Kringle Glycosylation in a Modified Human Tissue Plasminogen Activator Improves Functional Properties

By David T. Berg, Philip J. Burck, David H. Berg, and Brian W. Grinnell

Native tissue plasminogen activator (ntPA) has a variable glycosylation site on its kringle-2 domain. We have examined the effects of kringle glycosylation on functional properties by studying the simplified tPA molecule, tPA-6. tPA-6 is composed of kringle-2 and the serine protease domains and, like ntPA, cells expressing tPA-6 process it into two glycoforms: the monoglycosylated tPA-6-primary form, plasmin, another serine protease that degrades fibrinogen when compared to purified tPA-6P. However, in the presence of fibrin, tPA-6V unexpectedly exhibited a sixfold increase in selectivity toward Lys-plasminogen. In addition, tPA-6V was less susceptible than tPA-6P to plasmin-mediated conversion to the two-chain form. By site-directed mutagenesis of tPA-6, we eliminated variable glycosylation at Asn-184 and engineered a new glycosylation signal at a remnant site in the kringle. This derivative, designated tPA-6D, was secreted with complete kringle glycosylation. Like the naturally occurring tPA-6V, tPA-6D had lower rates of fibrin-stimulated Glu-plasminogen activation, increased specificity toward Lys-plasminogen, and greater resistance to plasmin digestion. Although the activity of tPA-6D could be stimulated by fibrin, its activity was not stimulated significantly by fibrinogen, and in human plasma the rate of fibrinogen depletion was reduced threefold. Although fibrin binding to kringle-2 of tPA-6D was slightly improved, there was a substantial increase in the dissociation constant (kd) for lysine binding, demonstrating a lack of correlation between these ligand-binding sites. Overall, our data demonstrate the marked effect of kringle glycosylation on functional properties. In addition, we have generated a derivative with properties that potentially improve clot specificity and single-chain half-life and reduce the potential for plasminogen activation in the plasma.

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to ntPA, but tPA-6 is of potential clinical interest because of its superior properties in the canine model of coronary artery thrombosis.16,17 Like ntPA, tPA-6 exists in two glycoforms differing in occupancy of glycosylation site 184. The monoglycosylated form, tPA-6P, is glycosylated only at Asn-448 in the serine protease domain. The diglycosylated form, tPA-6V, which is present in about 20% to 25% of the total secreted tPA-6, is glycosylated at Asn-448 as well as at Asn-184 in the kringle-2 domain. To more fully understand the role of glycosylation of kringle-2, we analyzed the in vitro biochemical properties of monoglycosylated and diglycosylated tPA-6 and a derivative that was engineered for secretion from mammalian cells as a homogeneous population of diglycosylated molecules. We show that the diglycosylated forms exhibit altered enzymatic activities, substrate specificity, and ligand-binding properties. In addition, we show that the engineered derivative has properties that are desired of an ideal fibrinolytic agent.

Fig 1. Structure of tissue plasminogen activator glycosylation derivatives. (A) schematic representation of the native tPA and domain-deletion derivative tPA-6. The functional domains are indicated with abbreviations as follows: EGF, epidermal growth factor homology; K1, kringle-1; K2, kringle-2. The sites of N-linked glycosylation are indicated by the inverted “Y” along with the percentage of secreted molecules with a glycosyl residue attached. (B) Diagram of the separated tPA-6 glycoforms indicating the sites of N-linked glycosylation and the percentage of molecules in the purified preparation with a glycosyl residue attached. (C) Diagram of derivative tPA-6D showing the amino acid changes made to eliminate the variable glycosylation site at amino acid 184 and the addition of Gly (G) to complete the remnant site at N205.

MATERIALS AND METHODS

Materials

The AV12-664 hamster tumor cell line is available from the American Type Culture Collection (CRL 9595). Restriction endonucleases were purchased from Boehringer-Mannheim (Indianapolis, IN), Bethesda Research Laboratories (Bethesda, MD), or New England Biolabs (Beverly, MA). T4 DNA ligase was from New England Biolabs. Biotinylated rabbit anti-goat IgG and the Vectastain avidin-biotin complex reagent were obtained from Vector Laboratories (Burlingame, CA). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL). Lysine-Sepharose and Sepharose 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Goat anti-human melanoma tPA IgG, Spectrolyte t-PA Activity and Inhibitor Assay Kit, Pam-1 on Sepharose 6 FF, human Glu-plasminogen, human Lys-plasminogen, PAI-1, and PAI-1-deficient human plasma were all obtained from American Diagnostica Inc (Greenwich, CT). The antibodies used for Western blot analysis and the fibrin/fibrinogen stimulators DESARB, CNBr fibrinogen digest, and FCB-2 were also obtained from American Diagnostica. The Mutagen Phagemid in vitro mutagenesis kit was purchased from Bio-Rad Laboratories (Richmond, CA). Human plasmin was from Boehringer-Mannheim. Aprotinin and amethopterin are Sigma Chemical Co (St Louis, MO) products. Cell culture media was from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). The synthetic substrate S2288 was purchased from KABI (Möln达尔, Sweden). All other reagents and materials were of the highest quality available.

Construction of tPA Variants

The recombinant cDNA for ntPA and tPA-6 was the same as that described previously.16 The nucleotide numbering system was that of Pennica et al,18 and the exons were numbered as described by Ny et al.19 All restriction endonuclease and ligation reactions were carried out following conditions recommended by the manufacturers. Oligonucleotide-directed site-specific and deletion mutagenesis experiments were performed using the Mutagene Phagemid in vitro Mutagenesis kit that is based on the method described by Kunkel et al.20,21 The oligonucleotides used for mutagenesis were designed with silent mutations that created unique restriction sites that were used for identifying clones that had received the mutagenic oligonucleotide. Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) DNA Synthesizer, Model 380 B.

Glycosylation site-derivative tPA-6D was made by introducing mutations into the 1375-bp BamHI fragment of the tPA-6 cDNA16 that had been subcloned into the BamHI site of phagemid pTZ19U to make template pTZ-6. The sequence giving rise to variable glycosylation at N184 was first mutated using the oligonucleotide N184Q (5'-CAGTGACTGCTACTTTGGCCAGGGGTCAGCCTACCGTGCCTCCCATGGA ATGGGTCCATGATCCTGATAGGC -3'). The resulting plasmid was called pTZ-N184Q. For tPA-6D, a new N-linked glycosylation consensus sequence was then made by inserting a codon for glycine between N205 and S206 using the template pTZ-N184Q and the mutagenic oligonucleotide G206 (5'-GGGTGCCT- CCTGCTTCCATGGAATG GGGTCATGTCCTGATAGGC-3'). After each round of mutagenesis, the inserts were sequenced to verify the presence of the desired mutation. The mutated fragment was then recovered and used for the cloning of expression vectors.

Vector Construction and Expression

Expression vector construction was preceded by cloning the BglII to Xmal restriction fragment of the tPA cDNAs into the vector pLP53-tLB. This step exchanged the native signal peptide and propeptide for a sequence that we have shown to be more efficiently processed.
by the cells in which the mutants were expressed. The tPA variants with their new 5' ends were then isolated as BamHI to XmaI restriction fragments from pLP53-LB-x (where x = the tPA variant fragment) for cloning into expression vectors. The tPA-6 and tPA-6D proteins expressed through this construct were each proteolytically processed as a homogeneous population beginning with serine +1.

The expression vectors were constructed from the following DNAs beginning at the EcoRI site in pBR322 and proceeding counterclockwise: the EcoRI to Acl restriction fragment of pBR322 containing ampicillin resistance and the origin of replication (pBR322 nucleotides 4363 to 2297); the Acl to Stul restriction fragment of BKV-P212 (BKV-P2 nucleotides 4339 to 5122); the poly GT-containing linker comprising 20 GT repeats and BamHI ends that were treated with the Klenow fragment of Escherichia coli DNA polymerase I and ligated to the Stul of BKV-P2; the Stul to Klenow-treated Acl restriction fragment of BKV-P2 that contains the BKV-P2 enhancer (BKV-P2 nucleotides 5122 to 5175 and 1 to 366); the adenovirus 2 major late promoter optimally spaced to the BKV-P2 enhancer for the most efficient transcription (adenovirus 2 nucleotides 5931 to 6071); a synthetic linker consisting of the spliced sequence of the adenovirus tripartite leader; a BclI linker; the 610-bp MboI fragment of SV40 containing the small-t-antigen splice site and the polyadenylation signal; the blunt-ended BamHI restriction fragment of pSV2-dhfr (ATCC no. 37146) containing the murine DHFR coding sequence and the SV40 early promoter/enhancer, small t-antigen splice site, and polyadenylation signal.

For DNA transcription, the adenovirus-transformed AV1-2-664 cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and transfected as described previously. Three days after transfection, culture medium was replaced with selection medium. The tPA variants expressed through this construct were each proteolytically processed as a homogeneous population beginning with serine +1.

Enzymatic Activity Assays

Direct amidolytic assay. The ability of the tPA-6 glycoforms to directly cleave a synthetic chromogenic substrate was performed with an amidolytic assay. The assay was performed in 96-well microtiter plates in a volume of 200 μL using the chromogenic substrate S2288 in a buffer consisting of 100 mmol/L NaCl, 20 mmol/L tris-hydroxymethylaminomethane (TRIS) pH 7.4, and 0.1% Tween 80 and 100 μg/mL BSA. BSA was added to reduce nonspecific binding of tPA to the polystyrene microtiter plates. Results are presented in amidochemical units (AU) that are defined as the amount of plasminogen activator required to release 1 μmol of p-nitroanilide in 1 minute at 24°C in 405 nm in a 1-cm path length, using an extinction coefficient of 9629 mol/L cm⁻¹ for p-nitroanilide.

Indirect amidolytic assay for plasminogen activation. Plasminogen activity was determined by an indirect assay using the American Diagnostica Spectrolyse tPA Activity and Inhibitor Assay Kit. The Spectrolyse tPA assay is based on the functional parabolic rate assay described by Ranby et al. The manufacturer's recommended procedure was modified for our microtiter plate assay as follows: The reaction volume was reduced twofold and instead of an endpoint assay, the optical density (OD₂₅₀) was read at 3- or 5-minute intervals over a 5-hour period by a Molecular Devices (Menlo Park, CA) Thermomax Kinetic microplate reader. The rates of plasmin generation were determined from the slopes of the derivative of plots of OD versus time and expressed as mOD/min⁻¹. As described previously, the derivative curve displayed the lag phenomena characteristic of this assay, in which the increase in absorbency at 405 nm was linear with reaction time only after an initial lag phase. The rates were determined from the second more active phase of the reaction. Plasminogen activity was determined using Glu-plasminogen or Lys-plasminogen and defined as rate of plasmin generation per milligram (mOD/min²/mg).

Deglycosylation of tPA Derivatives

Peptide-N4-(N-acetyl-β-glucosaminyl)asparagine amide, E.C. 3.4.1.52 (N-Glycanase), was obtained from Genzyme Corp (Boston, MA) and used to hydrolyze the asparagine-linked oligosaccharides from the tPAs using the following reaction conditions: 200 μg of each protein was digested at 37°C for 16 hours in a 0.3-mL reaction containing 30 U/mL N-Glycanase, 200 KIU/mL aprotinin, and 40 mmol/L NaPO₄. The extent of oligosaccharide removal from the glycoproteins was monitored electrophoretically using 12% SDS-PAGE under reducing conditions as described by Tarentino et al. The single-chain tPA form for each deglycosylated variant was then repurified using the PAM-1-Sepharose tPA MoAb column from American Diagnostica as described in the Purification section.

Effect of Variant Plasminogen Activators on Plasma Fibrinogen Levels

To determine the effect of the tPAs on plasma fibrinogen, 5 μL of an 80 μg/mL solution of each tPA in a buffer consisting of 50 mmol/L TRIS, pH 8.0, and 250 mmol/L NaCl plus 25 μL of PBS was incubated with 50 μL of human plasma for various times. The level of fibrinogen in the plasma was determined by the change in clotting time upon addition of 10 μL of 3 U/mL bovine thrombin (Miles...
Laboratories, Naperville, IL) essentially by the method of Clause. The effect of the tPAs on plasma fibrinogen during clot lysis was performed by adding a clot, formed from 25 μL of human plasma and washed in 50 mmol/L TRIS, pH 8.0, 150 mmol/L NaCl, 1 mg/mL BSA, to 200 μL of human plasma plus varying concentrations of each tPA. The level of fibrinogen remaining was determined from 50-μL aliquots as above.

Lysine and Fibrin Binding

Binding of the tPA derivatives to lysine was performed by incubating each tPA with varying amounts of lysine-Sepharose (Pharmacia) in Falcon 2063 tubes in 50 mmol/L TRIS–HCl pH 7.4; 150 mmol/L NaCl, 0.01% Tween 80 in a volume of 0.5 mL. The lysine concentration ranged from 0 to 1600 mmol/L, whereas the tPA concentration was held constant at 100 nmol/L. To eliminate any error caused from nonspecific binding to the Sepharose beads, equivalent levels of the Sepharose beads were maintained in each tube through addition of normal Sepharose 4B (Pharmacia). The tPA was incubated with the beads for 16 hours at 4°C on a clinical rotator. After pelleting the lysine-Sepharose and Sepharose 4B, the unbound tPA present in the supernatant was determined in an amidolytic assay using the synthetic substrate S-2288 and a Molecular Devices Thermomax Kinetic microplate reader. The specific activity for each variant was used to calculate the micrograms of tPA remaining unbound. Specific activities for the controls were compared before and after the incubation period under the same conditions as other samples to account for any changes in activity with the chromogenic substrate that might be caused by incubation. Dissociation rate constants and the number of binding sites were determined by Enzfitter (Elsevier Biosoft, Cambridge, UK), a weighted nonlinear, least square regression analysis program.

Fibrin-binding properties of the tPA variants were determined as described previously. Briefly, tPA at 180 mmol/L was incubated alone or with increasing concentrations of fibrin from 0.08 to 5 μmol/L in buffer containing 0.01% Tween 80, 150 mmol/L NaCl, 20 mmol/L TRIS pH 7.4 and 100 μg/mL BSA. The amidolytic activity was determined as described above, and the fibrin concentration was plotted versus the stimulated rate minus the nonstimulated rate at each fibrin concentration. The dissociation constant (kd) was determined as the concentration of fibrin that gave half-maximal stimulation as determined by nonlinear regression analysis.

Sensitivity of Plasminogen Activators to Plasmin Cleavage

Limited plasmin digestion was performed in multiple experiments with plasmin concentrations ranging from 0.6 to 11 nmol/L and tPA concentrations ranging from 120 to 200 nmol/L in a buffer comprising 50 mmol/L TRIS–HCl pH 7.4, 150 mmol/L NaCl, 0.01% Tween 80, and 100 μg/mL BSA in a volume of 0.6 mL. For each set of experimental conditions, the enzyme-substrate ratio was critically controlled for comparison among the mutants. The reactions were initiated by the addition of plasmin. At each time point, 95-μL aliquots were removed to microtiter wells containing 20 μL of a 200-KIU solution of aprotinin that quenched further plasmin activity. Negative controls (time 0) contained no plasmin and concentrations of tPA identical to the experimental levels. Complete plasmin digestion to the two-chain molecule was accomplished in simultaneous reactions containing 10 times the concentration of plasmin used for that experiment. The substrate S-2288 was used to monitor the progress of the reaction. From these data, the amount of tPA converted to two chain per minute was calculated. The linear data were plotted as micromoles of tPA converted to two chain versus the time of incubation with plasmin, and the initial velocity, v, was calculated from the resulting slopes. The second-order rate constant, kcat/Km, for each tPA was derived as previously described.

Clot Lysis Activity of Variant Plasminogen Activators

The ability of the tPAs to lyse clots in human plasma was determined using a modification of the microtiter plate method of Beebe and Aronson. Fifty microliters of assayed human reference plasma (Helena Laboratories, Beaumont, TX) was mixed with 50 μL of Helena APTT reagent and 30 μL of various concentrations of the variant plasminogen activators in a buffer consisting of 0.001 N HCl, 0.01% Tween 80, 50 mmol/L TRIS, pH 8.0, 250 mmol/L NaCl. Following incubation for 5 minutes at 37°C, the clot was initiated by the addition of 25 μL of 100 mmol/L CaCl2. The formation and subsequent dissolution of the clot were monitored by changes in absorbency at 405 nm read every 6 seconds using a Thermomax ( Molecular Devices) plate reader and SoftMax software for the Macintosh (Cupertino, CA) computer. Rates of clot lysis were determined from the slopes of the decay curve and correlated (r = .992) with the time to 50% clot lysis. Relative activities generated in this assay correlated to those generated previously with tPA-6 and ntPA by the Chandler loop protocol.

RESULTS

tPA-6 Variants

Figure 1A shows a schematic diagram of ntPA and the deletion derivative tPA-6. tPA-6 contains two sites that we have shown to be glycosylated, one in the serine protease domain that is always glycosylated and one in the kringle domain that is glycosylated on only 20% to 25% of the secreted molecules. Thus, as previously shown, two major proteins can be detected in conditioned culture medium from recombinant mammalian cells, displaying apparent molecular masses of about 40 and 42 Kd, representing the type II monoglycosylated tPA-6P and type I diglycosylated tPA-6V, respectively.

To study the role of glycosylation of the kringle on the simple plasminogen activator tPA-6, we separated the two glycoforms (represented schematically in Fig 1B) by differential lysine-Sepharose chromatography. In addition, by site-directed mutagenesis, we created tPA-6D, a derivative that was expressed with complete glycosylation of the kringle. Shown in Fig 1C are the site-directed changes in amino acids used to generate tPA-6D. The construction of tPA-6D began by disrupting the native consensus site that gives rise to variable glycosylation at Asn-184 in kringle-2 by exchanging the codon for asparagine at position 184 to glutamine. In the subsequent round of mutagenesis, a codon for glycine was inserted between amino acid codons N205 and S206, thus creating the glycosylation signal N-G-S in the protein. This glycine insertion reconstitutes an apparent remnant site in kringle-2, as N205 is in the same relative position in kringle-2 as is N117 of the fully glycosylated site in kringle-1.

The cDNAs for the tPAs were inserted into a multicistronic vector, introduced into AV 12-664 cells, and stable transformants were selected as described under the Materials and Methods section. The levels of tPA-6 protein secreted from the stable cell lines growing in serum-free medium in confluent 75-cm2 flasks ranged from 3 to 20 μg/mL/d. There were no differences in secretion rates between tPA-6D and tPA-6. AV 12-664 clones producing high levels of tPA-6 and tPA-6D were isolated for further analysis of the secreted protein.
The tPA-6 derivative was purified and subjected to SDS-PAGE under reducing conditions as described under the Materials and Methods section and is shown in Fig 2A, lane 3. As indicated above, the material secreted from mammalian cells consists of a primary ∼40-Kd band designated tPA-6P and a minor ∼42-Kd band designated tPA-6V. The size difference was previously shown to be due to the difference between one versus two sites glycosylated by amino acid and carbohydrate analyses. The monoglycosylated tPA-6P and diglycosylated tPA-6V were separated by lysine-Sepharose chromatography and are shown in lanes 1 and 2, respectively. The secreted glycosylation site derivative, tPA-6D, was purified from conditioned cell culture medium by immunoaffinity chromatography, which does not separate monoglycosylated from diglycosylated tPA-6 and was compared with purified tPA-6. As shown in Fig 2B, tPA-6D (lane 1) comigrated with the diglycosylated tPA-6V glycoform (lane 2). Western blot analysis of the conditioned medium from tPA-6D–secreting cells detected no monoglycosylated band. Following deglycosylation of the tPAs with N-glycanase, tPA-6V, tPA-6P, and tPA-6D comigrated at ∼37 Kd, the expected size of the nonglycosylated tPA-6 form (data not shown). These data indicated that the insertion of Gly into the remnant glycosylation site resulted in complete kringle glycosylation. Thus, this region of the kringle appears to be completely accessible for the transfer of the glycosyl core.

Enzymatic Activity of tPA-6 Glycoforms

By titration with dansyl-L-Glu-Gly-Arg chloromethylketone, each glycoform contained an intact serine protease domain. The ratio of active site per mole of the tPA-6 ranged from 0.73 to 0.92 among different preparations of each purified single-chain form. The rate of plasminogen activation with each derivative was determined by the indirect plasmin generation assay using both single-chain (one-chain) protein as well as two-chain protein (prepared by plasmin digestion as described under the Materials and Methods section). As shown in the example with tPA-6P and tPA-6V in Fig 3, there was little difference in the amount of total plasmin generated between the single-chain monoglycosylated and diglycosylated forms.

**Fig 3.** Determination of plasminogen-activating (PA) activity of one-chain and two-chain tPA-6V or tPA-6P. The tPA samples (0.4 µg/mL) were incubated with chromogenic plasmin substrate Spectrozyme PL and 40 µg/mL Glu-plasminogen, and the amount of plasmin generated was determined by changes in absorbency at 405 nm every 5 minutes in a Thermomax Kinetic microplate reader.
The ability of fibrin to stimulate plasminogen activation by the variant forms of tPA-6 was assessed using soluble fibrinogen (DESAFIB) or cyanogen bromide digests of fibrinogen (Table 2). Using Glu-plasminogen as substrate, the activity of each of the tPA glycoforms could be stimulated by fibrin, but the degree of stimulation was ∼10-fold less with both diglycosylated tPA-6V and tPA-6D. This trend is consistent with the results of Wittwer et al., who found that the type II glycoform of ntPA (which similarly lacks glycosylation at Asn-184 in kringle-2) had 2.2 to 2.4 times the fibrin-stimulated plasminogenolytic activity of the type I glycoform of ntPA (which has the glycosylation at Asn-184). However, the effect of glycosylation on fibrin stimulation appears to be more pronounced in our derivatives, which lack the fibrin-binding finger domain present in ntPA. Although the fibrin-stimulated activities of the diglycosylated derivatives were essentially the same, the fold increase in activity in the presence of fibrin was substantially lower for tPA-6D owing to its higher activity in the absence of fibrin. As described under the Materials and Methods section, activity for all the glycoforms was calculated from the slopes of the derivative plots made during the second more active phase of the assay, which followed the characteristic lag phase. The diglycosylated tPA-6 variants, tPA-6V and tPA-6D, showed a significantly longer lag phase than monoglycosylated tPA-6P; indicating a lower plasminogenolytic activity for the diglycosylated variants during the initial lag as well as during the linear portion of the assay (data not shown).

We also compared the fibrin-stimulated and unstimulated activities of the tPA derivatives using Lys-plasminogen as a substrate. As has been observed with ntPA, Lys-plasminogen was much more readily activated to plasmin in the absence of fibrin by each derivative than was Glu-plasminogen (Table 2). In the presence of fibrin stimulator, we observed no significant difference in the activity of tPA-6P with each substrate. However, the diglycosylated molecules, tPA-6V and tPA-6D, had fivefold to sixfold more activity with Lys-plasminogen as the substrate. Similar results also were obtained using CNBr-digested fibrin as a stimulator. Our data demonstrate an increase in selectivity for Lys-plasminogen with natural kringle glycosylation (tPA-6V), which also can be achieved by engineering a glycosylation site at a different location in the kringle (tPA-6D). Glu-plasminogen is the predominant circulating form of the molecule, whereas Lys-plasminogen makes up 50% of the plasminogen in a clot.

### Table 1. Comparative Enzymatic Activities of One- and Two- Chain tPA-6 Glycoforms

<table>
<thead>
<tr>
<th>Plasminogen Activator</th>
<th>Amidolytic Activity (AU/mg)</th>
<th>PA Activity (mol OD/min/mg)</th>
<th>PA/Amidolytic Activity (Relative to tPA-6P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPA-6P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One-chain</td>
<td>1.3 ± 0.1</td>
<td>9.5 ± 1.5</td>
<td>7.3 (1)</td>
</tr>
<tr>
<td>Two-chain</td>
<td>5.7 ± 0.9</td>
<td>66 ± 4</td>
<td>11.6 (1)</td>
</tr>
<tr>
<td>tPA-6V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One-chain</td>
<td>1.5 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>5.4 (0.74)</td>
</tr>
<tr>
<td>Two-chain</td>
<td>7.2 ± 1.1</td>
<td>57 ± 2</td>
<td>7.9 (0.7)</td>
</tr>
<tr>
<td>tPA-6D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One-chain</td>
<td>0.6 ± 0.05</td>
<td>21 ± 4</td>
<td>35 (4.8)</td>
</tr>
<tr>
<td>Two-chain</td>
<td>3.5 ± 0.6</td>
<td>82 ± 3</td>
<td>23 (2.4)</td>
</tr>
</tbody>
</table>

* AU, amidolytic activity unit. The amidolytic activity was determined with synthetic peptide substrate S2288. One unit is defined as the amount of plasminogen activator required to release 1 μmol of p-nitroanilide in 1 minute at 24°C at 405 nm in a 1-cm path length using an extinction coefficient of 9629 mol/L·cm·1 for p-nitroanilide. The values reported are the mean ± SD of data from five separate experiments each run in duplicate or triplicate.

† The plasminogen activation activity was determined using Glu-plasminogen. The level of plasmin generated was determined with the Spectrozyme chromogenic substrate (American Diagnostica) as described in the text. The values reported are the mean ± SD of data from three or four separate experiments.

‡ Ratio of specific amidolytic activity to specific Glu-plasminogenolytic activity. The number in parentheses is the relative ratio compared to the respective one-chain or two-chain tPA-6P value.

### Table 2. Activities of tPA-6 Glycoforms With Glu- and Lys-Plasminogen in the Presence or Absence of Fibrin

<table>
<thead>
<tr>
<th>Plasminogen Activator</th>
<th>Glu-Plasminogen</th>
<th>Lys-Plasminogen</th>
</tr>
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<tbody>
<tr>
<td>PA Activity (mol OD/min/mg)</td>
<td>− Fibrin</td>
<td>+ Fibrin</td>
</tr>
<tr>
<td>tPA-6P</td>
<td>9.5 ± 1.8</td>
<td>1,008 ± 164</td>
</tr>
<tr>
<td>tPA-6V</td>
<td>8.1 ± 0.1</td>
<td>115 ± 16</td>
</tr>
<tr>
<td>tPA-6D</td>
<td>21 ± 5</td>
<td>116 ± 12</td>
</tr>
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</table>

* The determination of PA activities were as described in Table 1. The fibrin stimulator was DESAFIB.
The above data, therefore, suggest that kringle glycosylation may result in greater specificity for the clot.

Although having a different glycosyl position in kringle-2 and two amino acid site changes, the engineered tPA-6D displayed essentially the same enzymatic properties as the natural kringle glycosylated tPA-6V. This strongly suggests that the observed differences in enzymatic properties of tPA-6D compared to tPA-6P were due to the kringle glycosylation. However, as an additional control, we tested the deglycosylated forms of each tPA. We found that the differences in fibrin-stimulated activities and the selectivity for substrate were no longer evident; i.e., the deglycosylated forms all had essentially the same activities with the respective substrates and were no longer selective for Lys-plasminogen. Thus, as indicated above, the differences observed with tPA-6D appear to be due to the glycosylation, not to the slight differences in amino acid sequence at the glycosylation sites.

**Effect of the tPAs on Plasma Fibrinogen**

The data in Table 2 show that each of the tPA-6 glycoforms' plasminogenolytic activity is stimulated by fibrin just as with ntPA. Although considered to be a relatively clot-specific protease, patients treated with tPA have demonstrable decreases in systemic fibrinogen as a result of activation of the circulating plasminogen. We determined relative fibrinogenolytic activities in vitro by measuring the fibrinogen levels in plasma incubated for various times with each tPA. As shown in Fig 4A and B, using levels of 23 nmol/L and 119 nmol/L of each tPA, respectively, tPA-6D was significantly less fibrinogenolytic compared to the other tPA-6 molecules and to ntPA used as a control. We determined the concentration of each tPA required to degrade 50% of the fibrinogen in 60 minutes to be 201 ± 30 for tPA-6D, 69 ± 17 for tPA-6V, 67 ± 14 for tPA-6P, and 59 ± 19 for ntPA. We also determined the effect of each tPA on fibrinogen degradation during clot lysis in human plasma, and we also observed that approximately three times more tPA-6D was required to obtain a similar degree of fibrinogen degradation compared with the other molecules. Even when using the more active two-chain tPAs, tPA-6D still did not deplete fibrinogen as fast as the other derivatives (data not shown).

We speculated that the reduced rate of fibrinogen depletion with tPA-6D could be the result of differences in the stimulatory effect of fibrinogen on this derivative. To test this, experiments were performed comparing the plasminogenolytic activities of the tPA-6 glycoforms using Glu-plasminogen in the presence of fibrinogen. As shown in Fig 5, the plasminogenolytic activities of tPA-6P and tPA-6V were stimulated sevenfold to eightfold by fibrinogen; however, the activity of tPA-6D was stimulated only ~80%. Therefore, it appears that the reduced fibrinogen depletion with tPA-6D is caused by a reduced stimulatory effect of fibrinogen on its plasminogen-activating activity.

**Clot Lysis Activity of the tPA Variants**

The ability of the tPA variants to lyse human clots was determined by a modification of the microtiter plate assay previously described.32 For these determinations, each tPA was incorporated into the clot during its formation and the subsequent rate of clot lysis was determined from decay curves. We used ntPA as a control. As shown in Table 3, the diglycosylated derivatives had slower rates of clot lysis at a concentration of 50 nmol/L and the rate with tPA-6P was lower than that observed for ntPA. However, at a concentration of 200 nmol/L, all of the derivatives displayed comparable activities to each other and were much closer to the activity of ntPA. Thus, the engineered diglycosylated derivative was capable of lysing human clots and at rates similar to the natural diglycosylated tPA-6V.

**Association of Variant tPAs With Lysine and Fibrin**

The kringle-2 domain of tPA contains both a lysine-binding and fibrin-binding site. To determine if the alterations in
koglobin glycosylation pattern affected the binding properties, the kds for lysine and fibrin were determined. As shown in Table 4, each of the tPAs bound lysine with varying affinities. When compared with ntPA with a determined kd for lysine of 119 (similar to the literature value of ~100), the tPA-6 glycoforms had reduced affinities ranging in magnitude from ~1.8-fold to 9-fold. The data suggested one binding site per molecule for the derivatives (n values of 0.7 to 1.2). Also shown in Table 4 are the kd values for fibrin binding to kringle-2. Each of the tPAs had values of n > 1.0. These fibrin-binding data are essentially identical to those previously ascribed to the lower-affinity fibrin-binding site on kringle-2 of ntPA. Interestingly, the differences in lysine-binding affinity among the derivatives did not correlate to the differences in fibrin-binding affinities. Most notably, tPA-6D had a slightly higher fibrin-binding affinity but the lowest affinity lysine-binding site. The data with these different glycoforms show no positive correlation between lysine-binding and fibrin-binding activities.

**Sensitivity of tPA Variants to Plasmin**

Wittwer et al. have shown that glycosylation at Asn-184 in ntPA reduced the sensitivity of the molecule to plasmin cleavage by approximately twofold. As described under the Materials and Methods section, we determined the sensitivity of the tPA-6 glycoforms to plasmin-mediated conversion to two-chain protein by monitoring changes in amidolytic activity with time of incubation with plasmin. As shown in the example in Fig 6, the rate of conversion of tPA-6V was slower than that observed for tPA-6P. These data indicate that glycosylation at Asn-184 in the kringle of tPA-6 increases the resistance of the molecule to plasmin cleavage, similar to the results obtained with ntPA. The engineered diglycosylated derivative tPA-6D was significantly more resistant to plasmin-mediated cleavage than the diglycosylated tPA-6V. The rates of plasmin-mediated conversion for each variant are summarized in Table 5. For comparative purposes, the plasmin sensitivity of ntPA also was determined. The second-order rate constants for tPA-6V and tPA-6D were approximately one half and one quarter of that obtained with the monoglycosylated tPA-6P or ntPA (as a mixture of its two glycoforms), respectively. Thus, glycosylation at the engineered site as well as the natural site at N184 on the kringle reduces plasmin-mediated cleavage.

**Inhibition of Variant tPAs by PAI-1**

The interaction and subsequent inhibition of tPA activity by its inhibitor, PAI-1, has been suggested to require interaction with kringle-2. We compared the rates of inhibition of each derivative using purified PAI essentially as described
times the plasmin was inhibited with aprotinin and the level of two-chain tPA was determined as described under the Materials and Methods section.

by Boose et al. In multiple experiments, we found that the rate of inhibition by PAI-1 was the same for each glycoform (data not shown). Thus, if kringle-2 is important for PAI-1 inhibitory activity, glycosylation appears to have no effect on the interaction.

The goal of this study was to determine the effect of kringle glycosylation on the functional properties of tPA. We took the approach of using a simplified derivative of tPA, tPA-6, which is composed of the kringle-2 and the serine protease domain. Previously, we demonstrated that such a derivative had activities similar to ntPA and was fibrin dependent. Like ntPA, tPA-6 is susceptible to variable glycosylation at Asn-X-Ser/Thr site. From recent structural analyses of the tPA kringle-2, the glycosylation site at N 184 is predicted to be either a half-turn by nuclear magnetic resonance (NMR) analysis or a type $\beta$-turn by crystal structure analysis. Residues C-terminal to the site have been predicted to be either helical (NMR) or in a type $\beta$-turn, and residues N-terminal to the site are predicted to be disordered. The discrepancy from these different structural analyses of this region may suggest that multiple conformations occur, only a proportion of which are acceptable for glycosylation. In support of this, we have observed no glycosylation at a new glycosylation signal created in the disordered region at N 177.

Using a number of functional assays, we have found a significant difference between the properties of tPA-6 with a glycosylated and nonglycosylated kringle. Most notably, the natural and the engineered diglycosylated tPA-6 molecules each had decreased Glu-plasminogen-activating activity and an increased selectivity for Lys-plasminogen. Moreover, the diglycosylated molecules had greater resistance to plasmin-mediated conversion to the two-chain molecule. The fact that both diglycosylated forms shared similar enzymatic properties, yet were glycosylated at distinctly different locations, suggests that the effects are not site specific. Although glycosylation in the kringle could alter the conformation of the molecule, the observed effects may simply result from added net negative charge, altering the affinities of substrates and stimulators. With regard to the reduced plasmin cleavage, a glycosyl side chain might also sterically or electrostatically block the plasmin cleavage site making it less accessible to the 84-Kd plasmin molecule.

Two binding domains of ntPA, the finger and kringle-2, contribute to the fibrin-binding activities of the protein. An analysis of the tPA-6 glycoforms allowed us to assess the binding properties of kringle-2 dissociated from the binding contribution by the finger domain. The kringle-2 domain contains a lysine-binding site that has been proposed to be important to the fibrin-binding properties of the kringle, especially following degradation of fibrin and generation of a fibrinolytic lysine residue. The fibrin and lysine binding sites have often been considered to be the same. However, there is some controversy over the exact role of lysine-binding activity based in part on the differences in dissociation constants between lysine and fibrin to isolated kringle-2. The failure of epsilon-aminocaproic acid to inhibit binding of tPA to plasmin-degraded fibrin and the lack of alteration of tPA-mediated clot lysis following predegradation of fibrin substrate also contribute to the debate. In this study, we found distinct differences in lysine-binding activities that did not correspond to changes in fibrin-binding activities, suggesting that these two sites are not equivalent or coupled.

By moving the glycosylation signal from N184 to N205, we increased the percentage of secreted molecules that are glycosylated from 20% to 25%, to 100%. The reason for the partial glycosylation at N184 in tPA is unknown. Studies have suggested that $\beta$-turns are correlated with glycosylation of the Asn-X-Ser/Thr site. From recent structural analyses of the tPA kringle-2, the glycosylation site at N184 is predicted to be either a half-turn by nuclear magnetic resonance (NMR) analysis or a type $\beta$-turn by crystal structure analysis. Residues C-terminal to the site have been predicted to be either helical (NMR) or in a type $\beta$-turn, and residues N-terminal to the site are predicted to be disordered. The discrepancy from these different structural analyses of this region may suggest that multiple conformations occur, only a proportion of which are acceptable for glycosylation. In support of this, we have observed no glycosylation at a new glycosylation signal created in the disordered region at N177. For our studies, we examined the two naturally occurring purified glycoforms of tPA-6, tPA-6P (monoglycosylated), and tPA-6V (diglycosylated). Additionally, we produced a novel glycoform of the kringle-serine protease derivative through site-directed changes resulting in a new glycosylation site on the kringle.

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nant glycosylation site is predicted by both NMR and crystal structure analysis to be $\beta$-turn. This may suggest a potential reason for the full glycosylation at N205 in tPA-6D, although the effect of the inserted Gly residue on local structure would be difficult to predict.

In recent years, there have been extensive efforts to modify tPA, both from the perspective of understanding the function of specific domains and also to improve the therapeutic efficacy of this potent thrombolytic agent. Major efforts have included attempts to improve clot specificity, maintain the single-chain form, and decrease inhibition by PAL 1.3,5,33-35 As we have shown, glycosylation of the kringle affects several of these desired biochemical and enzymatic properties, which could result in a potential improvement in therapeutic utility. We have shown that engineering an efficiently glycosylated derivative, tPA-6D, generated a molecule with several unique attributes that fulfill desired properties of the ideal thrombolytic. Possibly the combined effects of its decreased susceptibility to plasmin, its selectivity for Lys-plasminogen, and its decreased stimulation by fibrinogen will result in an enzyme that (1) could delay systemic conversion to the more active two-chain form and (2) reduce depletion of systemic plasminogen and fibrinogen, improving both efficacy and safety. An E. coli-produced, and thus nonglycosylated, form of tPA-6, BM06.022, currently is being studied in humans with promising results.33 However, animal studies with a tPA-6-like derivative lacking kringle glycosylation resulted in a dramatic increase in fibrinogen breakdown, supporting our in vitro results.44 Although we can allude to positive attributes based on biochemical and enzymatic assays, clearly further studies are needed to determine the importance of glycosylation for the therapeutic efficacy of the molecule. Nevertheless, glycosylation significantly affects the function of the kringle, and our data demonstrate the potential for developing an improved thrombolytic agent through glycosyl alterations.

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Kringle glycosylation in a modified human tissue plasminogen activator improves functional properties

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