Mutations in the Substrate Binding Site of Thrombin-activatable Fibrinolysis Inhibitor (TAFI) Alter Its Substrate Specificity*

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Lei Zhao‡§, Brad Buckman‡, Marian Seto, John Morser‡, and Mariko Nagashima‡

From the Departments of ‡Cardiovascular Research, ‡Medicinal Chemistry, and ‡Biophysics, Berlex Biosciences, Richmond, California 94806

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a zymogen that inhibits the amplification of plasmin production when converted to its active form (TAFIa). TAFI is structurally very similar to pancreatic carboxypeptidase B. TAFI also shares high homology in zinc binding and catalytic sites with the second basic carboxypeptidase present in plasma, carboxypeptidase N. We investigated the effects of altering residues involved in substrate specificity to understand how they contribute to the enzymatic differences between TAFI and carboxypeptidase N. We expressed wild type TAFI and binding site mutants in 293 cells. Recombinant proteins were purified and characterized for their activation and enzymatic activity as well as functional activity. Although the thrombin/thrombomodulin complex activated all the mutants, carboxypeptidase B activity of the activated mutants against hippuryl-arginine was reduced. Potato carboxypeptidase inhibitor inhibited the residual activity of the mutants. The functional activity of the mutants in a plasma clot lysis assay correlated with their chromogenic activity. The effect of the mutations on other substrates depended on the particular mutation, with some of the mutants possessing more activity against hippuryl-His-leucine than wild type TAFIa. Thus mutations in residues around the substrate binding site of TAFI resulted in altered C-terminal substrate specificity.

Carboxypeptidases (CPs) are enzymes that catalyze the hydrolysis of the C-terminal peptide bond in peptides and proteins. Although it might seem that removal of one or a few amino acids from the C terminus of a peptide or protein would be of limited importance, it can have profound effects on their biological activity (1). CPs perform many diverse functions in the body. These include digestion and assimilation of dietary proteins, processing of peptide hormone precursors, regulation of peptide hormone activity, and regulation of protein binding.

Thrombin-activatable fibrinolysis inhibitor (TAFI, EC 3.4.17.20) is a plasma protein that has basic carboxypeptidase activity upon activation. It is also known as plasma carboxypeptidase B, carboxypeptidase U, and carboxypeptidase R. TAFI is synthesized by the liver and circulates in plasma as a zymogen within a concentration range of 50–150 nM. It has been shown to play a role in the regulation of fibrinolysis both in vitro and in vivo (2–6). TAFI is a glycoprotein with a Mr ~58,000. Following its activation by the thrombin/thrombomodulin complex, activated TAFI (TAFIa) cleaves C-terminal basic residues of fibrin that are newly exposed by plasmin cleavage. Since these C-terminal basic residues are high affinity binding sites for both plasminogen and plasminogen activators, they serve as an amplification system for plasmin production. When TAFIa removes these C-terminal basic residues, plasmin production is reduced, leading to slower lysis of the clot. TAFIa may also be involved in plasmin-mediated cell migration (6).

In vitro TAFIa can cleave a number of peptides with biological activity in the circulation such as bradykinin and anaphylatoxins (7–9). In vivo, these peptides are degraded rapidly by the second CP present in plasma, carboxypeptidase N (CPN, EC 3.4.17.3). CPN is a Mr ~280,000 tetrameric enzyme consisting of two small catalytic subunits and two large glycosylated subunits. It is synthesized by the liver and circulates in plasma at 100 nM in a constitutively active form (10).

Both TAFI and CPN belong to a class of metallocarboxypeptidases that catalyze the hydrolysis of the C-terminal peptide bond in peptides and proteins. Based on sequence analysis, metallocarboxypeptidases can be divided into two groups: 1) carboxypeptidases A1, A2, and B, TAFI, and mast cell carboxypeptidase A and 2) carboxypeptidases N, H/E, M, D, and Z (11). The sequence similarity is high within each group (40–58%) but much lower between the two groups (14–20%). CPs from the first group are synthesized as inactive zymogens and require removal of a propeptide before they exert carboxypeptidase activity, optimally at neutral pH. Active CPs have a preference for aromatic or aliphatic residues, whereas active CPBs favor basic residues. A naturally occurring small protein, carboxypeptidase inhibitor from potato (CPI), and synthetic compounds such as guanidinoethylmercaptosuccinic acid inhibit the activity of these CPs. The three-dimensional structure and the mechanism of action for pancreatic carboxypeptidases A and B have been widely studied and are very similar (12–14). On the other hand, carboxypeptidases from the second group are constitutively active toward basic residues at various pH optima and possess a long C-terminal extension whose functions are unknown (11). Although they are inhibited by guanidinoethylmercaptosuccinic acid, they are not susceptible to inhibition by CPI. They perform important functions in regulation of biologically active peptides such as processing of propeptide hormones (15, 16), inactivation of biologically active peptides (10), and alteration of substrate specificity for receptor binding (17–19). Recently, the crystal structure of CPD has been elucidated and revealed an overall topological similarity to that of CPA and CPB but with unique structural features.

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‡ To whom correspondence should be addressed: Dept. of Cardiovascular Research, Berlex Biosciences, 2600 Hilltop Dr., Richmond, CA 94806. Tel.: 510-669-4725; Fax: 510-669-4246; E-mail: lei.zhao@berlex.com.

§ The abbreviations used are: CP, carboxypeptidase; CPI, potato carboxypeptidase inhibitor; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated TAFI; PPACK, D-Phe-Pro-Arg chloromethyl ketone.
that may explain differences in the activity of the two groups of CPs (20).

The current study was undertaken to investigate the structural basis for the differences in substrate specificity and susceptibility to CPI for CPs from the two groups. In this study, we employed site-directed mutagenesis to switch residues that may be involved in substrate binding between TAFI, a member of the first group of CPs, and CPN as a representative of the second group of CPs and then investigated the properties of the mutants.

**EXPERIMENTAL PROCEDURES**

**Materials**

QuikChange site-directed mutagenesis kit was from Stratagene, La Jolla, CA. The mammalian expression vector, pCEP 4, Lipofectin, and Opti-MEM were from Invitrogen. Polyclonal sheep anti-TAFI antibody, human TAFI-deficient plasma, human carboxypeptidase N, and TAFI enzyme-linked immunosorbent assay kit were from Enzyme Research Laboratories, South Bend, IN. SP-Sepharose fast flow was from Amersham Biosciences. Centriprep 10 was from Amicon, Inc., Beverly, MA. SDS-PAGE gels were from BioWhittaker Molecular Applications, Rockland, ME. Human α-thrombin and TAFI Developer were from American Diagnostica Inc., Greenwich, CT. Thrombin, plasminogen activator, tissue plasminogen activator, and Pefabloc SC were from Roche Applied Science.

**Methods**

Construction of TAFI Mutants—All TAFI mutants were constructed based on the previously described wild type sequence (21), except with alanine at position 147 of the zymogen (22). Mutations were introduced into the pCEP 4/TAFI, a plasmid coding for wild type TAFI cDNA, using a QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions. A pair of primers was used to construct each of four point mutants, one with Asp to Glu mutation at position 256 (TAFI D256E) in a modified pCEP 4 vector containing 5' and 3' untranslated sequence of TAFI mRNA. The primers used were 5'-ATAGATCCATGTGCCACCTCAGG-3' and 5'-CTGGAGGTGGCCAGGAGTTATCTATCTATCTATCTCTATCTCCAGG-3' as described previously (21), except with alanine at position 147 of the zymogen (22). Mutations were introduced into the pCEP 4/TAFI, a plasmid coding for wild type TAFI cDNA, using a QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions. A pair of primers was used to construct each of four point mutants, one with Asp to Glu mutation at position 256 (TAFI D256E) in a modified pCEP 4 vector containing 5' and 3' untranslated sequence of TAFI mRNA. The primers used were 5'-ATAGATCCATGTGCCACCTCAGG-3' and 5'-CTGGAGGTGGCCAGGAGTTATCTATCTATCTATCTCTATCTCCAGG-3'. The samples were applied to a 25-ml SP-Sepharose column equilibrated with 20 mM NaOH. The reaction was stopped by addition of 200 μl of 0.3 M iodoacetamide before analyzing denatured samples on SDS-PAGE followed by Western blotting using polyclonal sheep anti-TAFI antibody. A non-activated control was prepared by preincubating PPACK with thrombin for 10 min before recombinant wild type or mutant TAFI and thrombinomodulin were added.

Carboxypeptidase B activity of TAFI was measured in a chromogenic assay using hippuryl-arginine as the substrate. Carboxypeptidase activity of recombinant wild type and mutant TAFI was also measured using several other substrates: hippuryl-lysine, hippuryl-His-leucine, and hippuryl-phenylalanine. Carboxypeptidase N (3.6 nM) and pancreatic carboxypeptidase A (28.6 nM) were included in the assays as controls. Enzymes were incubated with these substrates for 30 min at room temperature, and the rate of hydrolysis was determined. The N-terminal sequence was determined by 241 amino acid sequencer (Agilent, Palo Alto, CA) that uses standard N-terminal activation. Recombinant wild type mutant TAFI (17 nM) was activated with 15 nM thrombin, 50 nM thrombomodulin, and 5 nM CaCl2 in 20 mM HEPES, 0.15 M NaCl, pH 7.4, at room temperature for 5–20 min. Activation was stopped by addition of 1 μl PPACK and was confirmed by analyzing denatured samples on SDS-PAGE followed by Western blotting using polyclonal sheep anti-TAFI antibody. A non-activated control was prepared by preincubating PPACK with thrombin for 10 min before recombinant wild type or mutant TAFI and thrombinomodulin were added.

Plasma Clot Lysis Assay—In a 96-well microtiter plate, 32 μl of human TAFI-deficient plasma containing 12 μl of recombinant TAFI was mixed with 12 μl of 40 mM HEPES buffer, pH 7.8, 12 μl of 25 mM NaCl, 12 μl of 2.5 mM CaCl2, and 24 μl of 0.5 μM tissue plasminogen activator in separate aliquots. The total volume of the mixture was made up to 120 μl with water. The final concentration of wild type or mutant TAFI in the solution was 3.4 nM. After mixing by pipetting, clot formation and lysis were monitored at 405 nm every 1 min at 37 °C using a SpectraMax 250 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). In some reactions, 5 μl of 24 μg/ml CPI was added to demonstrate the effect of inhibition of TAFI activity on clot lysis. Lysis time was defined as the time at which turbidity is one-half the difference between the plateau reached after clotting and the base line value achieved at complete lysis.

**RESULTS**

Construction of TAFI Mutants—Comparison of the TAFI sequence with those of other CPs suggested several residues that could be important for the substrate specificity of TAFI. Fig. 1 shows the amino acid sequence alignment between human TAFI and human CPN using SeqLab software based on secondary structure predictions. Numbering starts with the first alanine of the activated enzyme. Insertions and deletions were introduced to maximize the alignment. Although the overall identity between TAFI and CPN is only 14% (1), critical residues involved in zinc binding and catalysis, as well as many of the substrate binding residues, are conserved between the two proteins. From the alignment, we identified residues around...
the substrate binding site that may contribute to their differences in substrate specificity. We constructed five mutants of human TAFI to investigate these predictions. To test the importance of the negatively charged residues that define the P1 pocket, Asp-256 in TAFI was mutated to Gln as in CPN. Similarly, Asp-257 was mutated to Ala. Ser-207 in TAFI was mutated to Asp as in CPN since this negatively charged residue in CPN is close to the P1’ pocket. Another mutation converted Leu-248 to Trp to examine the effect of introducing a bulky residue adjacent to Tyr-249 that is known to have an influence on substrate binding. A double mutant of Asp to Gln at residue 256 and Ser to Asp at residue 207 was also constructed to test whether this double mutation would result in cancellation of the effects of the charge reversal caused by either of the single mutations. Following site-directed mutagenesis, the DNA sequence of the plasmids was confirmed. The residues that were mutated are shown in Table I with the corresponding residues in the wild type, as well as in CPN and CPA.

Expression and Purification of TAFI and Mutants—To compare the properties of TAFI mutants with wild type TAFI, the recombinant proteins were expressed in 293 cells. Stable clones were selected, and recombinant proteins were purified by SP-Sepharose chromatography. Homogeneity of recombinant wild type TAFI was demonstrated using silver staining and Western blotting with polyclonal sheep anti-TAFI antibody after separation on SDS-PAGE (Fig. 2, A and B). The purification profile of recombinant TAFI mutants D256Q, D257A, S207D, L248W, and D256Q/S207D was indistinguishable from that of wild type TAFI, implying that these mutations had not lead to gross alterations in their overall properties. As an example, the purification of D256Q is shown in Fig. 2, C and D, in which it can be seen that the mutant TAFI was eluted at the same salt concentration as the wild type TAFI. Wild type TAFI and the D256Q, D257Q, and D256Q/S207D mutants comigrated at the expected molecular weight of 58,000, which is similar to that of TAFI purified from plasma. TAFI L248W and S207D both showed a second band with a slightly lower apparent molecular weight than 58,000, both of which differ slightly from each other. From SDS-PAGE, the molecular weight of the second band from L248W and S207D was 49,000 and 50,000, respectively. Western blotting analysis showed that both bands in TAFI S207D and L248W reacted with polyclonal sheep anti-TAFI antibody, indicating that both bands were TAFI (see Fig. 4, lanes 9 and 11). Only a single band was observed in preparations of wild type TAFI and other TAFI mutants (see Fig. 4, lanes 1, 3, 5, and 7). Since the Mf 49,000 and Ml 50,000 bands in L248W and S207D preparations, respectively, was observed in Western blots of the crude medium that contained protease inhibitors, these smaller TAFI species were not degradation products produced during purification. Furthermore, the finding that the corresponding band was absent in preparations of wild type TAFI and other mutants including the double mutant that had been purified in the same way supports our hypothesis that the formation of these smaller TAFI species were a specific consequence of these particular mutations. Further analysis showed that the smaller TAFI species in S207D and L248W were not due to altered folding since reduction and alkylation of the samples prior to SDS-PAGE did not alter the pattern of the Western blots (data not shown).

The possibility that the smaller TAFI species was the product of a proteolytic cleavage prior to harvesting of the conditioned medium due to altered susceptibility to proteolysis was tested as follows. We first tested the integrity of the N terminus of the mutant L248W by N-terminal sequencing. To do this, we further purified a TAFI L248W preparation by chromatography on plasminogen-Sepharose. Both Mf 49,000 and Ml 50,000 bands were detected, indicating that both TAFI species bound to plasminogen. The fractions containing the two TAFI species were pooled and concentrated prior to N-terminal amino acid sequence analysis. Only one sequence, FQSGQVLAA, was de-

TABLE I

| Residue | TAFI WT* | Mutation | CPN | CPA |
|---------|---------|----------|-----|-----|
| 207     | Ser     | Asp      | Asp | Gln |
| 248     | Leu     | Trp      | Trp | Trp |
| 256     | Asp     | Gln      | Gln | Gln |
| 257     | Asp     | Ala      | Asp | Asp |
| 256     | Asp     | Gln      | Gln | Gln |

* WT, wild type.
To investigate the possibility that the C-terminal sequence had been truncated, we analyzed the TAFI preparations with an antibody specific for a C-terminal sequence. TAFI L248W and S207D and wild type TAFI were run on a 4–20% Tris-glycine gel, and Western blotting was performed with an antibody raised against a peptide with the sequence SEAVRAIEKTSKNT that corresponds to residues 224–237 in the C terminus of TAFI. This antibody recognized wild type TAFI with \( M_r \) 58,000 (Fig. 3A, lane 1), but it did not recognize the \( M_r \) 50,000 species in the preparation of the S207D mutant (Fig. 3A, lane 2). Instead it bound to a band of \( M_r \) 6,000. In the preparation of the L248W mutant, both the \( M_r \) 58,000 and the \( M_r \) 49,000 TAFI species were detected by the anti-peptide antibody (Fig. 3A, lane 3). Fig. 3B shows the same samples analyzed with polyclonal sheep anti-TAFI antibody in which the \( M_r \) 58,000 species can be seen in all three samples and, in addition, the \( M_r \) 49,000 species in the L248W mutant TAFI and the \( M_r \) 50,000 species in the S207D TAFI. The intensity of the higher band with \( M_r \) 58,000 in TAFI S207D (Fig. 3B, lane 2) was much lower than the lower band, indicating that the majority of S207D was in the lower form. This could explain the failure of the polyclonal antibody against the peptide to detect the \( M_r \) 58,000 band (Fig. 3A, lane 2). These data are consistent with the hypothesis that the \( M_r \) 50,000 and \( M_r \) 49,000 TAFI species present in S207D and L248W preparations differ in their C terminus and that the former lacked C-terminal residues containing the sequence SEAVRAIEKTSKNT, whereas the latter still contained this sequence. It is of note that the TAFI species with the longer sequence is migrating faster in SDS-PAGE.

**Activation of TAFI and Mutants**—To investigate whether mutations around the substrate binding site influenced activation, wild type and mutant TAFI were incubated with the thrombin/thrombomodulin complex at room temperature. After various times, aliquots were removed and analyzed by SDS-PAGE and Western blot using a polyclonal sheep anti-TAFI antibody. This antibody can only recognize the TAFI zymogen and TAFIa but not the activation peptide following its cleavage (data not shown). The time course of proteolytic activation of wild type and the TAFI mutants was similar when followed (data not shown). The time course of proteolytic activation of wild type and the TAFI mutants was similar when followed from 5 to 20 min, reaching the maximum after 10 min. The profiles after 10 min of activation are shown in Fig. 4. The TAFI zymogen with a molecular weight of 58,000 was cleaved to yield TAFIa with a molecular weight of 35,000 in all cases. Activation of the mutants was also calcium-dependent as shown for wild type TAFIa but not the activation peptide following its cleavage (Fig. 5A). The activity of the mutants against hippuryl-arginine was greatly reduced when 150 mM NaCl was used in the activation buffer rather than 300 mM NaCl (data not shown). Although all the mutants could be activated by the thrombin/thrombomodulin complex, TAFI L248W and the double mutant were more readily activated than wild type TAFI.

**Carboxypeptidase Activity of Recombinant Wild Type and Mutant TAFIa**—The effect of these mutations on TAFIa activity was measured using hippuryl-arginine as the substrate following their activation with the thrombin/thrombomodulin complex as described above. It is evident that the activity of all the mutants against hippuryl-arginine was greatly reduced (Fig. 5A). This reduction in activity is still significant when the data are normalized for differences in the extent of activation between wild type TAFI and TAFI mutants.

Substrates with different C-terminal residues, hippuryl-lysine, hippuryl-His-leucine, and hippuryl-phenylalanine, were used to investigate whether the carboxypeptidase activities of the mutant TAFIa had altered substrate specificity (Fig. 5A). CPN and CPA were tested in the same assays as controls (Fig. 5B). Wild type TAFIa had high activity against hippuryl-argi-
nine, lower activity against hippuryl-lysine, and even lower against hippuryl-His-leucine and hippuryl-phenylalanine, consistent with TAFIα being a basic carboxypeptidase with a preference for C-terminal arginine residues. All mutant TAFIαs had greatly reduced activity toward both hippuryl-arginine and hippuryl-lysine as compared with that of wild type TAFIα. However, the magnitude of the reduction in activity toward hippuryl-arginine was greater, and consequently, they showed a slight preference for hippuryl-lysine, as is the case for CPN. CPA had no basic carboxypeptidase activity. Unlike CPA, the mutant TAFIαs did not cleave hippuryl-phenylalanine at an appreciable rate. All mutant TAFIαs except D257A had activity toward hippuryl-His-leucine, as did CPN. CPA was inactive toward hippuryl-His-leucine. Some of the mutants, TAFI D256Q and the double mutant TAFI D256Q/S207D, had higher activity on hippuryl-His-leucine as the substrate than wild type TAFIα.

When wild type and mutant TAFIαs were assayed using TAFI developer, the percentage inhibition of TAFIα activity by CPI was measured. As the CPI concentration increased, the percentage inhibition of TAFIα activity also increased (Fig. 6), demonstrating that both wild type and mutant TAFIαs were dose-dependently inhibited by CPI. Although the mutants had significantly decreased TAFIα activity, their residual activity could be inhibited completely by CPI, unlike CPN, which is unaffected by CPI.

**Clot Lysis Assay**—The effect of the mutant recombinant TAFIαs on fibrinolysis was determined by testing each mutant in a plasma clot lysis assay in which TAFI-depleted human plasma was supplemented with the TAFI mutants without prior activation as described under “Experimental Procedures.” Clotting was initiated with thrombin, and subsequent fibrinolysis by tissue plasminogen activator was followed over time by measuring turbidity at 405 nm. In this assay, addition of thrombomodulin leads to enhanced activation of exogenous TAFI by thrombin and therefore prolongs the clot lysis time, but inclusion of both thrombomodulin and CPI together restores the clot lysis time to that observed without addition of TAFI. Thus inclusion of wild type TAFIα in the assay increased the lysis time to 45 min as compared with that obtained in the presence of exogenous TAFIα and CPI, which was 14 min (Fig. 7). This value was identical to that obtained in the absence of added TAFI (data not shown), indicating that the extension of lysis time in this experiment is due to the carboxypeptidase B activity of the TAFIα. The results of the clot lysis assay are shown in Fig. 8.
activity derived from TAFIas. TAFI D257A had plasma clot lysis times of 19 min, demonstrating that it could be activated in the plasma milieu but that it had much less functional TAFIa activity than wild type. The addition of CPI decreased the clot lysis time to 14 min in plasma with D257A, similar to that observed with wild type TAFI (Fig. 7). All the other TAFI mutants behaved similarly to D257A in the plasma clot lysis assay (data not shown). Thus the functional activity of the mutant TAFIas correlated with their enzymatic activity measured directly using a small molecule substrate as shown above.

**DISCUSSION**

TAFI and CPN are the two basic carboxypeptidases known to be present in the circulation. Although they show similar ac-
tivity toward a number of biologically active peptides with a C-terminal basic residue, they differ in many aspects including substrate specificity. The plasma level of constitutively active CPN remains relatively constant, whereas TAFI has been shown to be an acute phase protein, at least in rodents (9), and is a zymogen that requires removal of its activation peptide before it is active. Inflammatory conditions that generate thrombin such as disseminated intravascular coagulation lead to activation and increased expression of TAFI. The physiological role of TAFI is thought to be regulation of plasmin production as TAFIa readily cleaves C-terminal basic residues from partially degraded fibrin and reduces plasminogen binding from the cell surface. CPN, on the other hand, is less efficient toward these substrates (24). In the current study, we investigated the structural differences around the substrate binding sites in TAFI and CPN that could account for their subtle differences in substrate selectivity.

We built a model of TAFI based on the published crystal structure of human procarboxypeptidase B (CPB) (25). In our TAFI model, Ser-207, Leu-248, and Asp-257 are in the vicinity of residues known to be critical for substrate binding which include Asn-142, Arg-143, Asp-256, and Tyr-249. To investigate the importance of these residues for the substrate specificity of TAFIa, we produced recombinant TAFI mutants that have altered residues at these sites. The mutations were chosen based on the observation that although TAFI and CPN share a number of residues critical for their activity, they exhibit differences in substrate specificity. Therefore, our strategy was to replace the residues of interest in wild type TAFI with the corresponding residues from CPN.

Plasmids encoding the mutants were constructed by site-directed mutagenesis and transfected into 293 cells. The purification profile of the TAFI mutants was indistinguishable from that of wild type TAFI. The Mr 58,000 recombinant TAFI band was the major band on SDS-PAGE after purification. When the S207D and L248W mutants were purified, an additional band with lower molecular weight was observed. Based on the evidence using an anti-human TAFI peptide antibody and N-terminal amino acid sequencing, we hypothesized that the Mr 50,000 and Mr 49,000 form of recombinant TAFI in TAFI S207D and L248W, respectively, was generated by C-terminal cleavage, possibly at Arg-210, -228, or -238, which are known thrombin and trypsin cleavage sites in TAFI (21, 26). The data presented here can be explained if TAFI mutant S207D is partially cleaved during production at either Arg-210 or -228, forming the Mr 50,000 species that does not react with the anti-peptide antibody, whereas TAFI mutant L248W is cleaved at Arg-238, producing the Mr 49,000 form that still contains the epitope for the anti-peptide antibody. It is surprising that a single mutation around the substrate binding sites leads to the formation of recombinant proteins that are more susceptible to proteolytic cleavage. It is currently unknown which protease is responsible for these cleavages during cell culture.

Characterization of the purified TAFI mutants showed that they could be activated by the thrombin/thrombomodulin complex, generating the same Mr 35,000 band on SDS-PAGE as the wild type. Some mutants were more readily activated than wild type TAFI, which could be due to disruption of salt bridge and stacking interactions known to exist between the activation peptide and residues near the substrate binding sites (27). In general, the 10-min activation under the conditions used did not result in generation of an appreciable amount of inactive TAFI that has a molecular weight of around 25,000 except TAFI-L248W (3, 26). In the TAFI L248W mutant, however, a band corresponding to this inactive form was present, but insufficient quantities of these proteins prevented further characterization.

Similar amounts of full-length TAFI (Mr 58,000 species) were used for activation followed by characterization in activity assays. Since mutant TAFIas were activated at different rates, they resulted in the generation of slightly different amounts of mutant TAFIa when compared with wild type TAFIa. Allowing for the different amounts of TAFIa generated, our results clearly indicate that the TAFI mutants had very significantly reduced TAFIa activity when tested in a chromogenic assay using hippuryl-arginine as the substrate. The residual TAFIa activity was inhibited by CPI, which specifically inhibits members of the carboxypeptidase A and B family but not CPN. These results imply that the residues that were mutated here do not contribute to recognition by CPI but are involved in binding of specific TAFIa substrates.

In contrast to the results with the hippuryl-arginine substrate, all of the mutant TAFIas were active against substrates with different C-terminal residues. This demonstrates that the loss of activity of these TAFI mutants against substrates with basic C-terminal residues was not due to incorrect folding of proteins caused as a result of the mutations. Instead, the mutant TAFIas had an altered preference toward C-terminal residues, from Arg to Lys and Leu, similar to that of CPN. In our activity assay, a substrate has to be converted to hippuric acid that then reacts with cyanuric chloride to produce a chromogen. In the case of hippuryl-His-leucine, both the C-terminal leucine and the penultimate histidine have to be removed before any chromogen could be detected. From our study, it cannot be elucidated if TAFIa activity on hippuryl-His-leucine is due only to exopeptidase activity removing one residue at a time or if it is behaving as an endopeptidase.

To explain the altered substrate specificity, we compared the TAFI model with a model of CPN based on the published crystal structure of CPD, another regulatory carboxypeptidase that belongs to the family of CPN (20). In the TAFI model, Asp-257 and Ser-207 are in the proximity of the substrate binding residues Asp-256, Asn-142, and Arg-143 (Fig. 8), which are structurally equivalent to the conserved residues in the porcine pancreatic procarboxypeptidase B in complex with benzamidine, a known substrate binding site inhibitor. The negatively charged residues Asp-257 and Asp-256 and Ser-207 are important in stabilization of substrates with a positively charged C terminus. Mutations involving these residues resulted in drastically reduced activity toward substrates with basic C-terminal residues while gaining activity toward less
positively charged substrates such as hippuryl-His-leucine, demonstrating an altered substrate specificity. The double mutant where the total number of negatively charged residues around the critical site is preserved also exhibited altered substrate specificity, indicating the spatial requirement for these negatively charged residues.

The result of the L248W mutation may be explained based on the TAFI and CPN models. The homologous loop in the CPN model to Leu-248–Tyr-249 in TAFI is Trp-285–Tyr-286 (Fig. 8). In the CPN model, van der Waals contacts are present among hydrophobic residues Trp-285, Val-221, and Phe-275, involving aromatic-aromatic interactions of Phe-275, Trp-285, and Tyr-286. These CPN residues are conserved in the CPD structure (Fig. 1). Residue Phe-275 in the CPN model is from a unique loop insertion that is present in the CPD structure but not in the CPB structure or the TAFI model. In the TAFI model, not only is the corresponding Phe-275 residue not present, but the structurally equivalent residue to Val-221 is a positively charged residue, His-201. Although aromatic stacking between His and Trp occurs in some known structures (28), whether this interaction is favorable in the TAFI mutant is unclear given the results from the mutant. Steric hindrance may be introduced with the Leu-248 to Trp mutation involving the aromatic rings of Trp-248 and His-201, destabilizing the substrate binding pocket with Tyr-249. Residue Leu-248 in TAFI could also be important in substrate binding stabilization. Another possibility is that as compared with TAFI, there is a single-residue deletion N-terminal to the Trp-285–Tyr-286 dipeptide in CPN and CPD (Fig. 1). Deletion mutants of TAFI may provide additional insight into the effects of the L248W mutation.

In summary, we demonstrated that replacing residues around substrate binding sites in TAFI with the corresponding residues from CPN resulted in changes in substrate specificity, rate of activation, and stability of the mutant TAFIs. These changes, however, neither affect the ability of the TAFI mutants to be inhibited by CPI nor do they affect their inhibition of clot lysis in plasma. Thus the mutations described here, while modifying substrate specificity, do not alter the properties of TAFI such that it behaves as a member of the other subfamily of carboxypeptidases. Although further studies are needed to elucidate the mechanism for the altered substrate specificity observed with some mutants, a similar approach could be employed to investigate substrate specificity of other related carboxypeptidases and to screen for their inhibitors or enhancers.

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Mutations in the Substrate Binding Site of Thrombin-activatable Fibrinolysis Inhibitor (TAFI) Alter Its Substrate Specificity
Lei Zhao, Brad Buckman, Marian Seto, John Morser and Mariko Nagashima

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