Thrombin-activable fibrinolysis inhibitor (TAFI) is a human plasma zymogen similar to pancreatic pro-carboxypeptidase B. Cleavage of the zymogen by thrombin/thrombomodulin generates the enzyme, activated TAFI (TAFIa), which retards fibrin clot lysis in vitro and likely modulates fibrinolysis in vivo. In the present work we stably expressed recombinant TAFI in baby hamster kidney cells, purified it to homogeneity from conditioned serum-free medium, and compared it to plasma TAFI (pTAFI) with respect to glycosylation and kinetics of activation by thrombin/thrombomodulin. Although rTAFI is glycosylated somewhat differently than pTAFI, cleavage products with thrombin/thrombomodulin are indistinguishable, and parameters of activation kinetics are very similar with $k_{cat} = 0.55 \text{s}^{-1}$, $K_m = 0.54 \text{mM}$, and $K_D = 6.0 \text{nm}$ for rTAFI and $k_{cat} = 0.61 \text{s}^{-1}$, $K_m = 0.55 \text{mM}$, and $K_D = 6.6 \text{nm}$ for pTAFI. The respective TAFIa species also were prepared and compared with respect to thermal stability and enzymatic properties, including inhibition of fibrinolysis. The half-life of both enzymes at 37 °C is about 10 min, and the decay of enzymatic activity is associated with a quenching (to ~62% of the initial value at 60 min) of the intrinsic fluorescence of the enzyme. Stability was highly temperature-dependent, which, according to transition state theory, indicates both high enthalpy and entropy changes associated with inactivation ($\Delta H^\circ \approx 45 \text{ kcal/mol}$ and $\Delta S^\circ \approx 80 \text{ cal/mol/K}$). Both species of TAFIa are stabilized by the competitive inhibitors 2-guanidinoethylmercaptosuccinic acid and e-aminoacproic acid. rTAFIa and pTAFIa are very similar with respect to kinetics of cleavage of small substrates, susceptibility to inhibitors, and ability to retard both tPA-induced and plasmin-mediated fibrinolysis. These studies provide new insights into the thermal instability of TAFIa, a property which could be a significant regulator of its activity in vivo; in addition, they show that rTAFIa and rTAFIa are excellent surrogates for the natural plasma-derived species, a necessary prerequisite for future studies of structure and function by site-specific mutagenesis.

The balance between the activities of the coagulation and fibrinolytic cascades is essential to protect the organism from excessive blood loss upon injury as well as to maintain the fluidity of blood within the vasculature. Imbalances lead to a tendency toward either bleeding or thrombosis, the latter of which is manifested as heart attacks and strokes.

The coagulation and fibrinolytic cascades consist of a series of zymogen to enzyme conversions, terminating in the proteolytic enzymes thrombin and plasmin, which, respectively, catalyze the deposition and removal of fibrin. However, when in a complex with the endothelial cell-surface cofactor thrombomodulin, the specificity of thrombin is changed from fibrinogen to protein C (1), thus changing thrombin to an anticoagulant rather than a procoagulant enzyme.

When formed in the context of a fibrin clot, activated protein C was found to be profibrinolytic as well as anticoagulant (2–5), an observation that results from the ability of activated protein C to prevent the formation of a previously uncharacterized fibrinolysis inhibitor (6). Further investigations in our laboratory led to the isolation of this factor, a 60-kDa glycoprotein present in human plasma at a concentration of approximately 50 nm, which we termed TAFI (thrombin-activable fibrinolysis inhibitor (7)). Upon activation of TAFI by cleavage with thrombin, an active enzyme is formed (TAFIa) that possesses carboxypeptidase B-like activity and inhibits fibrinolysis, probably by removal of the C-terminal lysines which contribute to the development of positive feedback in the fibrinolytic cascade (8). Although thrombin itself is a weak activator of TAFI (8), the thrombin-thrombomodulin complex activates TAFI with a 1250-fold higher catalytic efficiency than thrombin alone, suggesting that thrombin/thrombomodulin is the physiological activator of TAFI (8).

We have shown that TAFIa inhibits tPA-mediated fibrinolysis in vitro half-maximally at a concentration of approximately 1 nM (8). This concentration is about 2% the level of the zymogen in plasma, indicating that enough TAFIa could be produced in plasma to have a significant effect on fibrin clot lysis in vivo. In support of this scenario, recent studies utilizing a canine model of thrombolysis indicated that inducible carboxypeptidase activity (probably TAFIa) is increased during...
thrombosis and thrombolytic therapy (9); in addition, a significant positive correlation is observed between inducible carboxypeptidase activity and the time required for restoration of blood flow (9). These studies strongly imply that regulation of TAFIa activity may play a significant role in modulating hemostasis and thrombolysis in vivo.

The protein that we have termed TAFI has been described previously by other groups. Hendriks et al. (10) detected an unstable carboxypeptidase B-like activity in human serum; the enzyme corresponding to this activity was subsequently isolated and named CPU (“unstable” carboxypeptidase (11)). In addition, Eaton et al. (12) discovered the zymogen as a contaminant in preparations of α2-antiplasmin and termed it plasma procarboxypeptidase B (pro-pCPB). These investigators also cloned a cDNA corresponding to pro-pCPB, the deduced amino acid sequence of which showed significant homology with pancreatic procarboxypeptidase B (12). Amino acid sequence analysis of TAFI and fragments derived from TAFI showed that TAFI and pro-pCPB are the same protein (7).

One of the striking features of the enzyme TAFIa is its inherent instability, a property for which it was given the name “unstable carboxypeptidase” U (11). The relatively short half-life of the enzyme at body temperature suggests that this inherent instability might be relevant in the down-regulation of the enzyme in vivo. The following studies were undertaken to both investigate the instability of TAFIa and to compare thoroughly the properties of recombinant and plasma-derived TAFI and TAFIa.

**EXPERIMENTAL PROCEDURES**

*Materials*—The synthetic carboxypeptidase substrates hippuryl-L-arginine (Hip-Arg), hippuryl-L-lysine (Hip-Lys), and N-[3-(2-furylcarboxyloxy)phenyl]alanyl-L-lysine (FA-Ala-Lys), as well as hippuric acid (HA), ε-aminocaproic acid (ε-ACA), potato carboxypeptidase inhibitor (PCI), and Sepharose CL-4B were purchased from Sigma. Q-Sepharose Fast Flow anion exchange resin was from Pharmacia (Uppsala, Sweden). The carboxypeptidase B inhibitor 2-guanidinoethylmercaptoacetic acid (GEMSA), 1,5-danaryl-Glu-Gly-Arg chloromethyl ketone (dEGRck), and p-Phe-Pro-Arg chloromethyl ketone (pPACK) were purchased from Calbiochem. N-Glycosidase F was purchased from Boehringer Mannheim (Laval, Quebec, Canada). DNA restriction and modification enzymes were obtained from New England BioLabs (Mississauga, Ontario) or Life Technologies, Inc. (Burlington, Ontario) and *Pyrococcus furiuso*rus (Pfu) DNA polymerase was obtained from Stratagene (La Jolla, CA). First-strand human liver cDNA preparations were the generous gift of Mona Rahman and Dr. Marlys Koschinsky (Queen’s University, Kingston, Ontario). Baby hamster kidney cells and the mammalian expression vector pNUT were provided by Dr. Ross MacGillivray (University of British Columbia). Newborn calf serum, Dulbecco’s modified Eagle’s medium, nutrient mixture F-12 (1:1) (DMEM/F-12), Opti-MEM, UltraSer G, penicillin/streptomycin/Fungizone mixture (PSF), and reduced glutathione were obtained from Life Technologies, Inc., and methotrexate was purchased from David Bull Laboratories (Vaudreuil, Quebec). For Western blot analysis, a monoclonal antibody (mAB 13) raised against purified TAFI (13) was employed; a secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase) was obtained from Sigma. Fibrinogen, Glu-plasminogen, prothrombin, and antithrombin III were isolated from human plasma, and plasmin and plasminogen were prepared from Glu-plasminogen and prothrombin, respectively, as described previously (7). TAFI was isolated from human plasma as described by Bajzar et al. (7), with the exception that the gel filtration step was omitted. Recombinant human α2-antiplasmin was purified from conditioned medium harvested from a stably expressing BHK cell line as described previously (7). Recombinant tPA (Activase) was generously provided by Dr. Gordon Vehar of Genentech, Inc. (South San Francisco, CA). Recombinant soluble thrombomodulin (Solulin) was the generous gift of Dr. John Morser (Berlex Biosciences Inc., Richmond, CA). A plasminogen-Sepharose column was prepared using purified human Glu-plasminogen and Sepharose CL-4B according to Bajzar et al. (7).

**Cloning of a TAFI cDNA and Construction of a TAFI Expression Vector**—Based on the cDNA sequence for plasma procarboxypeptidase
The reconstructed TAFI cDNA was excised by digestion with first-strand human liver cDNA library (16, 5 f fashion. in the reverse orientation (revTAFI-pNUT) was constructed in a similar otrexate (see below). An expression plasmid containing the TAFI cDNA select stable cell lines in the presence of a high concentration of methotrexate (see below). An expression plasmid containing the TAFI cDNA also contains a mutant version of the dihydrofolate reductase gene to the human growth hormone polyadenylation sequence. The plasmid Smal vector using the unique identical to that reported by Eaton et al. (16). The full-length TAFI cDNA was reconstructed in the pBluescript primer 1, 5'-CTGTTGGGATGAACTTGGC-3' and primer 2, 5'-GCGTCGAGGAAATGTGCTGTG-3'; primer 3, 5'-CTGG- CTGGTGTCGAGGAAATGTGCTGTG-3' and primer 4, 5'-GGTACCATCAGCATCGTC-3'; primer 5, 5'-GCGTACATCGCATCGTC-3'; primer 6, 5'-CAATGGCTGGTCTGCTGGT-3'. By using these primers, three overlapping PCR products were obtained by PCR amplification using a first-strand human liver cDNA library (1 μg) as the template. The PCR cycling conditions were as follows: 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 52 °C, and 30 s at 75 °C; and a final 15 min at 75 °C. The three primer pairs gave rise to 255 base pairs (nucleotides 10–346 of pro-pcPB sequence; Eaton et al. (12)), 665 base pairs (nucleotides 318–983), and 425 base pairs (nucleotides 953–1387), respectively. The three PCR products were individually inserted into the EcoR I site of pBluescript SK+ (Stratagene) and analyzed by DNA sequence analysis; the sequence of the cloned PCR fragments was identical to that reported by Eaton et al. (12).

The full-length TAFI cDNA was reconstructed in the pBluescript vector using the unique Bgl II and Sph I restriction sites that are located in the regions of overlap between the individual fragments (see Fig. 1). The reconstructed TAFI cDNA was excised by digestion with XbaI and XhoI; the ends were made blunt using the Klenow fragment of Escherichia coli DNA polymerase I, and the fragment was inserted into the pNUT plasmid that had been digested with SnaBI. The resultant ex- pression plasmid (TAFI-pNUT) contains nucleotides 10–1378 of the TAFI cDNA under control of the mouse metallothionein I promoter and the human growth hormone polyadenylation sequence. The plasmid also contains a mutant version of the dihydrofolate reductase gene to select stable cell lines in the presence of a high concentration of methotrexate (see below). An expression plasmid containing the TAFI cDNA in the reverse orientation (revTAFI-pNUT) was constructed in a similar fashion.

**Culture and Transfection of Mammalian Cells**—BHK cells were cultured in 100-mm dishes in DMEM/F-12 containing 5% newborn calf serum in a 37 °C humidified incubator (95% air/5% CO2 atmosphere). Cells were transfected by the method of calcium phosphate co-precipitation (14) using 10 μg of TAFI-pNUT plasmid per 100-mm plate. Six hours following transfection, the cells were washed and fed fresh DMEM/F-12 containing 5% newborn calf serum. The cells were allowed to recover overnight, after which the medium was replaced with DMEM/F-12 containing 5% newborn calf serum and 400 μM methotrexate. After a 2-week selection period, surviving foci were picked and analyzed for expression of TAFI by Western blot analysis.

**Recombinant Protein Expression**—BHK cell lines stably expressing TAFI were transfected in triple flasks (500 ml; Nunc, Roskilde, Denmark) in DMEM/F-12 containing 1% (v/v) UltraSer G and 200 μM methotrexate. Once the cells had become confluent, the medium was changed to Opti-MEM (5 ml/100-mm plate) and culture continued for a further 48 h. At this time, conditioned medium was har- vested from the cells and subjected to SDS-PAGE followed by Western blot analysis. TAFI-specific monomers and TAFI were subjected to recover overnight, after which the medium was replaced with DMEM/F-12 containing 5% newborn calf serum and 400 μM methotrexate. After a 2-week selection period, surviving foci were picked and analyzed for expression of TAFI by Western blot analysis.

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Fig. 2. Expression of rTAFI in BHK cells. BHK cells were transiently transfectected with the expression plasmids TAFI-pNUT or revTAFI-pNUT. The indicated volumes of conditioned serum-free medium (CM) from these cells were subjected to SDS-PAGE on a 5–15% polyacrylamide gradient gel followed by Western blot analysis using a TAFI-specific monoclonal antibody. Following incubation of the blot with a secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase), immunoreactive bands were visualized by chemiluminescence. Also shown on the blot is 10 ng of purified plasma-derived TAFI (pTAFI). The positions of molecular mass markers are shown to the left of the blot.

RESULTS

Expression and Purification of Recombinant TAFI (rTAFI)—Conditioned serum-free medium (CM) harvested from BHK cells transiently transfectected with the TAFI-pNUT expression plasmid was subjected to SDS-PAGE followed by Western blot analysis using a TAFI-specific monoclonal antibody (13) (Fig. 2). Also included in this blot was 10 ng of plasma-derived TAFI (pTAFI) and CM harvested from BHK cells transiently transfectected with the pNUT plasmid containing the TAFI cDNA in the reverse orientation (revTAFI-pNUT). An intense immunoreactive band similar in mobility to pTAFI was present in CM harvested from cells transfectected with the expression plasmid containing the TAFI cDNA in the forward, but not the reverse orientation, rTAFI and pTAFI were treated with trypsin (12), these are all relatively poor activators of the TAFIa at Arg-330 (7) were of identical size for rTAFI and pTAFI. In addition, the rates of appearance of the TAFIa activity (as measured by Hip-Arg hydrolysis following treatment with thrombin/thrombomodulin (Fig. 4. upper panel) revealed that the M$_r$ ~35,000 active enzyme (TAFIa) derived by cleavage of the zymogen at Arg-92 (7) and the M$_r$ ~25,000 and M$_r$ ~12,000 fragments derived from cleavage of TAFIa at Arg-330 (7) were of identical size for rTAFI and pTAFI. In addition, the rates of appearance of the M$_r$ ~35,000, M$_r$ ~25,000, and M$_r$ ~12,000 bands (Fig. 4, upper panel) and the rates of appearance of TAFIa activity (as measured by hydrolysis of hippuryl-L-arginine (Hip-Arg)) (Fig. 4, lower panel) were similar for rTAFI and pTAFI.

To assess if the difference in mobility on SDS-PAGE of rTAFI and pTAFI might be due to differences in N-linked glycosylation, rTAFI and pTAFI were treated with N-glycosidase F, an enzyme that removes N-linked glycans from protein substrates (17). Analysis of samples treated for various times with N-glycosidase F by electrophoresis on a Tris/Tricine gel (15), followed by silver-staining (Fig. 5), revealed that both rTAFI and pTAFI gave rise to identically sized (presumably fully deglycosylated) terminal products that corresponded closely in molecular weight to the predicted peptide molecular weight of rTAFI (M$_r$ 45,996 (12)). In addition, both rTAFI and pTAFI gave rise to three intermediately glycosylated species, indicating that all four potential N-linked glycosylation sites in the activation peptide of both rTAFI and pTAFI (12) are utilized. Both deglycosylated rTAFI and pTAFI species migrated as doublons on the gel, which may be the result of heterogeneity in O-linked glycosylation in the respective proteins.

Comparison of the Activation of rTAFI and pTAFI by Thrombin/Thrombomodulin—Although previous studies have shown that TAFI can be activated by thrombin (7), plasmin (12), and trypsin (12), these are all relatively poor activators of the TAFIa (14). To compare the activation of rTAFI and pTAFI (Fig. 6) purified rTAFI migrated marginally slower than pTAFI (Fig. 3). However, SDS-PAGE analysis of rTAFI and pTAFI activated by thrombin/thrombomodulin (Fig. 4, upper panel) revealed that the M$_r$ ~35,000 active enzyme (TAFIa) derived by cleavage of the zymogen at Arg-92 (7) and the M$_r$ ~25,000 and M$_r$ ~12,000 fragments derived from cleavage of TAFIa at Arg-330 (7) were of identical size for rTAFI and pTAFI. In addition, the rates of appearance of the M$_r$ ~35,000, M$_r$ ~25,000, and M$_r$ ~12,000 bands (Fig. 4, upper panel) and the rates of appearance of TAFIa activity (as measured by hydrolysis of hippuryl-L-arginine (Hip-Arg)) (Fig. 4, lower panel) were similar for rTAFI and pTAFI.

TABLE I

Comparison of the Activation of rTAFI and pTAFI by Thrombin/Thrombomodulin—Although previous studies have shown that TAFI can be activated by thrombin (7), plasmin (12), and trypsin (12), these are all relatively poor activators of the TAFIa (14). To compare the activation of rTAFI and pTAFI (Fig. 6) purified rTAFI migrated marginally slower than pTAFI (Fig. 3). However, SDS-PAGE analysis of rTAFI and pTAFI activated by thrombin/thrombomodulin (Fig. 4, upper panel) revealed that the M$_r$ ~35,000 active enzyme (TAFIa) derived by cleavage of the zymogen at Arg-92 (7) and the M$_r$ ~25,000 and M$_r$ ~12,000 fragments derived from cleavage of TAFIa at Arg-330 (7) were of identical size for rTAFI and pTAFI. In addition, the rates of appearance of the M$_r$ ~35,000, M$_r$ ~25,000, and M$_r$ ~12,000 bands (Fig. 4, upper panel) and the rates of appearance of TAFIa activity (as measured by hydrolysis of hippuryl-L-arginine (Hip-Arg)) (Fig. 4, lower panel) were similar for rTAFI and pTAFI.

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zymogen. Furthermore, at concentrations of these activators required to achieve significant cleavage at Arg-92, TAFIa itself is subsequently cleaved rapidly at Arg-330 to inactivate the enzyme. Recent studies from our laboratory, however, have shown that thrombin complexed with thrombomodulin activates TAFI with a 1250-fold higher catalytic efficiency than thrombin alone without appearing to enhance significantly the rate of the inactivating cleavage (8), suggesting that the physiologic activator is thrombin/thrombomodulin. Therefore, the ability of thrombin/thrombomodulin to activate rTAFI was investigated. The concentrations of substrate (rTAFI or pTAFI) and cofactor (Solulin) were systematically varied, and the initial rates of TAFIa formation were assessed by measurement of N-[3-(2-furylacryloyl)]-L-alanyl-L-lysine (FA-Ala-Lys) hydrolysis. Kinetic data were fit to an equation developed to describe the kinetics of pTAFI activation by thrombin/thrombomodulin (8). Presented in Fig. 6 are the rates of pTAFIa (Fig. 6A) or rTAFIa (Fig. 6B) formation plotted as a function of TAFI concentration. The solid lines in both panels represent the rates calculated from the fit parameters $k_{cat}$, $K_m$, and $K_d$. These kinetic constants are presented in Table II and are very similar for rTAFI and pTAFI.

Comparison of the Enzymatic Properties of rTAFIa and pTAFIa—To compare the enzymatic behavior of rTAFIa and pTAFIa, the ability of the respective enzymes to hydrolyze the synthetic substrates Hip-Arg (50–600 $\mu$M), Hip-Lys (100–1200 $\mu$M), and FA-Ala-Lys (0.1–2 mM) was assessed. Kinetic constants for rTAFIa and pTAFIa for these substrates were obtained by fitting the rates of substrate hydrolysis to the Michaelis-Menten equation using nonlinear regression; the results are presented in Table III. Inhibition constants ($K_i$) for three competitive inhibitors of TAFIa, $\epsilon$-aminocaproic acid ($\epsilon$-ACA (19)), 2-guanidinoethylmercaptosuccinic acid (GEMSA (11)), and potato carboxypeptidase inhibitor (PCI (18)) were also determined (Table III). The respective inhibitors were titrated ($\epsilon$-ACA, 0.1–5 mM; GEMSA, 2–50 $\mu$M; PCI, 2–50 nM) at three different concentrations of substrate (either Hip-Arg (200, 400, or 600 $\mu$M) or FA-Ala-Lys (200, 400, or 600 $\mu$M), see Table III), and the data were fit to a modified form of the Michaelis-Menten equation which describes competitive inhibition. The respective zymogens were activated by
thrombin/thrombomodulin; the thrombin was irreversibly inhibited with PPAck, and the activated enzymes were placed at 0, 22, 30, or 37 °C. Aliquots were removed at various times, and the FA-Ala-Lys hydrolytic activity in each sample was assessed. Whereas rTAFIa and pTAFIa are both stable at 0 °C, their activities decay with similar half-lives of about 120–150, 40–50, and 8–9 min at 22, 30, and 37 °C, respectively (Fig. 7A). The data of Fig. 7A exhibited first-order kinetics of decay. First-order decay constants were obtained by fitting the data to the equation $[\text{TAFI}]_0 \exp(-kT) = \text{TAFI}_0 \exp(-kT)$ by nonlinear regression. With plasma TAFI the first-order decay constants (min$^{-1}$) at 22, 30, and 37 °C were $0.0004 \pm 0.0003$, $0.013 \pm 0.001$, and $0.07 \pm 0.04$ respectively. The corresponding values with recombinant TAFIa were $0.0052 \pm 0.0003$, $0.016 \pm 0.01$, and $0.088 \pm 0.007$. The temperature dependence of the first-order decay constants was interpreted according to transition state theory whereby the forward rate constant ($k_1$) for a transition (e.g. denaturation) from one state to another is given by $k_1 = (kT/\hbar) \exp(-\Delta H^*/\Delta S^* R)$ where $k$ is Boltzmann’s constant, $T$ is the absolute temperature, $\hbar$ is Planck’s constant, $R$ is the molar gas constant, and $\Delta H^*$ and $\Delta S^*$ are the standard entropy and enthalpy changes for the presumed equilibrium between the initial state and transition state intermediate (20). The data were fit to the above equation by nonlinear regression with the values of the rate constants for decay and temperature as variables and $\Delta H^*$ and $\Delta S^*$ as fit parameters. The analyses returned values of $\Delta S^* = 82 \pm 19$ cal/mol/K, $\Delta H^* = 46 \pm 6$ kcal/mol for inactivation of plasma TAFIa and $\Delta S^* = 75 \pm 18$ cal/mol/K, $\Delta H^* = 43 \pm 5$ kcal/mol for recombinant TAFIa. The relatively high positive values for $\Delta H^*$ imply that inactivation is not enthalpically favored, possibly because of the need to break numerous noncovalent bonds. This is offset, however, by a highly favorable entropy change associated with inactivation. The high enthalpy change also accounts for the high sensitivity of inactivation to temperature. According to this interpretation, the process of TAFIa inactivation involves both the energetically unfavorable disruption of numerous noncovalent interactions within the protein and the energetically favorable assumption of a less ordered structure.

We also investigated the stability of rTAFIa and pTAFIa at 37 °C in the presence of saturating concentrations of the competitive inhibitors $\epsilon$-ACA and GEMSA. Interestingly, we found that the activity of both enzymes was stable in the presence of the inhibitors (Fig. 7B). In addition, the respective zymogens were stable at 37 °C; the potential TAFIa activity of the zymogens remained constant at this temperature over a 60-min period, as represented by the dashed (pTAFIa) and dotted (rTAFIa) lines in Fig. 7B.

To assess structural changes that may accompany the decay of enzymatic activity of the respective enzymes, the intrinsic fluorescence of pTAFIa and rTAFIa was measured at 37 °C (Fig. 8). The data show that incubation of the enzymes at this temperature elicits a marked quenching of the intrinsic fluorescence signal that correlates with the decline in enzyme activity and that reaches $-62\%$ of the initial value by 60 min. In control experiments, the intrinsic fluorescence of the respective zymogens was found to remain essentially stable over 60 min at 37 °C (data not shown).

**TABLE II**

| k/cat, $K_m$, and $K_i$ values for rTAFI and pTAFI activation by thrombin/thrombomodulin |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $K_i$ |
| s$^{-1}$ | $\mu M$ | s$^{-1}$ $\mu M^{-1}$ | nM |
| rTAFI | 0.551 $\pm$ 0.015 | 0.543 $\pm$ 0.037 | 1.01 $\pm$ 0.07 | 5.99 $\pm$ 0.30 |
| pTAFI | 0.611 $\pm$ 0.030 | 0.552 $\pm$ 0.065 | 1.11 $\pm$ 0.14 | 6.63 $\pm$ 0.62 |

**FIG. 7.** Comparison of the stability of rTAFIa and pTAFIa. A, rTAFIa (closed symbols) and pTAFIa (open symbols) were formed by incubation of the respective zymogens with thrombin/thrombomodulin, and the activated species were placed at 0 °C (circles), 22 °C (triangles), 30 °C (diamonds), or 37 °C (squares). At various times, aliquots were removed and the TAFIa activity measured using FA-Ala-Lys as the substrate. B, the effects of $\epsilon$-ACA (5 mM) and GEMSA (100 μM) on the stability of rTAFIa and pTAFIa at 37 °C were determined. The controls (no inhibitor) are indicated by circles, whereas results with GEMSA and $\epsilon$-ACA are indicated by squares and triangles, respectively. The dashed and dotted lines represent the total potential activity of pTAFIa and rTAFIa, respectively, incubated for 0, 10, and 60 min at 37 °C.
We have expressed a recombinant form of TAFI (thrombin-activatable fibrinolysis inhibitor; also known as CPU (11) and plasma procarboxypeptidase B (12)) in BHK cells. We chose to express rTAFI in mammalian cells primarily because a number of other proteins involved in the coagulation and fibrinolytic cascades have been successfully expressed in BHK cells. These include human prothrombin (22), human factor VII (23), human factor VIII (24), human antithrombin III (25), human plasminogen (26), and plasma procarboxypeptidase B (26). Unlike bacterial expression systems, recombinant proteins expressed in mammalian cells can be secreted in their native conformation and are subjected to post-translational modifications such as glycosylation.

In the present study, we found that while the recombinant version of TAFI migrates marginally more slowly on SDS-PAGE than its plasma-derived counterpart, rTAFI is virtually indistinguishable from pTAFI in terms of its ability to inhibit tPA- and plasmin-mediated clot lysis in vitro. A, clots were formed by adding a solution (194 μl) of fibrinogen (2.9 μM), Glu-plasminogen (0.66 μM), recombinant α2-antiplasmin (0.5 μM), antithrombin III (0.96 μM), and various concentrations of rTAFI [closed circles] or pTAFI [open circles] to the wells of a microtiter plate containing individual 2-μl aliquots of thrombin (7.7 nM, final), CaCl₂ (10 mM, final), and tPA (442 pM, final). Lysis of the clots was monitored by measurement of turbidity at 37 °C, and the time required for 50% lysis of the clots was determined. B, clots were formed by adding a solution (194 μl) of fibrinogen (2.9 μM) and various concentrations of rTAFI [closed circles] or pTAFI [open circles] to the wells of a microtiter plate containing individual 2-μl aliquots of thrombin (7.7 nM, final), CaCl₂ (10 mM, final), and plasmin (2 nM, final). Lysis of the clots was monitored as described for A.

**DISCUSSION**

We have expressed a recombinant form of TAFI (thrombin-activatable fibrinolysis inhibitor; also known as CPU (11) and plasma procarboxypeptidase B (12)) in BHK cells. We chose to express rTAFI in mammalian cells primarily because a number of other proteins involved in the coagulation and fibrinolytic cascades have been successfully expressed in BHK cells. These include human prothrombin (22), human factor VII (23), human factor VIII (24), human antithrombin III (25), human plasminogen (26), and human α2-antiplasmin (26). Unlike bacterial expression systems, recombinant proteins expressed in mammalian cells can be secreted in their native conformation and are subjected to post-translational modifications such as glycosylation.

In the present study, we found that while the recombinant version of TAFI migrates marginally more slowly on SDS-PAGE than its plasma-derived counterpart, rTAFI is virtually indistinguishable from pTAFI in terms of its ability to be activated by thrombin/thrombomodulin and the stability and enzymatic properties of TAFI including the ability to inhibit tPA- and plasmin-mediated fibrin clot lysis in vitro.

The mobility difference between rTAFI and pTAFI on SDS-PAGE (Fig. 3) is likely due to differences in the size and/or composition of N-linked glycans. Inspection of the cDNA sequence for plasma procarboxypeptidase B reveals the presence of four potential N-linked glycosylation sites (Asn-22, Asn-51, Asn-63, and Asn-86), all of which are located within the 92-amino acid activation peptide (12). Indeed, there is no apparent difference between the mobility of the M₉₋₅₃,000 TAFIa species derived from rTAFI and pTAFI (Fig. 4). Furthermore, treatment of rTAFI and pTAFI with N-glycosidase F, which specifically removes N-linked glycans, gave rise to terminal products of identical electrophoretic mobility (Fig. 5).
intermediately glycosylated species are present, indicating that all four potential N-linked sites are utilized in rTAFI and pTAFI.

Differences in the size and/or composition of N-linked glycans are frequently observed in the comparison of a recombinant protein and its naturally occurring counterpart (25, 27–29), which reflect the cell–, tissue–, and species-specificity of glycosylation (30). In some cases such differences have consequences for the functional properties of the recombinant protein. For example, BHK cells secrete a glycoform of ATIII that is not found in plasma and that differs in its affinity for heparin and in its rate of proteinase inhibition, in addition to two other glycoforms that are functionally similar to plasma-derived ATIII (25). However, in other cases differences in glycosylation have no apparent effect on the functional behavior of the recombinant protein. Human factor VIII expressed in BHK cells contains differences in the composition of its N-linked glycans relative to plasma-derived factor VIII (28), yet the two factor VIII preparations are similar with respect to cleavage by thrombin, factor Xα, and activated protein C (24), subunit association and dissociation (24), and pharmacokinetic parameters in baboons (28). Although we did not observe any functional differences between rTAFI and pTAFI attributable to differences in glycosylation, differences in, for example, the pharmacokinetics of rTAFI and pTAFI or in their binding to as yet undescribed substrates cannot be ruled out.

It should be noted that the fully N-deglycosylated TAFI species migrated as doublets, with both members of the doublet in similar proportions in both rTAFI and pTAFI (Fig. 5). These doublets are most likely the result of heterogeneity in usage of O-linked glycosylation sites. It is unlikely that the doublet is due to N-terminal sequence heterogeneity, since TAFI derived from plasma has been shown to possess a unique N-terminal sequence (7, 12).

We found that rTAFI is virtually indistinguishable from pTAFI in terms of its ability to hydrolyze small peptide substrates and to be inhibited by the competitive inhibitors e-ACA, GEMSA, and PCI (Table III). These data demonstrate the integrity of the active site of rTAFI and show that, when produced under the conditions reported in this study, rTAFI possesses a specific activity similar to that of its plasma-derived counterpart. Of note is the similarity in the \( K_c \) for PCI, a small (39 amino acid) inhibitor that has been shown to bind to carboxypeptidase A through regions distinct from those involved in substrate binding. Together with the similar kinetic constants obtained for the activation of rTAFI by thrombin/thrombomodulin (Table II), these data indicate that the overall structure of TAFI is likely to be faithfully represented by the recombinant protein.

A striking feature of TAFIa is that its activity is unstable, decaying rapidly at 37 °C both in vitro and in the absence of detectable proteolysis and in the serum milieu. In the present study, we found the stability of rTAFIa and pTAFIa to be very similar; although both enzymes were stable at 0 °C, their activities decayed with half-lives of about 10 min at 37 °C, about 40–50 min at 30 °C, and about 120–150 min at 22 °C (Fig. 7). The structural basis for the instability of TAFIa is not clear at present, but a thermodynamic interpretation suggests a highly unfavorable enthalpy change associated with inactivation (which may reflect the requirement to disrupt many noncovalent bonds), offset by a corresponding highly favorable entropy change (which in turn may reflect the adoption of a less ordered structure). Consistent with such changes in structure, we found a marked quenching of the intrinsic fluorescence of TAFIa at 37 °C which correlated temporally with the decay of enzymatic activity (Fig. 8). The quenching of the fluorescence signal is presumably attributable to the exposure of the solvent of residues (largely tryptophans) previously buried in the hydrophobic core of the enzyme. It is noteworthy that this presumptive structural change is not detectable by electrophoresis; the mobility of TAFIa species incubated for 60 min at 37 °C on SDS-PAGE is identical to that of TAFIa species incubated at 0 °C for 60 min (data not shown).

Whether an endogenous inhibitor of TAFIa exists is not known at present, although data exist that indicate that TAFIa interacts with \( \alpha \)-macroglobulin and pregnancy zone protein (31). The consequences of these interactions are not known, although binding of TAFIa to these proteins does not affect its enzymatic activity (31). Because carboxypeptidase U activity (TAFIa) in serum has a half-life similar to that of purified TAFIa shown here (10), the presence of a fast-acting endogenous inhibitor in vivo is not indicated. Therefore, the intrinsic instability of TAFIa at 37 °C may be physiologically relevant in the down-regulation of TAFIa in vivo. Furthermore, that the activity of TAFIa is stable at 37 °C in the presence of saturating concentrations of competitive inhibitors suggests that in the clot milieu, TAFIa activity may be maintained as long as there is sufficient substrate available.

Although the mechanism by which TAFIa inhibits fibrinolysis has yet to be conclusively determined, it likely involves removal of the C-terminal lysine residues from partially degraded fibrin that are required for maximal stimulation of tPA-mediated plasminogen activation (8). However, additional mechanisms for the antifibrinolytic effect of TAFIa may also be possible, since we have recently observed that TAFIa (albeit at higher concentrations) is capable of attenuating plasmin-mediated fibrinolysis (i.e., when plasminogen activation has been bypassed). Although the mechanism underlying this effect is unclear at present, plasmin itself may be the substrate for TAFIa in this instance (21). Based on the data presented in Fig. 9, which show that rTAFIa is capable of inhibiting both tPA and plasmin-mediated fibrinolysis in a manner identical to pTAFIa, it is clear that rTAFIa and pTAFIa are comparable in their ability to participate in the reactions involved in the antifibrinolytic effect. These reactions may include hydrolyzing C-terminal basic residues in fibrin and plasmin, as well as inactivation of TAFIa by plasmin cleavage at Arg-330.

In conclusion, these studies show that although plasma and recombinant TAFIa exhibit minor differences in glycosylation, recombinant TAFIa and TAFIa are excellent surrogates for the natural species and thus their properties can be used to interpret further differences obtained in structure-function studies utilizing site-directed mutagenesis.

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REFERENCES

1. Emon, C. T. (1995) FASEB J. 9, 946–955
2. Taylor, F. B., and Lockhart, M. S. (1985) Thromb. Res. 37, 639–649
3. Burdick, M. D., and Schaub, R. G. (1987) Thromb. Res. 45, 413–419
4. de Fouw, N. J., Haverkate, F., and Bertina, R. M. (1990) Adv. Exp. Med. Biol. 281, 235–243
5. Bajzar, L., Fordenburgh, J. C., and Nesheim, M. (1990) J. Biol. Chem. 265, 16948–16954
6. Bajzar, L., and Nesheim, M. (1993) J. Biol. Chem. 268, 8608–8616
7. Bajzar, L., and Nesheim, M. (1995) J. Biol. Chem. 270, 14477–14484
8. Bajzar, L., and Nesheim, M. (1996) J. Biol. Chem. 271, 16603–16608
9. Redlitz, A., Nielson, F. A., Malczyk, J. L., Topol, E. J., and Flow, E. F. (1996) Circulation 93, 1328–1330
10. Hendriks, D., Wang, W., Scharpe, S. S., Lommaert, M. P., and van Sande, M. (1990) Biochim. Biophys. Acta 1034, 86–92
11. Wang, W., Hendriks, D. F., and Scharpe, S. S. (1994) J. Biol. Chem. 269,
15937–15944
12. Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., and Drayna, D. (1991) *J. Biol. Chem.* 266, 21833–21838
13. Bajzar, L., Nesheim, M. E., and Tracy, P. B. (1996) *Blood* 88, 2093–2100
14. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* 7, 2745–2752
15. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368–379
16. Merrill, C. R., Goldman, D., Sedman, S. A., and Ebert, M. H. (1981) *Science* 211, 1437–1438
17. Plummer, T. H., Jr., Elder, J. H., Alexander S., Phelan, A. W., and Tarentino, A. L. (1984) *J. Biol. Chem.* 259, 10700–10704
18. Redlitz, A., Tan, A. K., Eaton, D. L., and Plow, E. F. (1995) *J. Clin. Invest.* 96, 2534–2538
19. Tan, A. K. and Eaton, D. L. (1995) *Biochemistry* 34, 5811–5816
20. Marshall, A. G. (1978) *Biophysical Chemistry*, pp. 102–107, John Wiley & Sons, Inc., New York
21. Wang, W., Boffa, M. B., Walker, J., and Nesheim, M. E. (1996) *Fibrinolysis* 10, Suppl. 3, 82
22. Côté, H. C. F., Stevens, W. K., Bajzar, L., Banfield, D. K., Nesheim, M. E., and MacGillivray, R. T. A. (1994) *J. Biol. Chem.* 269, 11374–11380
23. Pedersen A. H., Lund-Hansen, T., Bisgaard-Frantzen, H., Olsen, F., Olsen, F., and Petersen, L. C. (1989) *Biochemistry* 28, 9331–9336
24. Eaton, D. L., Hass, P. E., Riddle, L., Mather, J., Wiebe, M., Gregory, T., and Vehar, G. A. (1987) *J. Biol. Chem.* 263, 3285–3289
25. Fan, B., Crews, B. C., Turko, I. V., Choay, J., Zettlmeissl, G., and Gettins, P. (1993) *J. Biol. Chem.* 268, 17588–17596
26. Busby, S. J., Mulvihill, E., Rao, D., Kumar, A. A., Lioubin, P., Heipel, M., Sprecher, C., Hallpup, L., Prunkard, D., Gambee, J., and Foster, D. C. (1991) *J. Biol. Chem.* 266, 15286–15292
27. Parekh, R. B., Dwek, R. A., Rudd, P. M., Thomas, J. R., Rademacher, T. W., Warren, T, Wun, T.-C., Hebert, B., Reitz, B., Palmer, M., Ramabhadran, T., and Tiemeier, D. C. (1989) *Biochemistry* 28, 7670–7679
28. Hironaka, T., Furukawa, K., Emon, P. C., Fournel, M. A., Sawada, S., Kato, M., Minaga, T., and Kobata, A. (1992) *J. Biol. Chem.* 267, 8012–8020
29. Parkinson, J. F., Grinnel, B. W., Moore, R. E., Hoskins, J., Vlahos, C. J., and Bang, N. U. (1990) *J. Biol. Chem.* 265, 12602–12610
30. Rudd, P. M., Woods, R. J., Wermald, M. R., Opdenakker, G., Downing, A. K., Campbell, I. D., and Dwek, R. A. (1995) *Biochim. Biophys. Acta* 1248, 1–10
31. Valnickova, Z., Thogersen, I. B., Christensen, S., Chu, C. T., Pizzo, S. V., and Enghild, J. J. (1996) *J. Biol. Chem.* 271, 12937–12943