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Tissue-Type Plasminogen Activator Is a Multiligand Cross-β Structure Receptor

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Summary

Tissue-type plasminogen activator (tPA) regulates fibrin clot lysis by stimulating the conversion of plasminogen into the active protease plasmin [1]. Fibrin is required for efficient tPA-mediated plasmin generation and thereby stimulates its own proteolysis. Several fibrin regions can bind to tPA [1], but the structural basis for this interaction is unknown. Amyloid β (Aβ) is a peptide aggregate that is associated with neurotoxicity in brains afflicted with Alzheimer's disease [2]. Like fibrin, it stimulates tPA-mediated plasmin formation [3–5]. Intermolecular stacking of peptide backbones in β sheet conformation underlies cross-β structure in amyloid peptides [6]. We show here that fibrin-derived peptides adopt cross-β structure and form amyloid fibers. This correlates with tPA binding and stimulation of tPA-mediated plasminogen activation. Prototype amyloid peptides, including Aβ and islet amyloid polypeptide (IAPP) (associated with pancreatic β cell toxicity in type II diabetes [7]), have no sequence similarity to the fibrin peptides but also bind to tPA and can substitute for fibrin in plasminogen activation by tPA. Moreover, the induction of cross-β structure in an otherwise globular protein (endostatin) endows it with tPA-activating potential. Our results classify tPA as a multiligand receptor and show that cross-β structure is the common denominator in tPA binding ligands.

Results

tPA-Mediated Plasminogen Activation by Fibrin-Derived Peptides

One of the amino-acid sequences within the fibrin α chain that supports tPA binding and activation encompasses the 13 residues 148–160 [1]. Based on the amino-acid sequence of this region, we made two peptides: fibrin-derived peptide 13 (FP13) (148–160: KRLEVDIDI KIRS) and FP10 (148–157: KRLEVDIDIK). First, we tested the capacity of the peptides to substitute for fibrin in stimulating tPA-mediated plasminogen activation. The addition of FP13, but not FP10, to a mixture of plasminogen and tPA lead to a potent increase in the generation of plasmin activity over time (Figure 1A).

Next, we studied the ability of the peptides to bind to tPA by performing solid-phase binding assays. Surprisingly, we found that both peptides bound to tPA despite the fact that only FP13 activates tPA (Figures 1A–1C). Because FP10 has a C-terminal lysine residue (K), binding to this peptide could be mediated by the kringle domain(s) in tPA. In line with this notion, binding of FP10 to tPA is completely lost in the presence of the lysine analog ϵ-aminocaproic acid (ϵACA) (Figure 1D). In contrast, ϵACA inhibits the binding of tPA to FP13 by only about 40%, indicating that this interaction is largely lysine independent (Figure 1D).

Fibrin-Derived Peptides Form Cross-β Sheets

The above experiments show that the binding of tPA to the two peptides occurs through distinct types of interaction, one supporting tPA-mediated plasminogen activation, the other not. We considered the possibility that differences in the peptide structure could underlie the difference in cofactor activity. The relative contribution of random coils, β sheets, and α helices to the structure of the peptides in solution was studied via circular-dichroism measurements. Strikingly, we found that FP13 was in a 100% β sheet conformation with a characteristic minimum at 215 nm and a maximum at 190 nm (Figure 2A). In contrast, FP10 was completely randomly coiled, with a minimum observed at 198 nm (Figure 2A). Therefore, it seems likely that structural differences underlie the differences in the ability of these peptides to activate tPA.

Given the high β sheet content in FP13 and given the finding that amyloid β (Aβ) supports plasminogen activation by tPA [3–5], we tested whether FP13 is an amyloid peptide with cross-β sheet conformation. Congo Red binds to amyloid peptide aggregates, irrespective of the amino acid sequence [8]. Dried samples of FP10 and FP13 were stained with Congo Red and examined by light microscopy. Figure 2B shows that FP13 dried as aggregates that readily bound Congo Red; it displays the characteristic green birefringence when examined under polarized light. In contrast, FP10 dried as a uniform film that did not bind Congo Red (our unpublished data).

Thioflavin T (ThT) is a fluorescent indicator of cross-β sheets in solution [8]. By incubating the peptide solutions with increasing concentrations of ThT and subsequently measuring fluorescence emission, we found that FP13, but not FP10, enhanced ThT fluorescence (Figure 2C). Thus, the high content of β sheets in FP13 presum-

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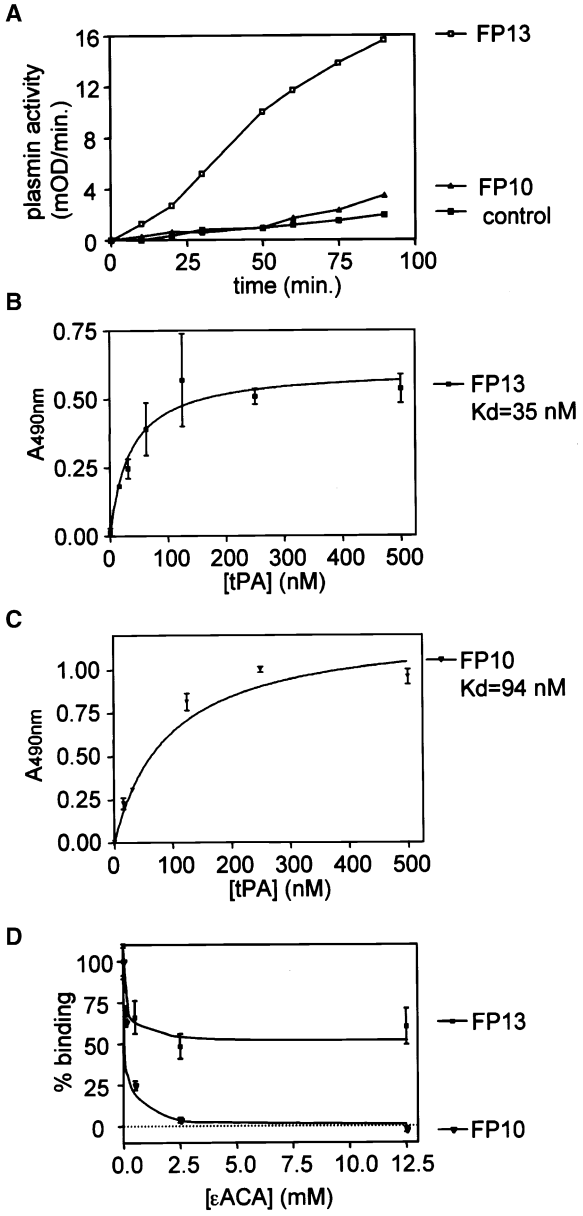

Figure 1. tPA Binding and Plasminogen Activation by Fibrin-Derived Peptides

(A) Plasminogen (0.1 mg/ml) and tPA (200 pM) were incubated with the fibrin-derived peptides (5 μM) or with control buffer. The conversion of plasminogen to plasmin was followed over a period of time by measurement of plasmin activity with a chromogenic substrate (S-2251).

(B) Binding of FP13 to tPA. FP13 was coated onto plastic and was overlayed with the indicated concentrations of tPA. tPA binding was then assessed with the polyclonal anti-tPA antibody 385R in an ELISA-type assay.

(C) Binding of FP10 to tPA was assessed as in (B).

(D) tPA binding to FP13 and FP10 was assessed as in (B) and (C) with 200 nM tPA in the presence of the indicated concentrations of εACA.

ably reflects cross-β sheet conformation. The presence of cross-β sheets can be demonstrated by X-ray diffraction analysis [9]. When analyzed by X-ray diffraction, FP13 caused a distinct diffraction pattern, with reflection maxima at 4.7, 11.7, and 23.4 Å. The fiber axis with its 4.7 Å hydrogen bond repeat distance is oriented along the vertical capillary axis. The 11.7 Å repeat is perpendicular to that. The perpendicular orientation of the 4.7 Å and 11.7 Å distances is typical for cross-β sheets. The 23.4 Å reflection that, like the 11.7 Å reflection, is oriented horizontally, signals higher ordering in the intersheet direction. Rough estimation of the crystallite sizes from the width of the reflections gives 120 Å for the fiber direction and 40–50 Å for the intersheet direction. This amounts to approximately 25 β strands being hydrogen-bonded into an array of about 4 cross-β sheets.

As a result of cross-β sheet formation, peptides may undergo fibrillogenesis [6], and such fibers can be visualized with transmission electron microscopy (TEM). We therefore examined single drops of peptide solutions by TEM. We found that FP13, but not FP10 (our unpublished data), formed unbranched fibrils that were about 5 nm thick, with lengths ranging from 250 to 1000 nm (Figure 2E). These dimensions are within the range of dimensions found for other amyloid peptides [10].

Thus, FP13 is an amyloid peptide with cross-β sheet conformation, it binds to tPA, and it stimulates tPA-mediated plasminogen activation. In contrast, FP10 is in a random-coil conformation and binds to tPA through its C-terminal lysine residue without stimulating tPA-mediated plasmin formation.

tPA Binding and Plasminogen Activation by Cross-β Sheet Peptides Irrespective of Their Amino Acid Sequences

The above results prompted us to investigate the binding characteristics of several amyloid peptides to tPA. Aβ stimulates tPA-mediated plasmin formation [3–5] but, to our knowledge, no binding data have been published. To test tPA binding to Aβ and to other amyloid peptides, we performed solid-phase binding assays and detected bound tPA by ELISA. Figure 3A shows that tPA binds to Aβ with high affinity (6.7 nM), as it does to the fibrin peptides. Human islet amyloid polypeptide (hIAPP) is a 37 amino acid peptide that has amyloidogenic properties and is found deposited in the islets of Langerhans in the pancreas of type II diabetic patients. It acts as a diabetogenic factor, presumably by being toxic to the insulin-producing β cells [7]. The hIAPP amino acid sequence is unrelated to that of Aβ or the fibrin peptides. We found that, like Aβ and the fibrin peptides, hIAPP binds to tPA with high affinity (K_d = 1.8 nM; Figure 3B).

Next, we tested the capacity of these two distinct preaggregated amyloid peptides (Aβ and hIAPP) to substitute for fibrin in stimulating tPA-mediated plasminogen activation. We found that both peptides greatly enhance tPA-mediated plasmin formation (Figure 3C). Interestingly, the same peptides did not stimulate tPA-mediated plasminogen activation when they were used immediately after solubilization (Figure 3C). Thioflavin T binding to both peptides increased gradually over a period of three weeks (our unpublished data). Thus, the formation of cross-β structure in the peptides is accompanied by an acquired ability to enhance tPA-mediated plasmin generation.

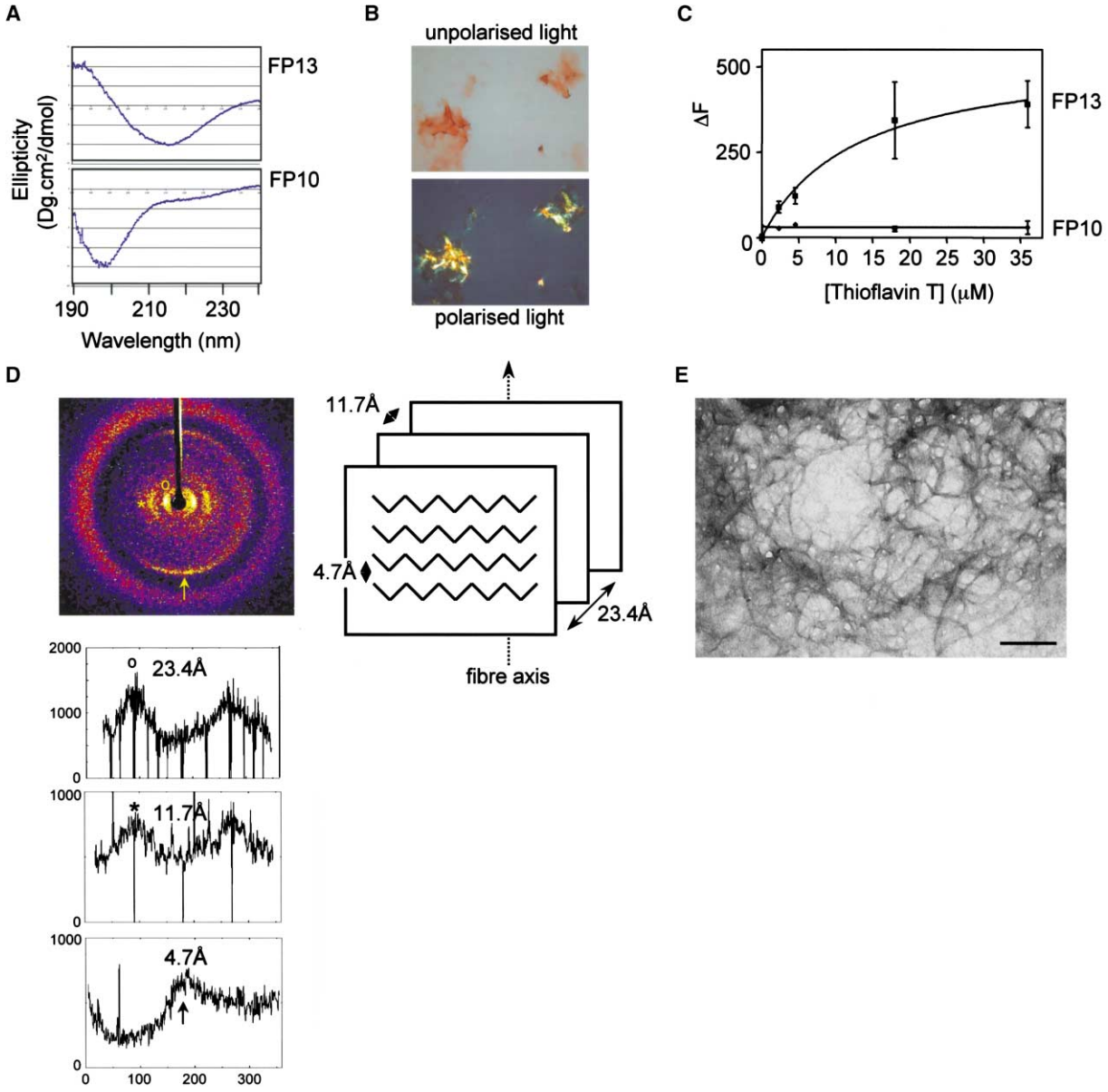

Figure 2. Structural Analysis of Fibrin-Derived Peptides

(A)The absorbance of circularly polarized light by solutions of FP13 and FP10 (0.1 mg/ml) was analyzed in a circular-dichroism spectropolarimeter at the indicated wavelengths.

(B) Single drops of FP13 and FP10 (1 mg/ml in H_2O) were air-dried and stained with Congo Red according to the manufacturer's protocol. Microscopic analysis with nonpolarized and polarized light shows that FP13, but not FP10 (not shown), binds Congo Red.

(C) Solutions of FP13 and FP10 were incubated with the indicated concentrations of thioflavin T, and fluorescence emission was measured at 485 nm.

(D) X-ray diffraction analysis of FP13. The scattering as obtained on the 2D detector is color-coded by intensity on a linear scale. The scattering intensity decreases as white-yellow-red-blue-black. The pattern shows diffraction maxima at 4.7, 11.7, and 23.4 Å. In the lower panels, tangential scans along the 2θ scattering angles corresponding to all three d-spacings show that the 4.7 Å scattering (hydrogen bonds) is perpendicular to that at 11.7 and 23.4 Å ($1\times$ and $2\times$ intersheet distance).

(E) Solutions of FP13 and FP10 were spotted on a carbon-coated grid and analyzed by transmission EM. FP13, but not FP10, shows unbranched amyloid-like fibers. The scale bar represents 200 nm.

Whereas the rate of Aβ-stimulated plasmin formation is further enhanced during the reaction, that induced by hIAPP is not. We have found that during Aβ-stimulated plasmin formation limited proteolysis generates free internal lysines in Aβ (O.K. et al., submitted). This may enhance the reaction by allowing more plasminogen and tPA binding. The lack of further rate-enhancement during reactions with hIAPP may be explained by the fact that it has only one lysine residue that is located at the extreme N terminus.

One of the amyloidogenic regions in hIAPP encompasses amino acid residues 20–29 [7, 11–14]. Diabetic

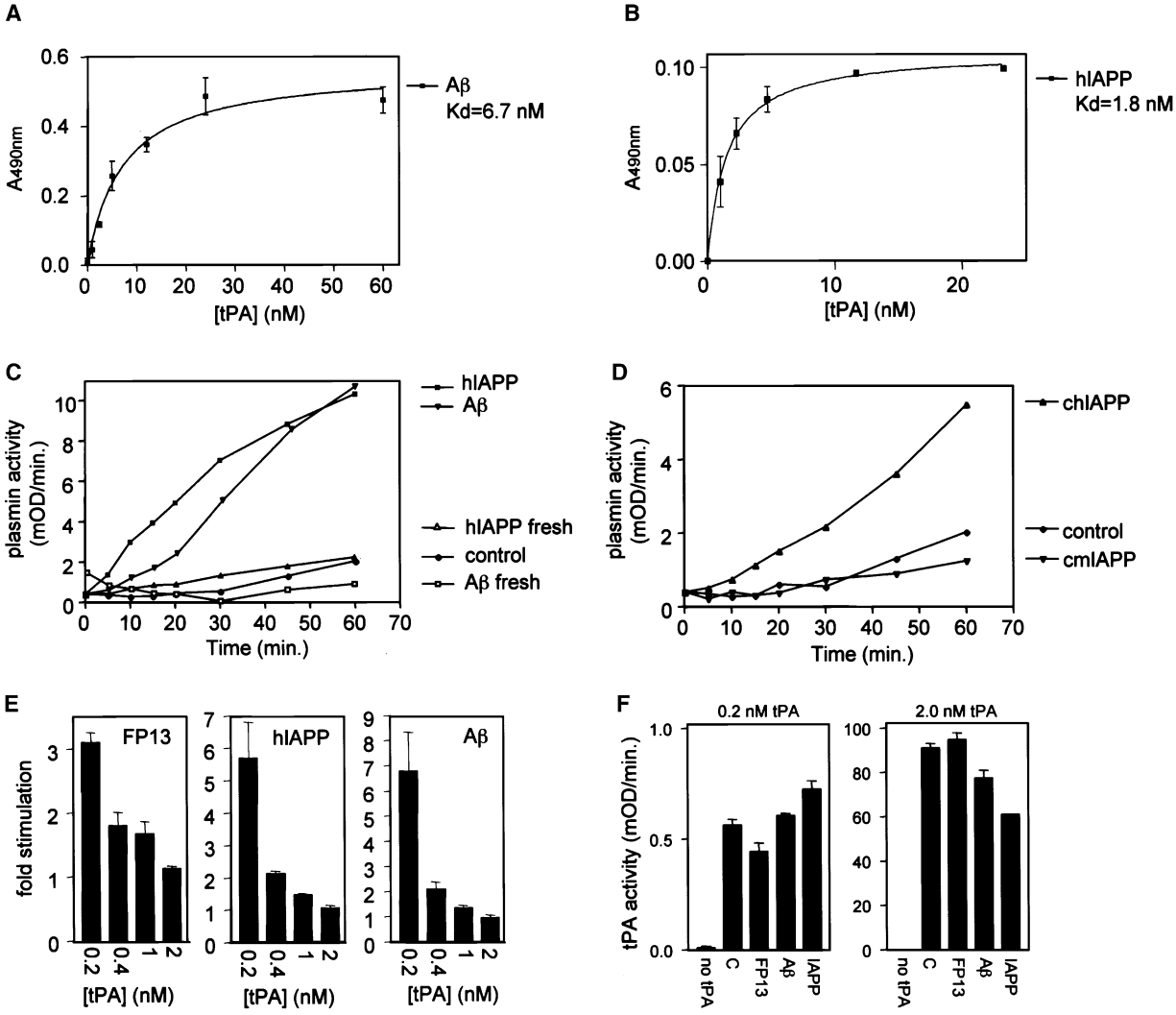

Figure 3. tPA Binding and Stimulation of Plasminogen Activation by Aβ and hIAPP

(A) The binding of tPA to Aβ was tested as in Figure 1B.

(B) The binding of tPA to hIAPP was tested as in Figure 1B.

(C) The stimulation of tPA-mediated plasminogen activation by Aβ and hIAPP, either freshly resuspended or pre-aggregated for three weeks, was tested as in Figure 1A.

(D) The activation of tPA by mouse and human "core" IAPP (cmIAPP, chIAPP) was tested as in Figure 1A.

(E) FP13, hIAPP, and Aβ were incubated with plasminogen and with increasing concentrations of tPA. Plasmin formation was then assessed as in Figure 1. The measured rates of plasmin generation in time with and without the peptides were then used for calculating the extent to which the peptides stimulated plasmin generation. These values were subsequently plotted against the tPA concentrations. The peptides loose their rate-enhancing effect at higher tPA concentrations.

(F) tPA, either at 0.2 nM or at 2 nM, was incubated with FP13, Aβ, and hIAPP together with S-2765, a chromogenic peptide substrate for tPA. tPA caused substrate conversion, but none of the cross-β peptides stimulated tPA enzymatic activity.

mice, in contrast to diabetic humans, do not develop pancreatic amyloid deposits because mouse IAPP (mIAPP) has a different amino-acid sequence in this region and lacks the propensity to adopt cross-β structure. We tested the presence of cross-β structure in these "core" regions in human and mouse IAPP (chIAPP, cmIAPP) by Congo Red binding and compared their ability to stimulate tPA-mediated plasminogen activation. As expected, Congo Red readily bound chIAPP, but not cmIAPP; it showed green birefringence under polarized light (our unpublished data). Figure 3D shows that chIAPP stimulated tPA-mediated plasmin formation, albeit less efficiently than the full-length IAPP, whereas cmIAPP was ineffective. When these results are taken together with the data on the fibrin peptides, we have shown that four amyloid peptides (FP13, Aβ, hIAPP, chIAPP) stimulate tPA-mediated plasmin formation, whereas two non-amyloid peptides (FP10, cmIAPP) do not. Therefore, the results strongly suggest that tPA binding and cofactor activity require the presence of cross-β structure in the peptides.

How do cross-β peptides cause enhanced plasminogen activation? Like fibrin, they could act as scaffolds by promoting the interaction between enzyme (tPA) and

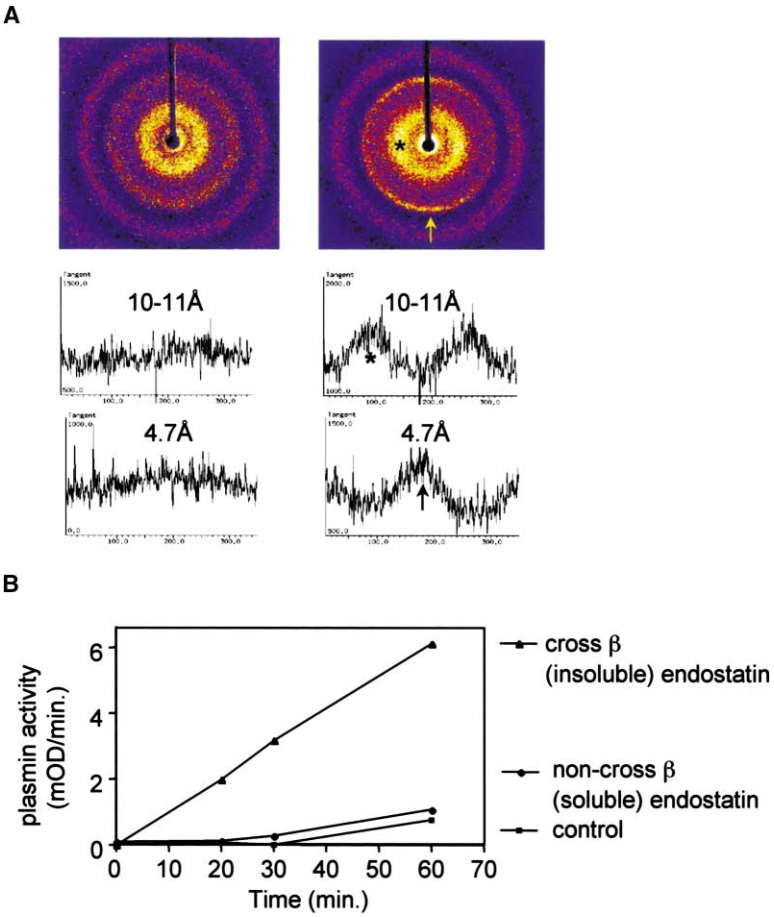

Figure 4. Enhanced Plasminogen Activation by Cross-β-Structured Endostatin

(A) X-ray diffraction analysis of two distinct forms of endostatin. The left panel shows globular endostatin with no detectable cross-β sheets. The right panel shows insoluble endostatin with abundant cross-β sheets. The scattering as obtained on the 2D detector is color coded as in Figure 2D. The pattern shows diffraction maxima at 4.7 and 10–11 Å. The fiber axis (hydrogen bond direction) with a 4.7 Å repeat is oriented vertically. The 10–11 Å repeat is preferentially oriented perpendicularly to that, as indicated by the asterisk. Tangential scans along the 2θ scattering angles corresponding to both d spacings in the lower panel show that the scattering at 4.7 Å is oriented vertically, with the maximum intensity at 180° indicated by the arrow. The reflection at 10–11 Å is oriented horizontally, with the maximum at 90° indicated by the asterisk.

(B) Stimulation of tPA-mediated plasminogen activation by the two distinct forms of endostatin was assessed as in Figure 1A.

substrate (plasminogen). Alternatively, cross-β peptides may stimulate tPA enzymatic activity. With increasing tPA and plasminogen concentrations, one would expect scaffold effects to diminish. In our assay, the tPA concentration is limiting. Therefore, we performed plasminogen activation assays with increasing concentrations of tPA by using FP13, hIAPP, and Aβ as cofactors. Figure 3E shows that the stimulatory effect of the cross-β peptides on plasmin generation is lost with increasing concentrations of tPA. Next, we tested whether the cross-β peptides would stimulate tPA enzymatic activity. Figure 3F shows that none of the tested peptides (FP13, IAPP, and Aβ) had a stimulatory effect on the conversion of the chromogenic peptide substrate S-2765 by tPA, neither at low (0.2 nM) nor at high (2 nM) concentrations of the enzyme. Thus, cross-β peptides act as scaffolds for efficient plasminogen conversion by tPA without modulating tPA activity.

Finally, we tested whether cross-β sheet formation in larger proteins would also support tPA-mediated plasminogen activation. Firstly, it should be noted that cross-β sheet formation occurs during the polymerization of fibrinogen into fibrin as measured by Raman spectroscopy [15] and that this is accompanied by tPA-activating potential. Secondly, we have recently found that endostatin, a 20 kDa fragment of the extracellular matrix protein collagen XVIII, has the propensity to form cross-β structure and to aggregate into amyloid deposits (Figure 4A). Endostatin can also be isolated in a soluble globular form that does not form cross-β sheets (Figure 4A). These two forms of endostatin allowed us to compare the capacity of a single larger protein (20 kDa) in two distinct conformations (cross-β versus non-cross-β) in the stimulation of tPA-mediated plasminogen activation. Figure 4B shows that endostatin with cross-β structure potently stimulates tPA-mediated plasminogen activation like all the other cross-β peptides but that globular endostatin has no effect. Thus, the correlation between cross-β structure and the ability to stimulate tPA-mediated plasminogen activation holds for (short) peptides as well as for larger proteins.

Discussion

Our results provide evidence that tPA is a multiligand receptor for proteins that display cross-β structure. Upon binding to tPA, all cross-β ligands tested stimulate tPA-mediated plasmin formation. The tPA binding peptide sequences in fibrin have been identified [1], but no structural data are available that show the basis of this interaction. Early data on the structural changes that are associated with the conversion of fibrinogen into fibrin show that this is accompanied by a general increase in β sheet content and by the formation of hydrogen bonds between lateral fibrin molecules [15]. This type of interaction within the fibrin meshwork is similar

to that underlying the cross-β sheet structure in amyloid peptide aggregates [6]. Furthermore, we show here that one of the isolated tPA binding regions in fibrin that supports plasminogen activation has cross-β structure. Thus, also in fibrin, tPA may bind to cross-β sheet-forming regions. It has long been known that aged fibrin deposits can bind Congo Red in tissue sections [16]. We have been able to corroborate these data in vitro by showing the selective staining of specific regions within a fibrin clot with Congo Red and by enhanced thioflavin T fluorescence during clot formation (O.K. and B.B., unpublished data). In addition, serum amyloid P component (SAP), as well as Aβ itself, modulates the assembly and lysis of fibrin clots [17–19]. Taken together, the available data suggest that the control of fibrin assembly and fibrinolysis is regulated by cross-β structures and amyloid binding proteins. The identification of tPA as a general cross-β sheet binding molecule strongly reinforces this notion. In contrast to tPA, urokinase-type plasminogen activator (uPA) is not activated by Aβ [4]. We are presently further investigating whether uPA is regulated by cross-β structure.

Like fibrin, partially denatured proteins can stimulate tPA-mediated plasminogen activation [20, 21] and are prone to form cross-β sheets [10]. Our finding that the interaction of tPA with cross-β sheet peptides and proteins invariably leads to tPA-mediated plasminogen activation strongly suggests that these two phenomena are causally related. Thus, the formation of cross-β sheets endows protein aggregates (for instance endostatin) with tPA binding and plasminogen-activating potential. tPA may play a general role in the removal of cross-β sheet-containing (improperly folded) proteins by inducing their plasmin-mediated proteolysis. In this way, tPA could prevent the accumulation of toxic protein aggregates in the circulation. Indeed, Aβ can induce its own destruction by activating the tPA/plasmin system in cultures of neuronal cells [22, 23].

An important new insight is that protein aggregates, not necessarily related to any disease, are toxic to cells irrespective of their identity [24]. This implies that a common structural element rather than a specific amino-acid sequence induces toxicity. Cross-β sheets are likely to represent such a sequence-independent structural element that can mediate toxic effects on cells. Hence, there must be molecular mediators of toxicity that recognize cross-β structure. With the discovery of tPA as a general cross-β sheet binding protein, we have identified a molecule that may contribute to the general cell toxicity induced by protein aggregates. Interestingly, tPA has been identified as an essential mediator of neuronal cell death following ischemia or excitotoxic injury in the brain [25, 26]. By analogy, tPA may play a role in neuronal and endothelial cell death induced by Aβ and/or in IAPP-induced β-cell death.

Further work is needed to assess whether activation of the tPA/plasminogen system by protein aggregates (including IAPP and Aβ) prevents toxicity of the aggregates through their destruction or whether tPA plays a role in mediating toxicity. These possibilities are not mutually exclusive. A critical variable between distinct aggregates will be their differential sensitivity to plasmin-mediated destruction.

Supplementary Material

Supplementary Material, including the Experimental Procedures, is available at http://images/cellpress.com/supmat/supmatin.htm.

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