at 37 °C. The efficiency of the oligosaccharide removal was assessed by Coomassie Blue staining. The molecular weights of the glycosylated (G) and deglycosylated (D) forms of TF proteins were determined by mass spectrometry.

Immunoblotting—Immunoblotting was performed as described previously (39). Three ng of TF was subjected to a 4–12% acrylamide SDS-PAGE gradient gel, transferred to nitrocellulose membrane, probed with a mix of anti-TF-5 mAb anti-TF-48 mAb, and detected by horseradish peroxidase-goat anti-mouse Ig.

Molecular Weight Determination by Matrix-assisted Laser Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry—Mass spectra of the G and D TFs were obtained on a Voyager-DE™ PRO MALDI-TOF mass spectrometer (Perceptive Biosystems, Framingham, MA). The spectra were obtained in positive ion mode with delayed extraction equipped with a 337-nm nitrogen laser. A calibration mixture contained rabbit muscle aldolose [M + H]+ = 39,212.28 (average) and bovine serum albumin [M + H]+ = 66,430.09 (average). Ten pmol of G and D TF were spotted onto a stainless steel sample plate (catalog number V700666, Applied Biosystems). One drop of sinapinic acid in 100% methanol was applied to the plate, followed by sample application in sinapinic acid reconstituted in 60% acetonitrile and 0.03% trifluoroacetic acid in water. Experimentally obtained protein masses were compared with the masses calculated from amino acid composition. The mass difference between the experimental (mass spectrometry) and the calculated mass (amino acid composition) provided the mass of posttranslational modifications on the protein.

FFVIIa Amidolytic Activity in the Absence of Phospholipids—The previously described procedure consisted of a reaction mixture of 2.0 ml of 0.5 mM FVIIa, varying concentrations of TF (0.05–6.0 nM), and 50 μM SN-17c fluorogenic substrate (44). TF was preincubated with FVIIa for 10 min at 37 °C. Substrate was added, and the rate of substrate hydrolysis as the change in fluorescence intensity over time (5 min) was measured at an excitation wavelength of 350 nm and emission wavelength of 400 nm.
470 nm using a 450-nm cut-off filter in Jobin Yvan-Spex FluoroMax-2 (Instruments S.A. Inc., Edison, NJ). Each point is the mean of two independent experiments ± 1 S.D. Kinetic constants of substrate hydrolysis were determined using the non-linear fitting program GraphPad Prism4 and the Michaelis-Menten equation.

Relipidation of TF—Five nm TF was incubated with 10 μM PCPS (25% 1,2-dioleoyl-sn-glycer-3-phospho-l-serine and 75% 1,2-dioleoyl-sn-glycero-3-phosphocholine) in HBS and 5 mM CaCl2 for 30 min at 37 °C, followed by extensive dialysis in HBS and 5 mM CaCl2 to remove detergent (45). The relipidated TFs were quantitated by FLI.

Extrinsic FXase Assay—TF (0.1 nm) rFVIIa for 10 min at 37 °C in HBS, 5 mM CaCl2, pH 7.4, and varying concentrations of FX were added (0.01–8.0 μM). At selected time points (0–5 min), aliquots were quenched in 20 mM EDTA in HBS (pH 7.4) containing 0.2 mM Spectrozyme Xa, and substrate hydrolysis was monitored by the change in absorbance at 405 nm using a Molecular Devices (Menlo Park, CA) Vmax spectrophotometer. The FXa generation rate was calculated from a standard curve prepared by serial dilutions of FXa. Each point is the mean of two independent experiments ± 1 S.D. Michaelis-Menten kinetic constants were calculated using the GraphPad Prism4 program.

Enzymatic Digest of TF Proteins—Samples of G and D TF proteins were subjected to enzymatic digestion using trypsin. Three μg of rTF1-243 and rTF1-263 and 1.5 μg of pTF in 50 mM ammonium bicarbonate at pH 8.3 were reduced with 10 mM DL-dithiothreitol during 1 h of incubation at 57 °C. The protein was alkylated with 17 mM iodoacetamide in the dark at room temperature for 20 min. Trypsin was added to each protein solution at a 1:50 enzyme/protein ratio, and the digestion mixture was incubated at 37 °C overnight. Digestion was stopped upon the addition of formic acid to a final concentration of 1.25%. A second sample of G and D TF proteins was subjected to enzymatic digestion using GluC. Three μg of rTF1-243 and rTF1-263 and 1.5 μg of pTF were reduced and alkylated as described for the trypsin cleavage. The buffer pH was reduced to 4.0, and the proteins were incubated with Glu-C enzyme at 37 °C overnight.

LC-MS/MS Analysis of TF Proteolytic Peptides—Liquid chromatography/mass spectrometry (LC/MS) analysis of 1 pmol of TF peptides was carried out using a Shimadzu SIL-20AC autosampler with two LC-20AD pumps (Columbia, MD) and a Thermo LTQ mass spectrometer (San Jose, CA). The LC was connected to a 100-μm inner diameter by 150-mm length nanospray column that was pulled and packed in house using a stainless steel pressure bomb and Magic C18 reverse phase material (5-μm beads, Michrom, Auburn, CA). We used a slightly modified form of the two-split configuration described previously (46). Instead of capillaries, we used a 1 × 150-mm Atlantis C18 column (Waters, Milford, MA) to act as a counter restriction to the nanospray column. Total flow from the pumps was 80 μl/min during the loading period and 40 μl/min during the running period. The resulting flow through the nanospray column was 1 μl/min during the load and 500 nl/min during the running period. We used water (solvent A) and acetonitrile (solvent B) with 0.1% formic acid in both solvents to load and elute the sample from the nanospray column. The LC gradient consisted of the following: 97% A and 3% B for 20 min, 40% B at 57 min, and 60% B at 59 min. Total time for the sample analysis was 65 min. Due to the presence of CHAPS in the TF samples, each sample analysis was followed by a 5-μl injection of 50% isopropyl alcohol (Fisher) to “wash” the column. The isopropyl alcohol injection was carried out in 60% solvent B followed by a decrease to 3% in 5 min; total time for the column wash was 25 min. The LTQ mass spectrometer was tuned according to the manufacturer’s recommended procedure. The nanospray column was placed in front of the mass spectrometer using an in-house built stage. The position of and voltage applied to the nanospray column were optimized to achieve a consistent spray. We applied 1.9 kV to the vent line on the tee at the head of the column. The transfer tube temperature was set to 150 °C. Our MS method consisted of a full MS scan from 250 to 2,000 Da in centroid mode, a zoom scan, and an MS/MS scan on the most intense ion from the full MS scan. We set the MS/MS parameters to the following: signal threshold = 6,000, isolation width = 2, normalized collision energy = 35, activation Q = 0.250, and activation time = 30. We chose the following data-dependent settings: recount = 2, repeat duration = 60 s, exclusion list size = 50, and exclusion duration = 100 s.

LC-MS/MS data files were viewed using Xcalibur Qualbrowser version 2.0. From Qualbrowser, we looked at the full MS in both the density map window and the spectrum window. The density map window allowed us to identify clusters of glycopeptides as described previously (47, 48). We also viewed the MS/MS spectra of the glycopeptides in Qualbrowser to identify glycan composition based on specific mass losses of different sugars as described previously (49–52).

Non-glycosylated and deglycosylated peptides were identified using the Sequest algorithm (53, 54). We made a reduced data base that only contained various forms of the TF protein: the TF protein predicted amino acid sequence and the TF protein where Asn was changed to Asp in N(S/T) motifs. Sequest used this data base to identify TF peptides in the glycosylated and deglycosylated samples. We included the following possible modifications on amino acids in the Sequest search: carboxyamidomethylation of cysteine (57.05 Da); phosphorylation of serine, threonine, and tyrosine (79.97 Da); and the addition of a GlcNAc or a fucosylated (Fuc) N-acetyl glucosamine on Asn (203.20 and 349.34 Da, respectively). We used the following filters for high confidence identifications: ΔCN = 0.100; Xcorr versus charge state 1 = 1.50, 2.00, and 2.50 for singly, doubly, and triply charged species, respectively; and peptide probability = 0.001. All Sequest-identified peptides were manually inspected to confirm correct b and y ion designations and to rule out possible false positive identifications.

RESULTS

Enzymatic Removal of Oligosaccharides from TF—Fig. 1a shows the mobility of G and D TF proteins by immunoblotting. All TFs presented in this figure were treated with PNGase F and used in the functional assays. Fig. 1b shows the mobility of G and D forms stained with Coomassie Blue. In the experiments of this figure the rTF1-263 was deglycosylated using the Endo F enzymes and was used for MALDI-TOF and carbohydrate
analysis by LC-MS/MS. The pTF in Fig. 1b was deglycosylated with PNGase F. Our first attempt at deglycosylation of both rTF₁₋₂₆₃ and pTF was done using the native protein deglycosylation kit, which includes the Endo F₁, F₂, and F₃ enzymes. The specificity of the endoglycosidases is as follows. Endo F₁ cleaves Asn-linked high mannose (Man) and hybrid but not complex carbohydrates, and Endo F₂ cleaves oligomannose and biantennary complex oligosaccharides, whereas Endo F₃ is specific for the cleavage of core fucosylated biantennary and triantennary complex sugars. All three Endo F enzymes cleave between the two GlcNAc residues in the diacetylchitobiose core of the carbohydrate generating an Asn-linked GlcNAc-truncated sugar molecule (55). Fig. 1, a and b, shows rTF₁₋₂₆₃G after deglycosylation (rTF₁₋₂₆₃D) with PNGase F and the Endo F enzymes, respectively. The increased mobility of rTF₁₋₂₆₃D in both figures suggests that deglycosylation took place.

Purification of pTF resulted in a protein with a higher apparent molecular weight and heterogeneity as compared with its full-length recombinant counterpart rTF₁₋₂₆₃G (Fig. 1, a and b). The natural TF purified from human placenta is comparable in mobility and heterogeneity with that purified from human monocytes and is different from the recombinant forms (39). The mobility of pTF after treatment with Endo F enzymes remained the same even after prolonged exposure (data not shown). We therefore proceeded to treat pTF with PNGase F. PNGase F is specific for oligomannose, hybrid, and complex type glycans. The enzyme releases the Asn-linked carbohydrate, converting the Asn into Asp (56). Deglycosylation of pTFG with the enzyme (pTFD) resulted in a higher mobility and an increased homogeneity relative to the glycosylated form (Fig. 1, a and b). After deglycosylation, pTFD had a slightly lower mobility than rTF₁₋₂₆₃D. The overall mobility of the proteins increased in the order rTF₁₋₂₆₃D > pTFG > rTF₁₋₂₆₃G > pTFD. The difference in mobility between rTF₁₋₂₆₃D and pTFD may be attributed to other posttranslational modifications besides glycosylation (12).

Treatment of rTF₁₋₂₄₃ with PNGase F, denoted as rTF₁₋₂₄₃”D”, did not affect the mobility of the protein, consistent with the knowledge that rTF₁₋₂₄₃ is not glycosylated as expected for a protein produced in E. coli (57). The presence and absence of carbohydrates on each protein, rTF₁₋₂₄₃, rTF₁₋₂₄₃”D”, rTF₁₋₂₆₃G, rTF₁₋₂₆₃D, pTFG, and pTFD, was also evaluated by carbohydrate staining, which showed positive staining for rTF₁₋₂₆₃G and pTFG and no staining was observed for rTF₁₋₂₄₃, rTF₁₋₂₄₃”D”, rTF₁₋₂₆₃D, and pTFD (data not shown).

Quantitation of TF Proteins—The concentration of the G and D proteins was measured by FLI (39), using rTF₁₋₂₄₃ as a calibrator for the glycosidase-treated rTF₁₋₂₄₃ and rTF₁₋₂₆₃G as a
TF-FFVIIa Amidolytic Activity—The affinities of the TF proteins for FFVIIa in the absence of phospholipids were evaluated in the fluorogenic assay (Fig. 3) and are summarized in Table 2. All $K_d/(app)$ values are in the range of those previously reported (43). Treatment of rTF$_{1–243}$ with PNGase F (Fig. 3a) does not alter the $K_d/(app)$ for FFVIIa or the maximum rate of substrate hydrolysis by the complex. Similarly, deglycosylation of rTF$_{1–263}$ (Fig. 3b) does not affect the affinity of the deglycosylated protein for FFVIIa or the $V_{max}$ of the complex. Similarly, after deglycosylation, pTF does not change in either the $K_d/(app)$ or $V_{max}$ (Fig. 3c). All TF forms exhibit a functional 1:1 stoichiometry with FFVIIa (Fig. 3, a–c, insets, and Table 2). Thus, functional concentrations are consistent with the mass concentrations of TF proteins determined by the immunoassay. The catalytic efficiency of various TF forms increases in the order rTF$_{1–243} < $rTF$_{1–263} < $pTF and is not changed by deglycosylation.

Factor Xa Generation—As demonstrated in Fig. 4a, natural human pTF is (5-fold) more active than any rTF. Comparison of rTF$_{1–243}$ and rTF$_{1–243}$D shows that treatment of the protein with the glycosidase does not alter the affinity ($K_m$) or the catalytic efficiency ($k_{cat}$) of the complex (Table 3). Deglycosylation of rTF$_{1–263}$ does not alter the $K_m$ but results in a slight decrease in the $k_{cat}$ (from 2.8 to 2.2 s$^{-1}$). A slight increase in $K_m$ was observed when pTF was deglycosylated (from 0.32 to 0.57 μM). In contrast, deglycosylation caused a pronounced reduction in the $k_{cat}$ (from 8.7 to 2.3 s$^{-1}$). This decrease in the $k_{cat}$ and $K_m$ of the pTF$_{1–263}$ complex resulted in ~6-fold reduction in the second order rate constant ($k_{cat}/K_m$) of the complex. The stoichiometry of TF-FFVIIa was unchanged (Table 3). When the kinetic constants of the three TF proteins were compared, an overall increase in $K_m$ of ~3-fold was observed in the order pTF$_G < $rTF$_{1–263}$G < $rTF$_{1–243}$. The catalytic efficiency ($k_{cat}$) increased in the reversed order rTF$_{1–263} < $rTF$_{1–243} < $pTF, with pTF being 5-fold more active than the most active recombinant counterpart (Table 3). These differences lead to the 18-fold difference in the second order rate constant between rTF$_{1–243}$ and pTF$_G$. After deglycosylation, the parameters of FX activation by both rTF$_{1–263}$D and pTF$_D$ were almost identical ($K_m = 0.57$ and 0.57 μM, and $k_{cat} = 2.2$ and 2.3 s$^{-1}$, respectively). The stoichiometry of the G and D forms of relipidated TFs was confirmed to be 1:1, as presented in Fig. 4b for pTF$_G$ and rTF$_{1–263}$D.

MS Analyses of TF Peptides—Digestion of full-length TF with trypsin should generate 30 fragments (supplemental Table 1). Included in the table are fragments generated by a Glu-C digest of fragment 10 (marked with an asterisk). These smaller peptides obtained from Glu-C digestion allowed for the increased recovery and identification of fragment 10. According to the previously determined amino acid sequence of a mature TF protein (4), the first 10 residues of the intact N terminus are Ser, Gly, Thr, Thr, Asn, Thr, Val, Ala, Ala, and Tyr. Amino acid sequence analysis of the rTF$_{1–263}$ by Edman degradation con-

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**TABLE 1**

| TF species | Source | Calculated$^a$ | MALDI-TOF | Difference$^a$ | SDS-PAGE |
|-----------|--------|---------------|------------|----------------|----------|
| rTF$_{1–243}$ | E. coli | 27,423 | 27,800 | 377 | 31,000 |
| rTF$_{1–243}$D | E. coli | 29,592 | 33,196 | 3,604 | 37,000 |
| rTF$_{1–263}$ | SF9 | 29,592 | 30,202 | 610 | 33,000 |
| pTF$_G$ | Human placenta | 29,592 | 36,179 | 6,605 | 45,000 |
| pTF$_D$ | Human placenta | 29,592 | ND | ND | 34,000 |

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$r_{TF1–263}$ or the mass of pTF after deglycosylation with the PNGase F enzyme. Any other deviations between the calculated masses and masses of glycosidase-treated proteins obtained by MALDI-TOF are probably related to glycation or other post-translational modifications, some of which were described previously (12).

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**FIGURE 2.** Fluorescence-linked immunoassay of TF proteins. Varying concentrations of rTF$_{1–263}$Gp, pTF$_G$, and pTF$_D$ were captured on anti-TF-S mAb-coated beads and probed with biotinylated anti-TF-48 mAb and R-phycocerythrin-streptavidine. The immunoassay detects both the G and D forms of TF proteins. Each point is recorded as a mean fluorescence intensity unit (MFU) and is an estimate of two experiments ± 1 S.D.
firms this sequence. The Edman degradation analysis also shows that the majority of the recombinant protein exists in a truncated form (80%) with the first two residues missing, resulting in the following sequence: Thr, Thr, Asn, Thr, Val, Ala, Ala, Tyr. Similarly, our MS analysis revealed that 75% exists as the truncated form. The natural pTF exists mainly in the full-length form (69%). The truncated form of TF was previously observed and reported (4, 31). No evidence of leader peptides or fragments were identified by Edman degradation or by MS analysis. The N- and C-terminal peptides of rTF1–263 and pTF were recovered and identified by MS. Sequest search result data identify the recovery of the N- and C-terminal peptides with high confidence, suggestive of the correct identity of the two terminals. Manual inspection of peptides also confirms the absence of any adducts. Likewise, the N terminus of rTF1–243 was recovered and identified. The truncated form was identified by MS in ratios similar to those of rTF1–263. The C terminus of rTF1–243, which constitutes the transmembrane domain of

| TF species     | $K_d$ (nM) | $V_{max}$ (pM/s) | Stoichiometry (TF/FVIIa) |
|---------------|-----------|----------------|-------------------------|
| rTF1–243      | 0.41 ± 0.40 | 59.7 ± 1.2     | 1.1:1.0                 |
| rTF1–243D     | 0.47 ± 0.03 | 61.2 ± 1.0     | 0.9:1.0                 |
| rTF1–263G     | 0.31 ± 0.02 | 75.8 ± 1.6     | 0.9:1.0                 |
| rTF1–263D     | 0.35 ± 0.02 | 78.3 ± 1.4     | 1.0:1.0                 |
| pTFG          | 0.92 ± 0.15 | 173.1 ± 8.4    | 0.9:1.0                 |
| pTFD          | 0.69 ± 0.09 | 172.8 ± 10.0   | 1.0:1.0                 |

$^a$ Maximum rate of substrate fluorogenic hydrolysis at 0.5 nM FVIIa and saturating TF.

3 R. Jenny, personal communication.
TF, was not recovered. The recovery of this peptide was not anticipated, based on our hypothesis that this very hydrophobic peptide would not elute from the column under our conditions or would elute with CHAPS, which would greatly diminish the ionization and identification of this peptide. rTF1–243 was constructed and purified as described previously (8, 31). Supplemental Table 1 presents the cleavage sites, peptides generated, and hypothetical masses of the peptides (acquired from the ExPASy Proteomics Server). The table presents the locations of the glycosylated sites in TF as identified in our study. It also serves as a comparison between the hypothetical mass of a non-glycosylated peptide versus the carbohydrate composition and mass of the corresponding glycopeptide (Tables 4 and 5). All four potential sites for N-linked glycosylation containing the Asn-X-Ser/Thr/Cys motif (boldface letters), where X could be any amino acid except Pro, are found on distinct peptides. Only one site is in the cytosolic domain, Asn-261, whereas the remaining three sites are in the extracellular domain. The TF peptides recovered by MS and identified by Sequest search results are marked with numbers 1, 2, and 3, which correspond to peptides recovered in rTF1–243, rTF1–263, and pTF, respectively (supplemental Table 1). These peptides accounted for 77.4% of the amino acid sequence coverage for TF1–243, 73.7% for TF1–263, and 70.7% for pTF. Small peptides and single amino acids were identified as miscleaved products attached to the larger, neighboring peptide. For example, peptides SK and DVK were identified as miscleaved products on the peptide CFYTTDTECDLTDIVK. Fragment 10 in supplemental Table 1 was too large for the mass range used in our method. However, digestion of TF with GluC (marked with an asterisk) produced Sequest-identified fragments within the sequence of fragment 10. The addition of these GluC peptides

**FIGURE 4.** Extrinsic FXase assay (a) and stoichiometry of relipidated TF (b). a, varying concentrations of FX were incubated with FVIIa (5 nM) in complex with rTF1–243 (∗), rTF1–243D (○), rTF1–263G (∆), rTF1–263D (□), pTF (■), and pTFG (□) (0.1 nM). FXa generation was measured and calculated from the FXa standard curve (inset). b, the stoichiometry of relipidated TF was analyzed in the TF-FVIIa amidolytic assay. Varying concentrations of rTF1–263G (∆) and pTFG (□) were incubated with FVIIa (0.05 nM) in HBS, 0.1% polyethylene glycol, 2 mM CaCl2, pH 7.4, for 10 min. Fluorogenic substrate was added (50 µM), and the rate of substrate hydrolysis was recorded. The graph represents the calculated 1:1 stoichiometry of the TF-FVIIa complex. Each point is a mean of two experiments ± 1 S.D.
increased our amino acid coverage. Our inability to obtain 100% coverage is not surprising. We also hypothesize that the other fragments not identified by MS were too hydrophilic (fragment 17) and not retained by the C18 column, resulting in these peptides eluting from the column with the other non-retained species in the sample, such as the salt in the digestion buffer. Our experience has shown that non-retained peptides also experience poor ionization due to the coelution with the digestion buffer and a decrease in sensitivity due to an inability to be concentrated on the head of the column (data not shown).

Carbohydrate Analysis—To determine the presence of carbohydrates at potential glycosylation sites of TF proteins, reduced and alkylated peptides from both the G and D TF proteins were manually inspected using density maps. Supplemental Fig. 1 shows the density maps of rTF1–263G in A and rTF1–263D in B. A number of doubly or triply charged peptide ions observed in the density map of the G form disappeared from the spectrum of the D form, suggesting that these peptides were glycosylated.

To determine the composition of carbohydrates at potential glycosylation sites, the MS/MS spectra of ions seen in supplemental Fig. 1A but not in supplemental Fig. 1B were manually investigated. For example, supplemental Fig. 2 shows the MS/MS scan for an ion with m/z of 1,382 that appeared in rTF1–263G but not in rTF1–263D. Two sets of fragmentation ladders can be distinguished. One ladder represents the oxonium ions that fragment off of the glycopeptides during collision-induced dissociation and show up as a singly charged species (49, 58). Starting at mass 528, which has been shown to be an oxonium ion for N-glycans (58), we observed increasing mass steps of 162 up to a mass of 1500. The mass steps of 162 represent a loss in the number of Man on the glycan that start out as Man6GlcNAc1 (m/z = 1500) and end as Man6GlcNAc2 (m/z = 528). The second observed ladder represents the doubly charged glycopeptide that shows losses of Man starting at mass 1301, which represents a loss of 81 from the selected ion 1382. Mass steps of 81 continue down to m/z 734, representing a loss of eight Man residues. A mass step of 101.7 from m/z 734 to 632.3 represents the loss of a GlcNAc, which we initially hypothesize as coming from the chitobiose core. The loss of this GlcNAc suggests that m/z 632 is the doubly charged glycopeptide with only one GlcNAc, commonly referred to as the Y1 ion (59). The most intense product ion in the spectrum is twice the mass of m/z 632 and potentially represents the singly charged Y1 ion.

The MS/MS spectra of the D forms allowed for the identification of ions that were observed in the MS/MS spectra of the G forms. For example, Sequest data analysis of the rTF1–263D sample identified m/z 632 as the peptide VNVTVEDER with a GlcNAc. The mass change by GlcNAc molecule, which remains attached to an Asn residue after treatment with Endo F enzymes, acts as a marker designating specific glycosylation sites in the D sample. This mass is seen in the MS/MS spectrum for ion 1,382 in supplemental Fig. 2 and thereby identifies ion 1,382 as being a glycopeptide with amino acid sequence VNVTVEDER. Supplemental Fig. 3 shows an example of a hybrid glycopeptide with sialic acid (SA) found in the pTF sample. As in supplemental Fig. 2, the most abundant ion is 1264, and the doubly charged ladder ends at m/z 632, which indicates that the peptide is VNVTVEDER.

N-Glycan Composition of rTF1–263—Table 4 presents the carbohydrate composition of rTF1–263. Three of the four potential sites for N-linked carbohydrates were found to be glycosylated including Asn-11, -124, and -137. Because all N-linked carbohydrates contain a specific trimannosyl diacetylchitobiose core structure composed of -Asn-GlcNAc2Man3, we will refer to that structure as the “core” and will discuss the carbohydrate composition and heterogeneity of sugar molecules that extend from and do not include the core. Proteolytic fragment 1 generated by the tryptic digest of TF (supplemental Table 1), which includes Asn-11, shows that the carbohydrate composition is dominated by high Man sugars, with the greatest number of 3 Man residues attached to the core (m/z = 1,666, z = 2). The smallest modification is one GlcNAc molecule on the Asn (m/z = 915, z = 2). Two fucosylated species are found, one a
fucosylated core ($m/z = 1333, z = 2$) and the other a fucosylated GlcNAc on the Asn ($m/z = 988, z = 2$). The range of glycan masses on Asn-11 in rTF$_{1–263}$ is from 202 to 1,866 Da.

Similarly to Asn-11, Asn-124 is also modified with high Man glycopeptides with the highest number of 8 Man residues ($m/z = 1,625, z = 2$). The smallest glycopeptide is composed of one GlcNAc molecule attached to the Asn ($m/z = 633, z = 2$). Unlike Asn-11, this site lacks fucosylated glycans. This site is also modified by hybrid carbohydrates ranging from one HexNAc or Hex$_2$HexNAc$_2$ residues attached to the core ($m/z = 1078, z = 2$ and $m/z = 1342, z = 2$, respectively). We define HexNAc as either GlcNAc or GalNAc, both with a mass of 203.20 Da. We define hexose as either mannose, glucose, or galactose, all three with a mass of 162 Da. The range of glycan masses on Asn-124 in rTF$_{1–263}$ is from 203 to 2188 Da.

Asn-137 is found in both the proteolytic fragment resulting from trypsin digest as listed in supplement Table 1 and a mis-cleaved fragment where Arg-135 from fragment 12 is attached to fragment 13 (RAsn). Asn-137 is modified by both high Man and fucosylated structures. The biggest glycan is composed of 6 Man residues attached to the core ($m/z = 1,415, z = 2$ and $m/z = 996, z = 3$). The fucosylated glycopeptides range from a
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The range of glycan masses is from 1,241 to 3,285 Da. The smallest is a fucosylated core plus one HexNAc (Asn-124 in rTF1–263). The largest glycan is a sialylated structure from 1,611 to 3,666 Da. Asn-11 is modified by one GlcNAc attached to the core. The range of glycan masses on Asn-11 in pTF is from 203 Da to 3,666 Da.

N-Glycan Composition of pTF—Table 5 presents the carbohydrate composition of pTF. Similar to rTF1–263, the natural protein is glycosylated on Asn-11, -124, and -137 in the extracellular domain, whereas Asn-261 in the cytosolic region is not modified. High Man glycans, a predominant feature of rTF1–263, are absent on all three sites in pTF. Asn-11 is modified predominantly by complex carbohydrates. All carbohydrates on this site have a fucosylated core. The majority of the structures are siaiylated with a minimum of 1 and a maximum of 2 SA residues. The largest glycan on Asn-11 is composed of Hex3HexNAc5SA2 (m/z = 1,211, z = 3). The smallest glycan consists of a fucosylated core plus Hex3HexNAc3 (m/z = 1,619, z = 2). The remaining carbohydrates have a wide heterogeneous composition with varying numbers of Hex, HexNAc, and SA molecules. The range of glycan masses on Asn-11 in pTF is from 1,611 to 3,666 Da.

Asn-124 is modified by hybrid and sialylated complex carbohydrates. This site is not fucosylated, as was also observed for Asn-124 in rTF1–263. The largest glycan is a sialylated structure consisting of Hex3HexNAc3SA2 (m/z = 1,211, z = 3). The smallest is one Hex attached to the core (m/z = 1,058, z = 2). The remaining carbohydrate compositions are heterogeneous and vary in the numbers of Hex, HexNAc, and SA molecules attached to the core. The range of glycan masses on Asn-124 in pTF is from 1,055 to 2,571 Da.

The glycan composition of Asn-137 of pTF is similar to that of Asn-11. As observed in rTF1–263, the glycopeptides exist as the correctly cleaved peptide and a miscleaved peptide with one Arg residue attached to Asn-137. The miscleavage could be a result of the inaccessibility of this site to trypsin due to the presence of carbohydrates on Asn-137, which is next to the assigned cleavage site. All of the carbohydrates on this site contain a fucosylated core. The largest glycan is composed of a fucosylated core plus Hex3HexNAc3SA2 (m/z = 1,469, z = 3). The smallest is a fucosylated core plus one HexNAc (m/z = 1,183, z = 2). The diversity of the other carbohydrate structures is wide and found on both the correctly cleaved and miscleaved peptides either doubly or triply charged. On Asn-137 in pTF, the range of glycan masses is from 1,241 to 3,285 Da.

DISCUSSION

In this study, we compare the function and carbohydrate composition of rTF1–263, rTF1–263G, and natural pTF. The data presented show that pTF is ~5-fold more active than any rTF, and a significant cause can be attributed to glycosylation of the protein. After deglycosylation, natural human pTF is virtually equivalent in activity to deglycosylated rTF1–263. It has been demonstrated that rTF is posttranslationally modified by N-linked glycosylation (12); however, the contribution of the carbohydrates to natural TF activity is controversial. It has been suggested that the activity of purified TF from bovine brain is inhibited by concanavalin A (37). It has also been observed that treatment of murine macrophages with tunicamycin inhibits TF activity in FXa generation (36). It has also been suggested that carbohydrates are not important for TF activity (30–34). These suggestions possibly arise from observations that the non-glycosylated recombinant forms are functional, as observed in this study for rTF1–263C1. One study compared the activity of truncated, soluble, recombinant forms of TF that do not interact with membrane and suggested that the activity is not carbohydrate-dependent (35). That observation is in line with our data where carbohydrates do not affect the activity of TF protein in a membrane-independent interaction (fluorogenic assay). A previously described mutagenesis study involving C186S and C209S demonstrated a decrease of activity in the mutant protein (34). The mutation also abolished the ability of the cell to fully glycosylate the protein, which could be a contributing factor in the decreased activity.

Despite the general consensus that TF proteins from different sources show heterogeneity in carbohydrate composition, based on their ability to bind concanavalin A (60), the extent and the type of glycans have not yet been studied. In the present study, we determined that N-linked carbohydrates modify both rTF and natural pTF. Three of the four potential sites for N-linked glycosylation, Asn-11, Asn-124, and Asn-137, contain glycans. An example of the analysis was shown in supplemental Figs. 2 and 3, where one can see the difference in MS/MS spectra between a high Man glycan attached to Asn-124 on rTF1–263 (Fig. 2) and a complex glycan attached to Asn-124 on pTF (Fig. 3). This type of MS analysis is limited to the glycan composition and cannot be used to define the structure of the glycans. Glycan structure analysis is best carried out by releasing the glycan from the protein, followed by permethylation and MS" analysis (61–63). Our goal in this study was to detect and define any differences in the type of glycans attached to each site that is glycosylated. The MS analysis we performed is consistent with previously published work that focused on similar goals (49, 64).

Our analysis could only identify N-linked glycans and not O-linked carbohydrates. The enzymes we used to remove carbohydrates from the protein do not remove O-linked carbohydrates. Hence, O-linked glycopeptides would remain unchanged in the D samples and would not be highlighted by their apparent disappearance, as was the case for N-linked carbohydrates (see supplemental Fig. 1). However, earlier work has found no evidence of O-linked carbohydrates (30). All oligosaccharides, thus studied, have a typical trimannosyl diacytethylchitobiose core composed of Hex3GlcNAc2 found in N-linked carbohydrates. Some carbohydrates contain a fucosylated core on the Asn-linked GlcNAc, and in the case of pTF, SA may be present.

We have chosen to convey the heterogeneity of glycosylation at each site for each protein by describing the composition of the glycan and the glycopeptide mass for rTF1–263G, and natural pTF (Tables 4 and 5, respectively). An earlier report by Jiang et al. (65) suggests that there will be a bias for detecting glycopeptides without SA over glycopeptides with SA when using electrospray ionization in positive ion mode. This bias would probably extend to the non-glycosylated form of the glycopeptide as well. The possible bias in detecting the non-glycosylated pep-
tides and glycopeptides without SA does not detract from our ability to define the differences in glycan composition between the two sources of TF. Further work is required to define how much of a bias exists in ionization efficiency between non-fucosylated, neutral, and SA-containing glycopeptides when using electrospray ionization in positive ion mode.

Three main differences exist between the carbohydrate composition of rTF1–263 and pTF. First, the carbohydrate composition of rTF1–263 predominates in high Man glycans on all three sites. In contrast, carbohydrates of pTF are highly processed and are completely devoid of high Man sugars. Hybrid structures composed of only Man and HexNAc molecules are found mainly on Asn-124 in pTF and to some extent on Asn-124 in rTF1–263. Interestingly, neither the recombinant nor the natural form of TF is fucosylated at this site, which is the only prominent feature that the two TFs share. The second main difference is the presence of SA on all three glycosylated sites of pTF including Asn-11, Asn-124, and Asn-137, a unique feature of the natural pTF. This sugar is present on both fucosylated (Asn-11 and Asn-137) and non-fucosylated (Asn-124) cores and is absent from rTF1–263. The inability of insect cells to sialylate carbohydrates was previously reported (66). Third, fucosylation is much more prevalent in pTF than in rTF1–263 where the majority of sugars contain a fucosylated core on Asn-11 and Asn-137. The overabundance of high Man glycans in rTF1–263 dominates over the few fucosylated glycopeptides in the recombinant protein. The range of glycans masses in TF is quite diverse in both the recombinant and natural form, with an extensively wider and higher numerical range in pTF.

As determined from the crystal structure of TF, generated with the extracellular domain of TF, two sites, including Asn-11 and Asn-137, are solvent-exposed, whereas Asn-124 is buried on the C-module close to the membrane (32, 67). Asn-137 is located within a region containing residues that are part of the extensive region of FVIIa ligand binding and include Arg-135, Leu-133, and Phe-140. Mutational analysis of Arg-135 and Phe-140 revealed that mutation of these residues results in a decreased ligand affinity but does not alter its catalytic function toward a small substrate (68). These data may support our observation where removal of the sugar moiety does not alter the catalytic efficiency of the complex, as demonstrated by the unaltered ability of FVIIa to cleave the fluorogenic substrate when complexed with either the G or D form of TF. The location of Asn-124 in the structure of TF renders it potentially important in the recognition and binding of FX. Because the residue is located close to the membrane and is in close proximity to the Cys-186–Cys-209 disulfide, where the interaction of TF and FX takes place, glycosylation on that residue of TF may influence its function in macromolecular substrate recognition (67).

Although contradictory reports exist as to the affect of glycosylation on the activity of TF protein, we have shown that carbohydrates significantly influence natural TF function. The unique carbohydrate compositions render the natural human protein different from the recombinant counterpart with respect to glycosylation. A common feature of pTF with other coagulation proteins, such as FV, anti-thrombin III, tissue factor pathway inhibitor, thrombin-activatable fibrinolysis inhib-
