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High Resolution Analysis of Functional Determinants on Human Tissue-type Plasminogen Activator*

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Sixty-four variants of human tissue-type plasminogen activator (tPA) were produced using recombinant DNA techniques. Charged residues were converted to alanine in clusters of from one to four changes per variant; these clusters spanned all the domains of the molecule. The variants were expressed by mammalian cells and were analyzed for a variety of properties. Variants of tPA were found that had reduced activity with respect to each tested property; in a few cases increased activity was observed. Analysis of these effects prompted the following conclusions: 1) charged residues in the nonprotease domains are less involved in fibrin stimulation of tPA activity than those in the protease domain, and it is possible to increase the fibrin specificity (*i.e.* the stimulation of tPA activity by fibrin compared to fibrinogen) by mutations at several sites in the protease domain; 2) the difference in enzymatic activity between the one- and two-chain forms of tPA can be increased by mutations at several sites on the protease domain; 3) binding of tPA to lysine-Sepharose was affected only by mutations to kringle-2, whereas binding to fibrin was affected most by mutations in the other domains; 4) clot lysis was influenced by mutations in all domains except kringle-2; 5) sensitivity to plasminogen activator inhibitor-1 seems to reside exclusively in the region surrounding residue 300. A model of the tPA protease domain has been used to map some of the critical residues and regions.

Tissue-type plasminogen activator (tPA)[1] is a multidomain, 60-kDa serine protease whose physiological role is to convert plasminogen to plasmin, and thus to initiate or accelerate the process of fibrinolysis. Recombinant human tPA (1, 2) is used therapeutically as a fibrinolytic agent in the treatment of acute myocardial infarction (3–6) and pulmonary embolism (7, 8); both conditions usually result from an obstruction of a blood vessel by a fibrin-containing thrombus. Initial clinical interest in tPA was raised because of its relatively high activity in the presence, compared to the absence, of fibrin (9). This unusual biochemical property of tPA is thought to translate clinically into a thrombolytic product that is less likely than non-fibrin-specific thrombolytics (such as streptokinase or urokinase) to induce systemic plasminogen activation (10).

In addition to serving as a useful therapeutic, tPA also provides a challenge for studies of protein structure-function relationships because of several distinguishing characteristics. (*a*) In the presence of fibrin, tPA becomes much more active in the conversion of plasminogen to plasmin (9). (*b*) tPA is unusual in the family of serine proteases in that it is active in both the single-chain and the two-chain forms (11–18). Most of the other members of this group of proteases exist as zymogens and require proteolytic cleavage to a two-chain form to release full enzymatic activity. (*c*) The action of tPA *in vivo* and *in vitro* can be inhibited by a serpin, PAI-1 (19, 20). (*d*) tPA binds to fibrin *in vitro* with a K_d in the μM range (21, 22); this binding has been proposed to facilitate the stimulation of activity toward plasminogen (23). Moreover, (*e*) tPA has a rapid *in vivo* clearance that is mediated by one or more receptors in the liver (24–27).

tPA has a modular structure (Fig. 1), consisting of five identifiable domains (1, 28): a fibronectin-like "finger," an epidermal growth factor homologous region, two "kringle" structures, and a serine protease domain. A central goal of tPA biochemistry is to relate the details of this structure to the diverse functionalities displayed by the enzyme. Physical analysis, for example by crystallography, has been difficult probably because of the large size of tPA and the fact that it is glycosylated in a heterogeneous way by mammalian cell expression systems. Enzymological and binding studies are complicated by the size, complexity, and heterogeneity of tPA's substrate (plasminogen) and its most important modulators (fibrin and PAI-1). Therefore, much of the hope for understanding the relationship between the structural elements of tPA and its various functional properties has become invested in the study of mutant proteins.

The tPA variants that have been studied and described in the literature thus far have fallen mainly into two categories: domain deletions and site-directed modifications. The domain-deletion studies have implicated the finger, growth factor, and kringle-2 domains in interactions with fibrin (23, 29–33), and the finger and growth factor domains in clearance by the liver (34–39). Mutants resulting from site-directed alterations have implicated glycosylation of tPA in clearance (40, 41), a particular region of the protease domain (around residue 300) with sensitivity to PAI-1 (42, 43), and residue 416 in the maintenance of activity in the one-chain form (44). Moreover, construction of mutants that cannot be cleaved by plasmin has allowed inferences about the relative functionality of the single-chain and two-chain forms of tPA (11, 12, 15–17).

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[1] The abbreviations used are: tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; MOPS, 4-morpholinopropanesulfonic acid; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; YPRck, tyrosylprolylarginyl chloromethylketone.

Relative to the central problem (*i.e.* mapping functional determinants of the protein to physical sites), analyses of the tPA variants so far described have proved to have limited value. Domain deletions, by definition, have a resolution of no better than one full domain, and in most cases only a few amino acid substitution variants have been constructed. Furthermore, the analysis usually has been limited to a small number of assays. The domain-deletion approach has had the additional inherent difficulty that such deletions are gross alterations that, in all likelihood, alter the relative positions of the remaining domains. Indeed such studies have resulted in considerable disagreement in the literature about issues as basic as the participation of kringle-1 in plasminogen activation (23, 30–32, 45). Thus, the central issues concerning the mapping of functional determinants to the structure of tPA, with a resolution better than one domain, remain largely open.

We have addressed this problem by constructing a synoptic set of clustered point mutations. These mutations were designed to produce tPA variants having an altered protein surface in restricted regions of individual domains, while avoiding gross alteration of the domains themselves, by a scheme called a "clustered charged-to-alanine scan"(46).[2] Wherever charged amino acids were found in proximity in the primary sequence, each of the charged residues in the group was changed to alanine. We endeavored in this way to avoid prior assumptions or bias about the functions of any of the domains by assaying this protein-spanning set of variants using a broad range of functional assays.

The set of mutations was designed using the following algorithm: regions of clustered charged amino acids (Glu, Asp, His, Lys, Arg) were mutated to alanine in groups of 1–4 residues (Table I). In regions of a scarcity of charged residues, *e.g.* K513, single changes to alanine were allowed; in addition, clusters were fashioned such that they did not span cysteine residues. Some overlapping mutants were made in which the same charged residues were included in adjacent clusters (*e.g.* K159A,K162A and K162A,E166A). Altogether 64 mutant tPA genes were constructed using the method of oligonucleotide mutagenesis (Table I and Fig. 1). Expression of these mutant genes was achieved by transient transfection of human embryonic kidney "293" cells with the respective phagemid expression vectors. The cell culture supenatants from these transfections were analyzed using a battery of assays. In this report, we present our analyses of these variants using assays to determine tPA concentration, plasminogen activation in the absence or presence of fibrinogen or fibrin, zymogen-like activity (activity in the two-chain form relative to the corresponding one-chain form), lysis of clots in the presence or absence of plasma, inhibition of tPA by PAI-1, and binding of tPA to lysine-Sepharose and fibrin.

EXPERIMENTAL PROCEDURES

Materials—RNase and aprotinin were purchased from Sigma. Human plasmin (KabiVitrum), *H*-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251; KabiVitrum), and *H*-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide-dihydrochloride (S-2288; KabiVitrum) were purchased from Helena Laboratories (Beaumont, TX). *H*-D-Tyrosyl-L-prolyl-L-arginyl-chloromethyl ketone was purchased from Bachem, Inc. (Philadelphia, PA). Human fibrinogen, human thrombin, and MOPS were purchased from Calbiochem (La Jolla, CA). Plasminogen acivator inhibitor-1 (PAI-1) and urokinase were purchased from American Diagnostica (Greenwich, CT). Sepharose CL-6B, cyanogen bromide-activated Sepharose 4B, and PD-10 columns were obtained from Pharmacia LKB, Uppsala, Sweden. Human plasma was purchased from Peninsula Memorial Blood Bank, Burlingame, CA. Millex GV and Sterivex GV filters were purchased from Millipore Corporation, Bedford, MA. Activase® tPA was from Genentech, Inc., South San Francisco, CA.

Mutagenesis—Oligonucleotide-directed mutagenesis was performed using the Amersham mutagenesis kit based on the method of Nakamaye and Eckstein (47). A single "all-in-one" phagemid vector (p1013) was used for the mutagenesis and transient expression. This vector contains pUC118 (47) and is a modification of pCIS vectors for transient expression (48). Cells transfected with p1013 express tPA variants using a human CMV enhancer and promoter and the SV40 early polyadenylation sequence. Single-stranded p1013 DNA was used as template for mutagenesis and was prepared by superinfection of plasmid containing cells with M13KO7 (49). Mutagenic oligonucleotides were designed to contain 12 nucleotides 5′ and 9 nucleotides 3′ of the mismatched region (consisting of from 3 to 21 nucleotides). Mutagenic oligonucleotides were synthesized by the hydrogen phosphonate procedure (50) and were purified on polyacrylamide gels prior to use. Mutant clones were identified by sequencing the region covered by the mutagenic oligonucleotides and at least 20 bases outward from either end, and they were analyzed further by digestion with several restriction enzymes into fragments of less than 500 base pairs to search for and eliminate spurious deletions or insertions that infrequently arise. Generally, the efficiency of mutagenesis was 75%. Table I shows the mutations that were obtained by sequencing. In the first variant, a threonine instead of an alanine was substituted mistakenly for arginine at position 7. DNA for transfection was prepared by a modified alkaline lysis miniprep procedure (51) from cultures of *Escherichia coli* strain 294 (tonA). Prior to transfection, the DNA was treated with RNase and then passed through a Sepharose CL-6B spin column.

Expression System—Transient transfection of human embryonic kidney "293" cells was performed in 6-well plates using a variation of the calcium phosphate procedure of Gorman *et al.* (49). Approximately 2 μg of plasmid DNA were used per 1×10^6 cells. The cells were incubated for 6 days in serum-free media after which the cell culture fluids (3 ml) were harvested by centrifugation. In experiments in which single-chain tPA was desired, the cells were plated prior to transfection in media which contained fetal bovine serum that had been depleted in plasminogen by chromatography over lysine-Sepharose. This medium was replaced with serum-free medium prior to transfection. The harvested cell culture fluids were aliquoted into 96-well microtiter plates and stored at −70 °C until use.

Preparation of Plasmin-Sepharose—Human plasmin (25 casein units) was reconstituted in 2 ml of water. The plasmin was chromatographed sequentially over two PD-10 columns according to the procedure recommended by the manufacturer. The columns were equilibrated in 0.05 M MOPS, 0.5 M NaCl, pH 7.0. The final eluate was filtered through a 0.22-μm Millex GV filter. Cyanogen bromide-activated Sepharose 4B (0.3 g) was hydrated and washed with 1 mM HCl. The plasmin solution was incubated at room temperature with the matrix for 1 h with gentle mixing. The matrix was washed with 15 ml of 0.05 M MOPS, 0.5 M NaCl, pH 7.0. Ethanolamine (2 ml of a 1.0 M solution, pH 9.0) was added to the matrix, and the matrix was mixed gently for 1 h at room temperature. The matrix was washed with 10 ml each of 0.1 M sodium acetate, pH 4.0; 0.5 M sodium chloride; 0.1 M Tris-HCl, pH 8.0; 0.5 M sodium chloride. The same wash sequence was then repeated two additional times. The matrix was adjusted to a settled volume of approximately 1 ml with PBS, and then 1 ml each of PBS and glycerol were added. The matrix was found to be stable for at least 1 month at 4 °C. Before use the matrix was washed extensively in PBS and suspended as a 5% slurry using cell culture medium.

Lysine-Sepharose Chromatography of Fibrinogen—The fibrinogen was treated with lysine-Sepharose to remove contaminating plasminogen. Four 100-mg vials of human fibrinogen were each reconstituted in 5 ml of water and 10 ml of PBS. The fibrinogen was pooled and dialyzed at 4 °C *versus* two 4-liter volumes of PBS. The dialysate was warmed to room temperature and filtered using 0.22-μm Sterivex-GV filters. The solution was chromatographed at room temperature over a lysine-Sepharose column (41.7 ml) equilibrated in PBS. The residence time on the column was approximately 50 min. The fibrinogen that flowed through the column was pooled and stored at −70 °C. The fibrinogen in the preparation was found to be greater than 90% clottable, and little or no plasmin activity could be detected in the preparation upon the addition of tPA.

Preparation of Human Glu-plasminogen—Human Glu-plasminogen was prepared from human plasma using a modification of the procedure described by Deutsch and Mertz (52). The entire procedure

[2] S. Bass and J. A. Wells, personal communication.

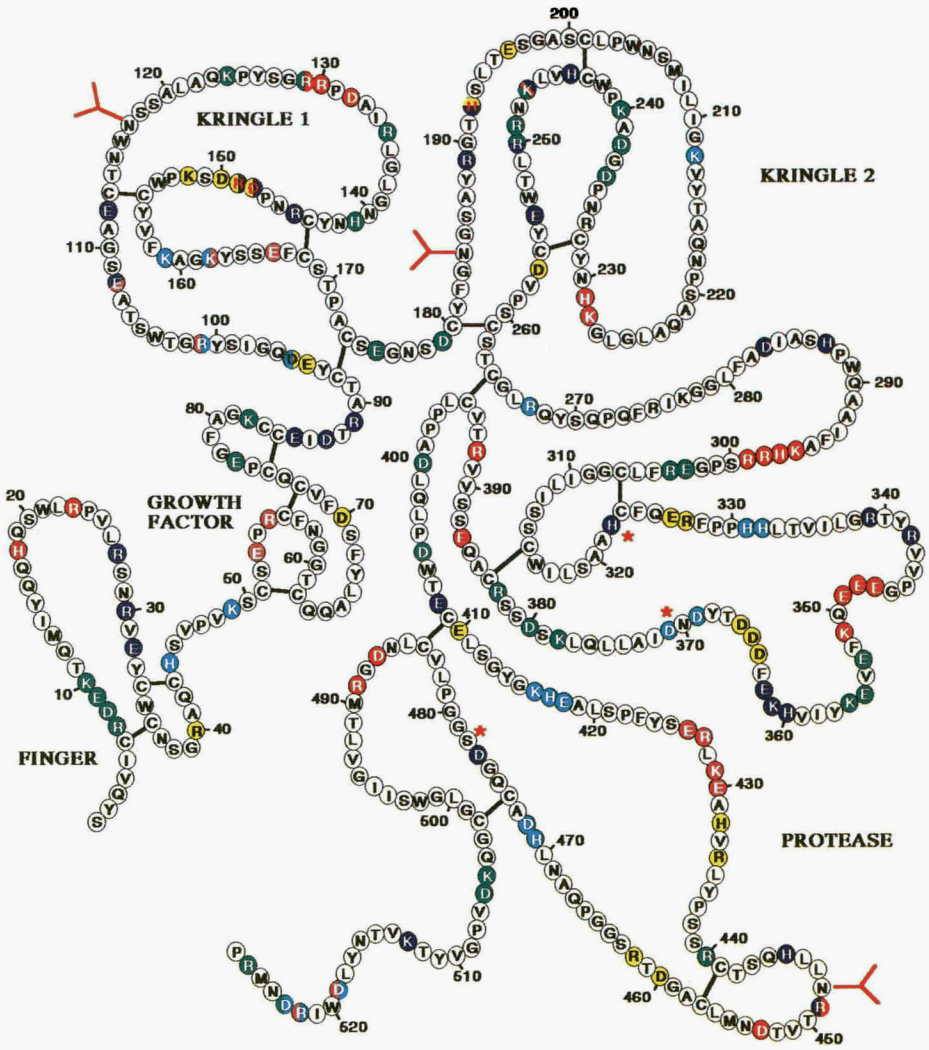

FIG. 1. **The primary structure of tPA (1).** Disulfide bonds are based on homology with other proteins (1, 28). Sites known to be glycosylated on wild-type tPA (62) are marked with a *Y*, and the active site residues (also based on homology) are marked with an *. Each charged-to-alanine scan mutant is represented by a sequential series of identically colored residues. Multicolored residues were changed in more than one variant.

was performed at 4–8 °C. Aprotinin was added to human plasma to a final concentration of 20 trypsin inhibitory units/liter. The plasma was filtered through a 0.2-μm filter and loaded onto a lysine-Sepharose column which was equilibrated in 200 mM potassium phosphate, pH 7.5, containing 20 trypsin inhibitory units/liter Aprotinin. Six column volumes of plasma were loaded onto the column using a residence time of 50 min. The column was washed with four column volumes of equilibration buffer followed by four column volumes of 200 mM potassium phosphate, pH 7.5. The column was washed with one column volume of 20 mM sodium phosphate, 100 mM sodium chloride, pH 7.5, and the plasminogen was eluted from the column using the same buffer containing 50 mM 6-aminohexanoic acid. The eluate was concentrated to approximately 1 mg/ml using a Millipore Pellicon Cassette System equipped with a 10,000 NMWL membrane. The solution was diafiltered using 10 diavolumes of 50 mM Tris-HCl, 100 mM sodium chloride, pH 8.0, and lyophilized. The plasminogen was stored at −70 °C. Amino-terminal sequence analysis indicated that the plasminogen was more than 95% in the glutamic acid form.

Amidolytic Activity Assay—The synthetic substrate, *H*-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide-dihydrochloride (S-2288), was used to assess the amidolytic activity of the alanine substitution variants. tPA variants and controls (15 μl) were added to the wells of a microtiter plate. If the assay was used to measure the activity of two-chain tPA, human plasmin (45 μl of a 0.176 casein units/ml solution) was added, and the samples were incubated at room temperature for 90 min. When assaying the samples in the single-chain form, PBS was added in place of plasmin, and the incubation step was omitted. Aprotinin (40 μl of a 2.5 trypsin inhibitory units/ml solution) was added to each well, and the samples were incubated for 15 min at room temperature. A 2.16 mM aqueous solution of S-2288 was diluted to 1.45 mM with 0.1 M Tris, 0.106 mM NaCl, 0.02% sodium azide, pH 8.4, and 100 μl of this solution was added to each well of the microtiter plate. Color development was monitored at 405 nm. Data were collected using an SLT Laboratories Model EAR 340 AT microtiter plate reader interfaced to an AST Premium/286 computer. The initial velocities of the reactions were determined using a computer program written in our laboratory which calculates the slope of the absorbance *versus* time curve for each sample. A standard curve was prepared by assaying Activase® tPA diluted in cell culture media. The concentrations of the variants based on enzymatic activity were obtained from the standard curve. The relative activities of the variants compared to Activase® tPA were obtained by dividing the "activity concentration" of each variant by its "mass concentration" obtained in the tPA ELISA. Wild-type tPA transfected at the same time as the variants consistently gave values of approximately 1.0 in this assay system. To compare results from assays performed at different times more accurately, four wild-type controls were included in every transfection. The results for the samples analyzed in each assay were normalized such that the mean of the the relative activity of the wild-type internal controls was equal to 1.0.

Fibrin-stimulated Plasminogen Activator Assay—This is a continuous, coupled assay wherein tPA activates plasminogen to plasmin, which in turn hydrolyzes the synthetic substate S-2251. The tPA variant and control samples were converted to the two-chain form by incubating plasmin-Sepharose (50 μl of a 5% slurry) with 150 μl of tPA sample for 90 min at room temperature with constant mixing. The matrix was removed by centrifugation, and 10 μl of sample were added to the wells of a microtiter plate. If the assay was used to assess the activity of one-chain tPA, medium was added in place of the plasmin-Sepharose and the incubation step was omitted. Human thrombin (10 μl of a 42 unit/ml solution) was added to each well. The plasminogen activation reaction was started by the addition of 130 μl of a mixture composed of 28 μl of human Glu-plasminogen (5.3 μM), 10 μl of lysine-Sepharose-treated human fibrinogen (10 μM), 30 μl of

3 mM S-2251, and 62 μl of PBS. Under these conditions, clot formation was rapid compared to the time course of the plasminogen activation reaction. Color development was monitored at 405 nm, and the absorbance at the reference wavelength of 492 nm was subtracted from each time point to correct for the effect of turbidity. Data collection and analysis were performed as described for the amidolytic activity assay except that the enzymatic activity was determined as the slope of the absorbance *versus* time squared curve for each sample.

Fibrinogen-stimulated Plasminogen Activator Assay—This assay was performed as described for the fibrin-stimulated assay except that PBS was substituted for thrombin and no reference wavelength was used.

Unstimulated Plasminogen Activator Assay—This assay was performed as described for the fibrin-stimulated assay except that PBS was substituted for both thrombin and fibrinogen and no reference wavelength was used.

Labeling of tPA for Binding Studies—The active site labeling agent was prepared by Chloramine-T-catalyzed radioiodination of tyrosyl-prolylarginyl chloromethylketone (YPRck), using a modification of the method described by Hunter and Greenwood (53). In a typical reaction, 50 μl of 1 M Tris-HCl at pH 7.5 was added to 40 μl of $Na^{125}I$ (4 mCi;1.8 nmol) in a capped reaction vessel. To this vessel, 8.3 μl of YPRck (1.8 nmol in 12 mM HCl) was added. Iodination was initiated by addition of 12.5 μl of Chloramine-T (1 mg/ml in 0.1 M NaP_i, pH 7.5). Iodination was quenched after 60 s at 24 °C with 25 μl of sodium metabisulfite (1 mg/ml in 0.1 M NaP_i, pH 7.5). The reaction was then diluted by addition of 2 ml of PBS, and 20-μl aliquots of the diluted labeled reagent were added to 1-ml aliquots of the culture supernatants of transiently transfected "293" cells. This mixture was incubated for 1 h, and the free ^{125}I was separated from the protein-bound ^{125}I by gel filtration on a PD-10 column (Pharmacia). Protein-bound ^{125}I was determined by precipitation with 10% trichloroacetic acid. These labeling conditions were optimized for a 1:1:1 molar ratio of iodine:YPRck:tPA. The specific radioactivity of the ^{125}I-labeled tPA variants, *i.e.* those that had an intact active site, was approximately 1 μCi ^{125}I/μg tPA.

Binding of Active Site-labeled tPA to Lysine-Sepharose or to Fibrin—Lysine binding of tPA variants was determined by mixing, at room temperature, 100 μl of YPRck-labeled tPA (10,000 cpm, about 10 ng) with a 50% slurry of PBS-washed lysine-Sepharose. The mixtures were shaken in microtiter plates or Micronics tubes (Flow Labs, McLean, VA) for 10 min, then centrifuged for 10 min at 3000 rpm. The supernatant liquid was removed and counted in a γ counter (LKB model 1277), and the fraction bound was determined.

Fibrin binding was determined by a modification of the method of Rijken *et al.* (18). Polystyrene removable wells (Immulon 1, Dynatech) in a microtiter format were pretreated with PBS containing 5% bovine serum albumin and 0.1% Tween 80 for 16 h at 4 °C to minimize nonspecific binding. Dilutions of lysine-Sepharose-treated human fibrinogen ranging from 2 μg/ml to 4 mg/ml were prepared in PBS containing 0.5% bovine serum albumin and 0.01% Tween 80. Aliquots of fibrinogen (50 μl) and YPRck-labeled tPA variants were added to microtiter wells and mixed briefly. Fibrin clots were formed by the addition of human thrombin (50 μl of 0.4 units/ml) and were visible within 60 s. After 60 min at room temperature, the clots were centrifuged at 3000 rpm for 2 h at 25 °C. Aliquots of the supernatants (50 μl) were transferred to individual tubes for counting. For determination of total radioactivity, the microtiter wells (containing the clots and the remaining liquid) were separated and counted. The amount of bound tPA was the difference between the total radioactivity (sum of the 50-μl aliquot and microtiter well with clot) and the unbound radioactivity which was calculated as 4-fold the amount in 50 μl of supernatant.

Clot Lysis Assays—The purified clot lysis assay was performed essentially as described (54), but in microtiter plates after conversion of samples to the two-chain form by plasmin-Sepharose. Plasma clot lysis was performed as follows: Plasma clots were formed in microtiter plate wells at room temperature by recalcification of 90 μl of pooled citrated human plasma by the addition of 10 μl of 0.15 M $CaCl_2$ and were used 6–8 h after formation. Samples were diluted appropriately and mixed with an equal volume of plasma, and 100 μl were overlaid on the clots and an initial turbidity reading (at 405 nm) was obtained. After incubation at 37 °C for 15 h, a final turbidity reading was obtained. The loss in turbidity for the standard curve (10–800 ng/ml) was plotted against log [tPA] and fitted by a nonlinear least squares 4-parameter fit program. Unknowns were calculated from this curve and normalized to identically treated wild-type controls.

RESULTS

Expression of the tPA Variants—The list of mutations is given in Table I. In addition to the specific amino acid substitutions, the table also provides the mean residue number for each mutation. For a given variant, this number is the rounded numeric average of the positions altered. On this basis, variants are identified in the graphical representations that follow.

Plasmids encoding the individual variant proteins were transfected into "293" cells in serum-free media; the variants were thus produced under serum-free conditions. Six days later the supernatants were harvested, and tPA in the cell culture medium was measured using a polyclonal ELISA. Within each transfection set, "tPA-minus" transfections were carried out using plasmids lacking the tPA coding region. The supernatants from these transfections were found consistently to contain less than 50 ng/ml tPA antigen by ELISA, and enzymatic activities of these supernatants were consistent with low plasminogen activator levels. To avoid problems with this background expression, results are reported only for those variants that were expressed in excess of 250 ng/ml. A total of five independent transfections were performed for most of the mutants listed in Table 1. Two of the transfections were performed under culture conditions which would produce mainly single-chain tPA. In general the expression of the variants ranged from 250 ng/ml (our lower limit) to about 5 μg/ml. Only four of the variants (mean residue numbers 30, 110, 147, and 370) were not expressed above the lower limit of 250 ng/ml.

A monoclonal antibody to tPA has been used to show that tPA is responsible for greater than 95% of the amidolytic activity in the cell supernatants, employing S-2288, which is hydrolyzed by either tPA or urokinase. This antibody does not inhibit low molecular weight urokinase, suggesting that urokinase is not produced to a significant extent by these cells, either before or after transfection. Sodium dodecyl sulfate-gel electrophoresis and Western blot analysis of the cell supernatants has shown that the protein produced in response to transfection is of the predicted mass for either one-chain or two-chain tPA and is not present, to any significant extent, in high molecular weight complexes.

Amidolytic Activity—The amidolytic activity of the variants was determined using the tripeptide chromogenic substrate S-2288 (D-Ile-Pro-Arg-*p*-nitroanilide). Fig. 2 shows the activity of the two-chain forms of the alanine scan variants toward this substrate, normalized to wild-type tPA. Most of the variant proteins (especially in the nonprotease domains) have wild-type specific activity. Elevated activity in variants near residue 150 in kringle-1 has been observed consistently in the activity assays, and it has been determined that these kringle-1 variants (and most kringle-1 variants generally) are underestimated by the ELISA assay.[3] When a correction for this error is made, the kringle-1 variants also have near wild-type activity levels. Very low amidolytic activity was observed in variants of the protease domain involving an active site residue (H322A) and several variants (positions 472, 477, and 488) near the active site serine at position 478.[4] Lower than wild-type amidolytic activity is also present in several other protease domain variants, whereas mutations to the nonprotease domains do not produce such effects.

Fibrin Stimulation and Specificity—A more physiological

[3] B. Keyt, D. Higgins, and D. Sinicropi, personal communication.

[4] The other active site mutants have been constructed: S478A, not a member of this set, has no detectable activity; variant 370, a member of this set containing the active site aspartate 371, has not been expressed to sufficient levels for assay.

TABLE I

The collection of mutants and the position of the mean residue of mutation for each

This table shows the mutations that were obtained by sequencing. In the first variant, a threonine instead of an alanine was substituted mistakenly for arginine at position 7. The positions of mutation were averaged, and rounded to the nearest integer.

Mutations	Mean residue	Mutations	Mean residue
R7T, D8A, E9A, K10A	9	D283A, H287A	285
H18A, R23A	21	K296A, H297A, R298A, R299A	298
R27A, R30A, E32A	30	E303A, R304A	304
R40A	40	H322A	322
H44A, K49A	47	E326A, R327A	327
E53A, R55A	54	H331A, H332A	332
D70A	70	R339A, R342A	341
E77A, K82A	80	E347A, E348A, E349A, K351A	349
E85A, D87A, R89A	87	E353A, E355A, K356A	355
E94A, D95A	95	H360A, K361A, E362A	361
D95A, R101A	98	D364A, D365A, D366A	365
R101A, E108A	105	D369A, D317A	370
E108A, E112A	110	K378A, D380A, R383A	380
K124A, R129A	127	E387A, R392A	390
R129A, R130A, D132A	130	D400A, D405A	403
R135A, H141A	138	E408A	408
R145A, D148A, R149A	147	E410A	410
D148A, R149A, D150A, K152A	150	K416A, H417A, E418A	417
K159A, K162A	161	E426A, R427A, K429A, E430A	428
K162A, E166A	164	H432A, R343A	433
E175A, D179A	177	R440A	440
R189A, H192A	191	H445A, R449A	447
H192A, E196A	194	R449A, D453A	451
K212A	212	D460A, R462A	461
K228A, H229A	229	H471A, D472A	472
D236A, D238A, K240A	238	D477A	477
H244A, K247A	246	D487A, R489A	488
K247A	247	K505A, D506A	506
K247A, R249A, R250A	249	K513A	513
R250A, E254A	252	D519A, R522A	521
D257A	257	D519A, R522A, D523A	522
R267A	267	D523A, R526A	525

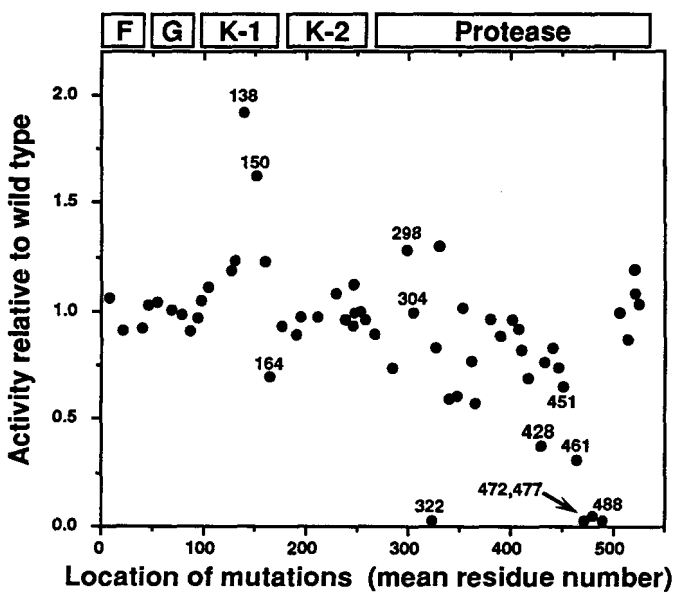

FIG. 2. **Amidolytic activity of alanine scan variants in the two-chain form.** The values are expressed as a ratio with wild-type recombinant tPA, which was transfected in parallel with the mutants and which was assigned a value of 1.0. Each sample was assayed in duplicate within each transfection set, and the data represent the means from five transfection sets. The average coefficient of variation for these samples across all tranfections was 14%.

activity of tPA was evaluated using an indirect chromogenic assay, in which plasminogen is converted to plasmin by the tPA variant, and plasmin activity is monitored continuously using the chromogenic substrate, *H*-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251) (55, 56). In this coupled assay, tPA produces an acceleration of the rate of S-2251 hydrolysis, from which a linear plot of optical density *versus* time squared is obtained. The slope of this plot is proportional to the tPA activity present. The assay was performed either in the absence of a stimulator, or in the presence of fibrinogen or fibrin. In this report, "fibrin stimulation" refers to the tPA activity in the presence of fibrin, compared to that in its absence. "Fibrin specificity" refers to the activity in the presence of fibrin, compared to that in the presence of fibrinogen.

Fig. 3 shows that, under the conditions chosen for this assay, the rate of plasmin production is about 40 times higher in the presence of fibrin than in its absence. tPA activity is also accelerated by fibrinogen, but to a considerably lesser extent (about six times the unstimulated value). The plasminogen-activating activities of the alanine scan variants were determined (relative to wild-type) and are represented in Fig. 4 as a function of position of mutation. *Panels A* and *B* give the results for the unstimulated and the fibrinogen-stimulated assays, respectively. In the absence of a stimulator, significant perturbations in plasminogen activation activity were observed in discrete regions throughout the protein. As was observed with the tripeptide substrate, the variants around residue 150 were elevated (in part due to the aforementioned ELISA error in that region), and significant increases and

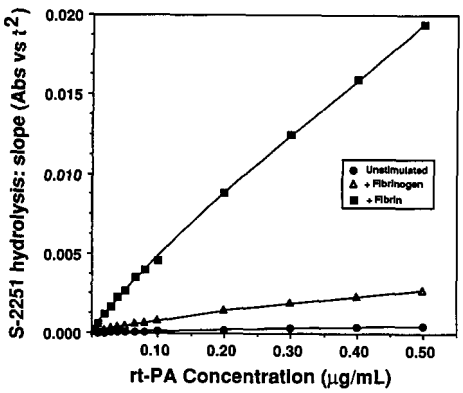

FIG. 3. **Plasminogen activation by tPA in the presence and absence of fibrinogen and fibrin.** Activase® tPA (*rt-PA*) was converted to two-chain and assayed as described under "Experimental Procedures." The activities are expressed as the slope of the absorbance *versus* time squared curves for each sample.

decreases in activity were observed in mutants of the kringle-2 domain. In the protease domain, in addition to the active site-related variants described earlier, mutations at charged residues throughout much of the protease domain generally reduced the activity in the unstimulated and the fibrinogen-stimulated assays. In contrast with those results (*panel C*), fibrin restored wild-type levels of activity to most of the variants. Variants with low amidolytic activity remained inactive, but for those with mutations flanking these areas, the loss of activity observed in the unstimulated and fibrinogen-stimulated assay was reversed by fibrin.

Activity greater than that of wild-type tPA was observed (in the presence of fibrin) for the three variants at positions 298, 304, and 332 (Fig. 4*C*). The largest of these effects was in a basic region (position 298), in which 2–3-fold greater activity than wild-type has been observed consistently with this assay. Since the activities of this variant and the one at position 304 were low in the presence of fibrinogen (Fig. 4*B*), the fibrin specificity, or ratio of activity in the presence of fibrin *versus* fibrinogen, was increased over wild-type tPA (Fig. 4*D*). Thus by producing charged-to-alanine modifications in certain regions of the protease domain, it was possible to generate several variants with 2–10-fold more fibrin specificity than wild-type tPA. (Note: These effects are in addition to the 6-fold fibrin specificity of wild-type tPA shown in Fig. 3.)

The most unexpected component of the fibrin stimulation and specificity results is the apparent lack of participation in these effects of charged residues in the nonprotease domains, because binding to fibrin has been ascribed repeatedly to these domains (23, 29–33).

"*Zymogenicity*"—tPA differs from most serine proteases in that the single-chain form of the molecule has appreciable enzymatic activity (11–18). Toward some small substrates, and toward plasminogen in the absence of fibrin, two-chain tPA has greater activity than one-chain. In the presence of fibrin, however, the two forms of tPA are equally active. The activity of the alanine scan variants toward both small and large substrates was evaluated in both the one- and two-chain forms. Fig. 5*A* shows the results of an S-2288 assay in which the variants either were converted to the two-chain form with plasmin, or were analyzed unconverted. Mutations in the nonprotease domains had little or no effect on the enzymatic activity in either the one- or two-chain form. In the protease domain, however, the activities of the single-chain forms of some variants were reduced markedly; these variants are thus more zymogenic than wild-type. This effect was most pronounced in variants of charged residues near position 275 (the conversion site) and in the vicinities of residues 339–351 and 410–440 (*arrows*, Fig. 5*A*).

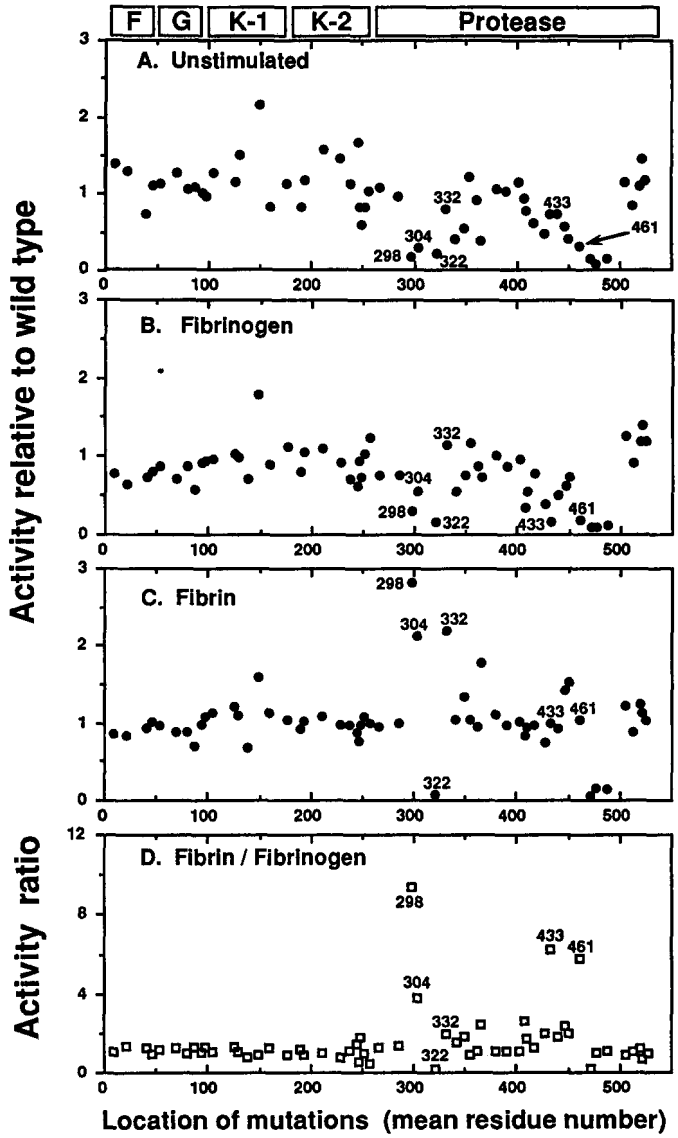

FIG. 4. **Plasminogen activator activity and fibrin specificity of alanine scan mutants.** The plasminogen activator assay was performed on the alanine scan mutants, as described under "Experimental Procedures," in the absence of stimulator (*panel A*), or in the presence of fibrinogen (*panel B*) or fibrin (*panel C*). All samples were assayed in the two-chain form. The activity of each variant is expressed as described in Fig. 2. The fibrin specificity (*panel D*) was determined by dividing the values shown in *panel C* by those in *panel B*. The average coefficient of variation for unstimulated, fibrinogen-stimulated, and fibrin-stimulated assays was 17, 14, and 11%, respectively.

In contrast with the behavior of wild-type tPA, some alanine scan variants are also zymogens in fibrin-stimulated plasminogen activation (see Fig. 5*B*). The ratios of the activities in two-chain *versus* one-chain form are presented in Fig. 5*C*. While it is evident that mutations in the nonprotease domains do not markedly affect the two-chain to one-chain ratios, mutations in two regions of the protease domain can elicit zymogen-like activity. In the region of residues 280–335, the magnitude of this effect is 2–3-fold and is due to increased activity in the two-chain form (see Fig. 4*C*). In contrast, the variants at mean residue numbers 417, 428, and 433 exhibit

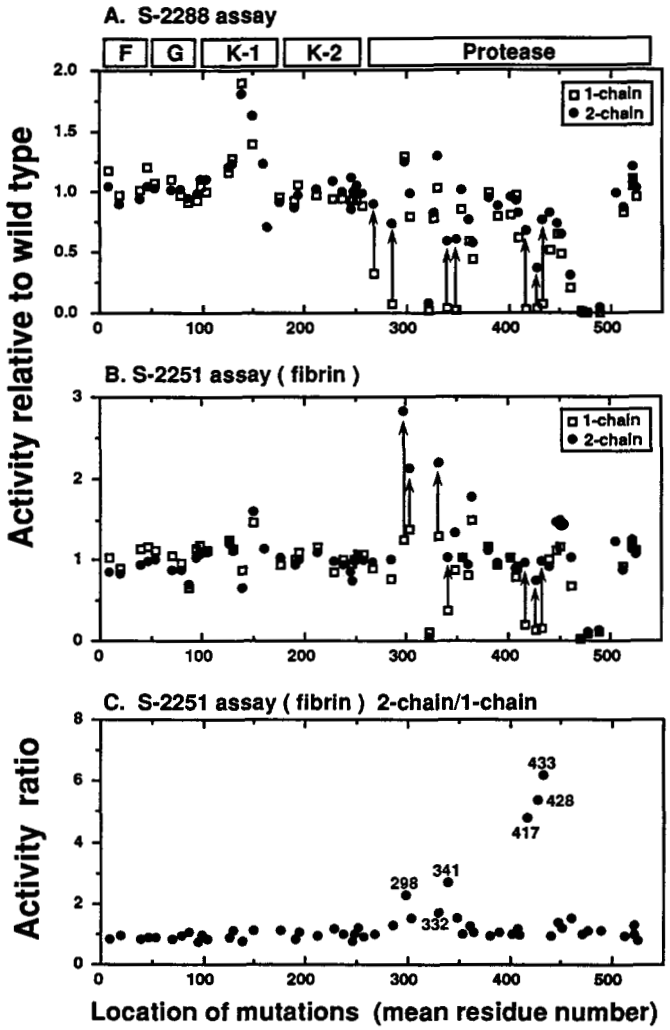

FIG. 5. **Difference between one-chain and two-chain activities of the alanine scan variants.** *Panel A*, the samples were assayed as in Fig. 2, except that for one-chain samples the incubation with plasmin was omitted. *Arrows* mark those variants whose increase in relative activity, upon conversion to two-chain, was greater than 5-fold. *Panel B*, the samples were treated as in Fig. 4*C*, except that for one-chain samples the incubation with plasmin-Sepharose was omitted. *Arrows* mark the variants whose increase in relative activity, on conversion to two-chain, was greater than 50%. *Panel C*, the values were obtained by dividing the two-chain values by the one-chain values from *panel B*.

5–7-fold greater zymogenicity than wild-type; this is the result of low activity in the one-chain form.

Inhibition by PAI-1—PAI-1 is a serpin which is thought to play a role in hemostasis via its fast-acting effects on tPA and/or urokinase (57). It binds to the tPA active site, is cleaved by tPA at its R346-M347 peptide bond, and forms a stable complex. Wild-type tPA from transfected "293" cell supernatants is inhibited completely with respect to its amidolytic activity by incubation with PAI-1. The set of alanine scan mutants was incubated with PAI-1 and tested for residual amidolytic activity toward S-2288. With only one exception, the alanine scan proteins were (like wild-type) totally inhibited under these conditions (data not shown). The exception was the mutant at position 298, in which alanines were substituted for the sequence KHRR. Considerable resistance to inhibition was evidenced by this variant; a quality that was attributed previously to the same region by Madison *et al.* (42, 43). It is noteworthy that, under the conditions chosen for our assays, this is the only charged site on the tPA molecule that appears to be critical for PAI-1 inhibition. We had expected that tPA and PAI-1 would have had more broadly based interactions (58), and we are currently investigating whether other parts of the tPA molecule are involved in its interaction with PAI-1, albeit less critically so, than the 296–300 region. In the reports by Madison *et al.* (42, 43), variants in this same region were claimed to be PAI-1-resistant, but otherwise unaffected. However, our results show that the KHRR 296–299 AAAA variant (position 298) also exhibits significantly enhanced fibrin stimulation, fibrin specificity, and zymogen-like behavior.

Binding to Lysine-Sepharose and Fibrin—In order to label tPA selectively for binding studies, without the need to purify the variant proteins from the cell culture supenatants, we used a reagent (^{125}I-iodotyrosylprolylarginyl chloromethylketone (59)) that allows ^{125}I-labeling of the active site of tPA even at concentrations of tPA as low as 0.1 μg/ml. Either one-chain or two-chain tPA can be labeled with this reagent, and our studies with labeled, purified tPA variants have shown that the ligand does not interfere with specific binding to fibrin, lysine, hepatocytes, nor with *in vivo* clearance of the protein. Because labeling of tPA in this way requires an active site, amidolytically inactive variants were not tested. The labeled proteins, in both the one-chain and two-chain forms, were tested for binding to fibrin or to lysine-Sepharose, and Fig. 6 shows the results (only the one-chain data are shown for lysine-Sepharose binding, since the conversion to two chain did not significantly alter lysine-Sepharose binding). Lysine binding (*panel A*) was essentially unaffected by mutations other than in kringle-2. This is the location of the lysine binding site of tPA (60, 61), and the data suggest that there is only one such site involving charged residues on the molecule. In contrast, fibrin binding (*panel B*), especially when tPA is in the two-chain form, was disrupted most by mutations in the first 150 residues and in the protease (at positions 408, 433, and 461). The figure indicates that mutations in kringle-2 seem to be less disruptive to fibrin binding than those in kringle-1, a peculiar result in view of the domain deletion experiments mentioned earlier (23, 29–33). Our results confirm that the kringle-2 domain of tPA does indeed bind lysine, and suggest that tPA's binding to lysine through kringle-2 constitutes only a small fraction of the total interaction of tPA with fibrin.

Three variants in the protease domain (at positions 408, 433, and 461), when in the two-chain form, bind poorly to fibrin. This result was unexpected, because fibrin binding has been linked almost exclusively to the nonprotease domains. While protease-mediated fibrin binding is inconsistent with the usual models of fibrin-tPA interaction, it is consistent with our observation implicating the protease domain in fibrin stimulation and specificity. These results, taken in context with the fibrin stimulation results given in Fig. 4, are difficult to reconcile with a simple causal connection between fibrin's binding to tPA and its stimulation of tPA's activity.

The figure also shows the binding of single-chain variants to fibrin and the magnitude of the changes in fibrin binding upon conversion to two-chain variants. It should be mentioned that more of the wild-type single-chain form of tPA binds fibrin than does the two-chain form (15, 21, 22). Since the one-chain and two-chain variants are normalized to one-chain and two-chain wild-type respectively, the observed effects are in addition to any intrinsic differences between one-chain and two-chain tPA . The trend is clearly in the direction of diminished fibrin binding in the two-chain forms; these effects are especially evident in the amino-terminal domains

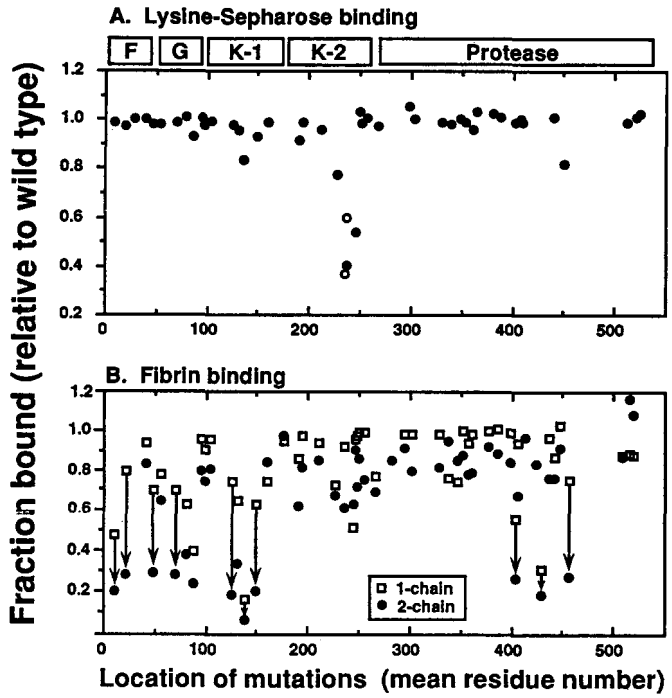

FIG. 6. **Binding of active site-labeled tPA to lysine-Sepharose (*panel A*) or to fibrin (*panel B*).** The variants and wild-type controls were labeled as described under "Experimental Procedures," then incubated with either lysine-Sepharose or with fibrinogen, which was converted to fibrin by treatment with thrombin. The bound and unbound fractions were analyzed for ^{125}I, and the fraction bound is presented as a ratio to wild-type tPA. Only the one-chain data are shown for lysine-Sepharose binding. The *open symbols* in *panel A* represent two variants not shown in the other data sets: D236A and D238A. These additional variants in the lysine binding site (60, 61) were constructed and expressed as described for the other variants. Although a range of fibrin concentrations from 1 mg/ml to less than 1 μg/ml was used to evaluate each variant, the data shown in *panel B* are for the fraction bound of one-chain and two-chain tPA variants at fibrin concentrations required for half-maximal binding (36 and 110 nM, respectively). The data shown for the variants have been normalized to an identically treated wild-type control. *Arrows* mark the positions of mutation where a difference of greater than 20% (bound to fibrin) was seen between the one-chain and the two-chain samples.

(finger, growth factor and kringle-1) and the latter half of the protease. Kringle 2 appears to be the least significant domain with respect to fibrin binding. Fibrin binding is evidently a complicated process which, in contrast with lysine binding, involves a considerable portion of the molecule. With respect to the protease domain at least, fibrin binding also seems to be related to fibrin specificity, because some mutations in the protease (positions 433 and 461) affect both these functions.

"Purified" Clot Lysis and Plasma-Clot Lysis—The previously described activity assays were designed to measure an early event in fibrinolysis, namely, conversion of plasminogen to plasmin. We also assayed the alanine scan mutants in two clot lysis systems, one with fibrin derived from purified components, the other with clotted plasma. Fig. 7 shows the activities of the variants in these microtiter-plate assays. In the "purified" clot lysis assay, the majority of the variants display near wild-type activity, the exceptions being primarily active site-related mutations in the protease domain. Several mutants in the 300–360 region show increased activity over wild-type, in general agreement with data from the fibrin-stimulated S-2251 assay (see Fig. 4*C*). Indeed there is a good correlation between the results of the fibrin-stimulated S-2251 assay and the purified clot lysis assay.

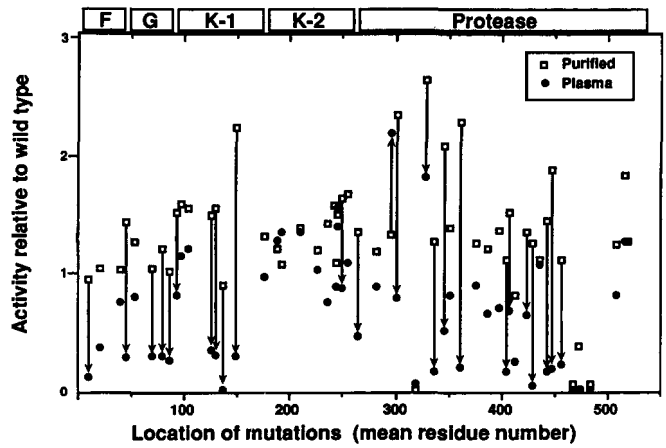

FIG. 7. **Activities of the variants in two clot lysis assays.** "Purified" clot lysis and plasma clot lysis assays were carried out as described under "Experimental Procedures." *Arrows* indicate positions where the difference in activity between the two assays was 0.7 or greater.

The lysis of plasma clots provides a marked and unexpected contrast with the purified clot lysis data. Fig. 7 also shows the activity of the alanine scan variants in this assay. The *arrows* in this figure highlight those positions of mutation in which the difference in activity between the purified and plasma results is 0.7 or greater. The surprising abundance of arrows testifies to the importance of plasma components in regulating the activity of tPA. The first 150 residues of the protein, as well as residues 330–450, appear to be the most sensitive to mutation when assayed in the plasma-based assay, with essentially no effects being observed in kringle-2. It is interesting to compare the fibrin binding properties and the plasma clot lysis activities of the mutants: there is a good correlation in the first 150 residues between fibrin binding and plasma clot lysis activity, while throughout the rest of the molecule there is little correlation. In the protease, however, the three mutants with diminished fibrin binding did show comparable losses in plasma clot lysis activity (see Fig. 6*B*). It seems clear from these data that a complete analysis of a tPA variant with an interesting property should include a survey of the effects of plasma on the variant's activity.

Localization of Mutations on a Three-dimensional Model of the Protease Domain—The linear structure of tPA (Fig. 1) does not allow one to place the target residues of the alanine-scan mutations in three-dimensional space. Such a correlation might aid in determining the functional role of a particular residue and might suggest additional mutants that could be made to test these hypotheses. While a crystal structure of tPA is not available, a three-dimensional model was generated based on known structures of seven related serine proteases. Fig. 8 shows a model for the protease domain of tPA, in which the residues that were mutated in three types of variants are highlighted.

The position 298 variant (highlighted in *light blue*, Fig. 8) is near the active site. Since these residues may interact directly with the substrate, the fact that substitution of alanines for these residues increases the activity toward plasminogen is understandable. As noted by Madison *et al.* (42, 43) and herein, some deletions and substitutions within this loop also render the protein resistant to PAI-1. This suggests that the interactions between these basic groups in tPA and complementary residue(s) in PAI-1 make a large contribution to the tPA-PAI-1 association. Furthermore, the position 298 variant exhibits nearly 10 fold greater fibrin specificity in plasminogen activation than wild-type tPA.

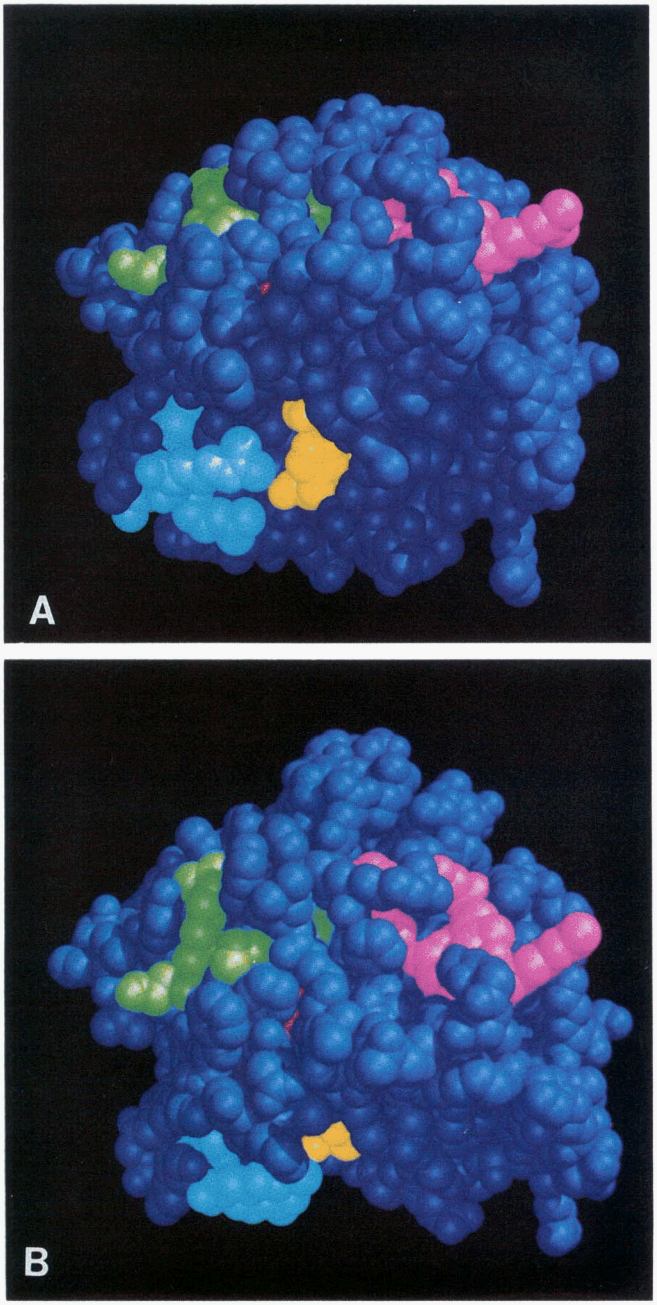

FIG. 8. **Three-dimensional model of the protease domain of human tPA and the positions of three types of variants.** Shown is a space-filling model of the protease domain of tPA (visualized in the two-chain form without hydrogens), in which the two views (*A* and *B*) differ by a rotation of about 45° about the *x* axis. The residues of the catalytic triad (H322, D371, S478) are shown in *yellow*, and the amino-terminal residue I276 is shown in *red* (partially visible), as it might exist in the two-chain configuration. The figure highlights the wild-type residues for which alanine was substituted in the variants. Shown are the fibrin specific/PAI-1-resistant variant at position 298 (KHRR296-299AAAA; *light blue*), the zymogen-like variant at position 428 (E426A, R427A, K429A, E430A: *green*), and the fibrin binding-deficient variants at positions 408, 433, and 461 (respectively: E408A; H432A, R434A; and D460A, R462A; *pink*).

The charged residues at positions 426–430 (position 428), when changed to alanine, result in a more zymogen-like tPA molecule. The positions of these residues within the model of the protease domain are shown in green in Fig. 8. The model is in the "two-chain" form, because it is based on the known structures of activated serine proteases. In tPA, conversion of one-chain to two-chain occurs by cleavage between R275 and I-276 (shown in *red*). The model shows that the positions of the residues comprising the position 428 variant cluster in this vicinity. It is possible that one or more of the alanine substitutions in the zymogen mutant results in a conformational shift of the residues near Ile-276 such that the one-chain form of the mutant is less active than the one-chain wild-type.

A third class of mutants on the protease domain are those that are reduced in fibrin binding. The *pink* residues in Fig. 8 are those comprising variants at positions 408, 433, and 461, located on a surface of the protease distinct from the others. These residues are clustered, which suggests that they might be part of a fibrin binding "patch" in the wild-type tPA molecule. These variants (in *pink*) are affected by the cleavage of tPA; all three show a lower affinity for fibrin in the two-chain chain form than in the one-chain form (Fig. 6). This suggests a possible cooperative role for the protease domain of tPA in its interaction with fibrin, which changes as the protease undergoes one-chain to two-chain conversion. The variants at positions 433 and 461 are also more fibrin-specific than wild-type tPA. Given the spatial separation of these variants from the other fibrin-specific variant on the model (position 298), they may be promoting fibrin specificity through a different mechanism of action.

DISCUSSION

When we began this work, most of the available information on structure-function relationships of tPA had come from a number of domain-deletion studies (16, 29–33, 45). We set out to construct a synoptic collection of substitution mutants of the protein, partly to determine whether the structural information that was available could be refined significantly by increasing the resolution toward functional determinants (and thus isolating some of the multiple effects likely brought about by deletion of a domain), and partly to search for new properties that might have been missed either because of a masking mutation or because of a less comprehensive analysis. Clustered charged-to-alanine scan mutagenesis, combined with transient transfection of human embryonic kidney "293" cells and analysis using a battery of assays proved to be a satisfactory method for approaching this problem. The mutant genes were constructed readily, and the variant proteins generally were expressed at acceptable levels. Using this strategy, the alanine scan variants have allowed us to confirm and refine a number of observations about the involvement of various domains in tPA function. More significantly, our results with these mutants have cast doubt on some of the current models of tPA function which were based on less subtle and/or less comprehensive mutational or biochemical analyses and have allowed us to make a number of novel observations as well.

Fibrin Binding, Stimulation, and Specificity—It was already clear from the literature that fibrin binding of tPA involves the finger and growth factor domains (29–33) and that kringle-2 has a lysine-binding site (60, 61); it is also believed generally that kringle-2 is important for fibrin binding (29–33). Our experiments confirm that mutations in the finger and growth factor domains affect fibrin binding, but they also suggest that kringle-2 is less involved in fibrin binding than expected. Furthermore, a functional lysine binding site on kringle-2 does not appear to be required for fibrin binding, fibrin stimulation, or clot lysis.

No evidence had yet been brought forward for an interaction of the protease domain either directly or indirectly with fibrin. Our results are consistent with such an interaction.

There are specific regions on the surface of the tPA protease where mutations affect both fibrin binding and specificity (possibly direct interactions), and other regions where only fibrin specificity is altered (probably indirect interactions).

The ability of fibrin to stimulate the activity of tPA is well documented (9, 11, 12, 15, 17) and implies that a signal from fibrin must be transmitted in some way to the protease. The aforementioned domain-deletion experiments had led to the widely accepted conclusion that this effect was mediated exclusively via the nonprotease domains. Our evidence points to a different and unexpected conclusion. Fibrin-stimulated activity in our plasminogen activation assay is affected very little by mutations at charged residues anywhere in the nonprotease domains. In fact, all of the mutations that significantly affected fibrin specificity were in the protease domain. The effects that we observe fall into two distinct categories: one type exhibits very low activity with fibrinogen, but is stimulated normally by fibrin. The other type has low activity with fibrinogen and increased activity with fibrin. Our results further indicate that there are multiple sites on the tPA protease that mediate the interaction with fibrinogen and fibrin. Variants of tPA that are more fibrin-specific than wild-type might be of therapeutic interest if it could be shown that increased fibrin specificity had a significant clinical correlate such as reduced fibrinogenolysis.

Zymogens—The ability to convert tPA into a more zymogen-like protease by the introduction of alanine-scan mutations is mechanistically interesting. The loss of certain determinants apparently constrains the protein to having either less activity in the one-chain form, or greater activity in the two-chain form, than wild-type tPA. The unusual phenotype found in tPA of being less of a zymogen than most serine proteases can evidently be reversed by mutations at selected sites in the protease domain. A tPA molecule that is more zymogen-like might be exploited clinically by administering the relatively inactive one-chain protein, which would be converted to a more active two-chain form upon exposure to plasmin at the site of a thrombus.

The Position 298 Variant—One alanine scan variant (KHRR 296–299 AAAA; mean residue 298) is singularly resistant to PAI-1. The basic loop in which the mutation occurs is likely to be the most important, possibly the only, site of PAI-1 recognition on tPA (other than the active site itself). It is noteworthy that this same region is the one which we found to be responsible for the most dramatic effects on fibrin stimulation and specificity. The position 298 variant has about three times the plasminogen-activating activity of wild-type tPA in the presence of fibrin, and its activity is reduced markedly in the absence of a stimulator or in the presence of fibrinogen, giving it 8–10-fold greater "fibrin specificity" than wild-type. This variant also has a greater differential activity between the one- and two-chain forms than wild-type tPA. It is also the only variant that exhibits an increase in activity against plasma clots *versus* fibrin clots (Fig. 7); all others show a decrease. This effect may be related to PAI-1 resistance. We want to refine even further the mutagenesis of this region to determine whether the effects of PAI-1 resistance, fibrin stimulation, and clot lysis are linked inextricably or whether they might be spatially or functionally separable by individual mutations within this relatively small locus.

In conclusion, we have used site-directed mutants to localize some of the functional regions of the tPA molecule in a more subtle and precise way than had been possible previously, with results that shed additional light on how tPA functions. We expect that this approach may foster a better understanding of the structure-activity relationships of tPA and that it may aid in the design of new types of thrombolytic proteins.

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