A key regulator of the coagulation process is activated protein C (aPC), which exerts its effects by proteolytically inactivating two cofactors involved in the clotting cascade, factors Va and VIIIa (1, 2). The physiological importance of aPC is shown by the thrombophilia associated with deficiencies in protein C, deficiencies in its cofactor (protein S), or mutations in its substrate (factor V Leiden) which make it resistant to cleavage by aPC (reviewed in Ref. 3).

Protein C circulates as an inactive zymogen whose conversion to its activated form is mediated by thrombin complexed to the endothelial cell membrane protein, thrombomodulin (TM). At physiological calcium levels, protein C is a poor substrate for activation by free thrombin, in part, because of a calcium-induced conformational change in PC. Rezaie et al. (4) have recently identified human protein C’s calcium-binding region as being comprised of protease domain residues Glu97 through Glu99. Binding of calcium to this surface loop alters protein C structure such that acidic P3 and P9 residues surrounding the cleavage site make unfavorable contacts with thrombin’s substrate-binding region. When thrombin binds to TM, its active site undergoes a “compensating” conformational change which reduces the inhibitory influence of protein C’s acidic P3/P9 residues (5–9). While the TM-induced changes in thrombin’s active site and the negative influence of these acidic P3/P9 residues are clearly important, they do not appear to completely account for the dramatic increase in protein C activation compared to that by free thrombin. Even under optimal conditions, for protein C activation by free thrombin (i.e. in the absence of calcium), the activation rate is still approximately 2 orders of magnitude slower than the activation rate by the TM-thrombin complex at physiological calcium levels.

Alignment of the amino acid sequences for the protease domains of numerous serine proteases serves to identify highly conserved and nonconserved regions (see Fig. 1). The conserved regions likely provide for a common structural framework, while nonconserved or “variable” residues impart properties unique to each molecule. In this study, we have focused on sequences in a region of the protease domain of protein C corresponding to variable region 1 (VR1) of the serine proteases. Interestingly, in the linear sequence of human protein C, VR1 is located between the activation peptide/cleavage site and the calcium-binding loop, two regions known to be involved in activation. The most striking feature of VR1 in protein C is the cluster of positively charged lysine residues (protease domain residues Lys97–99). Molecular modeling of PC by several groups (13–15) has suggested that Lys97–99 reside in a surface-exposed loop whose high concentration of positive charge is likely involved in protein-protein interactions. Therefore, we have analyzed what role the conserved tribasic Lys97–99 charge center might play in PC activation.

![Image](https://via.placeholder.com/150)

![Image](https://via.placeholder.com/150)
Activation of Protein C

**RESULTS AND DISCUSSION**

Expression and Preliminary Analysis of Lys37–39 Protein C Derivatives—Using site-directed mutagenesis, we changed the cluster of positively charged lysine residues (Lys37–39) in VR1 of human protein C’s protease domain to negatively charged amino acids by substitution with Asp37–Glu38–Asp39 (DED) and Glu37–Glu38–Glu39 (EEE) or to neutrality by substitution with Gly37–Gly38–Gly39 (GGG). Each of the derivatives was expressed and isolated as described under “Experimental Procedures.” We observed no significant effect of the Lys37–39 mutations on the secretion or processing of the protein from the 293 cell line. Purification of PCs by the pseudoaffinity method of Yan et al. (20), which selects for completely carboxylated form, resulted in equivalent recoveries (~90%) for wild-type and mutant PCs. SDS-PAGE analysis of zymogen and completely activated forms of each molecule (Fig. 2) shows similar patterns for the light and heavy chains, with all molecules exhibiting the typical α, β, and γ-glycoforms of the heavy chain. Likewise, there was no apparent effect of these mutations on the removal of the Lys-Arg dipeptide (which separates the light and heavy chains of PC) as there was little single chain material present in any of the zymogen molecules. In addition, activation of each molecule by thrombin was accompanied by a typical shift in mobility of the heavy chain glycoform.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant soluble human thrombomodulin (TMD1, CS, and CS ) was prepared essentially as described previously (16). Thrombin-Sepharose 4B was provided by Josephine Secnik of Eli Lilly & Co. All other chemicals used were of the highest purity commercially available.

Site-directed Mutagenesis and Isolation of HPC—Thrombin-Sepharose 4B was washed extensively with Buffer A, and then 300 μg of HPC in 1 ml of the same buffer was incubated with 200 μl of packed thrombin-Sepharose 4B for 4 h at 37 °C on a rotating platform. The course of this incubation, the degree of HPC activation was monitored by briefly pelleting the thrombin-Sepharose 4B and assaying an aliquot of the supernatant for aPC amidolytic activity using the chromogenic substrate S-2366. Following complete activation, the thrombin-Sepharose 4B was pelleted, the supernatant was collected, its protein concentration was verified by Pierce BCA assay, and aPC either was assayed directly or frozen in aliquots at −80 °C. The zymogen and activated forms of protein C were analyzed by SDS-PAGE (18).

Activation with Free and Tm-bound Thrombin—Reaction conditions were as described in Fig. 3 and Table I. Aliquots were removed at selected times and added to a 40-fold excess of hirudin (serving both to halt the activation reaction as well as quench background chromogenic activity arising from thrombin), then amidolytic activity and aPC generation were determined as described below.

Amidolytic Activity of aPC—The amidolytic activity of the recombinant aPCs was determined by hydrolysis of the tripeptide substrate Glu-Pro-Arg-p-nitroanilide (S-2366). Reactions were performed at 25 °C in Buffer A containing 1 mg/ml BSA, 3.0 to 3.5 mM CaCl2, and an initial concentration of 500 μM S-2366 chromogenic substrate. Assays were performed in a 96-well microtiter plate and amidolytic activity was measured as the change in absorbance units/min at 405 nm as monitored in a ThermoMax kinetic microtiter plate reader (Molecular Devices). Amounts of aPC formed were determined by comparison with standard curves generated using fully activated material. All data points were limited to cleavage of 15% or less of the chromogenic substrate. For determination of the kinetics of tripeptide-pNA substrate hydrolysis, reactions were performed at 25 °C with 0.5 nM aPC in Buffer A containing 1 mg/ml BSA, 3.0 mM CaCl2, and varying initial concentrations of chromogenic substrate (S-2366, S-2288, S-2238, S-2320, or S-2266). Reactions (200 μl/well) were carried out in a 96-well microtiter plate, and optical density at 405 nm was monitored as above. Kinetic constants were derived according to the Eadie-Hofstee Transformation (Molecular Devices Technical Applications Bulletin 008-A) using a path length of 0.13 cm (Molecular Devices Technical Applications Bulletin 4-1) and an extinction coefficient for pNA at 405 nm of 9620 m−1 cm−1 (19).

**FIG. 1.** Schematic representation of protein C and partial alignment with a serine protease consensus sequence. In the schematic, the following components of zymogen protein C are depicted: hatched boxes, light chain (Gla and epidermal growth factor domain factors) and heavy chain (serine protease domain) linked by a disulfide bond (S-S); solid box, the Lys-Arg dipeptide removed during cellular processing/secretion to generate the two-chain molecule; open box, activation peptide on the N terminus of the heavy chain; and oval, calcium-binding loop. Also shown is a partial alignment of residues in protein C from human (10), bovine (11), rabbit (B. Dahlback, personal communication), and murine (12) sources with the corresponding residues of a serine protease domain consensus sequence (amino acids 28–47, chymotrypsinogen numbering). Below the consensus sequence is an alignment of the same region in a number of other human serine proteases. Residues identical to the serine protease domain consensus sequence are outlined and shaded. The strictly conserved cluster of basic residues in protein C (Lys37–39) is also indicated (outlined, +).
the Lys37–39 charge center is in reasonably close proximity in molecular models of PC, the VR1 loop contains a wide variety of residues in this “variable” surface loop. Date indicated in Fig. 1 which indicates that serine proteases can accommodate a wide variety of residues in this “variable” surface loop.

Calcium Dependence of Activation by Free and TM-bound Thrombin—In molecular models of PC, the VR1 loop containing the Lys37–39 charge center is in reasonably close proximity to a calcium-binding loop (protease domain residues Glu70, Glu80) previously shown to be important for PC activation (4). The ligation of calcium by this surface loop results in a conformational change in PC which inhibits activation by free thrombin while favoring TM-thrombin activation. Thus, if alteration of the Lys37–39 charge center was to affect calcium-binding per se, one might expect to see changes in the calcium dependences for activation by both free and TM-bound thrombin. For activation with free thrombin (Fig. 3A), wild-type and mutant PCs displayed identical calcium inhibition profiles, with a half-maximal activation rate at approximately 0.13 mM CaCl2. These results suggest that there is no local disruption of PC’s ability to bind calcium, nor in the resulting conformational changes previously shown to be inhibitory for activation by free thrombin.

We next examined the calcium dependence for activation by the TM-thrombin complex (Fig. 3B) using chondroitin sulfate-modified (CS-modified) recombinant soluble human thrombomodulin (sTM). Limited activation by the sTM-thrombin complex was achieved in the absence of calcium, whereas activation rates increased with increasing calcium, finally plateauing as the calcium concentration reached physiological levels. In striking contrast, the Lys37–39 mutants all displayed poor activation by the CS-modified sTM-thrombin complex, regardless of calcium concentration. For the neutral substitution mutant (GGG), a stimulation of activation by the sTM-thrombin complex was noted with increasing calcium concentrations; however, the maximal rate reached a plateau value far below that of the wt-HPC activation rate. Thus, the alterations in the VR1 of protein C either eliminated or substantially reduced activation by the TM-thrombin complex, without altering activation by free thrombin.
Molecular modeling studies (14, 15) and experimental observations in our laboratory3 have suggested that the basic Lys37–39 charge center in PC might contribute to interactions with negatively charged glycosaminoglycans. Therefore, we examined whether the reductions in TM-thrombin activation were dependent on the presence of chondroitin sulfate on TM. Calcium dependence experiments employing CS-free sTM displayed similar reductions in TM stimulation as was shown in Fig. 3B with CS-modified sTM (data not shown). As shown in Table I, for wild-type and mutant PCs, there was little difference in activation rates at 3 mM calcium when using either CS-modified or CS-free sTM. Thus, it appears that the Lys37–39 charge center is not involved in interactions with the CS moiety of TM.

We also determined activation rates with CS-free sTM at 0.3 mM calcium, conditions which result in an approximately 3-fold higher activation rate (compared with that of the chondroitin-free sTM-thrombin complex at 3 mM calcium). This increased activation rate has previously been shown to be mediated by the Gla domain of protein C and the unoccupied anion-binding exosite 2 of thrombin (21). Although all substitutions of the Lys37–39 charge center either reduced or eliminated sTM-dependent stimulation of activation, activation of wild-type and mutant PCs with the CS-free sTM-thrombin complex at 0.3 mM calcium resulted in an approximately 3-fold higher activation rate compared with that at 3.0 mM calcium (Table I, D). Thus, the changes in VR1 had no effect on the Gla domain-mediated stimulation of activation rate at low calcium.

The major finding of this study is that mutation of the Lys37–39 charge center in VR1 of PC’s protease domain substantially reduces activation by the TM-thrombin complex, without affecting activation by free thrombin. Thus, it appears this basic charge center is integral to structural features in protein C that are recognized by the TM-thrombin complex but not used by free thrombin. The simplest mechanistic explanation for our results would be that the basic Lys37–39 charge center contributes directly to interaction the TM-thrombin complex via electrostatic attraction. Alternatively, the Lys37–39 charge center might be involved in the correct presentation of some other structural element in PC or a conformational change in PC induced by TM. Regardless of the exact mechanism, it is clear that the Lys37–39 charge center of protein C is critical for efficient recognition by the TM-thrombin complex. The strict interspecies sequence conservation for these VR1 amino acids in PC, which reside in a region of considerable diversity among different proteases, would also seem to support an important functional role. Although several studies have demonstrated a role for protease domain VR1 residues as they occur in the context of activated serine proteases (22–28), our results are unique in that they demonstrate that VR1 residues within the zymogen (substrate) form of a serine protease are essential for recognition by its physiological activator.

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Mutation of Protease Domain Residues Lys$^{37-39}$ in Human Protein C Inhibits Activation by the Thrombomodulin-Thrombin Complex without Affecting Activation by Free Thrombin

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