

## Detecting Activated Thrombin Activatable Fibrinolysis Inhibitor (TAFIa) and Inactivated TAFIa (TAFIai) in Normal and Hemophilia A Plasmas

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Thrombin activatable fibrinolysis inhibitor (TAFI) also known as plasma procarboxypeptidase B or U is a 60 kD glycoprotein, which is the major modulator of fibrinolysis in plasma. TAFI is a proenzyme, which is activated by proteolytic cleavage to an active carboxypeptidase B-like enzyme (TAFIa, 35.8 kD) by thrombin/thrombomodulin and plasmin. Modulation of fibrinolysis occurs when TAFIa enzymatically removes C-terminal lysine residues of partially degraded fibrin, thereby inhibiting the stimulation of tissue plasminogen activator (t-PA) modulated plasminogen activation. TAFIa undergoes a rapid conformational change at 37°C to an inactive isoform called TAFIai. Potato tuber carboxypeptidase inhibitor (PTCI) was shown to specifically bind to TAFIa as well as TAFIai. In this study, a novel immunoassay TAFIa/ai ELISA was used for quantitation of the two TAFI activation isoforms TAFIa and TAFIai. The ELISA utilizes PTCI as the capture agent and a double antibody sandwich technique for the detection. Low levels of TAFIa/ai antigen levels were detected in normal plasma and elevated levels were found in hemophilia A plasmas. TAFIa/ai antigen represents a novel marker to monitor fibrinolysis and TAFIa/ai ELISA may be a valuable assay for studying the role of TAFI in normal hemostasis and in pathological conditions.

**Key Words:** TAFIa/ai, TAFI, TAFIa, TAFIai, Fibrinolysis

### Introduction

Thrombin activatable fibrinolytic inhibitor (TAFI) [also known as plasma procarboxypeptidase B (PCPB), or U (PCPU)] is a 60 kD glycoprotein present in human plasma that modulates fibrinolysis *in vivo*. TAFIa modulates fibrinolysis by enzymatically removing C-terminal lysine residues of partially degraded fibrin, thereby inhibiting the stimulation of tissue plasminogen activator (t-PA) mediated plasminogen activation.<sup>1-6</sup> TAFI is a proenzyme which is activated by proteolytic cleavage at Arg-92 by thrombin to an 35.8 kD carboxypeptidase B-like enzyme (TAFIa, Figure 1). Activation of TAFI by thrombin is accelerated over one thousand-fold by addition of thrombomodulin and Ca<sup>2+</sup>. TAFI provides a link between the coagulation and fibrinolytic pathways through reliance on the thrombin/thrombomodulin complex which activates TAFI as well as Protein C. Plasmin, the major protease that mediates fibrinolysis, also activates TAFI by cleavage at Arg-92. However, plasmin also cleaves TAFI at a second site in the C-terminal region at Arg-307 resulting in a truncated 44.3 kD protein which lacks apparent biological activity.<sup>7</sup>

TAFIa rapidly loses enzymatic activity through a temperature-dependent ( $t_{1/2} = 10$  min at 37°C) inactivation process. The inactivation of TAFIa is not due to a proteolytic process, but rather to a conformational alteration in the protein structure that can be followed by a change in the fluorescence spectrum of the protein.<sup>8</sup> The conformationally inactive form of TAFIa is referred to as TAFIai (35.8 kD). The temperature-dependent inactivation of TAFIa to TAFIai is believed to be the major mechanism by which TAFIa activity is modulated as no known natural inhibitors of TAFIa have been found in human plasma.

Polymorphisms in the TAFI gene have been shown to

regulate the levels of TAFI protein in plasma.<sup>9-11</sup> TAFI plasma levels have been measured using functional and quantitative ELISA assays in normal<sup>12</sup> populations and in various pathological conditions including liver disease,<sup>13</sup> cancer,<sup>14</sup> DIC,<sup>15</sup> arterial and venous thrombosis.<sup>16-18</sup> The quantitation of TAFI in plasma has become problematic due to discordant results produced by the various assays used to measure TAFI polymorphisms.<sup>19-21</sup> Recently, it has been recognized that the Thr325Ile polymorphism can alter the value of TAFI depending on the ELISA used.<sup>20</sup> The differential reactivity of TAFI polymorphisms with some ELISAs causes in accurate reporting of the TAFI levels. Guimaraes *et al.*<sup>22</sup> showed that the Thr325Ile TAFI polymorphic proteins have the same reactivity using a commercial chromogenic functional assay or an ELISA made using carefully selected pairs of monoclonal antibodies. The quantitation of TAFI by ELISAs can also be skewed by the differential reactivity towards different forms of TAFI (e.g. TAFI, the amino terminal activation peptide, TAFIa, and TAFIai) that may be present in the plasma.<sup>23</sup>

A clearer understanding of the role of TAFI in fibrinolytic processes can be gained by specifically measuring the individual TAFI proteins. The measurement of TAFI, the procarboxypeptidase, provides an estimate of the *potential* TAFI-dependent anti-fibrinolytic activity in plasma. Functional assays and ELISAs specific for TAFI have been utilized in several studies for quantitating the procarboxypeptidase in plasma (9, 11, 20, 21, 23, 24). Recently, TAFI was found in human seminal plasma, where TAFI might be involved with the coagulation and liquefaction of human semen for the protection of the seminal clot against lysis.<sup>25</sup> Further insights into the biology of TAFI can be gained by measuring the TAFIa and TAFIai activation isoforms. The activation of TAFI is regulated by a number of factors including expression

of genetic variants with different specific activities, gene polymorphisms that regulate circulating TAFI levels, thrombin and plasmin generation, and thrombomodulin levels. Measuring TAFIa and/or TAFIai activation isoforms in plasma by immunoassay has been difficult because the protein sequences of the two activation isoforms are fully contained within the TAFI proenzyme protein sequence. Development of an immunoassay that specifically quantitates TAFIa and TAFIai isoforms in plasma would be a valuable tool for investigating the TAFI activation pathway.

In the present studies, TAFIa/ai ELISA was used for the specific quantitation of TAFIa and TAFIai antigens in plasma. The assay is based upon the finding that conformationally inactive TAFIai isoform as well as TAFIa specifically bind to potato tuber carboxypeptidase inhibitor (PTCI). The TAFIa/ai ELISA uses PTCI-coated microtiter wells as the solid phase capture agent. Detection of the bound TAFIa or TAFIai is done using a two-step sandwich immunologic technique. Both TAFIa and TAFIai react equally well in the ELISA, whereas, TAFI (pro-enzyme) does not crossreact in the assay. Low levels of TAFIa/ai antigen was detected in normal plasma and significantly higher levels were determined in plasmas from hemophilia A patients. The presence of TAFIa/ai antigen in plasma was confirmed by purification of the TAFIa/ai antigens using PTCI-Sepharose affinity column. The measurement of TAFIa/ai antigen by ELISA provides a novel plasma marker for monitoring the activation of TAFI and investigating the role TAFI plays in the regulation of fibrinolytic processes.

### Materials and Methods

**Materials.** Citrated plasmas from hemophilia A and B were obtained from Diagnostic Support Services (West Barnstable, MA) and Biochemed Inc. (Atlanta, GA). Plasma was obtained after centrifugation at 2000 x g for 15 minutes at 4°C and stored at -80°C before use. Pooled normal plasmas (PNP) were from Universal Reagents, Inc. (Indianapolis, IN). Acti-Chrome® TAFI kits, PPACK, thrombin and thrombomodulin were from American Diagnostica Inc. (Greenwich, CT). Purified human TAFI and anti-human TAFI monoclonal antibody was from Hematologic Technologies Inc. (Essex Junction, VT). TAFI-deficient plasma was from Affinity Biologics (Hamilton, Canada). PTCI was from Sigma Chemical Co. (St Louis, MO). Cyanogen bromide (CNBr)-activated Sepharose 4B was from Pharmacia (New Brunswick, NJ). Donkey anti-mouse IgG-HRP was from Jackson Laboratories (Jackson, ME). Precast SDS-PAGE gels were from Invitrogen (Carlsbad, CA).

**Preparation of TAFIa and TAFIai.** TAFI was activated by incubating alpha-thrombin (50 units/ml), thrombomodulin (3 units), Ca<sup>2+</sup> (5 mM), and TAFI (50 µg) in 500 µl of 100 mM TRIS-HCl pH 7.4 for 20 minutes at room temperature. The reaction was stopped by addition of PPACK (1 µg) to stop the proteolysis of TAFI by thrombin. The generation of TAFIa carboxypeptidase B activity was confirmed using the ACTI CHROME TAFI activity assay. TAFIa was used immediately or stored frozen at -20°C. TAFIai was prepared by incubating TAFIa for 2 hours at 37°C in a water bath. The TAFIai was determined to have lost >98% carboxypeptidase B activity

(data not shown) using the ACTI CHROME TAFI assay.

**Preparation of PTCI-Sepharose.** A PTCI-Sepharose affinity column was prepared by mixing PTCI (25 mg) with activated CNBr-Sepharose (3 ml of gel, Pharmacia, NJ) in 3 ml of 0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl, pH 8.3 coupling buffer with gentle rotation over night. The PTCI-Sepharose was washed with coupling buffer and the unreacted CNBr-sites were blocked by incubating with 0.1 M Tris-HCl at pH 8.0 for 2 hrs at room temperature. The PTCI-Sepharose column was sequentially washed with 0.1 M acetate buffer, 0.5 M NaCl, pH 4.0, and with 0.1 M Tris-HCl, 0.5 M NaCl, at pH 8.0 and equilibrated with 20 mM TRIS-HCl, pH 7.4 before use.

**Affinity Chromatography with PTCI-Sepharose.** TAFIa, prepared by treatment of TAFI with thrombin/thrombomodulin as described above, was applied onto the PTCI-Sepharose column. The column was washed with 20 mM Tris, pH 7.4 and the bound TAFIa protein was eluted with 0.5 M NaCl in 200 mM Tris, pH-7.4. Fractions (1 ml) were collected and protein monitored at OD 280 nm. TAFIa activity in the fractions was determined using ACTI CHROME TAFI kit. The fractions were analyzed by SDS-PAGE and Western Blotting using a goat anti-human TAFI IgG-HRP polyclonal antibody for detection. TAFIai was affinity purified using the same procedures as for TAFIa. TAFIa/ai antigen was affinity purified from hemophilia A plasma. The plasma was diluted 50-fold with 20 mM Tris-HCl, pH 7.4 before application to the PTCI-Sepharose affinity column. The same column washing and elution conditions were used as described above.

**TAFIa/ai ELISA.** Dynax Immulon 4 HBX microtiter plates

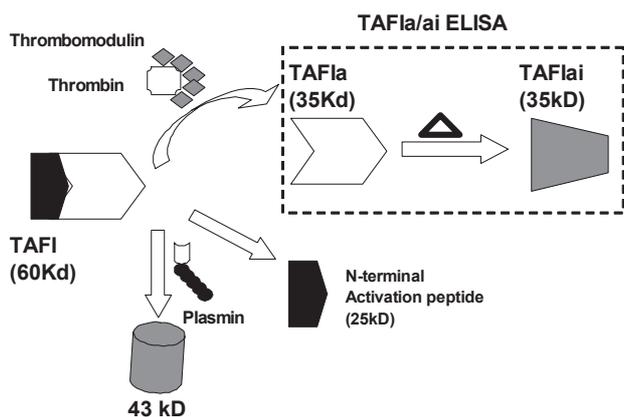
**Table 1.** Quantitation of TAFIa/ai antigen in normal plasma using TAFIa/ai ELISA

SAMPLE	N	MEAN ± STD
Pooled Normal Plasma	3	15.8 ± 19.0 ng/ml
Normal Plasma	16	18.6 ± 17.1 ng/ml

**Table 2.** FVIII and TAFIa/ai levels in hemophilia plasmas.

Sample	Age	Sex	TAFIa/ai (µg/ml)	FVIII (% activity)
1	22	M	30.6	< 1
2	37	M	39.0	23
3	17	M	141.9	< 1
4	31	F	178.0	18
5	27	M	81.7	< 1
6	15	M	80.7	5
7	20	M	125.3	17
8	33	M	138.2	11
9	41	M	154.8	35
10	13	M	122.2	3
11	NP <sup>a</sup>	NP	78.6	< 1
12	NP	NP	95.3	< 1
13	NP	NP	65.9	9
14	NP	NP	45.9	5
15	NP	NP	18.9	16
16	NP	NP	26.1	< 1

<sup>a</sup>not provided

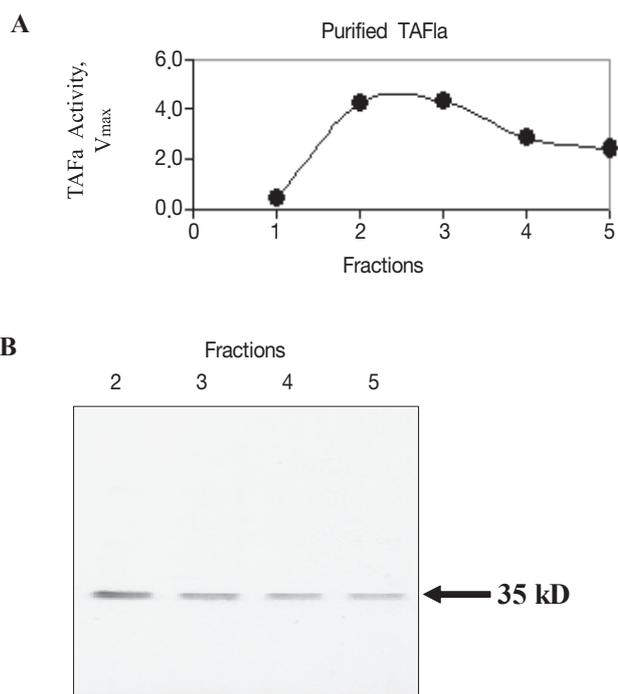


**Figure 1.** Major molecular forms of TAFI in plasma. TAFI (zymogen, 60 kD) could be activated by cleavage at Arg-92 with thrombin/thrombomodulin or plasmin, yielding TAFIa (active form, 35 kD) and non-enzymatic for of N-terminal activation peptide (25 kD). Plasmin could cleave at alternative site, Arg-307, yielding inactive enzyme with intact N-terminal activation peptide (43 kD). The open triangle indicated the inactivation factors, time and heat, of inactivating TAFIa to TAFIai. TAFIa/ai ELISA measured only TAFIa and TAFIai forms from alternative TAFI forms in plasma as indicated in the dotted box.

(Dynatech Lab. Inc.; Chantilly, VA) were coated with PTCI ( $1\mu\text{g}/\text{ml}$ ) and blocked with SuperBlock Blocking Buffer (Pierce Chemicals, IN). The standard curve was generated using two fold serial dilution of TAFIa in TAFI-deficient plasma or in phosphate buffered saline (PBS) containing 0.03% Tween 20. Human citrated plasma samples were diluted three-fold in buffer prior to being assayed. The TAFIa, TAFIai or diluted plasma samples ( $100\mu\text{l}$ ) were added into the PTCI-coated microwells and incubated for 2 hours at room temperature. The wells were washed four times with phosphate buffered saline containing 0.03% Tween 20. Antihuman TAFI monoclonal antibody ( $0.5\mu\text{g}/\text{ml}$ ) was added to each well and incubated for 1 h at room temperature. The microtiter wells were washed four times and  $100\mu\text{l}$  donkey antimouse IgG-HRP of (1:5,000) was added to each well. After incubation for 1 h the wells were washed and TMB substrate (Moss, MD) was added to each well. The color was developed for 10 minutes at room temperature and the reaction was stopped with  $50\mu\text{l}$  of 0.5 M sulfuric acid. The microtiter plate was read at 450 nm using a SpectroMax190 spectrophotometric plate reader (Molecular Devices Inc., Sunnyvale CA).

## Results

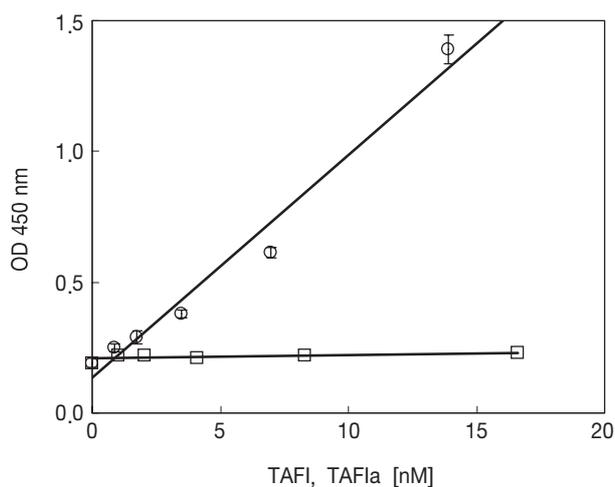
**Purification of TAFIa and TAFIai by PTCI-Sepharose Affinity Chromatography.** After activation of TAFI with thrombin/thrombomodulin, the reaction mixture containing TAFIa was applied to a PTCI-sepharose affinity column. After washing with buffer, TAFIa bound to the column was eluted with Tris-HCl pH 7.4 buffer containing 0.5 M NaCl. TAFIa carboxypeptidase activity was detected in fractions 2 to 5 (Figure 2A). SDS-PAGE analysis of fractions 2–5 showed a single protein band of molecular weight 35 kD (Figure 2B). Unactivated TAFI did not bind to the PTCI- column and was identi-



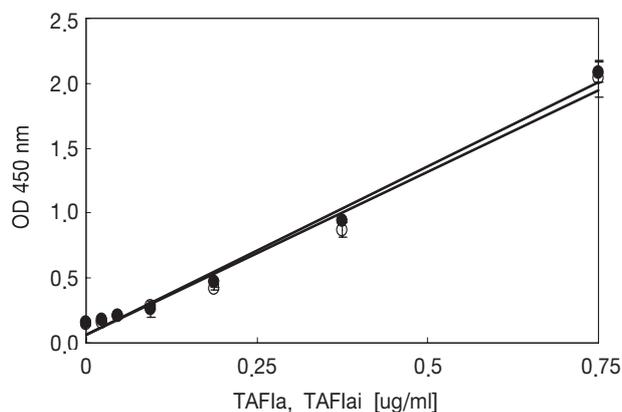
**Figure 2.** Affinity purification of TAFIa/ai antigen. TAFI was activated for 20 minutes with thrombin/thrombomodulin and the mixture applied to PTCI-Sepharose affinity column. The protein eluted in the 0.5 M salt fractions was assayed for TAFI activity (panel A). The protein in fractions 2-5 was applied to SDS-PAGE gel and silver-stained (Panel B).

fied by SDS-PAGE/Western blotting in the initial buffer wash (not shown). In a similar manner, TAFIai was prepared as described above and affinity purified on the PTCI-Sepharose column. The fractions from the 0.5 M NaCl wash did not contain carboxypeptidase B activity but a single 35 kD TAFIai protein was present (data not shown). These results indicate TAFIai retains the ability to bind to PTCI although it has no carboxypeptidase activity.

**Specificity of TAFIa/ai ELISA.** An ELISA assay for the two TAFI activation isoforms was used based on the ability of PTCI to bind strongly to both TAFIa and TAFIai. PTCI-coated microtiter plate was used as the solid-phase capture agent in the ELISA. Detection of TAFIa or TAFIai bound PTCI-coated microtiter well was accomplished using a dual antibody sandwich ELISA technique with an anti-human TAFI-IgG monoclonal antibody as the primary detection antibody and a polyclonal donkey antimouse-IgG-HRP labelled second antibody. TAFIa were spiked into TAFI-deficient plasma at different concentrations and run in the ELISA (Figure 3). TAFIa was readily detected in the ELISA in a concentration dependent manner. The specificity of the ELISA was determined by spiking the TAFI-deficient plasma with increasing concentrations of TAFI. TAFI was not detected in the ELISA demonstrating the ELISA is specific for the activated form of TAFI. Although TAFIa and TAFIai have the same molecular weights, they have significantly different conformations. We determined the reactivity of the two isoforms towards the anti-TAFI antibody used in the ELISA (Figure 4). Both TAFIa and TAFIai are readily detected in TAFI-deficient



**Figure 3.** Specificity of TAFIa/ai ELISA. TAFIa (O) or TAFI (□) were spiked into TAFI-deficient plasma and the samples were run in the TAFIa/ai ELISA. Each point is the mean of duplicates. R-square values for TAFIa and TAFI were 0.9745 and 0.4393, respectively.

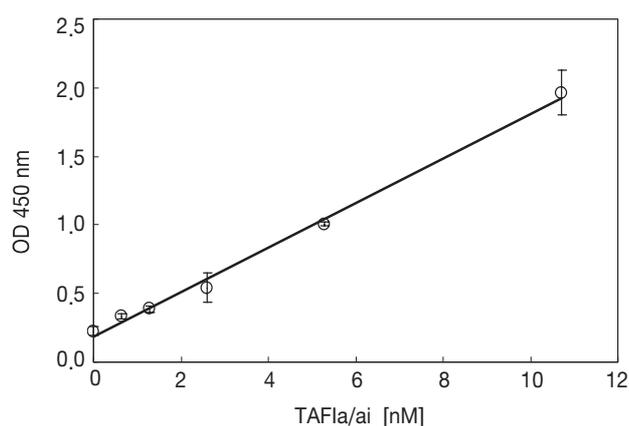


**Figure 4.** Reactivity of TAFIa and TAFIai proteins by ELISA. TAFIa (□) or TAFIai (●) protein was spiked into TAFI-deficient human plasma and run in the ELISA assay. Each point is the mean of triplicates. R-square values for TAFIa and TAFIai were 0.9827 and 0.9882, respectively.

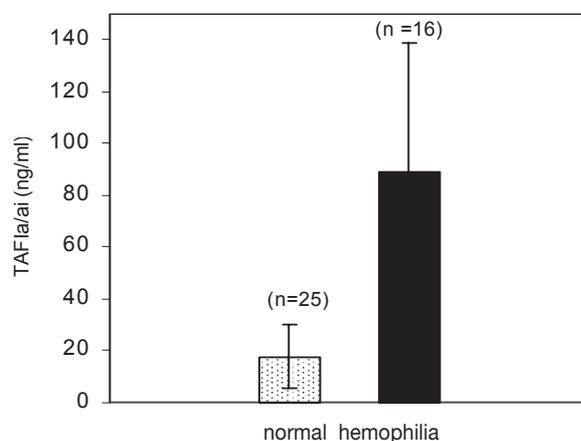
plasma and the reactivity of TAFIai is equal to that of TAFIa in the ELISA. Since both isoforms forms are recognized equally well in plasma, the antigen recognized by the ELISA would be referred as “TAFIa/ai antigen”.

Normal plasma contains high levels (approximately 200 nM) of TAFI. To validate the ability of the ELISA to measure TAFIa/ai antigen in plasma in the presence of high concentrations of TAFI, we spiked normal plasma with increasing amounts of TAFIa. As the amount of TAFIa spiked into normal plasma increased there was a corresponding increase in the amount of TAFIa measured in the ELISA (Figure 5). The sensitivity of the ELISA was approximately 1 nM TAFIa in normal plasma. These findings show that the ELISA is specific for TAFIa/ai and is able to quantitate very low levels of TAFIa/ai antigen in the presence of high levels of TAFI normally found in plasma.

**Measurement of TAFIa/ai Antigen in Normal Plasma by ELISA.** Initial studies were performed to determine if TAFIa/ai antigen was endogenously present in normal plasma. We test-



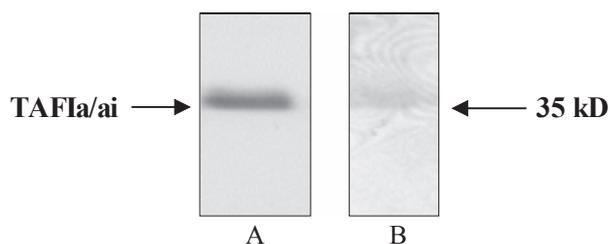
**Figure 5.** Measurement of TAFIa antigen spiked into normal plasma. Pooled normal human plasma was spiked with increasing concentrations of TAFIa and run in the ELISA assay. Each point is the mean of triplicates. R-square values was 0.9945.



**Figure 6.** Quantitation of TAFIa/ai antigen levels in hemophilia A plasma. TAFIa/ai antigen levels were quantitated in twenty-six normal plasmas (dotted bar) and in sixteen plasmas from hemophilia A (black bar) using TAFIa/ai ELISA.

ed three commercial pooled normal plasmas (PNP) and sixteen commercial citrated plasmas drawn from individuals for the presence of TAFIa/ai antigen. Low levels of TAFIa/ai antigen were determined in the three PNP samples tested (TABLE I). The TAFIa/ai antigen concentrations in individual normal plasmas ranged from 0 to 135 ng/ml. The mean concentration of TAFIa/ai antigen was  $18.6 \pm 17.1$  ng/ml [0.5 nM]. Thus, TAFIa/ai antigen is detectable at low levels in normal plasma using the TAFIa/ai ELISA.

**Elevated Levels of TAFIai in Hemophilia A.** Increased fibrinolytic activity has been reported in hemophilia.<sup>26</sup> It has also been observed that TAFI levels are depleted in hemophilia and may be related to up-regulation of TAFI activation.<sup>27</sup> If TAFI activation was enhanced in hemophilia then one might expect to find increased levels of TAFIa and/or TAFIai in hemophilic plasma. We tested sixteen plasma samples from patients with hemophilia A for elevated levels of TAFIa/ai antigen using the ELISA (Figure 6). The mean TAFIa/ai antigen levels in hemophilia A plasmas was  $88.9 \pm 49$  ng/ml. TAFIa/ai antigen was significantly elevated ( $p < 0.05$ ) as compared to



**Figure 7.** Identification of TAFIa/ai antigen in hemophilia plasma. TAFIa/ai was purified from hemophilia plasma by affinity chromatography on PTCI-Sepharose as described in Methods section. Protein eluted in high salt was applied to SDS-PAGE gel and silverstained for protein (lane A). Western blot of the same protein was immunostained with anti-TAFI antibody (lane B).

levels in normal plasmas (see above). There was no apparent correlation between TAFIa/ai levels and FVIII activity levels in hemophilia samples (TABLE II). The finding of almost 5-fold higher levels of TAFIa/ai antigen in hemophilia plasma suggests that TAFI activation pathway is up-regulated in hemophilia.

**Purification of TAFIa/ai Antigen from Plasma.** To confirm the presence of TAFIa/ai antigen in hemophilia plasma, we attempted to isolate the TAFIa/ai antigen from plasma using PTCI-affinity chromatography. A hemophilia plasma sample that contained high levels of TAFIa/ai antigen by the ELISA was applied to the PTCI-Sepharose affinity column. After washing away unbound protein, the column was eluted with 0.5 M NaCl high salt buffer as described above. The protein eluted in the high salt buffer was run on SDS-PAGE and the Western blot was immunostained with a goat anti-TAFI antibody. A 35 kD protein was immunostained using the TAFI-specific polyclonal antibody (Figure 7). These findings confirm the presence of the TAFIa/ai antigen (35 kD) in human plasma by ELISA.

## Discussion

Fibrinolysis is a highly complex hemostatic process involving multiple fibrinolytic enzymes and proteins. TAFI has been shown to be an important down-regulator of fibrinolysis through its ability to remove C-terminal lysine groups from partially degraded fibrin. Fibrinolysis is most commonly monitored by measurement of fibrin degradation products and D-dimer. The role that TAFI plays in pathological hemostatic conditions has been based mainly upon measurement of the 60 kD proenzyme TAFI. In order to modulate fibrinolysis, TAFI has to be activated by proteolytic enzymes (e.g. thrombin, plasmin) to TAFIa, the active carboxypeptidase B enzyme. A greater understanding of the function of TAFI may be achieved by quantitation of the endproducts of the TAFI activation pathway-TAFIa and TAFIai. The purpose of this study was to use a new method for the specific quantitation of TAFIa and TAFIai protein in plasma.

It has been well documented that PTCI binds to TAFIa with high affinity and blocks carboxypeptidase B activity. In this study enzymatically active TAFIa as well as its inactive isoform TAFIai was shown to bind to PTCI with high affinity (Figure 2). TAFIa undergoes significant conformational

change leading to the enzymatically inactive TAFIai isoform. However, the PTCI binding site of TAFIa remains intact as the protein undergoes conformational changes to the TAFIai isoform. Extensive protein structural analysis and crystallographic studies are required in order to fully elucidate how and where PTCI binds to TAFIa and TAFIai.

Two TAFI activation isoforms were measured based on the ability of PTCI to bind to TAFIa and TAFIai. The TAFIa/ai ELISA consists of a PTCI-coated microtiter well as the solid-phase capture agent and a double antibody sandwich technique for detection of the bound TAFIa/ai antigen. A TAFI-specific monoclonal antibody was used as the primary detection antibody. The immunoassay was specifically recognized TAFIa and TAFIai and as there was no crossreactivity with the proenzyme TAFI (Figure 3). An excellent linear correlation was obtained with either TAFIa or TAFIai with a lower limit of detection of approximately 1 nM. The TAFIa/ai ELISA measured TAFIa and TAFIai antigens with equal sensitivity (Figure 4). The presence of high levels of TAFI in normal plasma does not interfere with the measurement of TAFIa/ai antigen.

Measurable levels of TAFIa/ai antigen were found in several pooled normal plasmas as well as in some of the individual normal plasmas using the TAFIa/ai ELISA (TABLE I). The mean amount of TAFIa/ai antigen in normal plasma was  $18.6 \pm 17.1$  ng/ml [0.5 nM]. Neill *et al.*<sup>28</sup> using a highly sensitive fluorescent activity assay and plasma collected under carefully controlled conditions found very low levels (10 pM) of TAFIa to be constitutively present in normal plasma. The finding of TAFIa/ai antigen by ELISA in normal plasma would be consistent with TAFIa being constitutively produced during normal hemostasis. However, the level of TAFIa/ai antigen in plasma by ELISA is significantly higher than the amount of TAFIa determined using a fluorescence activity assay. The difference in the two results may be due to the nature of the TAFI activation isoform measured by the different assays. TAFIa constitutively generated *in vivo* is likely to be rapidly converted to TAFIai at body temperature. If TAFIai has a longer half-life than TAFIa in plasma, then one would expect TAFIai to be found at higher levels in plasma. Therefore, the TAFIa/ai antigen measured in plasma by ELISA is likely to be comprised mainly of the inactive TAFIai isoform rather than the active TAFIa enzyme. We did not detect enzymatically active TAFIa in the plasmas using a sensitive in-house TAFIa fluorescence activity assay (data not shown) which further supports that TAFIai is measured by the ELISA.

The TAFIa/ai antigen is the endproduct of the TAFI activation pathway and therefore should be a very useful new marker for monitoring fibrinolysis in various pathological conditions. It has been suggested that fibrinolytic activity is enhanced in hemophilia and may contribute to the excessive bleeding in hemophilia.<sup>26</sup> Mosnier *et al.*<sup>27</sup> restored the down regulation of fibrinolysis in hemophilia plasma by addition of TAFI to the hemophilic plasma. Reduction in TAFI plasma levels reported by Antovic *et al.*<sup>29</sup> provides further support that TAFI is involved in the regulation of hyperfibrinolysis in hemophilia A. Evidence of up-regulation of TAFI activation

in the sixteen hemophilia A plasmas was found by measuring TAFIa/ai antigen level using the ELISA. TAFIa/ai antigen was almost 5-fold higher (mean values  $88.6 \pm 49$  ng/ml) in hemophilia A plasmas ( $p < 0.05$ ) as compared to normal plasma (Figure 6). Isolation of the TAFIa/ai antigen from hemophilic plasma by PTCI-affinity chromatography confirmed the presence of the TAFIa/ai antigen detected in plasma by the ELISA (Figure 7). Similar findings of elevated TAFIa/ai antigen in hemophilia plasma were reported in a preliminary study that used a modified version of the current ELISA reported herein.<sup>30</sup> The finding of increased levels of TAFIa/ai antigen supports the hypothesis that TAFI activation is up-regulated in hemophilia A. It can be hypothesized that the enhanced fibrinolytic activity in hemophilia is counteracted by up-regulation of the TAFI activation pathway which leads to the depletion of TAFI.

The generation of thrombin is very low in hemophilia A due to a genetic defect in Factor VIII. The up-regulation of TAFI activation in hemophilia would seem unusual when thrombin, the major activator of TAFI, is not efficiently generated. Hyperfibrinolysis is likely mediated through enhanced plasmin generation via t-PA mediated activation of plasminogen. Plasmin is also known to be an effective activator of TAFI. Thus, enhanced plasmin generation associated with hyperfibrinolysis in hemophilia may also result in enhanced activation of TAFI and the elevation levels of TAFIa/ai antigen in hemophilia. High levels of plasmin-antiplasmin complex found in hemophilia plasmas support this hypothesis (unpublished results). Further work is needed to more fully understand the role TAFI activation plays in hemophilia and fibrinolysis.

In conclusion, a novel ELISA assay was used for measuring the two 35 kD activation isoforms of TAFI in plasma. The assay is specific for TAFIa and TAFIai but not TAFI proenzyme. We found low levels of TAFIa/ai antigen in normal plasmas and significantly elevated levels of TAFIa/ai antigen in hemophilia A plasmas. The finding of high levels of TAFIa/ai antigen indicates an upregulation of TAFI activation pathway and an ongoing fibrinolytic process in hemophilia. TAFIa/ai antigen measured by TAFIa/ai ELISA represents a novel marker for monitoring fibrinolysis. Quantitation of TAFIa/ai antigen levels in plasma should permit greater understanding of the role that TAFI plays in hemostasis and various pathological conditions such as hemophilia.

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