Structural Domains of Human Tissue-type Plasminogen Activator That Confer Stimulation by Heparin

Paul L. Stein, Anton-Jan van Zonneveld, Hans Pannekoek, and Sidney Strickland

From the Department of Molecular Pharmacology, State University of New York at Stony Brook, Stony Brook, New York 11794-8651, and the Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Department of Molecular Biology, Amsterdam, The Netherlands

Heparin has been shown recently to stimulate the activity of human tissue-type plasminogen activator (t-PA). To investigate this effect further, mutant proteins lacking various domains of t-PA were screened for the ability to be stimulated by heparin. Those mutants harboring either the finger domain or the 2nd kringle were found to have enhanced enzymatic activity in the presence of heparin. Only mutants containing these structures would bind to heparin-agarose beads; monoclonal antibodies directed against these domains blocked binding. The stimulatory effect of heparin was more pronounced in finger-containing mutants than kringle-2 proteins. Earlier results had localized the fibrin-binding domains to the same two structures. Unlike heparin, the 2nd kringle was shown to be more important than the finger for fibrin stimulation. Our results have implications for producing recombinant t-PA variants for use in thrombolytic therapy.

Considerable attention has been recently focused on the therapeutic value of tissue-type plasminogen activator (t-PA) as a thrombolytic drug (1). This enzyme converts the inactive proenzyme plasminogen into plasmin, a relatively potent trypsin-like protease. Because fibrin binds and can substantially increase the activity of t-PA, it has been suggested that t-PA might be targeted to the clot site where it could initiate digestion of the fibrin network (2). This property of clot selectivity might make t-PA superior to other types of plasminogen activators for the treatment of thrombotic disorders. It has been shown recently that the anticoagulant heparin can also stimulate t-PA activity, and in addition, can interfere with fibrin stimulation (3-5). Since heparin is used in conjunction with t-PA during thrombolytic therapy (6), it is possible that a t-PA-heparin complex could form in the blood, enhancing fibrin-independent degradation of plasma proteins including clotting factors.

The t-PA molecule is composed of several discrete domains (Fig. 1A). The amino-half of the protein consists of (i) a finger region which bears homology to the fibrin-binding fingers of fibronectin (7), (ii) a cysteine-rich domain which is partially homologous to epidermal growth factor (8) and is found in a number of serum proteins (9); and (iii) two triple-loop, three disulfide-bonded structures called kringles which have also been identified in several serum proteases (10-12). The carboxyl-half of the molecule consists of the catalytic portion of the enzyme and belongs to the trypsin family of serine proteases (8). Previous experiments have reported that the fibrin-binding domains of t-PA are localized in the finger and 2nd kringle. In mutant proteins stimulation by fibrin appears to be mediated primarily by the 2nd kringle, to a lesser degree by the finger (13-15), although in other constructs the 1st kringle also can participate in fibrin stimulation (16). Considering the potential significance of heparin use during thrombolytic treatment, we have investigated the regions of the protein that confer heparin stimulation.

MATERIALS AND METHODS

Construction and Assay of Mutant Proteins—The construction and expression of the t-PA variants has been described (13, 14). The mutants were quantitated by an enzyme-linked immunosorbent assay using a light chain-specific monoclonal antibody (13, 14). Aliquots of serum-free conditioned media were used in an amidolytic t-PA assay (3). The amount of mutant used was as follows: rt-PA, 2.5 ng; LF, 6.6 ng; LFE, 1.4 ng; LK1, 4.4 ng; LK2, 3.6 ng; and L, 2.4 ng. The assays were supplemented with either 15 μg/ml CNBr-digested fibrinogen (17) or 30 μg/ml (5 IU/ml) porcine intestinal heparin, Ca2+ salt (Sigma). Since the relative activities of the mutants differ slightly, the amount of enzyme used reflects the minimum concentration necessary to support the assays.

When the effects of the monoclonal antibodies on the activity of the mutant proteins were determined, the following conditions were employed. LF (1.7 ng) was incubated with 20 μg/ml anti-finger or anti-K2 antibodies (CLB-t-PA51 or CLB-t-PA74). For the mutant LK2, 1.8 ng of protein was preincubated with 1 μg/ml of the above antibodies. These concentrations were chosen after titration of the antibodies. Production and characterization of the monoclonal antibodies have been described previously (18).

Binding of Mutants to Heparin-Agarose—To measure binding, 20 μl of packed heparin-agarose (Sigma; 750 μg heparin/ml of gel) was incubated at room temperature with 60 μl of the various t-PA proteins in phosphate-buffered saline for 45 min. The amount of mutant used was as follows: rt-PA, 7.5 ng; LF, 6.6 ng; LFE, 2.8 ng; LK1, 13.2 ng; LK2, 18.8 ng. The beads were quickly pelleted and the supernatant removed. The supernatant was then adjusted to a final concentration of 2% SDS, 5% glycero. The packed material was washed once with 60 μl of PBS and the bound protein extracted by resuspending the beads in 80 μl 2% sodium doxycyl sulfate, 5% glycero in phosphate-buffered saline. After electrophoresis, the activity in the supernatant (S) and pellet (P) was visualized by casein-agar zymography (19, 20).

RESULTS

Using mutant proteins derived by expressing cDNAs with specific deletions in mouse L-cells t-PA (13, 14), we screened for enhancement of activity by heparin or fibrinogen fragments. The soluble fragments faithfully mimic the effects of
fig. 1. Structure and activity of t-PA mutants. A, schematic diagram of t-PA showing the positions of the different domains within the molecule. Each domain is indicated by a letter as follows: F, finger domain; E, epidermal growth factor homology; K1 or K2, 1st or 2nd kringle structures, respectively; and L, the catalytic light chain. The shaded portions indicate the regions where fibrin and heparin interact. Each mutant derives its name from the domains present in the protein. For example, LF contains only the finger and light chain regions. B, influence of heparin (open columns) and fibrinogen fragments (solid columns) on plasmin generation by t-PA mutants.

A mutant containing only the 2nd kringle (LK2) readily activated plasminogen in the presence of fibrinogen fragments. However, heparin only modestly increased the activity of LK2 compared to finger-containing mutants. The activity of mutants containing only the 1st kringle or the catalytic light chain alone (LK1 and L) were not enhanced by either fibrinogen fragments or heparin. It is interesting to note that intact t-PA is not stimulated by heparin as greatly as LF. Although the reason is not clear, it is possible that the presence of all the domains may partially mask the heparin-binding sites. These results suggested that the finger is primarily responsible for the heparin-mediated stimulation, whereas the 2nd kringle is of lesser importance.

Because the mutant LF was so dramatically stimulated by heparin, an important question was whether the mucopolysaccharide was stimulating plasminogen activation or facilitating the conversion of LF from the one-chain to the more active two-chain form. To address this question, we cleaved LF to the two-chain form by limited plasmin digestion since Western blots of secreted LF indicated that it was almost exclusively a single-chain molecule. The presence of two-chain LF after plasmin treatment was confirmed by Western blot analysis using a rabbit polyclonal antibody. The two-chain LF was also stimulated 4-fold by heparin (Fig. 2).

To determine the relative affinity of each mutant for heparin, the mutants were also analyzed for their ability to bind to heparin-agarose beads. LF and LK2 bind nearly quantitatively to the beads (Fig. 3). This is specific to heparin-agarose, since no binding occurred on unsubstituted beads.

Fig. 2. Effect of glycosaminoglycans on plasminogen activation by t-PA mutants. The chondroitin sulfate types A, B, and C were obtained from Sigma. A, LF. The increase in stimulation by high concentrations of chondroitin sulfate type B is probably due to trace amounts of heparin in the commercial preparation. B, LK2. C, t-PA. Note the difference in the scale of A (LF). □—□, chondroitin sulfate A; △—△, chondroitin sulfate B; ■—■, heparin.
of the epidermal growth factor domain to LF disrupts binding to a certain degree (see Fig. 3; LFE). The mutant LK1 which is not stimulated by heparin also does not bind to the heparin beads. This result eliminates both the light chain and 1st kringle as domains interacting with heparin. The relative affinities of the mutants for heparin-agarose correlate well with the activity measurements presented in Fig. 1. Despite the presence of both heparin binding domains, no all of the rt-PA activity is associated with the pellet (Fig. 3). In the presence of 0.1 M Tris-HCl binding of t-PA is quantitative (3); at physiologic NaCl concentrations, the salt reduces the affinity for heparin, and the extra domains can then interfere with efficient binding to heparin.

Monoclonal antibodies directed against either the finger or kringle-2 domains of t-PA (18) were used to probe for regions that might interact with fibrin or heparin. It was possible that the antibodies might interfere with stimulation by one ligand but not with the other. A finger-specific antibody reduced activity of LF by 40% when assayed in the presence of fibrinogen fragments, but actually increased the heparin-stimulated activity (Fig. 4A). The reasons for this increase in the presence of the monoclonal antibody are not known. A different anti-finger monoclonal antibody did not exhibit this stimulation. As expected, an antibody directed against K2, a domain not present in the mutant LF, had no effect on activity. When LK2 was assayed in the presence of an antibody recognizing the 2nd kringle, fibrin stimulation was inhibited 75% (Fig. 4B). In contrast, heparin-mediated activation was only slightly impaired by this antibody. An antibody directed against the finger domain had no effect on LK2. These data show that domain-specific monoclonal antibodies affect heparin and fibrin stimulation differently, implying that different sub-sites within each domain of t-PA are involved in these enhancements.

It has been shown previously that 20 mM ε-aminocaproic acid, a lysine analog, specifically blocks the binding of the K2 domain to fibrin (21). In contrast, this inhibitor has no effect on the interaction of LK2 with heparin-agarose.2 These results also suggest that the regions conferring fibrin or heparin binding are distinct.

Since the mutants in the absence of stimulator showed a loss of activity after antibody binding,2 it is possible that the activity measurements reflected integrity of the active site rather than specific disruption of a complex between the enzymes and heparin. To address this point, we analyzed the binding of the mutants to heparin-agarose in the presence of the monoclonal antibodies. Fig. 5 shows that binding to the heparin-agarose beads could be successfully blocked by the appropriate antibody. The mutant LK2 was found to be associated exclusively with the heparin-agarose pellet when

---

**Fig. 3. Binding of t-PA mutants to heparin-agarose beads.** The name of the protein used is indicated under each panel. S indicates supernatant (unbound fraction), P indicates the material eluted from the heparin-agarose pellet (bound fraction).

**Fig. 4. Effect of monoclonal antibodies on activity of t-PA mutants.** The activity was measured using a chromogenic assay (3) and is presented as the ratio of the -fold stimulation when the antibody is present, compared to an identical sample lacking antibody. This analysis is internally referenced for each mutant and is therefore independent of enzyme concentration. The ratio is derived from the initial rates of hydrolysis ± stimulators. It can be expressed as

\[
\frac{\text{Mutant + stim}}{\text{Mutant alone}} \times 100 = \% \text{ of control.}
\]

Stim represents the stimulators heparin (open columns) or fibrinogen fragments (solid columns); +Ab denotes that the assay was performed in the presence of either the anti-finger or anti-kringle-2 antibody; -Ab, no antibody present. A, LF, B, LK2. The finger-specific antibody used was CLB-t-PA51, and the 2nd kringle antibody was CLB-t-PA74 (18).

**Fig. 5. Interference of binding of t-PA mutants to heparin-agarose using monoclonal antibodies.** Each protein was incubated alone or with one of the monoclonal antibodies for 1 h at room temperature. The mixture was then analyzed for the ability to bind heparin-agarose. Anti-F, finger-specific antibody; Anti-K2, 2nd kringle-specific antibody. Top panel, 6.6 ng of LF incubated with 400 µg/ml anti-finger (CLB-t-PA70) or anti-K2 (CLB-t-PA74) antibodies. Bottom panel, 1.8 ng of LK2 incubated with 100 µg/ml anti-finger or anti-K2 antibodies. S indicates the unbound material in the supernatant, and P indicates the enzyme bound to the heparin-agarose pellet.
treated with a finger-specific antibody. However, when a K2 antibody was included during binding a significant portion of the enzyme was shifted to the unbound fraction instead. The same approach was used to analyze LF. The binding of this mutant could be specifically blocked by the finger antibody but not by the kringle antibody. It should be noted that the anti-finger monoclonal antibody in these experiments (CLB-t-PA70) is different from the one used in Fig. 4A; the concentration of CLB-t-PA70 necessary to inhibit binding precluded its use in the chromogenic assay since it would act as a competitive substrate for the plasmin generated during the assay.

These experiments localize the heparin-binding regions of t-PA to the finger domain and 2nd kringle. However, a recent report identified the catalytic light chain as the heparin-binding site of porcine t-PA (22). We have no explanation for the difference in results except for the possibility that the reduction and subsequent renaturation used to separate the heavy and light chains of porcine t-PA may have altered the heparin-binding properties.

**DISCUSSION**

Based on the stimulatory effect of heparin on the various t-PA mutants, we propose that the finger contributes most to the activation. In one sense this result is not surprising, since the homologous fingers from fibronectin also bind to heparin (12). It is not currently known if the heavy and light chains of porcine t-PA may have altered the heparin-binding properties.

Heparin-related compounds may also influence t-PA activity in other tissues. For example, rat ovarian follicular fluid contains an abundance of plasmin-sensitive proteoglycans as well as t-PA. Possibly, the heparin oligosaccharide units present on the proteoglycans stimulate plasminogen conversion, initiating digestion of the proteoglycans in preparation for fibrinolysis to provide a substrate for the plasmin generated during the process.

REFERENCES