Thrombomodulin (TM) functions as a cofactor to enhance the rate of protein C activation by thrombin—1000-fold. The molecular mechanism by which TM improves the catalytic efficiency of thrombin toward protein C is not known. Molecular modeling of the protein C activation based on the crystal structure of thrombin in complex with the epidermal growth factor-like domains 4, 5, and 6 of TM (TM456) predicts that the binding of TM56 to exosite 1 of thrombin positions TM4 so that a negatively charged region on this domain juxtaposes a positively charged region of protein C. It has been hypothesized that electrostatic interactions between these oppositely charged residues of TM4 and protein C facilitate a proper docking of the substrate into the catalytic pocket of thrombin. To test this hypothesis, we have constructed several mutants of TM456 and protein C in which charges of the putative interacting residues on both TM4 (Asp/Glu) and protein C (Lys/Arg) have been reversed. Results of TM-dependent protein C activation studies by such a compensatory mutagenesis approach support the molecular model that TM4 interacts with the basic exosite of protein C.

Thrombin is the final serine protease of the coagulation cascade that activates fibrinogen to form blood clots (1–3). Thrombin can up-regulate its own production by activating platelets (4), factor XI (5), and factors V and VIII (1). It can also down-regulate its own production by forming a complex with thrombomodulin (TM) on the surface of endothelial cells (6). Upon interaction with TM, thrombin can no longer clot fibrinogen but, instead, rapidly activates protein C to activated protein C (6, 7). Activated protein C is a vitamin K-dependent plasma serine protease that can shut down the coagulation cascade by degrading factors Va and VIIIa by limited proteolysis (8, 9). Structural and functional data have indicated that TM binds to exosite 1 of thrombin to change the specificity of thrombin in complex with these procoagulant proteins. It has been demonstrated that the binding of TM to exosite 1 competitively blocks the interaction of thrombin with these procoagulant proteins. Thus, the binding of TM to exosite 1 is sufficient to block all coagulant properties of thrombin; however, for catalyzing the activation of protein C, the fourth EGF domain of TM (TM456) is also required for its cofactor function (6, 10).

Calcium plays a key role in protein C activation by the thrombin-TM complex. Using Gla-domainless forms of protein C, it has been demonstrated that the Ca$^{2+}$-binding site critical for activation of protein C by the thrombin-TM complex is located in the 70–80-loop of the substrate (18, 19) (chymotrypsinogen numbering system (20)). This is the same site in trypsin that is also known to bind Ca$^{2+}$ (21). Occupancy of this site by Ca$^{2+}$ is a prerequisite for the ability of TM to catalyze the rapid activation protein C by thrombin (18, 19). Interestingly, the binding of Ca$^{2+}$ to this site of protein C is inhibitory for activation by thrombin in the absence of TM (18). It has been hypothesized that the binding of Ca$^{2+}$ to this site of protein C is associated with a conformational change in the activation peptide of the substrate and that the Ca$^{2+}$-stabilized conformer of the activation peptide is complementary for the active site pocket of thrombin in the presence of TM but non-complementary in the absence of the cofactor (6).

The exact molecular mechanism by which TM456 improves the catalytic efficiency of thrombin toward protein C in the presence of Ca$^{2+}$ is not known. Previously, it has been hypothesized that TM may function by inducing allosteric changes in the structure of residues in the extended binding pocket of thrombin (6). Thus, it is believed that the TM-altered conformer of thrombin optimally recognizes the Ca$^{2+}$-altered conformer of protein C (6). Recently, the x-ray crystal structure of thrombin in complex with TM456 was resolved (15). Surprisingly, structural data did not indicate any allostery changes in the active site pocket of thrombin upon binding to the TM fragment (15). Molecular modeling of protein C activation, based on the structure of the complex, suggested that binding of the TM5 domain of the cofactor to exosite 1 of thrombin positions the TM4 domain so that a negatively charged region on this domain juxtaposes a positively charged exosite of protein C (15). Several electrostatic interactions between these oppositely charged regions are postulated to facilitate a proper docking of protein C into the catalytic pocket of thrombin. Based on this model, it has been hypothesized that “substrate presentation” is the primary mechanism by which TM may function (15). A previous study proposed a similar function for TM in protein C activation by thrombin (22).

To examine the validity of this molecular model by a mutagenesis approach, we developed a bacterial periplasmic ex-
pression system for TM456 and constructed several mutants of TM456 in which the negative charges of five residues in TM4 (Glu94, Asp134, Glu157, Asp159, and Glu174) and two residues (Asp398 and Asp400) in the interface between TM4 and TM5 were reversed by substituting each residue with a Lys in separate constructs. In addition, a double mutant of TM456 containing Arg385 (R385E), Asp398 (D398K), and Asp400 (D400K) were prepared by substituting these residues individually or in a combination of two with Glu in a mammalian expression/purification vector system. The protein C mutants were prepared in Gla-domainless forms and expressed in HEK293 cells as described (23). Kinetic characterization of these mutant molecules by this compensatory mutagenesis approach supports the molecular model that the TM4 domain of TM456 in the complex interacts with the basic exosite of protein C. Moreover, analysis of results suggest that the 70–80-loop of protein C must be stabilized by the Ca²⁺ ion before it can productively interact with TM4. This accounts for the unique Ca²⁺ dependence of protein C activation by thrombin in the presence of TM.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression—The wild type and charge reversal mutants of TM456 including Asp398 (D349R), Glu94/Lys94 (E349R,D349K), Glu94 (E349R), Glu157/Lys157 (E57R,K57E), Glu157 (E57R), Arg385 (R385E), Arg398 (R398E), and Arg400 (R400E) were prepared by PCR methods and expressed in the periplasmic space of Escherichia coli using the pII-N-III-pelB expression/purification vector system as described previously (24). Wild type and mutant protein C derivatives were constructed in Gla-domainless forms (GD-PC) as described previously (23). The charge reversal mutants of GD-PC including Lys62 (K62E), Lys63 (K63E), and Arg74 and Arg75 (R74E,R75E) were prepared by PCR methods and expressed in HEK293 cells using RSV-PLA expression/purification system as described (23). Both mammalian and bacterial expression vectors contain the sequence of a 12-residue epitope for the Ca²⁺ -dependent monoclonal antibody, HPC4, for purification. Thus, both GD-PC and TM456 derivatives were purified by immunoaffinity chromatography using the HPC4 antibody linked to Affi-gel 10 (Bio-Rad) as described (23). TM456 derivatives were further purified by anion exchange chromatography on a Mono Q column developed with 30-ml linear gradient from 0.1 to 0.6 M NaCl and 0.02 M Tris-HCl, pH 7.5, as described for the purification of the mammalian TM456 (23). Accuracies of all mutant constructs were confirmed by DNA sequencing prior to their expression.

Protein C Activation—The initial rates of GD-PC activation by thrombin were measured in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5 (TBS), containing 1 mg/ml bovine serum albumin (BSA), 0.1% polyethylene glycol (PEG) 8000, and 2.5 mM CaCl₂, in both the absence and the presence of TM456. In the absence of TM456, the time course of activation of each GD-PC derivative was studied by incubating 1 μM substrate with 50 nM thrombin at room temperature. At each time point (5–80 min), samples of reactions were transferred to TBS buffer containing 500 μg/ml human antithrombin to inhibit thrombin activity. At this concentration of antithrombin, the activity of thrombin was rapidly inhibited, whereas the amidolytic activity of active GD-PC remained stable for more than 120 min. The amidolytic activities of active GD-PC derivatives in the activation reactions were monitored by hydrolysis of 400 μM Spectrozyme PCa (American Diagnostica Inc., Greenwich, CT) in the TBS buffer containing 1 mg/ml BSA and 0.1% PEG 8000. The rate of hydrolysis was measured at 405 nm at room temperature in a Vmax kinetic analyzer. Molecular Devices, Molecular Devices, Molecular Devices, Molecular Devices. The activation of active protein C derivatives in reaction mixtures was determined by reference to standard curves that were prepared by total activation of GD-PC derivatives with excess thrombin at the time of experiments. This was accomplished by total activation of 1 μM protein C with 10 nM thrombin in complex with 100 nM TM456 and 2.5 mM CaCl₂, for 90 min. Under these experimental conditions, the wild typezymogen was completely activated in less than 30 min. The GD-PC mutants, which were resistant to activation by the thrombin-TM456 complex in the presence of Ca²⁺, were totally activated with thrombin alone in 1 mM EDTA as described (23). In this case, 1 μM of each protein

The Fourth EGF-like Domain of TM

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Expression and Purification of Recombinant Proteins—Wild type and mutant GD-PC derivatives were expressed in HEK293 cells and purified to homogeneity on the HPC4 immunoaffinity column as described (23). The SDS-PAGE analysis of zymogens indicated that protein C derivatives were expressed as two closely migrating subforms that are glycosylation variants observed previously with this protein (23, 27, 28) (data not shown). TM456 derivatives were expressed in the periplasmic space of the XL-1B strain of E. coli using the pII-N-III-pelB expression/purification vector system as described (23). The relative positions of the mutant residues in the fourth epidermal growth factor domain of TM are schematically shown in Fig. 1A. TM456 expressed in this system contains a 12-residue epitope for the Ca²⁺ -dependent monoclonal antibody HPC4 at its N-terminal domain for an easy purification. The periplasmic extract was prepared by a hypotonic shock and passed through the HPC4 column in the presence of Ca²⁺ as described (24). The column was extensively washed with TBS containing 1 M NaCl and 2.5 mM Ca²⁺ followed by elution with TBS containing 5 mM EDTA as described (24). The HPC4 eluates were further purified by anion exchange chromatography on a Mono Q column as described (23). SDS-PAGE analysis under nonreducing conditions suggested that all TM456 derivatives had been purified to homogeneity, migrating as single bands with expected apparent molecular masses of ~15 kDa (Fig. 1B). Wild type forms of both mammalian and periplasmic TM456 exhibited comparable cofactor activities (within 80%) as determined by protein C activation assays described below.

Initial Rate of GD-PC Activation—The initial rates of thrombin-catalyzed wild type and mutant GD-PC activation were studied in both the absence and presence of TM456. GD-PC is known to contain a single Ca²⁺-binding site with an affinity of ~50 μM, which is located in the protease domain of protein C in a loop consisting of residues Glu70–Glu90 (19, 23). It is known that the binding of Ca²⁺ to this loop of protein C is inhibitory for activation by thrombin in the absence of TM (6, 19). However, the metal ion is an obligatory cofactor for activation by thrombin in the presence of the cofactor (6, 19). Since
as a function of varying Ca$^{2+}$ the initial rates of GD-PC activation by thrombin were studied due to the R75E mutation. Activation of the double mutant R74E,R75E is also probably impaired in the presence of TM456 (30). Consistent with these results, we found that the activation of a triple GD-PC K37E,K38E,K39E mutant by thrombin alone was normal in both EDTA and Ca$^{2+}$, but it was severely impaired in the presence of TM456 (data not shown).

**Initial Rates of Protein C Activation by Thrombin in Complex with TM456 Derivatives**—The initial rates of wild type and mutant GD-PC activation by thrombin in complex with different TM456 derivatives were measured as described above. As shown in Fig. 4, relative to wild type TM456, the cofactor activities of all TM456 mutants in catalyzing the thrombin activation of wild type GD-PC were impaired. The impairments ranged from a maximum of 90–95% for the E357K, D398K, and E382K mutants to a minimum of 50–60% for the E374K and R74E,K63E mutants (Fig. 3B). These results suggested that the basic residues of both 60- and 70-80-loops are critical for the thrombin activation of protein C in the presence of TM. It should be noted that in addition to these residues, it is known that basic residues of the 39-loop (Lys$^37$-Lys$^39$) are also required for the TM-dependent protein C activation by thrombin (30).

To test whether or not Ca$^{2+}$ is involved in this activation, we added increasing concentrations of Ca$^{2+}$ to apo-GD-PC and TM456 before activation. As shown in Fig. 2, the Ca$^{2+}$ concentration has no effect on the activation of GD-PC derivatives by thrombin. The initial rate of protein C (1 μM) activation by thrombin (10 nM) was measured in the presence of increasing concentrations of Ca$^{2+}$ (0–2.5 mM) at room temperature in TBS containing 1 mg/ml BSA and 0.1% PEG 8000. The activation reactions were stopped by adding 500 μg/ml antithrombin, and the rate of activation was measured from the concentration of activated protein C generated by an amidolytic activity assay using Spectrozyme PCA as described under “Experimental Procedures.” Data were normalized to maximal inhibition (100% at 2.5 mM Ca$^{2+}$) and plotted as a function of different concentrations of Ca$^{2+}$.

**Results**

**Residues of the 70-80-loop were targeted for the mutagenesis study, it was essential to evaluate the extent to which the interaction of these mutants with Ca$^{2+}$ has been altered. Thus, the initial rates of GD-PC activation by thrombin were studied as a function of varying Ca$^{2+}$ concentrations. To simplify the comparisons of the Ca$^{2+}$ dependence of activations, the data were normalized to maximal inhibition values as described (23). As shown in Fig. 2, the Ca$^{2+}$ ion inhibited the activation of all GD-PC derivatives with the exceptions of R73E and R74E,K75E with a similar half-maximal value of 30–40 μM (see legend of Fig. 2). However, these values were elevated ~2–3-fold for the R75E and R74E,K75E mutants. Since the half-maximal values for the activation of the R74E mutant were not affected, the altered effect of Ca$^{2+}$ in the thrombin activation of the double mutant R74E,R75E is also probably due to the R75E mutation.

Next, the time course of the initial rates of GD-PC activation by thrombin alone or by the thrombin-TM456 complex was studied at saturating concentration of Ca$^{2+}$ (2.5 mM). As shown in Fig. 3A, thrombin activated all GD-PC derivatives with comparable or improved initial rates. The greatest improvement (~6–7-fold) was observed for the thrombin activation of the R74E,R75E double mutant. A detailed kinetic analysis was not possible, since protein C exhibits a $K_m$ of greater than 60 μM for thrombin in the presence of Ca$^{2+}$ (29). In contrast to activation by thrombin alone, the TM-dependent activations of the GD-PC derivatives were all impaired at varying degrees. Under conditions described in the legend to Fig. 3, no TM456-dependent activation by thrombin was detected for the R74E and R75E mutants of GD-PC, and the rates were impaired ~2-fold for K63E and K79E and ~5-fold for the K62E and K62E,K63E mutants (Fig. 3B). These results suggested that the basic residues of both 60- and 70-80-loops are critical for the thrombin activation of protein C in the presence of TM. It should be noted that in addition to these residues, it is known that basic residues of the 39-loop (Lys$^37$-Lys$^39$) are also required for the TM-dependent protein C activation by thrombin (30). Consistent with these results, we found that the activation of a triple GD-PC K37E,K38E,K39E mutant by thrombin alone was normal in both EDTA and Ca$^{2+}$, but it was severely impaired in the presence of TM456 (data not shown).

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The rationale for the preparation of the R385E mutant of TM456 was that in the previous molecular model of protein C activation it was proposed that the interaction of Arg$^{580}$ of TM4 with a basic residue of the 70–80-loop of protein C (particularly Glu$^{80}$) might be responsible for the unique Ca$^{2+}$ dependence of activation (15). Thus, the initial rate of wild type GD-PC acti-
The initial rate of GD-PC activation by thrombin in complex with TM456 was measured at room temperature (data not shown). Interestingly, however, no Ca\(^{2+}\) dependence of GD-PC activation was observed by thrombin in complex with D349K, E357K, D398K, and D400K mutants of TM456. Although a similar \(K_{d(app)}\) of \(~20\ \mu M\) for GD-PC activation by thrombin in complex with other mutants of TM456 was observed, the extent of the cofactor effect of Ca\(^{2+}\) was, nonetheless, reduced to \(~2\)-fold with these mutants. Similarly, no Ca\(^{2+}\)-dependence of activation was observed for the thrombin activation of the R74E and R75E mutants of GD-PC in the presence of wild type TM456. Since the interaction of thrombin with TM456 is not known to be Ca\(^{2+}\)-dependent (6, 19), it follows that the cofactor defect of TM456 derivatives must primarily be caused by their inability to interact with the Ca\(^{2+}\)-stabilized conformer of GD-PC in the complex.

Next, the cofactor functions of the TM456 mutants in enhancing the thrombin activation of the compensatory GD-PC mutants were evaluated. Similar to wild type TM456, none of the mutants exhibited any cofactor activity toward thrombin activation of the 70–80-loop mutants of GD-PC (data not shown). However, the cofactor functions of both the D349K and E346R,D349K mutants in enhancing the activation of the compensatory 60-loop mutants of GD-PC were improved (Fig. 5, shown for the double mutant only). Relative to activation of wild type GD-PC, the activation of K62E, K63E, and K62E,K63E by the thrombin-TM456 complex were impaired 2–5-fold (Fig. 5). Interestingly, however, the defect in the activation of these mutants by thrombin was restored if the E346R,D349K mutant of TM456 was used as the cofactor in the activation reaction. As shown in Fig. 5, the initial rate of activation of K62E,K63E by thrombin in complex with the double mutant of TM456 was essentially a mirror image of the GD-PC activation rate by thrombin in complex with the wild type TM456. Thus, 100% of the cofactor activity of the Glu\(^{346}\) and Asp\(^{349}\) charge reversal mutants of TM could be restored if the E346R,D349K mutant of TM456 was used as the cofactor in the ternary complex. With the wild type GD-PC as a cofactor, the same as above, except that the thrombin activity by antithrombin, the rate of activated protein C generation was measured by an amidolytic activity assay as described under “Experimental Procedures.” The solid black bars are derived from activation studies in the presence TM456, and blank bars are derived from the same data in the presence of TM456 E346R,D349K.

Fig. 5. Restoration of the defective cofactor effect of the TM456 mutant by a compensatory GD-PC mutant. The initial rate of GD-PC (1 \(\mu M\)) activation by thrombin (1–10 \(\mu M\)) in complex with a saturating concentration of each TM456 derivative (500 \(\mu M\)) was measured at room temperature in TBS containing 1 mg/ml BSA, 0.1% PEG 8000, and 2.5 mM Ca\(^{2+}\). Following inhibition of the thrombin activity by antithrombin, the rate of activated protein C generation was measured by an amidolytic activity assay as described under “Experimental Procedures.” The solid black bars are derived from activation studies in the presence of TM456, and blank bars are derived from the same data in the presence of TM456 E346R,D349K.

TM456 by an order of magnitude with \(K_{d(app)}\) of \(~20\ \mu M\) at room temperature (data not shown). Interestingly, however, no Ca\(^{2+}\) dependence of GD-PC activation was observed by thrombin in complex with D349K, E357K, D398K, and D400K mutants of TM456. Although a similar \(K_{d(app)}\) of \(~20\ \mu M\) for GD-PC activation by thrombin in complex with other mutants of TM456 was observed, the extent of the cofactor effect of Ca\(^{2+}\) was, nonetheless, reduced to \(~2\)-fold with these mutants. Similarly, no Ca\(^{2+}\)-dependence of activation was observed for the thrombin activation of the R74E and R75E mutants of GD-PC in the presence of wild type TM456. Since the interaction of thrombin with TM456 is not known to be Ca\(^{2+}\)-dependent (6, 19), it follows that the cofactor defect of TM456 derivatives must primarily be caused by their inability to interact with the Ca\(^{2+}\)-stabilized conformer of GD-PC in the complex.

Evaluation of \(K_{d(app)}\) Values—The cofactor dependence of the initial rate of protein C activation by thrombin in complex with thrombin in the ternary complex. With the wild type GD-PC as a cofactor, the same as above, except that the thrombin activity by antithrombin, the rate of activated protein C generation was measured by an amidolytic activity assay as described under “Experimental Procedures.”

Fig. 4. Comparison of the cofactor effect of TM456 mutants. The initial rate of GD-PC (1 \(\mu M\)) activation by thrombin (1–10 \(\mu M\)) in complex with a saturating concentration of each TM456 derivative (500 \(\mu M\)) was measured at room temperature in TBS containing 1 mg/ml BSA, 0.1% PEG 8000, and 2.5 mM Ca\(^{2+}\). Following inhibition of the thrombin activity by antithrombin, the rate of activated protein C generation was measured by an amidolytic activity assay as described under “Experimental Procedures.”

The cofactor dependence of the initial rate of protein C activation by thrombin in complex with the wild type TM456 was essentially a mirror image of the GD-PC activation rate by thrombin in complex with the wild type TM456. Thus, 100% of the cofactor activity of the Glu\(^{346}\) and Asp\(^{349}\) charge reversal mutants of TM could be restored if compensatory mutations were made in residues of the 60-loop of protein C. These results clearly suggest that TM4 provides a site for interaction with protein C in the activation complex.

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substance, a $K_{d(app)}$ value of 5.7 nM for the interaction of mammalian TM456 with thrombin was observed, which is consistent with previously reported values in the literature (31). The bacterial TM456 exhibited a similar concentration of TM456 (~5 nM) and increasing concentrations of thrombin (1–60 nM) as described under “Experimental Procedures.” $K_{d(app)}$ and $V_{max}$ values were determined from the concentration dependence of the initial rate of activation of 1 μM GD-PC in the presence of a fixed concentration of TM456 (~5 nM) and increasing concentrations of thrombin (1–60 nM) as described under “Experimental Procedures.” $K_{m(app)}$ and $V_{max}$ values were determined from the concentration dependence of the initial rate of activation of wild type (WT) GD-PC activation by thrombin (1 nM) in complex with a saturating concentration of each TM456 derivative (500 nM) according to the Michaelis-Menten equation. The percentage increase in SpTH hydrolysis by thrombin (2 μM) in complex with each TM456 derivative (500 nM) was determined in TBS containing 0.1 mg/ml BSA, 0.1% PEG 8000, and 2.5 mM CaCl$_2$. ND, not determined.

| TM456            | $K_{d(app)}$ (nM) | $K_{m(app)}$ (μM) | $V_{max}$ (mol/min/mol) | SpTH hydrolysis increase % |
|------------------|-------------------|-------------------|------------------------|---------------------------|
| WT (mammalian)   | 5.7 ± 0.5         | 7.1 ± 0.7         | 24.1 ± 0.8             | 86                        |
| WT (bacterial)   | 4.2 ± 0.3         | 6.0 ± 0.3         | 20.4 ± 0.3             | 90                        |
| D349K            | 8.0 ± 1.3         | ND                | ND                     | 71                        |
| E346R/D349K      | 10.1 ± 0.9        | 43.1 ± 3.0        | 15.1 ± 0.8             | 72                        |
| E357K            | 7.2 ± 0.7         | 41.4 ± 3.5        | 21.3 ± 1.1             | 86                        |
| E374K            | 6.3 ± 0.3         | 22.3 ± 2.2        | 17.6 ± 0.9             | 92                        |
| E382K            | 5.2 ± 0.4         | 34.5 ± 2.0        | 17.4 ± 0.6             | 90                        |
| R385E            | 5.4 ± 0.3         | 27.2 ± 3.4        | 18.1 ± 0.8             | 83                        |
| D398K            | 15.8 ± 0.2        | 34.9 ± 3.1        | 19.9 ± 1.1             | 75                        |
| D400K            | 12.0 ± 0.9        | 39.1 ± 4.3        | 22.0 ± 1.2             | 83                        |

Previous molecular modeling of protein C activation, based on the structure of the thrombin-TM456 complex, predicted that the binding of TM56 domains of the cofactor to exosite 1 of thrombin may orient TM4 so that a negatively charged region in this domain could contact a basic exosite of protein C in the ternary activation complex (15). Based on this model, it was postulated that electrostatic interactions between the oppositely charged residues of these two regions could facilitate a proper docking of protein C into the catalytic pocket of thrombin (15). In this study, we tested the validity of this molecular model by a compensatory “gain of function” mutagenesis approach. Thus, we reversed the charges of the acidic residues of TM4 in TM456 constructs and expressed the mutant cofactor.

**DISCUSSION**

Previous molecular modeling of protein C activation, based on the structure of the thrombin-TM456 complex, predicted that the binding of TM56 domains of the cofactor to exosite 1 of thrombin may orient TM4 so that a negatively charged region in this domain could contact a basic exosite of protein C in the ternary activation complex (15). Based on this model, it was postulated that electrostatic interactions between the oppositely charged residues of these two regions could facilitate a proper docking of protein C into the catalytic pocket of thrombin (15). In this study, we tested the validity of this molecular model by a compensatory “gain of function” mutagenesis approach. Thus, we reversed the charges of the acidic residues of TM4 in TM456 constructs and expressed the mutant cofactor.

**TABLE I**

| TM456             | $K_{d(app)}$ (nM) | $K_{m(app)}$ (μM) | $V_{max}$ (mol/min/mol) | SpTH hydrolysis increase % |
|-------------------|-------------------|-------------------|------------------------|---------------------------|
| WT (mammalian)    | 5.7 ± 0.5         | 7.1 ± 0.7         | 24.1 ± 0.8             | 86                        |
| WT (bacterial)    | 4.2 ± 0.3         | 6.0 ± 0.3         | 20.4 ± 0.3             | 90                        |
| D349K             | 8.0 ± 1.3         | ND                | ND                     | 71                        |
| E346R/D349K       | 10.1 ± 0.9        | 43.1 ± 3.0        | 15.1 ± 0.8             | 72                        |
| E357K             | 7.2 ± 0.7         | 41.4 ± 3.5        | 21.3 ± 1.1             | 86                        |
| E374K             | 6.3 ± 0.3         | 22.3 ± 2.2        | 17.6 ± 0.9             | 92                        |
| E382K             | 5.2 ± 0.4         | 34.5 ± 2.0        | 17.4 ± 0.6             | 90                        |
| R385E             | 5.4 ± 0.3         | 27.2 ± 3.4        | 18.1 ± 0.8             | 83                        |
| D398K             | 15.8 ± 0.2        | 34.9 ± 3.1        | 19.9 ± 1.1             | 75                        |
| D400K             | 12.0 ± 0.9        | 39.1 ± 4.3        | 22.0 ± 1.2             | 83                        |
fragments in the periplasmic space of bacteria. We then prepared compensatory mutants of protein C in Glu-domainless forms by reversing the charges of certain basic residues of the putative exosite that is believed to interact with TM4. Based on results of previous TM456 (25, 31) and protein C (33, 34) mutants, we reasoned that such mutants would exhibit impaired cofactor or substrate activities if they were individually evaluated in appropriate functional assays. However, if the molecular model is correct, it was expected that the defective cofactor effect of certain TM456 mutants in catalyzing the thrombin activation of certain GD-PC mutants would fully, or at least partially, be restored. Indeed, the results suggested that the basic residues of the 60-loop of protein C interact with Glu46 and Asp49 of TM4 in the ternary activation complex. This was evidenced by the restoration of full cofactor activity of the double mutant of TM456 in catalyzing the thrombin activation of the K62E,K63E mutant of GD-PC.

In addition to basic residues of the 60-loop, the molecular model of the protein C activation predicts that the basic residues of the Ca$^{2+}$-binding 70–80-loop (particularly Arg74 and Arg75) also interact with the acidic TM4 domain of the cofactor. The results of this and other previous mutagenesis studies (33, 34) clearly suggest that these basic residues are essential for the TM-dependent activation of protein C by thrombin. However, by the compensatory mutagenesis approach of this study, we could not provide any information as to possible sites of the interaction of the 70–80-loop residues of protein C with TM4. The reason for the lack of success in mapping these interaction sites by this approach is not known. One possibility is that the guanidinium groups of Arg74 and Arg75 are involved in bifurcated salt bridges with two acidic residues in TM4, and thus the choice of Lys in the compensatory TM456 mutants may have flawed the strategy. The second possibility is that, with the exception of Glu346 and Asp349, other residues of TM4 mutated in this study are located on β-sheet structures and that the mutagenesis of these residues may cause subtle conformational changes in TM4, thereby hindering the proper alignment of these residues with basic residues of the Ca$^{2+}$-loop. Such adverse conformational effects in Glu46 and Asp49 residues of TM4 would be minimal, since these residues are more flexible and lie in the C-terminal loop and are also solvent-accessible (14). Finally, the other possibility is that Ca$^{2+}$ may have a complex electrostatic effect on the conformations of charged residues of the 70–80-loop in the protein C mutants. For instance, in addition to three basic residues, the 70–80-loop of protein C also has four acidic residues, two of which (Glu70 and Glu80) are involved in ligating the Ca$^{2+}$ ion. It is likely that, in the absence of Ca$^{2+}$, the acidic and basic residues of this loop are in random and disordered conformations and that the binding of the metal ion to the 70–80-loop leads to the internalization of acidic residues to ligate Ca$^{2+}$ and exposure of basic residues to facilitate their interaction with TM4 in the thrombin-TM complex. Thus, reversing the charges of Arg74 and Arg75 could interfere with such electrostatic steering of this loop by the Ca$^{2+}$ ion. In agreement with this hypothesis, previous spectroscopic studies have indicated that binding of Ca$^{2+}$ to the 70–80-loop of GD-PC is associated with a conformational change in the loop that is detected by quenching of the intrinsic fluorescence of two Trp76 and Trp79 residues in this loop (residues 231 and 234 in the protein C numbering) (35). Ca$^{2+}$ plays an intriguing role in protein C activation by thrombin. Whereas the metal ion is required for protein C activation by thrombin in the presence of TM, it inhibits the activation by thrombin alone (6). Based on the molecular model of protein C activation by the thrombin-TM456 complex, it has been proposed that the unique Ca$^{2+}$ dependence of protein C activation by thrombin may be the result of direct contacts between TM4 side chains and protein C residues that coordinate Ca$^{2+}$ (particularly between Arg385 of TM4 and Glu80 of protein C) (15). The observation that the Ca$^{2+}$ dependence of protein C activation by thrombin in complex with several mutants of TM456 (D349K, E357K, D398K, and D400K) was completely eliminated supports this hypothesis. However, the results do not agree with the proposal that interactions between Arg385 of TM and Glu80 of protein C account for this phenomenon, since protein C activation with the R385E mutant of TM456 remained Ca$^{2+}$-dependent. Moreover, we previously demonstrated that the substitution of Glu80 of protein C with Lys results in a protein C mutant whose activation by the thrombin-TM456 complex is independent of Ca$^{2+}$ (19). Thus, the unique Ca$^{2+}$ dependence of protein C activation is mediated by direct contacts between acidic residues of TM4 and basic residues of protein C (particularly Arg74 and Arg75). This is supported by the observation that the activation of both R74E and R75E mutants of protein C was severely impaired and that Ca$^{2+}$ did not stimulate the reaction.

It is known that TM activates the catalytic efficiency of thrombin toward activation of protein C in the presence of Ca$^{2+}$ by 3 orders of magnitude (6). Such an improvement in the rate of protein C activation by thrombin in the presence of TM is accounted for both by a 1-order of magnitude decrease in $K_m$ and a 2-order of magnitude increase in $V_{\text{max}}$. The mechanism by which TM improves both kinetic constants of protein C activation by thrombin in the presence of Ca$^{2+}$ is not known. Results of this mutagenesis study suggest that the phospholipid-independent improvement in the $K_m$ of activation is pri-
marily mediated by an interaction between the basic exosite of protein C and acidic residues of TM4. This is derived from the observation that the $K_m$ of activation was impaired with all mutants of TM456. Based on results presented, we believe that the Ca$^{2+}$ stabilization of the 70–80-loop of protein C culminates in the exposure of basic residues required for interaction with TM4 and that such an interaction leads to a 1-order of magnitude improvement in $K_m$. However, the result of this study does not provide insight into how TM improves the $k_{cat}$ of protein C activation by thrombin. The observation that the $k_{cat}$ values for the GD-PC activation by thrombin in complex with all mutants of TM456 were comparable, with only $K_m$ values being impaired suggests that the interaction of basic residues of protein C exosite with the acidic residues of TM4 may not contribute to the $k_{cat}$ of the activation reaction. Thus, the open question is how does TM improve the $k_{cat}$ of protein C activation by thrombin? Previously, it was hypothesized that TM456 may induce conformational changes in the active site pocket of thrombin that leads to preferential improvement in the catalytic efficiency of thrombin toward protein C activation (6). Although numerous kinetics (36), mutagenesis (37), and spectroscopic (38) studies have supported this hypothesis, no significant structural rearrangement in the active site pocket of protein C activation by thrombin in the presence of Ca$^{2+}$ is needed to understand exactly how TM improves the $k_{cat}$ of protein C activation by thrombin in the presence of Ca$^{2+}$. In summary, our results clearly show that TM4 in the thrombin-TM456 complex provides a binding site for interaction with the basic exosite of protein C. The interaction of the basic exosite of protein C with acidic residues of TM4 improves the $K_m$ of activation in the presence of Ca$^{2+}$, and this interaction also accounts for the unique Ca$^{2+}$ dependence of protein C activation by the thrombin-TM complex.

Acknowledgment—We thank Audrey Rezaie for proofreading of the manuscript.

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