Site-directed mutagenesis of the 40 N-terminal residues (γ-carboxyglutamic acid domain) of blood clotting factor VII was carried out to identify sites that improve membrane affinity. Improvements and degree of change included P10Q (2-fold), K32E (13-fold), and insertion of Tyr at position 4 (2-fold). Two other beneficial changes, D33F (2-fold) and A54E (1.5-fold), may exert their impact via influence of K32E. The modification D33E (5.2-fold) also resulted in substantial improvement. The combined mutant with highest affinity, (Y)4P10Q/K32E/D33F/A54E, showed 150–296-fold enhancement over wild-type factor VIIa, depending on the assay used. Undercarboxylation of Glu residues at positions 33 and 34 may result in an underestimate of the true contributions of γ-carboxyglutamic acid at these positions. Except for the Tyr4 mutant, all other beneficial mutations were located on the same surface of the protein, suggesting a possible membrane contact region. An initial screening assay was developed that provided a faithful evaluation of mutants in crude mixtures. Overall, the results suggest features of membrane binding by vitamin K-dependent proteins and provide reagents that may prove useful for research and therapy.

Interaction between the vitamin K-dependent plasma proteins and a membrane surface is essential for hemostasis (1, 2). This interaction is mediated through contact of the γ-carboxyglutamic acid (Gla) domains of these proteins with membranes containing acidic phospholipids (3, 4). The Gla domain consists of ~40 N-terminal residues of which 9–12 glutamic acid residues are post-translationally modified to Gla (5–7). In the presence of calcium, the Gla domain adopts structure and binds to membranes by a mechanism that is not fully understood.

The Gla domains of the vitamin K-dependent plasma proteins show striking sequence homology, yet have quite different affinities for phospholipid membranes (8). If the Gla domain constitutes the membrane contact region, individual amino acid residues within this domain should contribute to these different affinities. This suggestion has been supported by mutations of the Gla domain that affect membrane affinity. For example, the P10H mutant of bovine protein C shows an ~10-fold enhancement in membrane affinity, whereas the HI10P mutant of protein C shows an ~3-fold decline in membrane affinity (9). Although a major difference among the vitamin K-dependent proteins is a Gla residue versus another amino acid at position 32, the Q32E mutation of protein C has no impact on membrane affinity (10, 11). Similarly, replacement of Glu12 in factor X has little impact on protein function (12), and Glu32 of human prothrombin was described as only moderately important (13), having little impact on either K_m or V_max of the prothrombinase reaction. However, a double mutant of human protein C, S11G/Q32E, shows an ~10-fold enhancement in membrane affinity (11), and a double mutant of factor VII, P10Q/K32E, has a 25-fold enhancement in membrane affinity (14). Under appropriate conditions, these proteins show similar improvement in function (11, 14–16).

Although previous studies showed that proteins with enhanced membrane affinity can be created, the contribution of individual amino acid residues was not determined, and possible improvement by changes in other sites was not shown. This study used human factor VII (FVII) as a model Gla domain to investigate these questions and presents a simple functional assay that allows evaluation of minute quantities of impure protein as an initial screen of mutant protein activity. Single-site mutants as well as those with the highest affinity were purified and characterized. The results indicate that nearly all functional improvements arose from membrane binding affinity. The largest enhancements in membrane affinity resulted from mutation in the region of residue 32, a position that is located quite far from the N-terminal end of the protein, where membrane contact is generally suggested (39). The mutant with highest function, (Y)4P10Q/K32E/D33F/A34E, showed 150–296-fold improvement over wild-type FVII. Overall, the proteins described in this study contribute to a better understanding of the membrane contact site and the binding forces involved while providing novel reagents to probe coagulation reaction mechanisms that may be of use in therapy.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**Cloning and mutagenesis were performed by ATG Inc. (Eden Prairie, MN) following standard procedures (17). Human FVII cDNA was cloned from a human liver cDNA library and then subcloned into the vector pRC-CMV. Mutagenesis was verified by sequencing of the entire Gla domain of all variant FVII proteins, including untranslated pre- and propeptide segments. Proteins were expressed in fetal human kidney 293 cells that were stably transfected using the agent LipofectAMINE™ 2000 (Invitrogen) following the manufacturer’s instructions. Following previously outlined procedures (9), Geneticin-resistant colonies were selected, and high pro-

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The abbreviations used are: Gla, γ-carboxyglutamic acid; FVII, factor VII; WT, wild-type; WT-FVIIa, active-site-blocked wild-type factor VIIa; TF, tissue factor; dasyI, 5(dimethylaminomethyl)phenyl-1-sulfon-yl; PS, phosphatidylserine; PC, phosphatidylcholine; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
during cloning were grown to confluence in three layered flasks (Nunc) containing Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1.0 mg/ml nonessential amino acids, 50 units/ml penicillin, 10 microgram/ml vitamin K<sub>1</sub> and 100–200 microgram/ml Geneticin. Confluent cells were rinsed with and then cultured in serum-free Dulbecco’s modified Eagle’s medium containing 1.0 mg/ml nonessential amino acids, 10 microgram/ml vitamin K<sub>1</sub> and 0.5 mg/ml benzamidined hydrochloride. EDTA (pH 7.4) and benzamidine hydrochloride were added to conditioned medium intended for purification to concentrations of 5.0 and 2.0 mg/ml, respectively. The conditioned medium was vacuum-filtered twice then eluted with Whatman double-filter paper (qualitative No. 1) and then through a 0.22-mm polyether sulfone membrane (Corning Inc.). If not immediately purified, the filtered medium was stored at −70 °C.

The filtered medium was diluted 1:1 with distilled and deionized water containing 4.0 mM EDTA (pH 7.4) and 2.0 mg/ml benzamidined hydrochloride and then applied to a High Q Macro-Prep anion-exchange column (Bio-Rad). The column was equilibrated prior to loading and washed extensively with Tris buffer (50 mM Tris, 100 mM NaCl, and 0.2% (w/v) NaN<sub>3</sub> (pH 7.4)) containing 2.0 mM EDTA and 2.0 mg/ml benzamidined hydrochloride. The column was eluted isocratically with the same buffer containing 400 mM NaCl and no EDTA.

Eluted fractions containing FVII activity were pooled and divided 1:1 with Tris buffer containing 30 mM CaCl<sub>2</sub> and 2.0 mg/ml benzamidined hydrochloride. The pooled and diluted fractions were subjected to immunoaffinity chromatography using a calcium-dependent anti-human FVII antibody (Biosciences). Eluted fractions containing vesicles with average diameter of 32–38 nm (LSA2 photon correlation spectrometer, Langley Ford Co.) were pooled for use in membrane binding studies. The concentrations of phospholipid were determined by phosphorus analysis (18) using a phospholipid/phosphorus weight ratio of 25:1.

**Light Scattering Measurements**—Protein-membrane binding was determined by relative light scattering at 90° using methods previously described (19). Briefly, for a constant particle concentration and for particles that are small compared with the wavelength of light, the ratio of the light scattering intensity of a protein-vesicle complex (I<sub>S</sub>) to that of the vesicles alone (I<sub>V</sub>) is related to the ratio of the molecular weight of the protein-vesicle complex (M<sub>0</sub>) to that of the vesicles alone (M<sub>V</sub>) by the relationship in Equation 1,

\[
I_S = \frac{M_0}{M_V} \times I_V \quad \text{(Eq. 1)}
\]

where I<sub>S</sub>/I<sub>V</sub> is the change in refractive index as a function of concentration of the light scattering species and was estimated as described (19). Light scattering contributions of the buffer and protein were subtracted to obtain I<sub>B</sub> and I<sub>P</sub>. Values of M<sub>0</sub>/M<sub>V</sub> were estimated at various amounts of added protein and were plotted versus the protein (P)/phospholipid (PL) ratio (w/w). Dissociation constants (K<sub>D</sub>) for protein-membrane binding were calculated from the relationship in Equation 2.

\[
K_D = \frac{[P][PL]}{[P][PL] + [P]_B} \quad \text{(Eq. 2)}
\]

The amount of free protein ([P]<sub>B</sub>) was estimated from the known weight concentrations of phospholipid and protein in the solution and the difference between M<sub>0</sub>/M<sub>V</sub> and the theoretical value of M<sub>0</sub>/M<sub>V</sub> if all of the added protein were bound. Weight concentrations of bound and free FVII were converted to molar concentration with a M<sub>0</sub> of 50,000 for all FVII variants.

**Matrix-assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Analysis**—MALDI-TOF mass spectrometry was used to confirm the identity of the variant FVII proteins tested and to assess the extent of carboxylation. Purified proteins were incubated at 37 °C for 30 min in the presence of either chymotrypsin or trypsin at a ratio of 500:1 (w/w) FVII protein/protease. Reaction solutions were dried by vacuum centrifugation and solubilized in 5:95 acetonitrile/water solution containing 0.1% trifluoroacetic acid. The solutions were desalted using reverse-phase chromatography (ZipTip, Millipore Corp.), mixed 1:1 with a saturated matrix solution (5-methoxyanisallic acid in 50:50 ethanol/water solution), spotted on the spectrometer target, allowed to dry, and then subjected to MALDI-TOF mass spectrometry (Biflex® III, Bruker). Minimum laser power was used to obtain spectra. Moderate increases in power above this minimum did not result in changes in the distribution of the various carboxylated species observed. The percentage of each carboxylation species was determined by measurement of peak areas using integration software provided by the spectrometer manufacturer.

**Factor X Activation by FVIIa**—The relative activities of FVIIa variants were determined by a method outlined previously (15). Full activation of FVII was first ensured by incubation with TF (18 pM; Innovin) in 50 μl of Tris buffer containing 5.0 μM CaCl<sub>2</sub> and 1.0 mg/ml bovine serum albumin. The mutant FVII proteins with higher membrane affinity (K32E, P10Q/K32E, P10Q/D33E, and (Y4)P10Q/K32E/D33F/A34E) were added to a final protein concentration of 3.0 nM. WT-FVIIa, afIIa, and afIIa were added to a concentration of 200 nM to initiate the reaction. After 10 min, the reaction was stopped by addition of excess EDTA (15 mM). The concentration of factor Xa was measured by monitoring the absorbance change at 405 nm in a Beckman DU-70 UV-visible spectrophotometer. The amount of FVIIa that was bound to TF was measured using integration software provided by the spectrometer manufacturer.

**Enhancement of the Gla Domain of Human Factor VII**

\[
\log([WT-FVIIa]/[FVIIa] - TF) = \log([WT-FVIIa]/[FVIIa]) + K_{FVIIa}/K_{WT-FVIIa} \quad \text{(Eq. 3)}
\]
where $K_{FVIIai}^P$ is the dissociation constant for the WT-FVIIai/TF complex and $K_{FVIIai}^{SH}$ is the dissociation constant for the FVIIai/TF complex. Comparison of a plot of log([WT-FVIIai]/[FVIIai]) versus log([WT-FVII])/[TF] for two FVIIia variants at an identical and constant FVIIa concentration in an unpurified sample. It was important that TF was present in excess over FVII. Activation of FVII was allowed to proceed to completion (60 min at 37 °C). Activated FVII solution (containing 37.5 nM FVII) was added to Innovin (0.1 ml) in an amount to generate ~0.3 nM FVII. This concentration of pure FVII provided a final coagulation time of 25 s for all samples. Because all FVII variants have the same clotting time in the absence of inhibitor (see pure protein analysis below and Ref. 15), use of a constant clotting time allowed the determination of the FVII concentration in an unpurified sample. It was important that TF was maintained in excess over FVII. Activation of FVII was allowed to proceed to completion (60 min at 37 °C). Activated FVII solution (containing 2.5 μl of FVIIa) plus varying amounts of WT-FVIIa were mixed with Tris buffer containing 8.67 mM CaCl$_2$ and 1.0 mg/ml bovine serum albumin to create 112.5-μl aliquots, which were incubated for 1 h at 37 °C to achieve equilibrium between TF, WT-FVIIa, and FVIIa. Coagulation was initiated by addition of 37.5 μl of prewarmed FVII-deficient plasma. Clotting times (CT) were determined, and results were evaluated by a plot of log[CT/CT$_{0.3}$] versus log([WT-FVIIai]), where CT$_{0.3}$ is the clotting time without inhibitor. Relative function of the different FVIIa variants was estimated by offset of the plots for the two proteins.

**RESULTS**

Analysis of Protein Carboxylation States by MALDI-TOF Mass Spectrometry—The identities of purified recombinant FVII proteins were verified by mass spectrometry of Gla domains released from the intact proteins by limited protease digestion. The Gla domains consisted of either 1–40 (containing all Gla residues) or 1–32 (less one carboxylation site at residue 35) N-terminal residues. Use of the methoxyxysaliclyl acid matrix and the lowest practical laser power resulted in a low level of undecarboxylated peptide species (Fig. 1). In most cases, the fully carboxylated peptide (theoretical m/z of 5235 for the +1 charge state of K32E) was the most abundant peak (Fig. 1A). However, in-source decarboxylation of Gla residues occurred during MALDI-TOF analysis and reached quantitative levels when the sinapinic acid matrix was used (20).

Undecarboxylation was detected by peaks separated by 44 mass units and a small peak corresponding to the fully decarboxylated peptide (m/z 4751) (Fig. 1A). The quantitative distribution among the different species was very consistent for replicate samples as well as for many plasma-derived versus recombinant proteins (20). This consistency suggested that comparison of quantitative MALDI-TOF data could be used to detect relative differences in the carboxylation states of various proteins. For example, repeated measurement of different preparations of plasma-derived bovine factor X gave 77 ± 4% signal intensity in the fully carboxylated peptide. This level of fully carboxylated peptide corresponded to 97–98% carboxylation of all Gla residues. Similar results were obtained for bovine prothrombin and human protein C. Consequently, the somewhat lower yield of the fully carboxylated peptide of recombinant WT-FVIIa (46%) suggested undercarboxylation of the parent protein (Fig. 1B). In fact, undercarboxylation at position 35 of recombinant WT-FVIIa has been previously observed (21, 22). That position 35 of recombinant WT-FVIIa was the major site of undercarboxylation was also suggested by MALDI-TOF analysis of peptide 1–32, which gave a high yield of the fully carboxylated state (70%) (Fig. 1B). Undercarboxylation at position 35 of FVII and 10.7 of 11 theoretical residues for P10Q/D33E and 10.9 of 11 theoretical residues for P10Q/K32E (Table I). Undercarboxylation at position 35 of recombinant WT-FVIIa, it was possible that the additional Glu

**FIG. 1.** MALDI-TOF mass spectrometry of Gla domains. Recombinant FVII proteins were digested with either chymotrypsin or trypsin (500:1, w/w) at 37 °C for 30 min. The peptides were desalted by the ZipTip procedure, mixed with matrix, and subjected to MALDI-TOF mass spectrometry. A, a representative spectrum for K32E. B, amino acid sequences and yield of different carboxylation states. Shaded residues indicate the sites of mutation and the amino acids that have been incorporated. Gla residues, converted from glutamic acid, are indicated by X. The percentages of the fully carboxylated peptides or peptides minus one carboxyl group (m/z ~44) or minus two carboxyl groups are also shown. Observed carboxylation versus theoretical number of Gla residues is shown in the last column. Q10, P10Q; E32, K32E; Q10E32, P10Q/K32E; Q10E33, P10Q/D33E; Y4Q10E32F33E34, (Y4P10Q/K32E/D33F/A34E).
residues at positions 33 and 34 were undercarboxylated as well. If correct, the functional impact of Gla residues at positions 33 and 34, detected in the following studies, may underestimate the true impact of Gla at these positions.

Interaction of Purified FVII Variants with Phospholipid Vesicles—When small unilamellar vesicles of 25:75 PS/PC were used to measure protein binding, the variant proteins displayed increasing membrane affinity in the order WT-FVII < P10Q < K32E (Fig. 2A). Mutants with higher affinity (K32E) bound at the theoretical limit (Fig. 2A), so equilibrium binding constants could not be estimated. Because binding affinity is dependent on PS content of the membrane, use of a lower PS content (10:90 PS/PC) (Fig. 2B) provided an equilibrium of bound and free protein for most mutants. Affinity increased in the order WT-FVII < P10Q < K32E < P10Q/D33E < P10Q/K32E. Dissociation constants estimated from these results are reported in Table I. The $K_D$ values obtained for P10Q/K32E (0.16 ± 0.08 μM) compared well with the value of 0.22 μM for small unilamellar vesicles of 10:90 PS/PC reported by Shah et al. (14). Even lower PS content was needed to estimate the binding constant for the highest affinity mutant (5:95 PS/PC) (Fig. 2C). Estimated $K_D$ values indicated a 3-fold enhancement in membrane affinity for the (Y4)P10Q/K32E/D33F/A34E mutant over the P10Q/K32E variant.

Activation of Factor X—Functional evaluation of the FVIIa mutants was carried out in a purified system that detected factor X activation. Experiments were performed under equilibrium competition conditions in which the FVIIa variants must compete with inhibitor, WT-FVIIai, for TF (described in Ref. 15). To allow comparison of results for different proteins, the FVIIa species and WT-FVIIai concentrations were maintained in great excess over TF, so the total FVIIa and FVIIai concentration approximated the respective free concentration. The ability of the lowest affinity FVIIa variants (10 nM) to displace WT-FVIIai increased in the order WT-FVIIa < P10Q < K32E (Fig. 3A). Mutants with higher function were evaluated at 3 nM and gave increasing affinity in the order K32E < P10Q/D33E < P10Q < K32E < (Y4)P10Q/K32E/D33F/A34E (Fig. 3B). The WT-FVIIai concentrations required to reach 50% inhibition (FVIIai/TF/FVIIa:TF = 1:0) are reported in Table I. In agreement with the higher affinity of WT-FVIIai for TF (15, 24, 25), its concentration at 50% inhibition was lower than that of WT-FVIIa.

The data in Fig. 3 are presented in the manner of a Hill plot. The plots have slopes of ~1.0, as anticipated. Validity of this analysis was supported by titration at different FVIIa levels. For example, a 3.4-fold difference in inhibitor concentration (Table I) was observed for titration of K32E at 3 nM (Fig. 3B) versus 10 nM (Fig. 3A). The concentration of WT-FVIIa at the midpoint of each titration curve was used to estimate the relative binding affinity of FVIIa variants. The K32E, P10Q/K32E, and (Y4)P10Q/K32E/D33F/A34E mutants displayed 13.8-, 45-, and 149-fold increases in activity over WT-FVIIa, respectively. If the sites make independent contributions to membrane affinity, the 13.8-fold enhancement for K32E and the 3.1-fold enhancement for P10Q would suggest a 42.8-fold enhancement for P10Q/K32E. This was very nearly the value that was observed. Thus, this functional assay suggested that the P10Q and K32E modifications functioned in a manner that was independent of each other.

Overall, the results of the functional assay in the purified system under conditions in which the protein ligands (FVIIa and WT-FVIIai) were in large excess over TF mirrored the differences in membrane affinity observed in the phospholipid binding studies (Fig. 2). It appeared that all improvements in function arose from changes in the membrane contact site.

Functional Evaluation by Coagulation—Coagulation assays were conducted under conditions in which TF was more abundant than FVIIa. To compare the relative function of different FVIIa variants versus inhibition by WT-FVIIai, the concentration of free FVIIai needed to approximate the concentration of total FVIIai. Low affinity variants such as WT-FVIIa were displaced at low inhibitor concentration, at which most of WT-FVIIai was bound to TF. Consequently, comparison of protein function by this assay was limited to FVIIai variants with high affinity. This included K32E and better. These variants were displaced at WT-FVIIai concentrations that greatly exceed the TF concentration, so total WT-FVIIai approximated free WT-FVIIai in the solution.

The coagulation assay showed the same sequence of function observed by other measurements: K32E = P10Q/D33E < P10Q/K32E < (Y4)P10Q/K32E/D33F/A34E (Fig. 4, A and B). Comparison of inhibitor levels needed to increase clotting time by 60% (log(C/CT)) = 0.2) suggested that (Y4)P10Q/K32E/D33F/A34E had up to 6.9-fold higher function than P10Q/D33F/A34E.
Use of this value and the enhancement of P10Q/K32E over WT-FVIIa (43-fold) resulted in a total enhancement for (Y4)P10Q/K32E/D33F/A34E of 296-fold (Table I).

This coagulation assay appeared useful for screening mutants in conditioned media (Fig. 4B). In fact, results obtained using this crude protein source were very reproducible for different batches of media and were indistinguishable from the results obtained for purified proteins (Fig. 4A). Screening of FVIIa variants in conditioned media was used as a facile first method to estimate protein function to identify the best proteins for purification. This assay appeared amenable to high throughput analysis and might be used in future studies to identify beneficial mutants from large libraries of cells expressing proteins with different modifications. A logical modification would be to quantify FVIIa activity by measuring the product, factor Xa, by amidolytic assay.

TABLE I

| Impact of mutagenesis on FVII activity and membrane affinity |
|-------------------------------------------------------------|
| Factor X activation (relative function) | Clotting assay | Potassium dissociation constant ($K_D$) | Change in $K_D$ |
| --- | --- | --- | --- |
| WT-FVII | 0.8 (1.0) | ND (1.0) | ND | 5.8 ± 0.6 (1.0) | ND | 0.0 |
| P10Q | 2.4 (3.1) | ND | ND | 3.2 ± 0.3 (1.8) | ND | -0.52 |
| K32E | 10.4 (43) | 0.31 (43) | 0.25 | 0.16 ± 0.08 (36) | 1.5 ± 0.3 (36) | -2.06 |
| P10Q/K32E | 3.8 (16) | 0.15 (21) | 0.19 | 0.48 ± 0.08 (12) | ND | -1.52 |
| (Y4)P10Q/K32E/D33F/A34E | 35 (146) | 1.2 (166) | 2.9 (296) | ND | 0.6 ± 0.2 (95) | -2.8 to -3.5 |

- WT-FVIIai concentration at 50% inhibition of 10 nM (lightface) and 3.0 nM (boldface) factor VIIa. Values in parentheses indicate the -fold change relative to WT-FVIIa.
- Inhibitor concentration required to increase clotting time by 60% (log(CT/CT$_0$) = 0.2). Fold changes in mutant activity relative to WT-FVIIa are in parentheses and are based on a 43-fold improvement of P10Q/K32E (first column).
- Values of the average of the $K_D$ values determined from each titration point. Values in parentheses indicate the -fold change relative to WT-FVIIa.
- Values determined from the difference in $K_D$ for WT-FVII and variants.

K32E. Use of this value and the enhancement of P10Q/K32E over WT-FVIIa (43-fold) resulted in a total enhancement for (Y4)P10Q/K32E/D33F/A34E of 296-fold (Table I). This coagulation assay appeared useful for screening mutants in conditioned media (Fig. 4B). In fact, results obtained using this crude protein source were very reproducible for different batches of media and were indistinguishable from the results obtained for purified proteins (Fig. 4A). Screening of FVIIa variants in conditioned media was used as a facile first method to estimate protein function to identify the best proteins for purification. This assay appeared amenable to high throughput analysis and might be used in future studies to identify beneficial mutants from large libraries of cells expressing proteins with different modifications. A logical modification would be to quantify FVIIa activity by measuring the product, factor Xa, by amidolytic assay.

The Tyr$_4$, D33F, and A34E mutants were introduced sequentially into P10Q/K32E to estimate their individual impact.

**FIG. 3.** Factor X activation by FVIIa variants. Analysis of low (A) and higher (B) affinity proteins. In A, 10 nM WT-FVIIa (●), P10Q (□), or K32E (●) was added to a solution containing 18 pM TF (Innovin), 5.0 mM CaCl$_2$, and 1.0 mg/ml bovine serum albumin in Tris buffer. In B, 3.0 nM K32E (●), P10Q/K32E (○), P10Q/D33E (△), or (Y4)P10Q/K32E/D33F/A34E (■) was added to the same solution. After an incubation time that assured full activation of FVIIa in the solution (30 min), WT-FVIIa was added at the concentrations shown, and the reactions were allowed to equilibrate for 2 h at 37 °C. Factor X was added to a final concentration of 200 nM, and the incubation was continued for 10 min. The reaction was halted by addition of excess EDTA (15 mM, pH 7.4), and factor Xa activity for the chromogenic substrate S-2222 was measured. Activity was converted to the ratio of FVIIai:TF to FVIIa:TF and plotted. All experiments were performed in triplicate and S.D. are shown.

**FIG. 4.** Clotting activity of FVII variants from purified (A) and unpurified (B) sources. FVIIa proteins K32E (●), P10Q/K32E (○), P10Q/D33E (△), and (Y4)P10Q/K32E/D33F/A34E (■) were activated by incubation with Innovin for 1 h. The activation mixture was diluted to ~5.5 pM FVIIa and 45 pM TF, and various levels of WT-FVIIa were added (112.5-μl total volume). After sufficient time to allow equilibrium binding (1 h at 37 °C), 37.5 μl of prewarmed FVII-deficient plasma was added to initiate the clotting reaction. Experiments were performed in triplicate, and all data points plotted.
These proteins were evaluated only in the preliminary screening test. The result showed that the Tyr4 insertion conferred a 2-fold functional enhancement (data not shown) and that this was independent of other changes in the Gla domain. Introduction of D33F also increased the function of P10Q/K32E by 2-fold. Incorporation of D33F into WT-FVIIa was not attempted, so it was not possible to determine whether this change was independent of other changes. Introduction of A34E into proteins that did not contain K32E had no detectable influence on protein function, but showed a 1.5-fold increase in function when introduced into P10Q/K32E/D33F. The order of observed enhancements of function generally followed changes in membrane affinity, but appeared to slightly exceed changes in Kd values (Table I).

**DISCUSSION**

Human FVII displays one of the lowest affinities for phospholipid membranes among the vitamin K-dependent plasma proteins (8). Modifications within the Gla domain of FVII (P10Q/K32E) have been shown to enhance membrane affinity (14) and, under appropriate conditions, to increase function of the protein (15). This study identified several new sites that also enhance membrane affinity and protein activity. Mutations were designed to test the independence and relative contribution of each site. A simple assay, amenable to screening large numbers of variant proteins, that used unpurified FVIIa was shown to faithfully report changes in activity due to mutagenesis of the Gla domain.

The interactions responsible for binding of the Gla domain of vitamin K-dependent plasma proteins to phospholipid membranes are not fully understood. The location of amino acids that enhance affinity as well as the magnitude of the changes can offer information about the membrane contact site and binding mechanism. All but one of the beneficial changes reported here were clustered on the same surface of the Gla domain (Fig. 5), suggesting a possible membrane contact surface.

The exception was the Tyr4 insertion, which is located on nearly the opposite surface in the protein, adjacent to the N-terminal structure referred to as the ω-loop (26). Insertion of an additional hydrophobic residue at position 4 was motivated by sequence comparison with bovine protein Z, a vitamin K-dependent protein with very high membrane affinity. The ω-loop of the Gla domain provides three solvent-exposed hydrophobic side chains (Phe4, Leu5, and Leu8 for human FVII) (Fig. 5) (27). Although this region in bovine prothrombin fragment 1 is a contact point in the crystal (26) and may be subject to some distortion, these hydrophobic groups have been suggested to constitute a central feature of the membrane-binding site (28–30). Insertion of tyrosine (or phenylalanine; data not shown) at this position resulted in a 2-fold increase in protein function. One possible explanation for enhanced membrane binding is contact of the Tyr4 side chain with the hydrophobic region of the phospholipid membrane. However, hydrophobic insertion by the ω-loop has been questioned on many grounds (3, 31–35). The impact of the Tyr4 insertion was small (2-fold; ΔΔG = 0.4 kcal/mol) compared with the free energy transfer for a large hydrophobic group such as the phenyl side chain from aqueous solution (to octanol; ΔΔG = −3.6 kcal/mol for ΔKd = 400-fold) (36). The small impact of the Tyr4 insertion suggested an indirect role in membrane association.

Replacement of Pro33, a residue present only in vitamin K-dependent proteins displaying low membrane affinity, also increased protein affinity by 2–3-fold. Assay by the preliminary screen showed that any of a number of amino acid substitutions (Gln, Tyr, Glu, Leu, or Asn) at this position gave similar enhancement. The small effect of Pro33 replacement and the large range of permissible residues suggested a relatively peripheral effect. Thus, this level of affinity enhancement may result from increased mobility within this region and improved packing of hydrophobic groups near H-bonding networks. Complete removal of a H-bond from an aqueous environment results in a change in free energy of approximately −2.4 to −3.6 kcal/mol (37).

The largest single benefit was provided by introduction of glutamic acid at position 32, producing a 13-fold enhancement as a single independent site (ΔΔG = −1.6 kcal/mol). Basic residues occur at this position in human and bovine FVII. This change in membrane affinity, which resulted in a large improvement in protein function, equates with a free energy change of −15% of the total binding energy for the protein (Kd = 10⁻⁷ M; ΔΔG = −10 kcal/mol). Although not measured as independent changes, it was likely that the D33F (2-fold enhancement) and A34E (1.5-fold enhancement) mutations exerted their impact by indirect means such as by increasing the pKₐ of Gla32, thereby increasing affinity for ion pairs such as calcium binding. Gla32 has been proposed to associate with an extra calcium ion during membrane binding (8). It was reasoned that another Gla residue at position 34 may enhance this interaction, whereas addition of a large hydrophobic side chain in this vicinity may serve to isolate this charge complex from water molecules. The total impact of changes at positions 32–34 (39-fold; ΔΔG = −2.2 kcal/mol) may be sufficient to suggest a direct role for this region in membrane association. This conclusion is necessarily tentative and must be confirmed by additional study.

Completely independent contribution as a result of change at positions 10 and 32 was surprising. In the case of human protein C, simultaneous changes at residues 11 and 32 are required to realize enhancement (11). It is possible that Ser11 of human protein C hinders development of a structure, allowing contact of position 32 with calcium or the membrane.

Another surprising benefit was the D33E mutation. This change introduced a 5.2-fold increase in function over P10Q. Taking into consideration the observed undercarboxylation of the P10Q/D33E mutant, possibly at position 33 (see below), it is possible that Gla32 offers a significantly larger enhancement and may rival the impact of K32E. This was surprising for several reasons. First of all, the charge difference and electrostatic effect of the D33E mutation (Δcharge = −1) were much smaller than those of the K32E mutation (Δcharge = −3). Furthermore, the D33E mutation would leave a cationic group at position 32 and would move the Gla residue relatively far from any of the suggested membrane contact regions. Clearly, additional effort is needed to identify the mechanism and binding site of vitamin K-dependent proteins.
The highest affinity mutant that has been identified to date, (Y4)P10Q/K32E/D33F/A34E, provided 146–296-fold enhancement over WT-FVIIa (ΔΔG = –3.0 to –3.4 kcal/mol). The range of functional enhancement arose from use of different assays. The benefit of P10Q/D33E also differed with these assay methods. It is possible that these method-dependent differences arose from undercarboxylation of these two mutants, producing preparations with different functional states. Assays that used excess FVIIa would detect an average function for the entire preparation, resulting in an overestimate of the function of the low affinity species and an underestimate of the function of the high affinity species.

The coagulation assays also suggested heterogeneity of these preparations. Titration of K32E and P10Q/K32E gave plots with parallel slopes, indicating similar differences between these proteins at all levels of inhibition. Parallel plots would arise if both protein preparations were homogeneous but had these proteins at all levels of inhibition. Parallel plots with parallel slopes, indicating similar differences between the low affinity species and an underestimate of the function of the excess FVIIa would detect an average function for the entire preparation. Assays that used different proteins may allow for investigation of surfaces within this region of the Gla domain encompassing residues 32–34. We have found that amino acid changes within the centering around hydrophobic residues near the N terminus (39), we have found that amino acid changes within the Gla domain encompassing residues 32–34 resulted in the greatest enhancements in membrane affinity (Fig. 5). Access to proteins with a range of function may prove useful in investigation of blood clotting reactions both in vitro and in vivo. The large enhancements obtained may also provide benefit for therapies that are based on the vitamin K-dependent proteins (38).

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REFERENCES

1. Furie, B., and Furie, B. C. (1998) Cell 53, 505–518
2. Kalafatis, M., Swords, N. A., Rand, M. D., and Mann, K. G. (1994) Biochim. Biophys. Acta 1227, 113–129
3. Nelsestuen, G. L., Shaw, A. M., and Harvey, S. B. (2000) Vitam. Horm. 58, 355–389
4. Neuschwander, P. F., and Morrisey, J. H. (1994) J. Biol. Chem. 269, 8097–8013
5. Magnusson, S., Sotrup-Jensen, L., Peterson, T. E., Morris, H. D., and Dell, A. (1974) FEBS Lett. 44, 189–193
6. Nelsestuen, G. L., Zytokovicz, T. H., and Howard, J. B. (1974) J. Biol. Chem. 249, 6147–6150
7. Stenflo, J. (1974) J. Biol. Chem. 249, 5527–5535
8. McDonald, J. F., Shaw, A. M., Schwalbe, R. A., Kisiel, W., Dahlback, B., and Nelsestuen, G. L. (1997) Biochemistry 36, 5120–5127
9. Shen, L., Shah, A. M., Dahlback, B., and Nelsestuen, G. L. (1997) Biochemistry 36, 16025–16031
10. Zhang, L., Jiang, A., and Castellino, F. J. (1992) Blood 80, 842–952
11. Shen, L., Shah, A. M., Dahlback, B., and Nelsestuen, G. L. (1998) J. Biol. Chem. 273, 31086–31091
12. Larson, P. J., Camire, R. M., Long, D., Fasana, N. C., Monroe, D. M., Tracy, P., and High, K. A. (1998) Biochemistry 37, 5029–5038
13. Ratcliffe, J. V., Furie, B., and Furie, B. C. (1993) J. Biol. Chem. 268, 24339–24435
14. Shah, A. M., Kisiel, W., Foster, D. C., and Nelsestuen, G. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4229–4234
15. Nelsestuen, G. L., Stone, M., Martinez, M. B., Harvey, S. B., Foster, D., and Kisiel, W. (2001) J. Biol. Chem. 276, 39825–39831
16. Henderson, N., Key, N. S., Christie, B., Kisiel, W., Foster, D., and Nelsestuen, G. L. (2002) Thromb. Haemostasis 88, 98–103
17. Cornbach, B. (1991) in Current Protocols in Molecular Biology (Ausubel, F. M., ed) pp. 8.5.7–8.5.9, John Wiley and Sons, Inc., New York
18. Chen, P. S., Törbára, T. Y., and Warner, H. (1956) Anal. Chem. 28, 1756–1758
19. Nelsestuen, G. L., and Lim, T. L. (1977) Biochemistry 16, 4164–4170
20. Martinez, M. B., Harvey, S. B., Higgins, L., Krick, T., Shen, T., Kisiel, W., Foster, D., Brown, T., Evans, T. C., Jr., Shah, A. M., and Nelsestuen, G. L. (2001) Proceedings of the 49th Conference on Mass Spectrometry and Allied Topics, Chicago, May 27–31, 2001, Abstr. A011052, American Society for Mass Spectrometry, Santa Fe, NM
21. Jurlander, B., Thim, L., Klausen, N. K., Persson, E., Kjaaki, M., Rezen, P., Jørgensen, T. B., Ostergaard, P. B., Erdhardsen, E., and Bjorn, S. E. (2001) Semin. Thromb. Hemost. 27, 373–383
22. Thim, L., Bjørn, S., Christensen, M., Nicolaisen, E. M., Lund-Hansen, T., Pedersen, A. H., and Hedner, U. (1988) Biochemistry 27, 7765–7770
23. Gillis, S., Furie, B. C., Furie, B., Patel, H., Huberty, M. C., Switzer, M., Foster, B. W., Scoble, H. A., and Bond, M. D. (1997) Protein Sci. 6, 185–196
24. Sorensen, B. B., Persson, E., Feskgard, P., Kjaaki, M., Ehrman, M., Williams, T., and Rao, V. M. (1997) J. Biol. Chem. 272, 11863–11868
25. Dickinson, C. D., and Ruf, W. (1997) J. Biol. Chem. 272, 19875–19879
26. Sornaraj-Garcia, M., Padmanabhan, K., deVos, A. M., and Tulinsky, A. (1992) Biochemistry 31, 2554–2561
27. Banner, D. W., D’Arcy, A., Chene, C., Winkler, F. K., Guha, A., Koningsberg, W. H., Nemerson, Y., and Kirchhofer, D. (1996) Nature 380, 41–46
28. Zhang, L., and Castellino, F. J. (1994) J. Biol. Chem. 269, 3590–3595
29. Sunnerhagen, M., Drakenberg, T., Forsen, S., and Stenflo, J. (1996) Biochemistry 35, 193–200
30. Furie, B. C., Furie, B., Jacobs, M., and Rigby, A. C. (2001) J. Biol. Chem. 276, 23985–23992
31. Mayer, L. D., Nelsestuen, G. L., and Brockman, H. L. (1983) Biochemistry 22, 316–321
32. Mayer, L. D., Pusey, M. L., Greip, M. A., and Nelsestuen, G. L. (1983) Biochemistry 22, 6226–6232
33. Evans, T. C., Jr., and Nelsestuen, G. L. (1996) Biochemistry 35, 8210–8215
34. McDonald, J. F., Evans, T. C., Jr., Emegwali, D. B., Harrihan, M., Allewell, N. M., Pusey, M. L., Shah, A. M., and Nelsestuen, G. L. (1997) Biochemistry 36, 15589–15598
35. Lu, Y., and Nelsestuen, G. L. (1996) Biochemistry 35, 8193–8200
36. Persh, A. (1985) Enzyme Structure and Mechanism, 2nd Ed., pp. 299–300, W. H. Freeman & Co.
37. Toner, L., Jr., Sauer, K., and Wang, J. (1995) in Physical Chemistry: Principles and Applications in Biological Sciences (Young, D., ed) pp. 99–100, Simon and Schuster, Englewood Cliffs, NJ
38. Grier, S., Hedner, U., and Falch, J. F. (1995) Adv. Exp. Med. Biol. 386, 163–174
39. Freedman, S. J., Blistone, M. D., Baleja, J. D., Jacobs, M., Furie, B. C., and Furie, B. (1996) J. Biol. Chem. 271, 16227–16236
40. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254

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