Identification of fibronectin type I domains as amyloid-binding modules on tissue-type plasminogen activator and three homologs

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Abbreviations: tPA = tissue-type plasminogen activator; FXII = coagulation factor XII; HGFA = hepatocyte growth factor activator; Fn = fibronectin; Af = amyloid β peptide; ATTR = transthyretin; ELISA = enzyme-linked immunosorbent assay; F = fibronectin type I (finger) domain; EGF = epidermal growth factor; E = EGF-like domain; K = kringle domain; εACA = epsilon-amino caproic acid; BSA = bovine serum albumin; AGE = advanced glycation end-product; GST = glutathione-S-transferase; LAM12 = laminin γ1-chain (2097–2108) amyloid core peptide

Abstract
The serine protease tissue-type plasminogen activator (tPA), a key enzyme in hemostasis, is activated by protein aggregates with amyloid-like properties. tPA is implicated in various pathologies, including amyloidoses. A major task is to further elucidate the mechanisms of amyloid pathology. We here show that the fibronectin type I domain of tPA mediates the interaction with amyloid protein aggregates. We found that in contrast to full-length tPA, a deletion-mutant of tPA, lacking the first three N-terminal domains (including the fibronectin type I domain), fails to activate in response to amyloid protein aggregates. Using recombinantly produced domains of tPA in direct binding assays, we subsequently mapped the amyloid-binding region to the fibronectin type I domain. This domain co-localized with congophilic plaques in brain sections from patients with Alzheimer’s disease. Fibronectin type I domains from homologous proteases factor XII, hepatocyte growth factor activator and from the extracellular matrix protein fibronectin also bound to aggregated amyloidogenic peptides. Finally, we demonstrated that the isolated fibronectin type I domain inhibits amyloid-induced aggregation of blood platelets. The identification of the fibronectin type I domain as an amyloid-binding module provides new insights into the (patho-)physiological role of tPA and the homologous proteins which may offer new targets for intervention in amyloid pathology.

Introduction
Tissue-type plasminogen activator (tPA) is a serine protease which has a key role in hemostasis. It initiates the destruction of blood clots via activation of the fibrinolytic system. The fibrinolytic system functions by proteolysis (fibrinolysis) of fibrin polymers, which are the main constituents of blood clots. Upon binding to fibrin, tPA is activated and converts the zymogen plasminogen into plasmin, which efficiently cleaves the fibrin polymers. Besides its role in fibrinolysis, roles for tPA and plasmin have been described in many and diverse processes, such as chemotaxis [1], cellular activation [2], remodeling of extracellular matrix [3], tissue repair [4], angiogenesis [5] and inflammation [6]. Surprisingly, these processes are not necessarily related to the presence of fibrin.

Many groups have reported that, in addition to fibrin, tPA is activated by numerous unrelated proteins, including denatured forms of albumin, fibrinogen and endostatin and other proteins [7–12]. We showed that tPA is activated by a common structural denominator that is present in amyloid fibrils containing cross-β structure and misfolded protein aggregates with amyloid-like properties [13,14], observations which help to explain how tPA can bind such diverse ligands. Amyloids are well known from a wide variety of protein misfolding diseases, in which tissues and organs become affected by cytotoxic protein deposits. Examples of...
these diseases are Alzheimer’s disease, diabetes type II, systemic amyloidoses and Creutzfeldt–Jakob’s disease. Recent observations also suggest that protein misfolding and consequent amyloid formation may contribute to the etiology of atherosclerosis [15,16]. A role for activation of tPA has been implicated in Alzheimer’s disease as well as in other misfolding diseases and cardiovascular disease on various occasions [17–23].

In this study, we aimed to identify the binding site through which tPA interacts with proteins that display amyloid properties. We found that the fibronectin type I domain of tPA, mediated binding to proteins with amyloid-like properties. The individual fibronectin type I domain of tPA, as well as those of homologous domains in factor XII (FXII), hepatocyte growth factor activator (HGFA) and fibronectin (Fn) all bound to aggregated amyloidogenic peptides.

Methods

Amyloid peptides and proteins

Human amyloid-β (1–40) Dutch type (Aβ, DAEFRHDSGYEVHHQKLVFFAQDVGSNKGAIIGLMVGGV), islet amyloid polypeptide (IAPP, KCNTATCATQRANFLVHSNNF-GAILSTNVGSNTY), amyloid fragment of transthyretin (TTR11, YTIAALLSPYS) [24], laminin x1-chain (2097–2108) amyloid core peptide (LAM12, AASIKAVAASDR) [25], mouse non-amyloidogenic IAPP (20–29) core (mIAPP, SNNLGVPVLLP), non-amyloid fragment FP10 of human fibrin x-chain (148–157) (KRLEVIDIK) [13] and human fibrin x-chain (148–160) amyloid fragment with Lys157Ala mutation (FP13, KRLEVIDIDAIERS) [13,26] were synthesized by the Netherlands Cancer Institute NKI (Amsterdam, the Netherlands) or Pepscan Systems Inc. (Lelystad, the Netherlands) or Pepsyn Systems Inc. (Lelystad, the Netherlands). For pull-down experiments, Aβ (1–40) and IAPP were dissolved in phosphate-buffered saline (PBS) pH 7.2, at 1 mg/ml. IAPP, LAM12 were kept at room temperature for at least 3 weeks. Alternatively, amyloid Aβ, IAPP, FP13 and LAM12 were disaggregated in a 1:1 (v/v) mixture of 1,1,3,3,3-hexafluoro-2-isopropyl alcohol and trifluoroacetic acid, air-dried and dissolved in H2O (Aβ, IAPP, LAM12: 10 mg/ml; FP13: 1 mg/ml). After 3 days at 37°C, peptides were kept at room temperature for the presence of amyloid conformation by thioflavin-T (ThT) or Congo red fluorescence as described earlier [14]. By the treatment described above, ThT and Congo red fluorescence was enhanced for amyloid peptides, and not for non-amyloid mIAPP, FP10 or freshly dissolved Aβ and BSA (not shown).

Recombinant and synthesized proteins

Cloning of human tPA, factor XII, HGFA and fibronectin domains was performed using polymerase chain reactions (PCR) and standard recombinant DNA techniques. Each construct was designed with a carboxy terminal GST-tag (GST). Factor XII, HGFA and fibronectin domains were preceded by two amino acids (GA), following the C-terminus of the tPA propeptide. All constructs, except fibronectin type I domains 10–12 of fibronectin, are followed by a carboxy terminal GST-tag (GST). Factor XII, HGFA and fibronectin domains were preceded by two amino acids (GA), following the C-terminus of the tPA propeptide. All constructs, except fibronectin type I domains 10–12 of fibronectin, are followed by a carboxy terminal GST-tag (GST).
The tPA fibronectin type I domain (F, finger domain) and the Finger-EGF (FE) region, together with the tPA propeptide, were amplified using 1 ng vector Zpl7 containing tPA [27], the product was digested with SalI and NotI and cloned into vector Zpl7 containing tPA [27], the N-terminus (GARRP). tPA cDNA was a kind gift of M. Johannessen (NOVO Research Institute, Bagsvaerd, Denmark). The cDNA encoding for HGFA, for an N-terminal fragment of human fibronectin, comprising fibronectin type I domains 4–5, and for a C-terminal fibronectin fragment, comprising fibronectin type I domains 10–12, respectively. For protein expression, baby hamster kidney (BHK) cells were transfected and stable cell lines were selected. GST-tagged constructs, as well as the free GST tag, were purified from cell culture medium using a 5-m glutathione-Sepharose 4B column (Pharmacia LKB, Uppsala, Sweden). Alternatively, a biotinylated finger domain of tPA (residue 39–81: tPA F-biotin) was prepared by chemical synthesis. In brief, two polypeptides (CH3CONH-Val39-Tyr68-COSR and NH2-Cys69-Val81-Lys-NH2) (residues numbered according to SwissProt entry for tPA) were prepared by solid-phase peptide synthesis [32]. NH2-Cys69-Val81-Lys-NH2 was biotinylated at the side chain of the non-native C-terminal lysine. The two peptides were joined by native chemical ligation [33,34], folded, HPLC purified, lyophilized and frozen until use.

**Enzyme-linked immunosorbent assay**

Immobilizer plates (Exiqon, Dahlbaek, Denmark), or Microlon high-binding plates (Greiner, catalogue number 655092, Frickenhausen, Germany) were used. Peptides were coated at a concentration of 5 μg/ml in coat buffer (100 mM NaHCO3, pH 9.6, 0.05% (m/v) NaNO3) for 1 h at room temperature. Immobilizer plates were blocked with 1% (v/v) Tween20 in PBS, Microlon plates were blocked with blocking reagent (Roche Diagnostics, Almere, the Netherlands). Binding studies with full-length tPA (Actilyse, Alteplase; Boehringer-Ingelheim, Alkmaar, the Netherlands) or a truncated form of tPA (K2P-tPA; Retepase; Rapilysin, Roche Diagnostics GmbH, Mannheim, Germany), were performed in buffer containing PBS, 0.1% v/v Tween20 and 10 mM ε-amino caproic acid (εACA). Binding studies with GST or GST-fusion constructs to immobilized peptides were performed using purified proteins in PBS. tPA was detected with monoclonal antibody 374b or polyclonal antibody 385R (American Diagnostica, Instrumentation Laboratory, Breda, the Netherlands), factor XII with polyclonal antibody Z-5 (Santa Cruz Biotechnology, CA, USA), and negative control for amyloid binding, human hemoglobin, with polyclonal antibody 3700, directed against the FE domain of tPA was purchased from American Diagnostica, (Calbiochem, Schwalbach, Germany), fibronectin with polyclonal antibody A0245 (Dako Diagnostics, Glostrup, Denmark), GST-tagged constructs with polyclonal antibody Z-5 (Santa Cruz Biotechnology, CA, USA), and negative control for amyloid binding, human hemoglobin, with polyclonal antibody A0118 (Dako Diagnostics, Glostrup, Denmark). Antibody 3700, directed against the FE domains of tPA was purchased from American Diagnostica. Binding of soluble BSA-AGE aggregates to tPA F-biotin was assayed by coating 5 μg/ml tPA F-biotin in immobilizer plates (Exiqon, Dahlbaek, Denmark), followed by incubation with BSA-AGE, which was detected using a monoclonal antibody against advanced glycation end-products (4B5) [35]. Primary antibodies were detected using monoclonal peroxidase-labeled rabbit anti-mouse or...
Amyloid pull-down assay

Aβ or IAPP aggregates were incubated with conditioned medium obtained from stably transfected BHK cells. Since the aggregates are insoluble, unbound proteins can be removed from the pelleted peptides following centrifugation and washing. Starting material, bound protein and unbound protein in the supernatant were analyzed by SDS-PAGE followed by immunoblotting. Blots were analyzed with anti-GST antibody Z-5. Control binding studies were performed with 4 μg/ml full-length human tPA (Actilyse, Boehringer-Ingelheim, Germany), 0.8 μg/ml full-length human factor XII (Calbiochem, catalogue no. 233490), 0.5 μg/ml human fibronectin (Harbor Bio-Products, Tebu-bio, Heerhugowaard, the Netherlands, catalogue no. 2003) and endogenous HGFA in 200 times diluted human serum (Sigma-Aldrich, catalogue no. DPVR-55AP) was applied and sections were stained with 3,3'-diaminobenzidine (Sigma-Aldrich, catalogue no. D-5905) and hematoxylin. Finally, sections were incubated with Congo red, according to the manufacturer’s recommendations (Sigma Diagnostics).

Platelet aggregation assay

Freshly drawn venous blood from healthy volunteers with informed consent was collected into 0.1 volume 130 mmol/l trisodium citrate. The donors stated they had not taken any medication during 2 weeks prior to blood collection. After centrifugation of the whole blood (15 min, 150 x g, 20°C), the platelet-rich plasma (PRP) was carefully removed and the pH was lowered to 6.5 by adding 10% ACD buffer (2.5% trisodium citrate, 2% D-glucose and 1.5% citric acid) to avoid platelet activation. Following centrifugation (15 min, 300 x g, 20°C), the platelet pellet was resuspended in HEPES-Tyrode buffer (145 mmol/l NaCl, 5 mmol/l KCl, 0.5 mmol/l Na2HPO4, 1 mmol/l MgSO4, 10 mmol/l Hepes, 5 mmol/l D-glucose, pH 6.5), 10 ng/ml PGI2 (final concentration) was added and the wash step was repeated. The platelet pellet was resuspended in HEPES-Tyrode buffer pH 7.2 to a final platelet count of 2 x 1011 platelets/l. Before the start of the experiments, the platelets were kept at 37°C for at least 30 min to achieve a resting state. Platelet aggregation was recorded in an aggregometer (Chrono-Log Corporation, Havertown, PA, USA) at 37°C, at 900 rpm. A volume of 270 μl platelet suspension was activated with 30 μl of 100 μg/ml of BSA-AGE or 30 μM thrombin receptor activating peptide (TRAP) as activator. For inhibition experiments, tPA, K2P-tPA or tPA F-biotin was added to the agonist solution directly prior to testing in the platelet aggregation assay.

Results

tPA contains a lysine-independent amyloid-binding site, located in its N-terminal region

The domain architecture of tPA consists of a fibronectin type I domain (finger) domain (F), an...
epidermal growth factor like domain (E), two kringle domains (K1, K2) and a serine protease domain (P) (Figure 1). In terms of this domain architecture, tPA is homologous to factor XII (FXII), the initiating enzyme of the intrinsic coagulation cascade [36,37], as well as HGFA, which is involved in tissue repair [38,39] and organ formation [40]. Both these enzymes have a very similar finger-EGF-kringle (FEK) region, but are preceded by a fibronectin type II (II) and E-domain (Figure 1), which are not present in tPA. A fourth homolog is fibronectin (Fn), which contains 12 finger domains, but that lacks kringle or protease domains.

To localize a possible binding site for amyloid aggregates in tPA, we first compared full-length tPA (fl-tPA) to a truncated form of tPA (K2P-tPA), that

<table>
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<tr>
<th>Protein/Construct</th>
<th>Domain Architecture</th>
<th>Amino-acid sequence</th>
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<tr>
<td>tPA</td>
<td>NH₃-F-E-K1-K2-P</td>
<td>-</td>
</tr>
<tr>
<td>K2P-tPA</td>
<td>NH₃-F-E-K1-K2</td>
<td>-</td>
</tr>
<tr>
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<td>NH₃-F-E-GST</td>
<td>NH₂-G33-S85-GRP-tag</td>
</tr>
<tr>
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<td>NH₂-G33-T126-RP-tag</td>
</tr>
<tr>
<td>tPA FEK1-GST</td>
<td>NH₃-F-E-GST</td>
<td>NH₂-G33-D214-RP-tag</td>
</tr>
<tr>
<td>tPA E-GST</td>
<td>NH₃-F-E-GST</td>
<td>NH₂-G33A34-V81-T126-RP-tag</td>
</tr>
<tr>
<td>tPA K1-GST</td>
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<td>NH₂-G33A34-D122-D214-GRP-tag</td>
</tr>
<tr>
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</tr>
<tr>
<td>HGFA</td>
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<td>-</td>
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<tr>
<td>HGFA F-GST</td>
<td>NH₃-I-E-F-K1-P1</td>
<td>NH₂-GA-G198-A244-GRP-tag</td>
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lacks three N-terminal domains, for their capacity to induce plasmin formation in the presence of amyloid protein preparations. For these experiments, we prepared amyloid preparations of Aβ (1–40), a peptide of the amyloid core fragment of transthyretin (TTR11) and an amyloidogenic fibrin peptide (FP13). We found that amyloid Aβ (1–40) was only capable of inducing plasmin generation in the presence of fl-tPA and not in the presence of K2P-tPA (Figure 2A). Similarly, only fl-tPA could induce plasmin generation in the presence of FP13 and TTR11 (Figure 2B, C, respectively). These experiments show that the N-terminal FEK1 region of tPA is required for plasmin formation in the presence of various aggregated amyloid peptides. Although K2P-tPA was not activated by these amyloid proteins, its protease domain was equally functional to that of fl-tPA, as determined by conversion of the chromogenic substrate S2756 (Figure 2D).

We next investigated whether this region of tPA was involved in binding of proteins with amyloid structure. Where fl-tPA had significant affinity for immobilized Aβ (1–40), as well as the amyloid peptides FP13 and TTR11, truncated K2P-tPA had lost all binding capacity for these preparations (Figure 2E). This shows that the FEK1 region of tPA supports the binding of tPA to proteins with amyloid features and suggest that this binding is a requirement for tPA activation in the presence of amyloid peptide aggregates.

Kringle domains bind to lysine residues, as is the case in the binding of plasminogen and tPA to C-terminal lysines in fibrin. We therefore investigated whether tPA binding to immobilized Aβ (1–40) could be inhibited by addition of the soluble lysine analogue ε-amino caproic acid (εACA) to the binding interaction. We found that tPA–Aβ interaction could be inhibited only for approximately 50% with εACA (Figure 2F). This indicates a clear, although partial, functional role for lysine-dependent interactions. These data correspond to earlier findings on amyloid FP13 binding by tPA, which could be inhibited for 40% by εACA [13]. The remaining 50% of the tPA binding to Aβ (1–40) was attributed to lysine-independent binding, which indicates the presence of another type of amyloid-binding site in tPA. Since kringle domains are involved in lysine binding, amyloid binding most likely occurs through either the finger- or EGF-like (FE) domains.

We investigated the role of the FE region by studying the effect of a monoclonal murine antibody (Ab3700) directed against this region of tPA and compared the induced loss of binding with that of a monoclonal murine antibody (Ab374b) directed against the tPA protease domain. tPA binding to Aβ (1–40) was detected with a polyclonal rabbit antibody (Ab385R). Because the affinity constants of both monoclonal ε-tPA antibodies were not comparable (Kd for Ab3700 and Ab374b are 700 and 70 ng/ml, respectively), we tested potential inhibition of tPA binding to Aβ (1–40) at 33 and 100 times Kd of the antibodies. This experiment shows that targeting of the FE region of tPA results in significantly reduced binding, whereas a control antibody directed at the protease domain had no effect (Figure 2G). In control experiments, preincubation of immobilized Aβ (1–40) with equal amounts of Ab3700 had no effect on tPA binding (not shown) and binding of the polyclonal rabbit Ab385R was not inhibited by Ab3700 antibody (not shown). Together, these experiments indicate that binding of Ab3700 to the FE region of tPA, inhibits binding of tPA to Aβ. At the highest antibody concentrations, the inhibitory effect of Ab3700 surpassed 50%; we attribute this difference with the lysine-independent binding shown in Figure 2F to an additional sterical effect of the Ab3700 that interferes with the kringle-lysine interactions. These experiments point towards the presence of a lysine-independent binding site for amyloid in one or more of the three N-terminal domains of tPA.

Amyloid binding of tPA requires the finger domain

Since tPA may contain an amyloid-binding site in the FEK1 region (Figure 2), that was likely to be responsible for the multiligand receptor function that we described earlier [13], we investigated the role of these domains in amyloid binding in more detail. Here, we recombinantly produced the F, E and K1 domains of tPA, as well as a combined FE and FEK1 constructs in mammalian cells. All constructs were GST-tagged for analytical and purification purposes. Next, conditioned media from each transfection were incubated with amyloid aggregates of Aβ (1–40) or IAPP and binding of the produced proteins to the amyloid aggregates was analyzed by a centrifugation pull-down, followed by Western blotting against GST. In this experimental setup, as expected, fl-tPA, as well as the FEK1-GST, bound to both amyloid preparations (Figure 3A). All constructs with the finger domain, including the F-GST construct by itself, were capable of binding to both amyloids, whereas the E-GST and K1-GST or GST-tag alone did not display significant amyloid binding (Figure 3A). We conclude from these experiments that an amyloid-binding site is present in the finger domain of tPA.

In a next series of experiments, we compared the affinity of the purified F-GST construct to that of tPA for various immobilized amyloid proteins (and where possible, their native counterparts) to investigate the multiligand receptor capabilities of the finger domain. We found that both F-GST and tPA bound to amyloid Aβ (1–40) with comparable affinity.
Figure 2. A lysine-independent amyloid-binding site is located in the first three N-terminal domains of tPA. Amyloid-induced plasmin formation by Aβ (1–40), FP13 and TTR11 is only activated in the presence of full-length tPA, but not truncated tPA (K2P; A–C, respectively). However, the amyloidolytic activity of both tPA variants for the chromogenic substrate S-2765 was comparable (D), indicating that the protease domain of K2P is functional. Full-length tPA, but not truncated tPA (K2P), binds to immobilized amyloid preparations of Aβ (1–40), FP13 and TTR11 (E). Binding of tPA to immobilized amyloid Aβ (1–40) can only be inhibited up to 50% by lysine analog εACA, indicative of a lysine-independent binding site for amyloid in tPA (F). tPA binding to immobilized amyloid Aβ (1–40) can be inhibited by a monoclonal antibody directed against the finger-EGF-like domains (α-tPA-FE; Ab3700), but not by a monoclonal antibody directed against the protease domain (α-tPA-P; Ab374b) (G).
No binding was observed to freshly dissolved Aβ (1–40). In line with this experiment, tPA and F-GST binding to amyloid preparations of IAPP, FP13, TTR11 was comparable (Table I). No binding of tPA or F-GST was detected to murine mIAPP, which does not form amyloid structure. Interestingly, no fl-tPA binding was detectable to an amyloid preparation of the laminin peptide LAM12, whereas the F-GST construct bound to it with high affinity. This suggests that there is a finger-binding site present in amyloid LAM12, which may be unavailable to the much larger tPA molecule. Indeed, amyloid LAM12 was only capable of stimulating tPA-dependent plasmin formation with very low capacity (Figure 4). These data suggest that the amyloid-binding capacity of tPA transcends amino acid sequence dependence, since it can bind several unrelated amyloid peptides, and can be mediated by the finger domain alone.

**Finger-domains of homologous proteins have conserved amyloid-binding capacity**

As described above, tPA has several homologs, each of which contains one or more finger domains (Figure 1). These homologs have been suggested to interact with amyloid on various occasions. For example, amyloid structure has been suggested to be a regulatory motive in activation of FXII [41,42]. Since we had found that the finger domain of tPA is involved in amyloid binding, we next tested whether finger domains of FXII, HGFA and Fn had similar capacities. Hereto, we recombinantly produced F-GST constructs of FXII and HGFA. We also produced GST-tagged constructs of fibronectin containing fingers 4 and 5 (Fn F4–5-GST), and fingers 10 to 12 (Fn F10–12-GST). We next analyzed amyloid binding by pull-down assay.

Amyloid aggregates of IAPP associated with full-length FXII, Fn and HGFA in medium and could be retrieved from the amyloid pellets after pull-down (Figure 5). Finger domains of FXII and HGFA, as well as fingers 4–5 and 10–12 of Fn, bound to amyloid IAPP in a similar manner. Amyloid aggregates of Aβ (1–40) bound to coagulation factor XII, as well as its finger domain alone. However, little or no full-length protein was associated with amyloid Aβ for Fn or HGFA, respectively. In the case of full-length Fn, we could not retrieve Fn in the supernatant of the pull-down experiment, indicating full
removal of Fn from the medium via the amyloid aggregates. Since the amyloid pellet only contained a small fraction of the initial Fn present, it is likely that the washing procedures used in these experiments had eluted the Fn from the amyloid Aβ. Similar to IAPP aggregates, Aβ aggregates efficiently bound to Fn F4–5 and F10–12. In the case of full-length HGFA, almost no HGFA was bound to the amyloid pellet and nearly all HGFA could be retrieved in the pull-down supernatant. The finger domain of HGFA, however, displayed significant amyloid Aβ binding when tested. Control constructs, containing the GST-tag, failed to associate with the amyloid pellets. We conclude from these experiments that amyloid binding by full-length FXII, HGFA or Fn may vary, but their finger domains possess amyloid-binding activity in all cases.

Table I. Binding of tPA and F-GST is comparable to various amyloids. The binding of full-length tPA (fl-tPA) and tPA F-GST to immobilized proteins was studied by ELISA. Affinity constants and maximal binding were determined by nonlinear regression (one-site binding) in Graphpad Prism 4.00. – indicates no (saturated) binding could be detected. In most cases, the F-GST construct had a slightly higher affinity for the immobilized amyloid proteins than did fl-tPA. In case of LAM12, fl-tPA binding was completely absent. This suggests that in most cases, the binding site for the finger domain on proteins with amyloid structure has better availability to the smaller F-GST construct than to fl-tPA.

<table>
<thead>
<tr>
<th>Immobilized protein/peptide</th>
<th>tPA Affinity constant (nM)</th>
<th>tPA B_{max} (OD)</th>
<th>F-GST Affinity constant (nM)</th>
<th>F-GST B_{max} (OD)</th>
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<tr>
<td>Aβ (1–40) amyloid</td>
<td>3.46</td>
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<td>7.61</td>
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<td>Aβ (1–40) fresh</td>
<td>0.55</td>
<td>0.41</td>
<td>0.22</td>
<td>0.44</td>
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<tr>
<td>IAPP</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
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<td>mIAPP</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>FP13</td>
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<td>0.85</td>
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<tr>
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<td>0.72</td>
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<td>LAM12</td>
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<td>–</td>
<td>0.22</td>
<td>0.98</td>
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Figure 4. Activation of tPA-dependent plasmin formation by amyloid laminin peptide LAM12 is minimal, which corresponds to lack of tPA binding, determined by ELISA.

**Functional use of finger domains**

So far, we had investigated the amyloid-binding site of tPA and homologs using aggregated amyloid Aβ and IAPP peptides. From these experiments, it became clear that these interactions required at least the finger domain. Next, we investigated whether a finger domain would recognize pathological amyloid that had formed in vivo. We incubated brain sections of a person that had deceased from Alzheimer’s disease with the amyloid-specific dye Congo red as well as a construct containing tPA FE-GST, followed by incubation with an anti-GST antibody. The tPA FE-GST construct was selected because it had displayed the highest affinity for amyloid Aβ (1–40) in our earlier experiments (Figure 3A). The tPA FE-GST was visualized with an antibody against the GST-tag, which is displayed as a brown staining on the section. As can be seen in Figure 6A and B, Congophilic plaques present in the section could be stained by the FE-GST construct. Since Congo red and FE-GST apparently co-localize on the plaques, they may have different binding sites for the same amyloid. As a control, GST-tag by itself, followed by incubation with anti-GST antibody, did not stain Congophilic plaques at all (Figure 6C, D). These data indicate that the finger domain of tPA is capable of binding to pathological amyloid that has formed in vivo.

In a recent publication by our group, we demonstrated that platelets activate and aggregate in the presence of proteins with amyloid characteristics [15]. Advanced glycation end-products of albumin (BSA-AGE) form soluble amyloid-like aggregates [43] that have been reported to stimulate tPA-dependent plasmin generation [8]. Additionally, advanced glycated proteins are implicated in diabetic vascular dysfunction and nephropathy [35,44]. BSA-AGE, but not native albumin, was found to be one of the most potent inducers of platelet aggregation. It was also shown that fl-tPA could significantly inhibit this process. We used this knowledge to investigate the role of the finger domain. We first compared the binding of fl-tPA and K2P-tPA to immobilized BSA-AGE. As expected from Figure 2E, fl-tPA, but not K2P-tPA could bind BSA-AGE with high affinity (Figure 7A). Next, we evaluated whether immobilized tPA finger domain could bind to soluble BSA-AGE aggregates and observed a high-affinity association (Figure 7B). We next performed BSA-AGE induced platelet aggregation experiments. We observed that tPA, but not K2P-tPA, could inhibit BSA-AGE-induced aggregation (Figure 7C), which is in good agreement with the binding experiments. As a control for specificity of this observation, platelet activation by TRAP, which follows a different platelet activation mechanism by activation.
of the thrombin receptor PAR-1, was unaffected by both forms of tPA (at a suboptimal concentration of 3 μM; Figure 7E). This shows that the inhibitory activity of tPA remains solely in the tPA FEK region and is specific for platelet activation by BSA-AGE. Thus, we next investigated whether the tPA finger domain was capable of inhibiting BSA-AGE-induced platelet aggregation. Albeit somewhat less efficient compared with tPA, the isolated finger domain could reduce BSA-AGE-induced platelet aggregation with an estimated maximal capacity of 50% compared with fl-tPA (Figure 7D). Again, no effect was seen on TRAP-induced platelet aggregation (Figure 7E). Taken together, these experiments indicate that
Figure 7. Platelet aggregation by BSA-AGE is inhibited by full-length tPA (fl-tPA) and its finger domain, but not by K2P-tPA. Amyloid-like aggregates of BSA-AGE bind to fl-tPA, but not K2P-tPA, with high affinity, indicating a role for the FEK1-region in this interaction (A). Immobilized tPA finger domain (tPA F-biotin) binds to BSA-AGE in solution (B). Washed platelets were activated by a final concentration of 10 µg/ml of BSA-AGE (C, D); freshly dissolved BSA did not activate platelets (not shown). This activation could be inhibited by addition of 50 nM of fl-tPA, but not by addition of 50 nM K2P-tPA (C). Similarly, titration of a construct consisting of tPA biotin-labeled finger domain (tPA F-biotin) inhibited this activation up to 50% (D). As a control, activation of platelets by a final concentration of 3 µM TRAP was unaffected by either fl-tPA, K2P-tPA or F-biotin (E). Experiments shown are representative for at least three individual experiments.
tPA and its finger domain recognize a binding site on BSA-AGE aggregates that overlaps at least partially with that of the involved platelet receptors and can be used to intervene in platelet activation.

Discussion

The etiology of protein misfolding diseases is an area of intense research as these diseases are generally highly deleterious and of enormous social and economical impact. While much is known about the folding and degradation of proteins within the cell, little is known about the removal of damaged or obsolete proteins in the extracellular environment. Novel insights into the clearance of proteins outside cells can open new avenues in the development of therapeutics for protein misfolding diseases.

Earlier work from our group showed that tPA autoactivates in the presence of proteins with amyloid structure, and requires binding for this process [13]. We here demonstrate that this binding capacity resides in the finger domain of this molecule, which can independently support binding to various proteins and peptides with unrelated amino acid sequences. Additionally, isolated finger domains of FXII, HGFA and Fn have similar amyloid-binding capacities. In our studies, binding of fl-tPA to amyloid Aβ (and in an earlier report, FP13 [13]) could be inhibited up to 50% with εACA, which indicates a role for kringle-dependent interactions. In this context, it is noteworthy that neither K2P-tPA nor the kringle 1 domain of tPA supported independent binding to amyloid. Based on these findings, we propose a model in which tPA first binds to proteins with amyloid characteristics via its finger domain, after which kringle-dependent interactions are supported. Interestingly, the same two-step binding model has been proposed for the interaction between tPA and fibrin polymers [29]. The binding site for the finger domain in fibrin has not been clarified after those reports. As several fibrin peptides possess the intrinsic capacity to adopt amyloid structure (when aggregated) it is attractive to think that this structure is a primary target for tPA-mediated clearance of fibrin. In line with these considerations, Raman spectroscopic studies have shown that fibrin polymerization is accompanied by the stacking of β-sheets in the polymers [45], which corresponds to increased tPA-activating potential. Together with our findings here, these data suggest that the interaction between fibrin and tPA may correspond to the interaction between amyloid protein aggregates and tPA.

At this point, the specific details of the interaction between the finger module and proteins with amyloid structure remain to be elucidated. In fact, we only know that finger-binding sites are abundantly present in amyloid proteins, but not in native proteins, which does not directly imply that it is the 4.7 Å cross-β structure that is specifically present in amyloid. Given the number of ligands with unrelated amino acid sequences, the interactions are likely to be mediated by structure over amino acid sequence. The presence of a hydrophobic core in the finger domain [46], as well as the presence of hydrophobic regions in amyloid precursors, suggests that hydrophobic interactions could play a role.

The presence of amyloid proteins in vivo has various deleterious effects. The formation of amyloid plaques can cause organ damage or cognitive dysfunction in protein deposition diseases. In the case of Alzheimer’s disease, activation of tPA and plasmin has been described on various occasions. The resulting activation of the plasmin system is thought to be directly protective by being responsible for reduced amyloid plaque formation [17,18,21,22,47] and, as was shown in a recent paper, indirectly by decreased fibrin deposition [48]. In contrast to these protective functions of tPA and plasmin, activation of the plasmin system by tPA is suggested to contribute to cerebral hemorrhage in Alzheimer’s disease [49], while tPA mediates amyloid cytotoxicity in Alzheimer’s disease [50]. In addition to the interaction of tPA with amyloid in Alzheimer’s disease, FXII has also been shown to colocalize with amyloid plaques in Alzheimer’s disease [51] and is activated by it, both in vitro and in vivo [42,52,53]. From these reports, it is suggested that the kallikrein-kinin system, which is activated by FXII, may play a role in the inflammatory processes seen in Alzheimer’s disease. Also, fibronectin [54,55] has been found associated with pathological amyloid in Alzheimer’s disease and activation of HGFA is reported to take place [56]. In all, all four finger domain-containing proteins are implicated in Alzheimer’s disease. However, these proteins are also implicated in other amyloidoses. For example, increased levels of HGF, which is controlled by HGFA, appear to be a good prognostic marker for various types of systemic amyloidosis [57].

An important question that arises is, for what reason the finger domain has evolved as a conserved amyloid-binding module and thus, in what physiological systems it may play its role. It is possible that tPA-dependent plasmin generation may also be activated by non-pathological misfolded proteins in vivo, resulting in clearance of these proteins before they have a chance to accumulate. The physiological activators of FXII and HGFA are not known. We therefore hypothesize that FXII and HGFA may have protective roles on sites of injury by their capacity to activate inflammatory processes and tissue repair mechanisms in the presence of misfolded proteins.
An especially interesting role for finger domains is present in Fn, which consists of no less than 12 of these type I domains (Figure 1). Finger domains 1–5, most notably domains 4–5, are involved in the binding of Fn to fibrin [30,58], and play a key role in self-association of Fn [59]. Additionally, Fn F6 and F7–9, are together present in a functional domain that binds to denatured collagen (gelatin), whereas Fn F10–12 form a second stretch of finger domains with affinity for fibrin. For Fn to exert its 'matrix assembly' function, the Fn F1–5 region first has to bind to cells and associate with the first type III domain (III-1) on another Fn molecule, which needs to be unfolded for this binding [60]. Indeed, unfolded type III domains are required for Fn assembly in general [61] and it has been suggested that this occurs by self-assembly of type III-1 domains [62], which can form amyloid-like fibrillar structures when isolated [63]. An interesting model known as 3D domain swapping has been suggested as an explanation for self-association of molecules, which may be applicable in this setting [64,65]. In this model, domains that normally interact with each other within a single protein molecule, interact with equal domains from other molecules in the same microenvironment. However, such a model in which only unfolded III-1 associate with each other does not explain the critical dependence on the Fn F1–5 region, which can inhibit Fn matrix formation on cells. Our findings, as well as findings of others [66], in which proteins with unfolded or amyloid structural properties interact with Fn finger domains may be suggestive for a 3D domain swapping model in which finger modules of Fn interact with unfolded type III domains during self-association.

We show here that the finger module can bind to amyloid plaques and demonstrate that we can inhibit amyloid-induced cell activation. A recent publication elegantly demonstrated the use of a soluble form of low-density lipoprotein (LDL) receptor-related protein 1 (LRP) in clearance of circulating Aβ peptide, thereby reducing the Alzheimer’s disease-related pathology in vivo [67]. Besides its known ligands (LDL and coagulation FVIII), LRP-1 has been described to bind to Aβ peptide in vitro [68,69]. Further studies will decide whether finger domain-containing constructs can be applied to enhance clearance of amyloidogenic peptides from plasma and/or prevent amyloid-related pathology in a similar fashion.

In conclusion, we propose that the interaction between finger domains and amyloid proteins reflects a mechanism for recognition and clearance of misfolded proteins from the extracellular compartment. Our findings may offer new targets for therapeutic interventions to conquer protein misfolding diseases.

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Declaration of interest: B. Schiks, M. F. B. G. Gebbink and B. Bouma are employees of CrossBeta Biosciences BV. Additionally, B. N. Bouma, M. F. B. G. Gebbink and B. Bouma are shareholder of this company. Other authors declare no financial interests. The authors alone are responsible for the content and writing of the paper.

References


41. Maas C, Govers-Riemslag JWP, Bouma B, Schiks B, C. Maas et al.


