Tissue factor (TF) is an integral membrane protein cofactor for factor VIIa (fVIIa) that initiates the blood coagulation cascade during vascular injury. TF has two fibrinonecitin type III-like domains, both of which make extensive interactions with both the light and heavy chains of fVIIa. In addition to interaction with fVIIa, the membrane proximal C-terminal domain of TF is also known to bind the natural substrates factors IX and X, thereby facilitating their assembly and recognition by fVIIa in the activation complex. Both fVIIa and TF are elongated proteins, and their complex appears to be positioned nearly perpendicular to the membrane surface. It is possible that, similar to fVIIa, the N-terminal domain of TF also contacts the natural substrates. To investigate this possibility, we substituted all 23 basic and acidic residues of the TF N-terminal domain with Ala and expressed the mutants as soluble TF2–219 in a novel expression/purification vector system in the periplasmic space of bacteria. Following purification to homogeneity, the cofactor properties of mutants in promoting the amidolytic and proteolytic activity of fVIIa were analyzed in appropriate kinetic assays. The amidolytic activity assays indicated that several charged residues spatially clustered at the junction of the N- and C-terminal domains of TF are required for high affinity interaction with fVIIa. On the other hand, the proteolytic activity assays revealed that none of the residues under study may be an interactive site for either factor IX or factor X. However, it was discovered that the Arg74 mutant of TF was defective in enhancing both the amidolytic and proteolytic activity of fVIIa, suggesting that this residue may be required for the allosteric activation of the protease.

Tissue factor (TF) is an integral membrane cofactor that upon exposure to circulating blood binds with high affinity to factor VIIa (fVIIa) to catalyze the rapid activation of procoagulant zymogens factors IX and X, thereby initiating the blood clotting cascade (1–3). The structure of TF is composed of two fibrinonecitin type III-like extracellular domains, a single membrane-spanning domain and a short cytoplasmic tail (4, 5). Although all three domains of TF are required for the physiological function of the cofactor, previous studies have indicated that the two extracellular domains expressed by recombinant DNA methods as a soluble protein (sTF) can bind to fVIIa with a high affinity to enhance both the amidolytic and proteolytic activities of the protease (6–9). The crystal structure of sTF either alone or in complex with fVIIa has been determined (4, 10). The structural data have indicated that the extracellular domains of TF make extensive interactions with both the light and heavy chains of fVIIa (10). It is believed that these interactions allosterically change the conformation of the active-site pocket of fVIIa, leading to a dramatic improvement in the catalytic efficiency of the protease toward both synthetic and natural macromolecular substrates (1). Most of the functionally critical residues of TF have been mapped by the Ala-scanning mutation approaches (7, 9, 11). The characterization of these mutants has indicated that, in addition to interaction with fVIIa, the C-terminal membrane proximal domain of TF also provides binding sites for the γ-carboxyglutamic acid and/or the first epidermal growth factor-like domains of factors IX and X thereby facilitating the assembly and optimal recognition of these substrates by fVIIa in the activation complex (11, 12). Thus, it has been demonstrated that the interaction of the γ-carboxyglutamic acid domains of both substrates with the two basic residues Lys365 and Lys366 in the membrane proximal C-terminal domain of TF makes a significant contribution to the specificity of the zymogen activation by fVIIa (11).

The structural and fluorescence energy transfer studies data have further indicated that the binding of TF to fVIIa stabilizes the protease in a topological orientation in which the active site pocket of fVIIa is maintained far above the membrane surface in the activation complex (10, 13). Thus, for effective activation by fVIIa, both factors IX and X are also required to assemble into the activation complex in a similar extended conformation to maintain the activation peptide of the substrates at the same distance above the membrane surface. Noting that several charged residues of the N-terminal domain of TF make extensive interactions with the protease domain of fVIIa, we hypothesized that these residues may also interact with the protease domains of the substrates in the activation complexes. To test this hypothesis, we substituted all 23 basic and acidic residues of the TF N-terminal domain with Ala and expressed the mutant constructs in soluble sTF2–219 forms in a newly developed expression/purification vector system in the periplasmic space of bacteria. The Ala substitution mutants of two constructs (Glu56 and Asp58) were not expressed to a high yield, thus these residues were substituted with Asn. All of the mutants were purified to homogeneity and characterized with respect to their ability to function as cofactors to enhance the amidolytic and proteolytic activities of...
fVIIa using standard kinetic assays. The results of the amidolytic activity assays indicated that a number of basic and acidic residues of the N-terminal domain, centered spatially on the middle side of the cofactor near its junction with the C-terminal domain, are required for the high-affinity interaction of the cofactor with fVIIa. This finding is consistent with previous similar mutagenesis studies (7). On the other hand, detailed kinetic analysis in the presence of saturating concentrations of the sTF2–219 mutants suggested that the N-terminal domain of the cofactor may have no interactive site for either factor IX or factor X in the related activation complexes. However, we identified Arg74 as a unique residue that is required for the catalytic function of fVIIa in reaction with both synthetic and macromolecular substrates. The mutagenesis of Arg74 markedly impaired both the amidolytic and proteolytic functions of fVIIa, suggesting that this residue may be required for the allosteric activation of the catalytic pocket of the protease.

EXPERIMENTAL PROCEDURES

Construction, Mutagenesis, and Expression of Soluble Tissue Factor in Bacteria—Construction and expression of soluble TF lacking both the trans-membrane and cytoplasmic domains (sTF2–219) in the pIIIHI-pelB bacterial periplasmic expression/purification vector system has been described previously (6). To improve the expression yield of sTF2, this vector was modified by inserting a promoterless neomycin cDNA lacking the Shine-Dalgarno sequence immediately downstream of the sTF2–219 stop codon. This construction strategy translationally coupled the expression of the neomycin cDNA to that of sTF2. To facilitate purification, the 12-residue epitope for the Ca2+–dependent monoclonal antibody, HPC4, was also linked in-frame to the pelB signal peptide as described previously (6). The Ala substitution mutants of sTF2–219 were prepared by PCR mutagenesis methods in the same vector system as described previously (6). After confirmation of the accuracy of the mutagenesis by DNA sequencing, the mutant constructs were transformed into the BL2 strain of Escherichia coli and selection was carried out in kanamycin as described previously (6). Bacterial colonies harboring the wild-type and mutant sTF2–219 derivatives were grown in LB medium containing 50 μg/ml kanamycin in 1-liter flasks at 37°C for 12 h) at room temperature (–25°C). The supernatant was collected by centrifugation at 4000 × g for 15 min. The supernatant and the periplasmic extract were mixed and containing 50 μM EDTA. In the second stage, the amidolytic activity of each sample was determined by the subsequent addition of Spectrozyme FXa (a final concentration of 0.1 μM). The absorbance at 405 nm was monitored over 5 min using a V-max kinetic microplate reader, and the initial rates of chromogenic substrate hydrolysis (ΔA405/min) were converted to the nanomolar of product by reference to a standard curve prepared with purified human factor Xa. The apparent Km and kcat values for substrate hydrolysis were calculated from the Michaelis-Menten equation, and the catalytic efficiencies were expressed as the ratio of kcat/Km.

Factor IX Activation—Steady-state kinetics of factor IX activation by fVIIa in complex with wild-type and mutant sTF2–219 derivatives was performed by the same methods described above for factor X. fVIIa (5 nM), PC/PS vesicles (0.3 mM), and saturating concentrations of sTF2–219 mutants (at least 10× Kapp values) were incubated for 10 min in 96-well assay plates and the reactions then were initiated by adding increasing concentrations of factor X (20–2630 nM). After 50 min of incubation at room temperature, the reactions were terminated by adding 50 μl EDTA. In the first stage, the amidolytic activity of each sample was determined by the subsequent addition of Spectrozyme FXa (a final concentration of 0.1 μM). The absorbance at 405 nm was monitored over 5 min using a V-max kinetic microplate reader, and the initial rates of chromogenic substrate hydrolysis (ΔA405/min) were converted to the nanomolar of product by reference to a standard curve prepared with purified human factor Xa. The apparent Km and kcat values for substrate hydrolysis were calculated as described above.

Factor IXa Binding Studies—The competitive effect of the Arg74–1219 to Ala mutant of sTF2–219, exhibiting impaired cofactor activity toward macromolecular substrates, on factor X activation by the wild-type fVIIa–sTF2–219 complex was studied. In this case, the activation of factor X (500 nM) by a limiting fixed concentration of fVIIa (2 nM) in complex with PC/PS vesicles (100 μg/ml) and 100 nM sTF2–219 was monitored in TBS/Ca2+ in the presence of increasing concentrations of sTF2–219 mutant (0.156–10 μM). Following incubation for 5 min at room temperature, the reactions were terminated by adding 50 μl EDTA and the rate of factor Xa generation was determined as described above.

RESULTS AND DISCUSSION

Expression and Purification of sTF2–219 Derivatives—All of the sTF2–219 derivatives were expressed in the periplasmic space of bacteria using the pIIIHI-pelB-neomycin expression/purification vector system as described under “Experimental Procedures.” This vector system, which incorporates a 12-residues epitope for the Cα2+–dependent monoclonal antibody HPC4 to the N-terminal of the expressed protein, is an improved version of pIIIHI-pelB, which we previously used to express and purify sTF2–219 from bacterial culture (6). In the current study, we inserted a promoterless neomycin cDNA immediately downstream of the sTF2–219 stop codon. This strategy improved the expression yield to greater than 20 mg/liter sTF2–219. Wild-type and mutant proteins were separated from the bacterial culture supernatants and periplasmic extracts by a single step immunofinity chromatography on immobilized HPC4 as described previously (6). With the exception of Glu56 and Asp58, all of the basic and acidic residues of the N-terminal domain of sTF2–219 were substituted with Ala in individual constructs. The Ala substitution mutants of both Glu56 and Asp58 did not express to high yield; thus, these residues were substituted with Asn. SDS-PAGE analysis of the purified proteins under non-reducing conditions indicated that the isolated proteins were essentially homogenous (Fig. 1). We previously demonstrated that the bacterial sTF2–219 with or without an N-terminal HPC4 epitope exhibits identical cofactor activity as the mammalian sTF1–219. The generation of product (factor Xa) was monitored by a two-stage discontinuous assay. In the first stage, fVIIa (0.3 mM), PC/PS vesicles (0.3 mM), and saturating concentrations of sTF2–219 mutants (at least 10× Kapp values) were incubated for 10 min in 96-well assay plates and the reactions then were initiated by adding increasing concentrations of factor X (20–2630 nM). After 10 min of incubation at room temperature, the reactions were terminated by adding 50 μl EDTA. In the first stage, the amidolytic activity of each sample was determined by the subsequent addition of Spectrozyme FXa (a final concentration of 0.1 μM). The absorbance at 405 nm was monitored over 5 min using a V-max kinetic microplate reader, and the initial rates of chromogenic substrate hydrolysis (ΔA405/min) were converted to the nanomolar of product by reference to a standard curve prepared with purified human factor Xa. The apparent Km and kcat values for substrate hydrolysis were calculated from the Michaelis-Menten equation, and the catalytic efficiencies were expressed as the ratio of kcat/Km.

Factor IX Activation—The steady-state kinetics of factor IX activation by fVIIa in complex with wild-type or mutant sTF2–219 derivatives were studied in TBS/Ca2+ at room temperature as described previously (16). with fVIIa in complex with wild-type or mutant sTF2–219 derivatives was performed by the same methods described above for factor X. fVIIa (5 nM), PC/PS vesicles (0.3 mM), and saturating concentrations of sTF2–219 mutants (at least 10× Kapp values) were incubated for 10 min, and the reactions then were initiated by adding increasing concentrations of factor IX (27–3510 nM). After 50 min of activation at an ambient temperature, the reactions were terminated by adding 50 μl EDTA. The rate of factor IXa generation was determined by an amidolytic activity assay using CBS 31.29 containing 33% ethylene glycol as described previously (17). The initial rates of chromogenic substrate hydrolysis (ΔA405/min) were converted to nanomolar of product by reference to a standard curve prepared with purified human factor IXa. The apparent Km and kcat values for substrate hydrolysis were calculated as described above.

Factor IXa Binding Studies—The competitive effect of the Arg74–1219 to Ala mutant of sTF2–219, exhibiting impaired cofactor activity toward macromolecular substrates, on factor X activation by the wild-type fVIIa–sTF2–219 complex was studied. In this case, the activation of factor X (500 nM) by a limiting fixed concentration of fVIIa (2 nM) in complex with PC/PS vesicles (100 μg/ml) and 100 nM sTF2–219 was monitored in TBS/Ca2+ in the presence of increasing concentrations of sTF2–219 mutant (0.156–10 μM). Following incubation for 5 min at room temperature, the reactions were terminated by adding 50 μl EDTA and the rate of factor Xa generation was determined as described above.

RESULTS AND DISCUSSION

Expression and Purification of sTF2–219 Derivatives—All of the sTF2–219 derivatives were expressed in the periplasmic space of bacteria using the pIIIHI-pelB-neomycin expression/purification vector system as described under “Experimental Procedures.” This vector system, which incorporates a 12-residues epitope for the Ca2+–dependent monoclonal antibody HPC4 to the N-terminal of the expressed protein, is an improved version of pIIIHI-pelB, which we previously used to express and purify sTF2–219 from bacterial culture (6). In the current study, we inserted a promoterless neomycin cDNA immediately downstream of the sTF2–219 stop codon. This strategy improved the expression yield to greater than 20 mg/liter sTF2–219. Wild-type and mutant proteins were separated from the bacterial culture supernatants and periplasmic extracts by a single step immunofinity chromatography on immobilized HPC4 as described previously (6). With the exception of Glu56 and Asp58, all of the basic and acidic residues of the N-terminal domain of sTF2–219 were substituted with Ala in individual constructs. The Ala substitution mutants of both Glu56 and Asp58 did not express to high yield; thus, these residues were substituted with Asn. SDS-PAGE analysis of the purified proteins under non-reducing conditions indicated that the isolated proteins were essentially homogenous (Fig. 1). We previously demonstrated that the bacterial sTF2–219 with or without an N-terminal HPC4 epitope exhibits identical cofactor activity as the mammalian sTF1–219.
The N-terminal Domain of TF

The sTF2–219-mediated improvement in the amidolytic activity of fVIIa

The amidolytic activity (milli optical density (mOD)/min at 405 nm) of 5 nM fVIIa toward Spectrozyme VIIa in the presence of increasing concentrations of wild type or mutant cofactors was determined in TBS/CaCl2 as described under “Experimental Procedures.” The values are the average of 2–3 independent measurements ± S.D. WT, wild type. ND, not determinable since the amidolytic activity of fVIIa in the presence of the mutant cofactors, fVIIa exhibited a loss of activity for up to 12 μM sTF2–219 mutant. As shown in Table I, the mutants exhibited nearly similar maximal amidolytic activity. It should be noted that, with the D58N mutant, no Kd(app) could be measured because the amidolytic activity of fVIIa in the presence of the mutant cofactor remained linear for up to 12 μM sTF2–219 mutant. Thus, the results with this mutant cannot be analyzed with certainty. The functionally important residues of the TF N-terminal domain were found to be spatially clustered at the junction of the N- and C-terminal domains of TF (Fig. 2).

**Table I**

| sTF   | Maximal velocity (mOD/min) | Kd(app) (nM) |
|-------|---------------------------|--------------|
| WT    | 7.3 ± 0.4                 | 5.3 ± 1.5    |
| K15A  | 7.1 ± 0.1                 | 4.2 ± 0.8    |
| K20A  | 8.5 ± 0.1                 | 12.68 ± 60   |
| E24A  | 7.3 ± 0.4                 | 44.3 ± 5.7   |
| E26A  | 9.3 ± 0.3                 | 13.0 ± 1.7   |
| K28A  | 6.9 ± 0.5                 | 8.8 ± 0.5    |
| K41A  | 7.7 ± 0.3                 | 2.5 ± 0.5    |
| D44A  | 6.4 ± 0.1                 | 267 ± 61     |
| K46A  | 7.9 ± 0.5                 | 20.9 ± 2.3   |
| K48A  | 7.8 ± 0.2                 | 88.5 ± 9.5   |
| D54A  | 9.7 ± 0.1                 | 4.1 ± 0.9    |
| E56N  | 9.8 ± 0.3                 | 26.3 ± 3.5   |
| D58N  | ND                        | >12,000      |
| D61A  | 11.0 ± 0.1                | 1.1 ± 0.2    |
| E62A  | 11.0 ± 0.1                | 3.5 ± 0.4    |
| K65A  | 8.8 ± 0.4                 | 2.9 ± 0.3    |
| D66A  | 8.9 ± 0.5                 | 1.5 ± 0.2    |
| K68A  | 13.1 ± 0.3                | 9.1 ± 1.4    |
| R74A  | 3.1 ± 0.2                 | 950 ± 150    |
| E84A  | 8.8 ± 0.1                 | 6.2 ± 0.3    |
| E91A  | 8.2 ± 0.2                 | 78.0 ± 3.9   |
| E95A  | 2.9 ± 0.6                 | 10.3 ± 0.2   |
| E99A  | 7.6 ± 0.8                 | 10.0 ± 0.2   |
| E105A | 5.8 ± 1.3                 | 7.9 ± 0.3    |

Fig. 1. SDS-PAGE analysis of sTF2–219 mutants. Under non-reducing conditions, molecular mass standards (lane 1), wild type sTF2–219 (lane 2), and the 23 mutants of sTF2–219 (lanes 3–25), which are listed in Table I, are shown.

Fig. 2. The space-filling model of the crystal structure of the sTF. Acidic residues are shown in yellow, and the basic residues are in red.

(6). Thus, the cofactor activities of all of the sTF2–219 derivatives were evaluated without cleaving the HPC4 epitope from the N termini of the recombinant proteins.

**Evaluation of the Kd(app) of sTF2–219 Mutants for Binding to fVIIa—** TF is known to enhance the amidolytic activity of fVIIa toward tripeptidyl chromogenic substrates 30–100-fold (1). This property of the cofactor was utilized to evaluate the affinity of sTF2–219 derivatives for binding to fVIIa. In agreement with previous results, wild-type sTF2–219 interacted with fVIIa with a Kd(app) of 5.3 nM (Table I). On the basis of their importance for interaction with fVIIa and improving the catalytic efficiency of the protease, the sTF2–219 mutants can be functionally classified into three general categories: 1) mutants with impaired affinity but with normal cofactor activity; 2) mutants with both impaired affinity and cofactor activity; and 3) mutants with improved cofactor activity. Among the first class, at least five mutants (Lys20, Glu24, Asp44, Lys48, and Asp58) exhibited the greatest loss of affinity for fVIIa, suggesting that these residues play a critical role either directly or indirectly in interaction with fVIIa (Table I). However, at saturating concentrations of the mutant cofactors, fVIIa exhibited nearly similar maximal amidolytic activity. It should be noted that, with the D58N mutant, no Kd(app) could be measured because the amidolytic activity of fVIIa in the presence of the mutant cofactor remained linear for up to 12 μM sTF2–219 mutant. Thus, the results with this mutant cannot be analyzed with certainty. The functionally important residues of the TF N-terminal domain were found to be spatially clustered at the junction of the N- and C-terminal domains of TF (Fig. 2).

The mutagenesis of these residues and the use of synthetic peptides based on this region have been reported to dramatically impair the cofactor activity of fVIIa (7, 18–20). Results of this study suggest that fVIIa has a normal amidolytic activity in the presence of saturating concentrations of these sTF2–219 mutants, suggesting that the catalytic defect with these mutants is solely due to their loss of interaction with the protease. In the case of Asp58, substitution with Asn dramatically reduced the affinity of sTF for fVIIa. Interestingly, when Lee and Kelley (21) used phase-display technology to create an altered version of sTF that retained wild-type binding affinity for fVIIa but which failed to enhance the proteolytic activity of fVIIa, they found that their mutants contained exclusively a substitution of Trp for Asp at position 58 of sTF.

In the second class of mutants, we identified a new functionally important residue, Arg74, which was not included in the previous Ala-scanning mutagenesis study of TF (7). The mutagenesis of this residue resulted in both impaired affinity and cofactor activity for the sTF2–219 mutant. As shown in Table I, relative to wild-type, the Kd(app) of fVIIa interaction with the R74A was elevated ~190-fold. Moreover, unlike a normal amidolytic activity with the other mutants, the maximal activity of fVIIa in the presence of a saturating concentration of R74A was also impaired ~2-fold (Table I). These results suggest that the interaction of the side chain of Arg74 with fVIIa may be responsible for allosterically changing the conformation of the active site pocket of fVIIa, leading to improvement in the catalytic efficiency of the protease. Thus, a cofactor-mediated
conformational change in the active site pocket of fVIIa may be, at least partially, mediated through protease interaction with Arg

219 of TF. Indeed, from the crystal structure of the sTF–fVIIa complex, residue Arg

219 appears to make contact with residues in the protease domain of fVIIa (11). In contrast to the loss of affinity and cofactor function for most of the sTF

219 mutants, the mutagenesis of three acidic residues and one basic residue of sTF

219 around the 61–66 sequence resulted in an improved affinity for fVIIa and also improved maximal amidolytic activity (Table I). These residues are located on the opposite surface of the cofactor and thus are not expected to contact fVIIa (although Asp

61 might be able to make contacts with fVIIa). It is not known how the neutralization of the charges of these residues improves the cofactor function of the molecules, although interestingly, these residues are all located within one of the very few α-helical regions on sTF.

Proteolytic Function—The cofactor function of the sTF

219 mutants in enhancing the proteolytic activity of fVIIa toward the natural substrates factors IX and X was also studied. The kinetic parameters $K_a$ and $k_{cat}$ for the activation of both substrates by fVIIa in complex with the sTF

219 mutants are presented in Table II for factor IX and Table III for factor X. Note that $K_a$ and $k_{cat}$ values for the activation of factors IX or X by the fVIIa–sTF

219 complex are reported here as apparent values. This is because both $K_a$ and $k_{cat}$ change as a function of phospholipid concentration due to partitioning of enzyme and substrate between the solution and membrane-bound phases as described in detail by Fiore et al. (22). All of the cofactor mutants with the exception of D58N and R74A exhibited near normal or improved cofactor activity in promoting the fVIIa activation of both substrates. The kinetic basis for the defect with the D58N mutant cannot be readily analyzed, because the affinity of this mutant for fVIIa was dramatically impaired and the activation assay was carried out at a subsaturating concentration of the mutant due to a limited amount of the cofactor. However, a similar previous study with the same mutant of TF in the full-length form has indicated that the Asp

58 mutant has only impaired affinity for fVIIa with a normal cofactor function (23). This has been evidenced by the observation that fVIIa in complex with a saturating concentration of the Asp

58 mutant exhibits a normal maximal activity (23). On the other hand, as shown in Fig. 3, the proteolytic cofactor function of the R74A mutant in activation of both factors IX and X by fVIIa was markedly impaired. The extent of impairment in the cofactor function of the R74A mutant was ~2.5- and ~6-fold for the activation of factors IX and X, respectively. In both cases, the primary defect in the cofactor function was due to impairment in the $k_{cat}$ of the activation reaction (Tables II and III). These results further support the hypothesis that the side chain of Arg

219 may be in contact with a region of fVIIa that is allosterically linked to the active site pocket of the protease. The Ala substitution mutants of an acidic region in most C-terminal end of the TF N-terminal domain resulted in the improvement in the cofactor function of the TF mutants in the factor X but not factor IX activation assay (Table III). Thus, the catalytic efficiency of fVIIa in complex with the E105A mutant of sTF

219 was improved 3.5-fold (Table III). It is likely that the charge neutralization of this residue eliminates a repulsive interaction of an acidic region of factor X (most likely γ-carboxyglutamic acid and/or EGF1 domain) with the fVIIa–sTF complex.

To provide further evidence that Arg

219 is critical for the proteolytic function of fVIIa, the R74A mutant was used as a competitive inhibitor of factor X activation by fVIIa in complex with wild-type sTF

219. As shown in Fig. 4, R74A inhibited the activation of factor X by fVIIa in complex with wild-type sTF

219 in

\[ \text{WT} \]

\[ \text{K15A} \]

\[ \text{K20A} \]

\[ \text{E24A} \]

\[ \text{K28A} \]

\[ \text{K41A} \]

\[ \text{D44A} \]

\[ \text{K46A} \]

\[ \text{K48A} \]

\[ \text{D54A} \]

\[ \text{E56N} \]

\[ \text{D58N} \]

\[ \text{D66A} \]

\[ \text{D66A} \]

\[ \text{K41A} \]

\[ \text{K46A} \]

\[ \text{K48A} \]

\[ \text{D54A} \]

\[ \text{E56N} \]

\[ \text{D58N} \]

\[ \text{D61A} \]

\[ \text{E62A} \]

\[ \text{K55A} \]

\[ \text{D54A} \]

\[ \text{E84A} \]

\[ \text{E91A} \]

\[ \text{E95A} \]

\[ \text{E99A} \]

\[ \text{E105A} \]

\[ \text{K15A} \]

\[ \text{K20A} \]

\[ \text{E24A} \]

\[ \text{K28A} \]

\[ \text{K41A} \]

\[ \text{D44A} \]

\[ \text{K46A} \]

\[ \text{K48A} \]

\[ \text{D54A} \]

\[ \text{E56N} \]

\[ \text{D58N} \]

\[ \text{D61A} \]

\[ \text{E62A} \]

\[ \text{K55A} \]

\[ \text{D54A} \]

\[ \text{E84A} \]

\[ \text{E91A} \]

\[ \text{E95A} \]

\[ \text{E99A} \]

\[ \text{E105A} \]

\[ \text{WT} \]

\[ \text{K15A} \]

\[ \text{K20A} \]

\[ \text{E24A} \]

\[ \text{K28A} \]

\[ \text{K41A} \]

\[ \text{D44A} \]

\[ \text{K46A} \]

\[ \text{K48A} \]

\[ \text{D54A} \]

\[ \text{E56N} \]

\[ \text{D58N} \]

\[ \text{D61A} \]

\[ \text{E62A} \]

\[ \text{K55A} \]

\[ \text{D54A} \]

\[ \text{E84A} \]

\[ \text{E91A} \]

\[ \text{E95A} \]

\[ \text{E99A} \]

\[ \text{E105A} \]
a concentration-dependent manner, and at a high concentration of the cofactor mutant, which was sufficient to displace most of the wild-type cofactor, the rate of factor X activation declined ~2-fold, paralleling the rate of the substrate activation by the FXIIa in complex with the R74A mutant. These results suggest that the role of Arg74 in the cofactor function of TF is distinct from other charged residues under study and may confirm the hypothesis that it interacts with a site of FXIIa that is in allosteric linkage with the active site pocket of the protease. Previously, a similar cofactor role for the Asp44 of TF has also been postulated (8). In that study, an ~4-fold impaired cofactor function for the D44A mutant of TF in factor X activation by FXIIa has been reported (8). However, in the current study, we noted an ~2-fold impairment in the kcat of factor X activation by FXIIa in complex with the D44A mutant of TF and this defect was partially compensated by an improvement in the Kcat(app) of the activation reaction (Table III). Moreover, unlike the defective cofactor function of the R74A mutant in the activation of both macromolecular substrates, the D44A mutant exhibited near normal cofactor function in the activation of factor IX (Table II). The differences in the results of two studies may be due to different experimental conditions used to evaluate the cofactor effect of this mutant. The previous study used a limiting concentration of TF in complex with excess FXIIa to evaluate the cofactor function of the mutant at 27 °C whereas our studies were carried out with a limiting concentration of FXIIa in complex with an excess of the TF mutants at room temperature. However, despite these differences, the observation that the kcat of factor X activation with D44A mutant was impaired ~2-fold is in agreement with the previous hypothesis that Asp44 of TF is critical for the proteolytic function of FXIIa in the extrinsic factor Xase complex.

In summary, we have mapped the functionally important basic and acidic residues of the N-terminal domain of TF and demonstrated that most of the charged residues critical of FXIIa binding are spatially located at the junction of the N- and C-terminal domains of TF. The results further suggest that, unlike the C-terminal domain of TF, which contains direct binding sites for interaction with both factors IX and X, the N-terminal domain of the cofactor may not interact with either substrate of FXIIa in the activation complex. Finally, we have identified Arg74 as a critical residue that appears to directly interact with a region of FXIIa that is in allosteric linkage with the active site pocket of the protease.

REFERENCES
1. Morrissey, J. H., Neueneschwander, P. F., Huang, Q., McCallum, C. D., Bixia, S., and Johnson, A. E. (1997)Thromb. Haemostasis 78, 112–116
2. Jackson, C. M., and Nemerson, Y. (1980) Ann. Rev. Biochem. 49, 765–811
3. Morrissey, J. H., Fakhrai, H., and Edgington, T. S. (1987) Cell 50, 129–135
4. Harlos, K., Martin, D. M. A., O’Brien, D. P., Jones, E. Y., Stuart, D. I., Polikarpova, I., Miller, A., Tuddenham, E. G. D., and Boys, C. W. G. (1994) Nature 370, 662–666
5. Muller, Y. A., Ulltsch, M. H., Kelley, R. F., and de Vos, A. M. (1994) Biochemistry 33, 10864–10870
6. Rezaie, A. R., Fiore, M. M., Neueneschwander, P. F., Emon, C. T., Morrissey, J. H. (1992) Protein Expression Purif. 3, 453–460
7. Ruf, W., Schullek, J. R., Stone, M. J., and Edgington, T. S. (1994) Biochemistry 33, 1565–1572
8. Kelly, C. R., Schullek, J. R., Ruf, W., and edgington, T. S. (1996) Biochem. J. 315, 145–151
9. Kirchofer, D., Lipari, M. T., Moran, P., Eigenbrot, C., and Kelley, R. F. (2000) Biochemistry 39, 7380–7387
