Tissue Factor residue Asp⁴⁴ regulates catalytic function of the bound proteinase Factor VIIa

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The coagulation pathways are initiated by the cell-surface receptor Tissue Factor (TF), which binds the serine proteinase coagulation Factor VIIa (VIIa), resulting in enhanced catalytic function, both amidolytic, towards small pseudo-substrates, and proteolytic, towards macromolecular substrates. Here we implicate Asp⁴⁴ in TF as a ligand-interactive residue that, in contrast with previously characterized binding residues, is involved in the enhancement of VIIa catalytic function. Whereas charge neutralization by replacement of Asp⁴⁴ with Asn did not reduce function of human TF, the exchange by Ala resulted in mutants with 8-fold reduced affinity for binding of VIIa. Enhancement of VIIa amidolytic function by TF Ala⁴⁴ was reduced

by 20–25 % relative to wild-type and support of proteolytic function was diminished 6-fold indicating that this cofactor residue is significantly enhancing proteolysis of the macromolecular substrate by VIIa. Replacement of Asp⁴⁴ by Glu, Thr and Arg exhibited a less severe phenotype with an approx. 4-fold reduced affinity for VIIa and a 2–3-fold diminished activation of Factor X. The improved activity of these mutants as compared with the Ala replacement is consistent with functional importance of an extended side chain at this position. The specific influence of the Asp⁴⁴ exchange on catalytic function of the TF·VIIa complex indicates fine specificity of the TF ligand interface in mediating receptor and cofactor function.

INTRODUCTION

In vivo, the blood-coagulation pathways are initiated upon docking of the plasma serine proteinase Factor VIIa (VIIa) to the cell surface receptor and cofactor Tissue Factor (TF) [1,2]. TF is a 263-residue transmembrane glycoprotein with a 219-amino-acid extracellular domain which folds into two tandem structural modules of fibronectin-type III topology [3,4]. Binding of VIIa to TF results in the formation of an active TF·VIIa complex which efficiently activates the macromolecular substrates Factors IX and X. The acquisition of catalytic competence of VIIa upon assembly with TF results in a 10–100-fold increase in the catalysis of small peptidyl pseudo-substrates [5–7] and in a > 1000-fold increase in the activation of the macromolecular protein substrates Factors IX and X [5,8–11]. The cofactor-dependent enhanced catalytic function of VIIa is inferred to result from cofactor interactions which stabilize the insertion of the VIIa-catalytic-domain N-terminal Ile in a site appropriate for activation of the catalytic triad [12]. The hydrolysis of small peptidyl pseudo-substrates may reflect this catalytic activation of the primary specificity site of VIIa. However, the degree of enhancement of macromolecular substrate hydrolysis is considerably greater than that observed for peptidyl pseudo-substrates, inferring that cofactor interactions also influence extended recognition of macromolecular substrate [13].

In previous studies we have identified ligand-binding residues in the TF extracellular domain by alanine scanning mutagenesis [14,15]. A cluster of residues involving Lys²⁰, Ile²², Asp⁵⁸, Arg¹³⁵ and Phe¹⁴⁰ mapped to the boundary of the N- and the C-terminal modules on the crystallographically resolved three-dimensional structure of the TF extracellular domain [3,4]. Although these residues accounted for approximately one-third of the calculated

free energy of binding of VIIa, Ala exchange at these positions did not result in reduction of catalytic function of the TF·VIIa complex [16], indicating that the tightness of VIIa binding by TF is insufficient to solely account for TF cofactor function. The TF ligand docking site in the N-terminal module includes residues from the patch of solvent-exposed hydrophobic residues [15,17] and the spatially close residues Gln³⁷ [15,17] and Asp⁴⁴ [15,17,18]. The latter residue has been suggested to be involved in TF cofactor function, on the basis of a subtle reduction in the enhancement of small substrate hydrolysis by VIIa [18] and a < 2-fold reduction in procoagulant function [17] of soluble TF-(1–218)-peptide (TF_{1–218}) incorporating the Asp⁴⁴→Ala exchange. In the present study we provide the functional characterization of different replacement mutants at the Asp⁴⁴ position which were expressed as both, full-length transmembrane and soluble, proteins. We further analyse the cofactor function of these mutants in supporting the catalytic activity of VIIa towards various small peptidyl *p*-nitroanilide pseudosubstrates and the macromolecular substrate Factor X. This provides novel evidence that Asp⁴⁴ predominantly influences the catalysis of macromolecular substrate with only minor effects on small-peptidyl-substrate hydrolysis.

EXPERIMENTAL

Proteins

The TF coding sequence, cloned into the expression vector CDM8, was mutated by oligonucleotide-directed mutagenesis as previously described [19]. Mutated DNA sequences were identified by restriction-endonuclease digestion of introduced cleavage sites, and the mutations were confirmed by DNA

Abbreviations used: TF, Tissue Factor; VIIa, Factor VIIa; CHO, Chinese-hamster ovary; TF_{1–n}, TF-(1–n)-peptide.

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sequencing. The coding sequences were further subcloned into the yeast expression vector pMF α , and the entire coding region was sequenced on a model 373A automated DNA sequencer (Applied Biosystems) to detect potentially unrecognized mutations introduced during mutagenesis or subcloning procedures. Full-length wild-type and mutant TF (TF₁₋₂₆₃) proteins were transiently or stably expressed in mammalian Chinese-hamster ovary K1 (CHO-K1) cells as described in [19]. Wild-type control and mutant proteins comprising the soluble extracellular domain of TF (TF₁₋₂₁₈) were expressed in *Saccharomyces cerevisiae* as previously described [14]. For most experiments, the wild-type control was an Asn¹³⁷ → Asp exchange mutant which had reduced N-linked glycosylation [20]. The TF₁₋₂₁₈ Asn¹³⁷ → Asp is functionally indistinguishable from wild-type TF₁₋₂₁₈, consistent with previous analysis of soluble TF₁₋₂₁₈ expressed in yeast, indicating that the carbohydrate structures do not influence function [21]. Soluble TF₁₋₂₁₈ proteins were purified by sequential immunoaffinity and ion-exchange chromatography [14]. All purified TF₁₋₂₁₈ proteins were dialysed against TBS (20 mM Tris/HCl/130 mM NaCl, pH 7.4), and protein concentrations were determined using the micro BCA assay (Pierce Chemical Co.). Recombinant human Factor VIIa was purchased from Novo Nordisk. Human Factors X and Xa were purified from human plasma as described previously [5].

Amidolytic function of VIIa in complex with soluble TF₁₋₂₁₈

Complexes of wild-type or mutant TF₁₋₂₁₈ with recombinant VIIa were analysed for amidolytic activity towards chromogenic peptidyl substrates as described in [16]. A fixed concentration (5 nM) of VIIa was saturated with increasing concentrations of TF₁₋₂₁₈ at ambient temperature in TBS/0.1% BSA/5 mM CaCl₂. Initial reaction rates were measured on addition of chromogenic substrate at 1.25 mM. In order to evaluate the effect of substitutions at positions P1–P3 [22] of the peptidyl substrate on the observed catalytic rate, three different substrates were used to measure TF·VIIa activity. These were L-pyroglutamyl-L-prolyl-L-arginine *p*-nitroanilide dihydrochloride (S2366; Kabi Pharmacia Hepar, Franklin, OH, U.S.A.), methoxycarbonyl-D-cyclohexylglycyl-L-glycyl-L-arginine *p*-nitroanilide acetate (Spectrozyme FXa; American Diagnostica, Greenwich, CT, U.S.A.), and methanesulphonyl-D-cyclohexanylaniline-butyl-Arg *p*-nitroanilide acetate (Pefachrome VIIa; Pentapharm, Basel, Switzerland). Double-reciprocal plots of rate versus TF concentration were found to be linear for TF concentrations in excess of the fixed VIIa concentration. This is expected from the equilibrium equation when the free ligand concentration (unbound TF) approximates to the total variable ligand concentration (total TF in the assay). Maximum catalytic rates were calculated from these data using non-linear curve-fitting analogous to the Michaelis–Menten equation using Enzfitter (Elsevier Biosoft). Experimentally determined rates were converted into concentrations of TF·VIIa complexes based on the maximum catalytic rate at the fixed VIIa concentration in the assay. Free ligand was calculated by subtracting the bound TF from the initial TF concentration in the assay. These data were used for Scatchard analysis to calculate $K_{D,app}$ as described in [14,16]. The free energy of binding (ΔG) was calculated from $K_{D,app}$ according to the equation:

$$\Delta G = -RT \cdot \ln(1/K_{D,app})$$

Proteolytic function of VIIa in complex with full-length TF₁₋₂₆₃

The proteolytic function of recombinant VIIa in complex with wild-type or mutant TF₁₋₂₆₃ was determined in an enzyme-linked

functional assay measuring activation of macromolecular substrate Factor X, as previously described in detail [16]. Briefly, CHO cells expressing wild-type or mutant TF₁₋₂₆₃ on cell surfaces were lysed by vortex-mixing with 15 mM octyl β -D-glucopyranoside (Calbiochem, San Diego, CA, U.S.A.) in HBS (20 mM Hepes/130 mM NaCl, pH 7.4), followed by incubation at 37 °C for 15 min, and a final dilution of 1:3 in HBS. A fixed concentration of TF₁₋₂₆₃ (5 pM) was saturated with increasing concentrations of VIIa (up to 15 nM), and the TF·VIIa complexes formed were incubated with substrate Factor X at 100 nM for 10 min at 37 °C, then quenched with 100 mM EDTA. Factor Xa formation was quantified by measuring rates of chromogenic-substrate hydrolysis using a kinetic microtiter plate reader. Extrapolated maximum rates were calculated essentially as described above and normalized for the effective enzyme concentration given by the limiting TF concentration in the assay. We have observed increased catalytic efficiency after cell lysis resulting in catalytic rates approx. 3-fold higher than previously reported [16]. This was observed for both wild-type and mutant TF and has been attributed to batch differences of the octyl β -D-glucopyranoside used for solubilization. Catalytic rates for solubilized cells reported here are consistent with the rates determined for purified TF₁₋₂₆₃ re-lipidated into charged phospholipid [16]. The functional $K_{D,app}$ was evaluated for these experiments as described in [16].

Determination of kinetic parameters $K_{m,app}$ and k_{cat}

Kinetic parameters $K_{m,app}$ and k_{cat} for protein substrate Factor X hydrolysis by the TF·VIIa complex were determined using wild-type TF₁₋₂₆₃ and Asp⁴⁴ → Ala TF₁₋₂₆₃ stably expressed in CHO cells. The concentrations of wild-type and mutant Asp⁴⁴ → Ala TF₁₋₂₆₃ expressed in stable lines were 750 ng/10⁷ cells and 145 ng/10⁷ cells respectively as determined by immunoassay. These concentrations are consistent with cell-surface immunoreactivity determined by flow cytometry using a panel of monoclonal antibodies to non-overlapping epitopes [15]. For functional analysis, CHO cells were solubilized as described above, and TF₁₋₂₆₃ (50 pM) was incubated with excess VIIa (2 nM) in TBS/5 mM CaCl₂/0.1% BSA, pH 7.4, to allow for complex assembly for 5 min. Increasing concentrations of Factor X (0.0078–1.0 μ M) were added for 1–5 min at 37 °C and the reaction stopped with 100 mM EDTA. Factor Xa generation was assessed by Spectrozyme FXa hydrolysis, and data were fitted to the Michaelis–Menten equation using the Enzfitter program (Elsevier Biosoft).

Proteolytic function of VIIa in complex with TF₁₋₂₁₈

Complexes consisting of soluble TF₁₋₂₁₈ mutants and Factor VIIa were evaluated for proteolytic activation of the macromolecular substrate Factor X. Factor VIIa (2 nM) was assembled with excess purified mutant or wild-type TF₁₋₂₁₈ (15 μ M) in the presence of 5 μ M Ca²⁺ and 100 mM phosphatidylcholine/phosphatidylserine vesicles (7:3, w/w) [5] for 10 min at 37 °C. Factor X (1 μ M) was added and the reaction stopped after 1 min. Factor Xa generation was quantified by Spectrozyme FXa hydrolysis essentially as described above. Under the reaction conditions employed, depletion of the substrate Factor X was < 10%.

Competition of the TF Asp⁴⁴ → Ala mutant with wild-type TF

A limiting concentration of Factor VIIa (50 nM) was incubated with a molar excess of wild-type TF₁₋₂₁₈ (250 nM) in TBS/0.1% BSA/5 mM CaCl₂/100 μ M phosphatidylcholine/phosphatidyl-

serine vesicles (7:3) in the presence of increasing concentrations of either TF₁₋₂₁₈ mutant Asp⁴⁴ → Ala (0–31 μ M) or as control, wild-type TF₁₋₂₁₈, for 5 min at 37 °C. Factor X was added to a final concentration of 1.25 μ M, and the reaction was stopped with 100 mM EDTA after 1 min. Factor Xa generation was determined by hydrolysis of Spectrozyme FXa (0.2 mM). The conditions for this experiment were initially based on a previous study [5] and adjusted empirically by choosing the concentrations of VIIa, Factor X and phospholipid as such that mutant and wild-type complexes exhibited a significant difference in the catalytic rates at very high concentrations (30 μ M) of TF₁₋₂₁₈. The fixed concentration of wild-type TF₁₋₂₁₈ was then chosen based on a dose titration in order to yield approx. 70–80 % of the maximum rate of the wild-type TF₁₋₂₁₈·VIIa complex.

RESULTS

Expression and purification of soluble truncation mutants

The coding sequences for the mutants TF Ala⁴⁴ and TF Glu⁴⁴ or wild-type TF were subcloned into the vector pMF α for expression of soluble TF₁₋₂₁₈. The entire coding sequences in the expression constructs were confirmed by DNA sequencing. Mutants and wild-type control TF₁₋₂₁₈ were expressed in *S. cerevisiae* and purified using sequential immunoaffinity and ion-exchange chromatography. The concentrations of purified proteins were determined by protein assay, based on calibration by amino acid analysis. The purified mutant proteins were compared with wild-type in a sandwich immunoassay using conformation-dependent monoclonal antibodies to non-overlapping sites. In this immunoassay, the TF₁₋₂₁₈ mutants produced titration curves indistinguishable from those of wild-type soluble TF₁₋₂₁₈, providing evidence that the conformation-dependent antibodies are fully reactive with the mutant proteins. This is consistent with proper global folding of the mutant proteins, as also inferred from the efficient cellular expression by eukaryotic cells.

Analysis of binding function of soluble mutant TF₁₋₂₁₈

A fixed concentration of VIIa (5 nM) was saturated with increasing concentrations of soluble wild-type or mutant TF₁₋₂₁₈ (Figure 1a). In comparison with wild-type TF₁₋₂₁₈, much higher concentrations of the Asp⁴⁴ → Ala or the Asp⁴⁴ → Glu mutants were required to achieve saturation. Dissociation constants were estimated from the data in Figure 1(a). A $K_{D,app}$ of 3–4 nM was found for wild-type TF₁₋₂₁₈, and this value was independent of the chromogenic substrate used to monitor TF·VIIa complex formation (Table 1). The $K_{D,app}$ for each of the mutants was increased to 200–280 nM or 55–70-fold compared with wild-type TF₁₋₂₁₈. The substrate chosen for analysis of complex formation did not influence the determination of $K_{D,app}$ within the error of the method. These data demonstrate a significantly reduced affinity for binding of VIIa by the soluble TF₁₋₂₁₈ mutants at the Asp⁴⁴ position with a calculated loss in the free energy of ligand binding of approx. 10.46 kJ (2.5 kcal)/mol.

The saturation curves illustrated as Figure 1(a) suggest decreased catalytic function of the mutant TF·VIIa complexes in comparison with wild-type binary enzyme, as also reported by Gibbs et al. [18]. Based on the $K_{D,app}$ (Table 1), VIIa should be saturated to 98 % at the highest concentration of the mutants displayed in Figure 1(a), indicating that, at near-saturation, catalytic rates of the mutant complexes remain reduced as opposed to wild-type TF. Analysis of the data by double-reciprocal representation resulted in linear plots (Figure 1b), consistent with acceptably precise extrapolation of data. The

Figure 1 Amidolytic function of VIIa in complex with wild-type or mutant soluble TF₁₋₂₁₈

(a) A fixed concentration of VIIa (5 nM) was assembled with increasing concentrations of soluble wild-type ($\square$), Asp⁴⁴ → Ala ($\triangle$) or Asp⁴⁴ → Glu ($\circ$) and the amidolytic activity towards the chromogenic substrate Pefachrome VIIa was analysed. (b) Double-reciprocal representation of the data from (a) for TF₁₋₂₁₈ concentrations much greater than the fixed VIIa concentration. The displayed data points were used for the extrapolation which had an S.E.M. of less than 5%.

calculated maximum rates obtained from the curve fit (Table 1) indicated subtle differences in the maximum rates when mutant and wild-type complexes were compared. With each of the three chromogenic substrates utilized, a reproducible 20–25 % reduction in catalytic function was observed. This is considered significant because, in all experiments, mutant and wild-type TF₁₋₂₁₈ were compared with the identical dilution of enzyme VIIa which is the limiting reactant. These data therefore indicate an influence of amino acid substitutions at the TF Asp⁴⁴ positions on the catalytic function of the TF·VIIa complex.

Effect of Asp⁴⁴ substitutions on the proteolytic function of the TF·VIIa complex

Mutant TF·VIIa complexes were also analysed with respect to catalytic function towards the macromolecular substrate Factor X. In these experiments, a fixed concentration of full-length TF₁₋₂₆₃ expressed in mammalian cells was saturated with increasing concentrations of VIIa. The maximum rate obtained for the activation of Factor X by full-length TF₁₋₂₆₃ Asp⁴⁴ → Ala in complex with VIIa was reduced in comparison with wild-type binary complexes (Table 2). The 7-fold reduction in proteolytic activity was selective for the Asp⁴⁴ → Ala replacement, since the incorporation of additional mutations at other residue positions

Table 1 Amidolytic function of soluble TF₁₋₂₁₈ replacement mutants for Asp⁴⁴

The amidolytic function of 5 nM VIIa towards the indicated *p*-nitroanilide peptidyl substrate was determined in the presence of increasing concentration of soluble wild-type or mutant TF₁₋₂₁₈ at ambient temperature and pH 7.4. The maximum rates were obtained from linear transformations, as shown in Figure 1(b). Means and S.D. values were calculated from three independent experiments. ΔG was calculated from the $K_{D,app}$ and $\Delta\Delta G$ is the loss in the free energy of binding for the respective mutants in comparison with wild-type TF₁₋₂₁₈. Note that 1 kcal $\equiv$ 4.181 kJ.

Mutation	Substrate	Maximum rate of peptide hydrolysis (relative absorbance/min)	$K_{D,app}$ (nM)	ΔG (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
Wild-type	Spectrozyme FXa	0.72 $\pm$ 0.08	3.7 $\pm$ 1.7	-11.4	
	S2366	1.48 $\pm$ 0.47	3.9 $\pm$ 0.8	-11.3	
	Pefachrome VIIa	2.09 $\pm$ 0.10	3.6 $\pm$ 0.4	-11.4	
Asp ⁴⁴ $\rightarrow$ Ala	Spectrozyme FXa	0.53 $\pm$ 0.05	253.4 $\pm$ 54.3	-8.9	2.5
	S2366	1.09 $\pm$ 0.30	256.7 $\pm$ 78.2	-8.9	2.5
	Pefachrome VIIa	1.71 $\pm$ 0.10	200.9 $\pm$ 25.4	-9.0	2.4
Asp ⁴⁴ $\rightarrow$ Glu	Spectrozyme FXa	0.51 $\pm$ 0.07	218.5 $\pm$ 103.6	-9.0	2.4
	S2366	1.10 $\pm$ 0.33	276.5 $\pm$ 92.4	-8.9	2.5
	Pefachrome VIIa	1.76 $\pm$ 0.07	209.6 $\pm$ 16.8	-9.0	2.4

Table 2 Proteolytic function of full-length TF₁₋₂₆₃ mutants at the Asp⁴⁴ position

Full-length, membrane-inserted TF₁₋₂₆₃ proteins (5 pM) were saturated with increasing concentrations of VIIa and the activation of factor X (100 nM) was analysed at 37 °C. The maximum rate of factor X activation was determined from extrapolation at high ligand concentrations, and the $K_{D,app}$ was derived from Scatchard analysis. Means and S.D. values were calculated based on the indicated number (*n*) of experiments. $\Delta\Delta G$ is the calculated change in the free energy of binding of the mutants as compared with wild-type TF. Note that 1 kcal $\equiv$ 4.181 kJ.

Mutation	Maximum rate of Factor X activation (mol of Factor Xa/s per mol of TF·VIIa)	$K_{D,app}$ (pM)	$\Delta\Delta G$ (kcal/mol)	<i>n</i>
Wild-type	3.2 $\pm$ 0.8	2.8 $\pm$ 1.2	8	
Asp ⁴⁴ $\rightarrow$ Ala	0.43 $\pm$ 0.06	23.4 $\pm$ 8.3	1.4	4
Gln ³⁷ $\rightarrow$ Ala	2.4 $\pm$ 0.2	9.7 $\pm$ 2.1	0.8	4
Lys ²⁰ /Asp ⁴⁴ $\rightarrow$ Ala	0.57 $\pm$ 0.10	2288 $\pm$ 646	4.1	6
Phe ¹⁴⁰ /Asp ⁴⁴ $\rightarrow$ Ala	0.68 $\pm$ 0.13	514 $\pm$ 19.3	3.2	7
Asp ⁴⁴ /Trp ⁴⁵ $\rightarrow$ Ala	0.66 $\pm$ 0.19	33.1 $\pm$ 3.4	1.5	3
Asp ⁴⁴ $\rightarrow$ Glu	1.2 $\pm$ 0.6	9.7 $\pm$ 4.9	0.8	4
Asp ⁴⁴ $\rightarrow$ Thr	1.1 $\pm$ 0.2	8.5 $\pm$ 1.4	0.7	3
Asp ⁴⁴ $\rightarrow$ Arg	1.0 $\pm$ 0.3	14.7 $\pm$ 7.3	1.0	4
Asp ⁴⁴ $\rightarrow$ Asn	3.1 $\pm$ 0.2	3.0 $\pm$ 1.3	0.0	3

which affect VIIa binding without influence on proteolytic function did not further diminish this function. This is illustrated by double mutants, which incorporated, in addition to the Ala replacement for residue Asp⁴⁴, mutations of Lys²⁰ or Phe¹⁴⁰ (Table 2). These double mutants had the same reduction in proteolytic function as the individual Asp⁴⁴ exchange, despite the fact that the double mutants have a considerable further decrease of affinity for ligand. Ala replacement for Asp⁴⁴ together with the adjacent Trp⁴⁵ did not reduce proteolytic function more than the single Asp⁴⁴ exchange, consistent with the previous conclusion that the Trp⁴⁵ exchange does not produce a mutational effect which is independent from Asp⁴⁴ [15]. The exchange of Gln³⁷, which is a binding residue in spatial vicinity to Asp⁴⁴ and Trp⁴⁵, resulted in slightly reduced proteolytic function of the mutant TF·VIIa complex, suggesting a spatially confined locus involved in the change in catalytic function with a major participation of the Asp⁴⁴ side chain.

To define more precisely the proteolytic defect observed with the Asp⁴⁴ $\rightarrow$ Ala mutant, kinetic parameters for the proteolytic activation of Factor X were determined using limiting concentration (50 pM) of cell surface expressed full-length TF with excess VIIa (2 nM) to compensate for the reduced affinity of the mutant (Figure 2). The determined apparent K_m for substrate Factor X activation was slightly increased for VIIa in complex

with TF₁₋₂₆₃ Asp⁴⁴ $\rightarrow$ Ala ($K_{m,app}$ = 167.4 $\pm$ 22.7 nM, *n* = 3) compared with wild-type TF₁₋₂₆₃·VIIa complexes ($K_{m,app}$ = 100.2 $\pm$ 8.2 nM, *n* = 3). The k_{cat} in the presence of the mutant was decreased by close to 4-fold (1.3 $\pm$ 0.11 mol of Factor Xa/s per mol of TF·VIIa) compared with wild-type TF (4.8 $\pm$ 0.46 mol of Factor Xa/s per mol of TF·VIIa). These results demonstrate that the observed functional defect of the Asp⁴⁴ $\rightarrow$ Ala mutant is mostly attributable to a decreased k_{cat} for Factor X substrate activation, with only a minor alteration in the $K_{m,app}$ for Factor X binding to TF·VIIa complexes.

The effect of alternative substitutions for TF Asp⁴⁴ was further analysed. When substituted by Asn, the residue found as consensus in most mammalian species [23], there was no perturbation of function (Table 2). This demonstrates that charge *per se* is not a requirement for function of the Asp⁴⁴ side chain in human TF. Consistent with a minor importance of charge is the functional defect resulting from charge conversion by Arg replacement. This mutant exhibited a functional defect which was less pronounced than the Ala exchange (Table 2). Attenuated functional defects were also observed for the Glu and Thr exchange. The Arg, Glu and Thr mutants were characterized by 2–3-fold decreased support of proteolytic function. The improved function of these exchanges as compared with the Ala replacement suggests that atoms in the extended aliphatic side chains partially

Figure 2 Proteolytic function of VIIa in complex with wild-type (□) or mutant Asp⁴⁴ → Ala (○) full-length TF₁₋₂₆₃

The effect of variations in the substrate concentration on Factor Xa generation by 2 nM VIIa in the presence of 50 pM TF is shown. Kinetic parameters determined by fitting of data from three independent experiments to the Michaelis–Menten equation were as follows. TF₁₋₂₆₃ Asp⁴⁴ → Ala: $K_{m,app} = 167.4 \pm 22.7$ nM, $k_{cat} = 1.3 \pm 0.11$ mol of Factor Xa/s per mol of TF·VIIa, wild-type TF₁₋₂₆₃: $K_{m,app} = 100.2 \pm 8.2$ nM, $k_{cat} = 4.8 \pm 0.46$ mol of Factor Xa/s per mol of TF·VIIa. For the calculation of the k_{cat} , it is assumed that the limiting concentration of TF is saturated and equals the concentration of enzymic TF·VIIa complexes.

normalize function. However, the extension of the side chain in the Glu exchange mutant was not tolerated without functional alteration, indicating that even some subtle changes at the Asp⁴⁴ position are incompatible with fully normal cofactor function of TF.

The apparent dissociation constants calculated from these experiments demonstrated normal binding function for the Asp⁴⁴ → Asn replacement analysed as full-length transmembrane TF₁₋₂₆₃ (Table 2). The affinity for VIIa was reduced approx. 8-fold for the Ala exchange, 5-fold for the Arg replacement and 3-fold for the Glu and Thr mutants (Table 2). The estimated loss of free energy of binding of VIIa by TF Glu⁴⁴ and Ala⁴⁴ was 3.34 kJ (0.8 kcal) and 5.85 kJ (1.4 kcal)/mol respectively. This contrasts with an estimated 10.46 (2.5 kcal)/mol loss of free binding energy estimated from the analysis of the same mutations within the context of soluble truncated TF₁₋₂₁₈ (Table 1). In the analysis of TF₁₋₂₁₈, both the Glu and the Ala exchanges for Asp⁴⁴ had similarly reduced affinity for VIIa and a concordant defect in enhancing amidolytic function of the bound proteinase. However, in the analysis of the full-length TF₁₋₂₆₃ protein in the presence of Factor X the binding defect appeared to parallel the proteolytic defect which was most pronounced with the Ala substitution. This could indicate functional differences of the exchange mutants which are specific to the full-length TF₁₋₂₆₃ molecules. To exclude this possibility and to further substantiate that the reduced proteolytic function of the Asp⁴⁴ → Ala exchange as compared with the Glu replacement was a result of the amino acid exchange at this position, the soluble TF₁₋₂₁₈ mutants were analysed for the support of proteolytic activation of Factor X by VIIa.

Proteolytic function of complexes with soluble Asp⁴⁴ mutants

The proteolytic activity of soluble mutant and wild-type TF in complex with a fixed concentration of VIIa was analysed in the presence of negatively charged phospholipid vesicles. VIIa (2 nM) was saturated with a large excess of soluble wild-type or mutant TF₁₋₂₁₈ (15 μM) and the rate of activation of 1 μM Factor X was monitored. Wild-type TF₁₋₂₁₈·VIIa complexes generated 45.3 ± 2.1 nM of Xa/min ($n = 3$). The rate of Factor Xa gen-

Figure 3 Competition of Asp⁴⁴ → Ala TF₁₋₂₁₈ with wild-type TF₁₋₂₁₈

The effect of increasing concentrations of soluble Asp⁴⁴ → Ala TF₁₋₂₁₈ (○) on the proteolytic function of VIIa (50 nM) in the presence of 250 nM wild-type TF₁₋₂₁₈ is shown. TF₁₋₂₁₈ Asp⁴⁴ → Ala (△) supported activation of 1 μM Factor X by 50 nM VIIa in the presence of 100 μM charged phospholipid vesicles inefficiently compared with very high concentrations of wild-type TF₁₋₂₁₈ (□).

eration was reduced to 21.5 ± 2.7 nM/min for the Asp⁴⁴ → Glu mutant and to 10.4 ± 1.6 nM/min for the Asp⁴⁴ → Ala replacement. Thus the loss of cofactor function analysed by Factor X activation exhibited a similar rank order for the soluble TF₁₋₂₁₈ and the full-length TF₁₋₂₆₃ mutants at the Asp⁴⁴ position. Notably, the loss of function appeared to be less pronounced for the soluble in comparison with full-length TF complexes. However, the kinetic analysis presented above demonstrated a slight increase in the $K_{m,app}$ for the Asp⁴⁴ mutant when compared with wild-type TF·VIIa complexes. Since the data for TF₁₋₂₆₃ mutants presented in Table 2 were obtained at Factor X concentrations below the $K_{m,app}$ for the mutant, the loss of function will be larger than for analysis at near saturating Factor X concentrations (1 μM), as employed for analysis of the soluble mutants. These experiments thus establish the generation of a substantial defect in cofactor function resulting from replacements at the Asp⁴⁴ position in TF.

Competition of the Asp⁴⁴ → Ala mutant with wild-type TF

The data presented so far predict that functionally homogeneous mutant TF₁₋₂₁₈ should form catalytic complexes with VIIa with reduced proteolytic function. In competition experiments, increasing concentrations of mutant should overcome the difference in affinity relative to wild-type TF resulting in progressive reduction of the rate of Factor X activation. Wild-type control TF₁₋₂₁₈ (250 nM) was assembled with 50 nM VIIa in the presence of increasing concentrations of TF₁₋₂₁₈ Asp⁴⁴ → Ala mutant or wild-type TF₁₋₂₁₈ (Figure 3). The rate of substrate activation slightly increased when wild-type TF₁₋₂₁₈ was added and full saturation was achieved at > 2 μM. With the addition of TF₁₋₂₁₈ Asp⁴⁴ → Ala, no increase in the rate of Factor X activation was observed. This is inconsistent with the idea that mutant molecules with binding and cofactor function equivalent to wild-type TF are responsible for the functional activity observed with the mutant preparation. Furthermore, at very high concentrations of the mutant TF₁₋₂₁₈, the rate of substrate hydrolysis progressively declined and approached levels observed for the mutant TF₁₋₂₁₈·VIIa complex in the absence of wild-type TF. This experiment thus demonstrates that the Asp⁴⁴ → Ala

mutant of TF can compete with wild-type TF to form complexes with reduced proteolytic function.

DISCUSSION

In this study we analysed the role of TF residue Asp⁴⁴ in the enhancement of amidolytic and proteolytic function of the bound proteinase Factor VIIa. Asp⁴⁴ is implicated in the interaction with VIIa based on reduced affinity for binding of ligand upon residue exchange. The substitutions of Asp⁴⁴ → Ala and Asp⁴⁴ → Glu were found to similarly reduce affinity of soluble TF₁₋₂₁₈ for ligand VIIa by 55–70-fold compared with wild-type TF, when characterized in an assay which monitored TF·VIIa complex formation by the change of catalytic activity towards small chromogenic substrates. The energetic contributions attributed to the Asp⁴⁴ → Ala exchange are consistent with previous analysis using similar methodology [18]. When analysed in the presence of Factor X, the binding defect observed for both mutations was less pronounced, indicating that extended substrate interaction may influence the affinity of the TF·VIIa interaction, as previously suggested [24]. These data and the direct characterization of the interaction of VIIa with the Asp⁴⁴ → Ala mutant using surface plasmon resonance technology [17] clearly implicate the Asp⁴⁴ side chain as an important energetic contact for binding of the ligand VIIa.

Unlike mutations of previously identified ligand binding residues of TF [16], substitutions at the Asp⁴⁴ position were found to be significantly defective in enhancing the catalytic function of the bound VIIa. Although three different *p*-nitroanilide peptidyl pseudo-substrates were hydrolysed with slightly reduced rates by the mutant TF·VIIa complexes, the more significant effect was on the activation of the macromolecular substrate Factor X. Moreover, the mutant TF was shown to compete with wild-type TF for limited enzyme, resulting in the formation of TF·VIIa complexes with reduced proteolytic function. These data confirm both the affinity difference and the loss of cofactor function attributable to the Asp⁴⁴ → Ala mutation. Mutation of surrounding residues on the protein surface identified the Gln³⁷ → Ala substitution to be slightly diminished in proteolytic function. Analysis of the double mutation of Asp⁴⁴/Trp⁴⁵ → Ala showed no additional defect in proteolytic function in excess of that of the Asp⁴⁴ → Ala mutation, consistent with the previously shown inter-dependence of these neighbouring residue side chains in the interaction with VIIa [15]. The substantial effect of Asp⁴⁴ substitutions on the proteolytic function of the TF·VIIa complex and the potential subtle contribution of Gln³⁷ suggest that this relatively restricted region serves an important role in the cofactor enhancement of VIIa. In contrast with the findings reported by Gibbs *et al.* [18], we find no evidence for a role of Lys²⁰ or other previously identified binding residues in the catalytic enhancement of VIIa. Consistent with our previous study [16], double Ala replacements of Lys²⁰ and Asp⁴⁴ or Phe¹⁴⁰ and Asp⁴⁴ resulted in TF·VIIa complexes deficient in proteolytic function comparable with the single Asp⁴⁴ → Ala substitution, although a further decrease in affinity for VIIa was observed for the double mutants.

Ala substitution for Asp⁴⁴ produced the most pronounced functional defect among the residue replacements tested, indicating a specific functional role of certain Asp⁴⁴ side-chain atoms. The functionally normal Asn substitution most closely maintains the side-chain geometry present at the Asp⁴⁴ position and provides compelling evidence that the charge of the Asp⁴⁴ side chain is not required for function. The most substantial defect in ligand binding and cofactor function was observed with the Ala substitution, which results in deletion of side-chain

atoms distal to the C β -position. In comparison with the Ala replacement, Thr and Glu substitutions exhibited less severe reductions in proteolytic function which may be attributed to additional side-chain mass, such as the presence of the C β -hydroxy group of the Thr residue and of a similar aliphatic side chain in the case of the Glu replacement. The Arg replacement mutant had a reduction in function intermediate between the Ala and the Thr or Glu replacement, indicating that the bulk of the guanidinium group may perturb TF function. The side chain of Asp⁴⁴ is well exposed to solvent, and replacement with more extended side chains, such as Glu and Arg, should not perturb the local packing on the protein surface. One likely explanation of the functional defect resulting from these replacements is steric hindrance introduced by the additional side-chain mass in the protein interactive site. However, it appears that the large Arg side chain can be somehow accommodated when VIIa assembles. This may indicate that Asp⁴⁴ is located towards the periphery rather than in the centre of a binding contact for the VIIa proteinase domain.

VIIa assembly with cofactor has been shown to increase primarily the catalytic efficiency of the bound proteinase with respect to both small peptidyl [5–7] and macromolecular substrate hydrolysis [6,10]. Concordantly, our kinetic analysis of the Asp⁴⁴ → Ala mutation indicated that the observed deficiency in proteolytic activation resulted predominantly from a 4-fold reduction in k_{cat} with only a slight effect on the $K_{m,app}$. Notably, the amidolytic activity of the complexes of VIIa formed with either the Ala or Glu exchange mutants were reduced similarly by 20–25%, with no significant differences observed between *p*-nitroanilide substrates which vary at the P2 and P3 positions. In contrast, the same two residue exchanges exhibited significant differences in the activation of macromolecular substrate Factor X, which was reduced by 2-fold for the Glu exchange and 4-fold for the Ala exchange in the analysis using soluble TF₁₋₂₁₈. These data suggest that cofactor interactions not only influence the activity of catalytic triad [12], but, more importantly, the extended recognition of the macromolecular substrate. This cofactor influence on macromolecular recognition may, in part, explain the relative enhancement differences observed between amidolytic and proteolytic hydrolysis by the complex [5,6,10]. The present study thus provides novel insight into the structural basis of cofactor-dependent enhancement of catalytic function of the serine proteinase VIIa.

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