Molecular Characterization of an Extended Binding Site for Coagulation Factor Va in the Positive Exosite of Activated Protein C*

Andrew J. Gale‡, Alexander Tsavaler, and John H. Griffin

From the Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

The anticoagulant human plasma serine protease, activated protein C (APC), inhibits blood coagulation by specific inactivation of the coagulation cofactors factorVa (FVa) and factor VIIIa. Site-directed mutagenesis of residues in three surface loops of a positive exosite located on APC was used to identify residues that play a significant role in binding to FVa. Eighteen different residues were mutated to alanine singly, in pairs, or in triple mutation combinations. Mutant APC proteins were purified and characterized for their inactivation of FVa. Three APC residues were identified that provide major contributions to FVa interactions: Lys 191, Arg 229, and Arg 230. In addition, four residues made significant minor contributions to FVa interactions: Lys 191, Lys 192, Asp 214, and Glu 215. All of these residues primarily contributed to APC cleavage at Arg 506 in FVa and play a small role in the interaction of APC with the Arg 506 cleavage site. In conjunction with previously published work, these results define an extensive FVa binding site in the positive exosite of APC that is primarily involved in binding and cleaving at Arg 506 on FVa.

Activated protein C (APC)† provides a critical feedback mechanism to inhibit the coagulation pathway. Protein C circulates in the blood as an inactive serine protease zymogen that is activated by thrombin when it is complexed with thrombomodulin on the surface of endothelial cells (1, 2). APC then inactivates the procoagulant cofactors factor Va (FVa) and factor VIIIa via specific cleavages of these proteins (3–5).

Recent evidence suggests that a basic exosite located on APC is critical for recognition and inactivation of FVa. This exosite was first recognized in a molecular model of the serine-protease domain of APC and was proposed to be a likely candidate for protein-protein interactions (6). The presence of the exosite was confirmed in the x-ray crystallographic structure of the gla-domainless APC (7). The exosite is generally located in the same position as anion binding exosite I of thrombin, an exosite implicated in a variety of thrombin-protein interactions. Subsequently, studies have been done to characterize the interactions of this exosite with the APC substrate, FVa. Peptide inhibition studies clearly implicated the autolysis loop (residues 301–316, chymotrypsin (CHT) residues 142–153) in binding of APC to FVa (8). Alamine scanning of individual residues in this loop was used to identify residues Arg 306, Lys 311, Arg 312, and Arg 314 as critical residues in the interaction of APC with FVa. Furthermore, these residues are primarily involved in interactions with the Arg 506 cleavage site on FVa, and mutation of them has little effect on Arg 506 cleavage (9). Another study determined that the naturally occurring type II APC mutation of Arg 310 to Trp (CHT 187) results in defective FVa inactivation as well (10). Residues in loop 37 (CHT 37–39) were also determined to be important for FVa Arg 506 cleavage, whereas one mutation in the calcium binding loop (CHT 70–80) at Arg 223 (CHT 74) and mutations in loop 60 (CHT 60–67) had little effect on FVa inactivation (11). Studies have also implicated this positive exosite in interactions with thrombomodulin and heparin (12, 13).

As a continuation of our autolysis loop studies, we undertook a complete alanine-scanning mutagenesis of the remaining surface loops in the basic exosite of APC (Fig. 1). We inspected the crystal structure of gla-domainless APC to verify that candidate residues were on the surface of APC and mutated every surface-exposed residue in these loops to alanine, either singly or in pairs. Additionally we made double and triple mutation combinations of some individual mutations that had negative effects. In loop 37, which contains residues 190–193 (CHT 36–39), residues Ser 190, Lys 191, Lys 192, and Lys 193 were mutated. In loop 60, which contains residues 214–222 (CHT 60–68), residues Asp 214, Glu 215, Lys 217, Lys 218, and Arg 222 were mutated. In the calcium-binding loop, which contains residues 223–235 (CHT 70–80), residues Asp 227, Arg 229, Arg 230, Trp 231, Glu 232, Lys 233, and Trp 234 were mutated. Combined with previously published work (9, 10), analysis of these mutations allowed us to define an extensive binding site for FVa in the positive exosite of APC.

MATERIALS AND METHODS

Proteins and Reagents—FVa and Glu 225–FVa were prepared as described (14, 15). FXa was purchased from Enzyme Research Laboratories (South Bend, IN). Phospholipid vesicles (80% phosphatidylcholine, 20% phosphatidyserine) were prepared as described (16). The chromogenic substrate H-0-lysyl (g-Chl)-prolyl-argininyl-p-nitroanilide (Pefachrome PCa) was purchased from Centerchem, Inc. (Norwalk, CT). The chromogenic substrate CBS 34-47 was purchased from American Bioproducts (Parsippany, NJ). Normal human citrate-anticoagulated plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS).

Expression and Purification of Recombinant Protein C—Mutant protein C expression vectors were constructed as described (17). Purified recombinant protein C was prepared as described using a fast flow Q-Sepharose column (9, 17). Some preparations of recombinant protein C were purified further by chromatography on a calcium-dependent
sheep polyclonal anti-protein C antibody-Sepharose column (18) as described (9). Final concentration of protein C was determined using the Asserachrom Protein C enzyme-linked immunosorbent assay (ELISA) assay from American Bioproducts (Parsippany, NJ).

**Functional Assays.—**Protein C was activated by thrombin. Protein C in HBS (50 mM HEPES, 150 mM NaCl) with 2 mM EDTA and 0.5% BSA, pH 7.4, at a concentration of 600 μg/ml was incubated for 2.5 h with 12 μg/ml thrombin at 37 °C followed by the addition of 1.1 units of hirudin per unit of thrombin to inactivate the thrombin. Controls were done in amiodolytic assays, clotting assays, and FVa inactivation assays to verify that the thrombin and hirudin used had no effect on subsequent assays. All APC mutants were quantitated using an active site titration adapted from Chase and Shaw (19) using APC at ~8 μM in HBS and p-nitrophenol-guanidine benzoate at 0.1 mM with an extinction coefficient for p-nitrophenol of 11,400 M⁻¹ cm⁻¹ calculated for pH 7.4. Km and kcat for the chromogenic substrate, Pefachrome PCa, were determined by varying substrate concentration from 1.43 to 0.0446 mM in HBS, 0.5% BSA, 5 mM CaCl₂, pH 7.4 with APC at 5.7 nM. Michaelis constants were derived using Eadie-Hofstee plots. Alternatively, the 5 mM CaCl₂ was replaced with 5 mM EDTA for similar determinations. Color development was measured with an Optimax microplate reader (Molecular Devices, Sunnyvale, CA) (16).

Dilute prothrombin time clotting assays were performed according to the following procedure. Plasma (50 μl) was incubated with 50 μl of APC in HBS with 0.5% BSA at APC concentrations from 8 to 32 nM (2.7–11 mM final concentration) for 3 min at 37 °C. Then clotting was initiated by adding 50 μl of Innovin (Dade Behring Inc., Newark, DE) diluted 1:60 in HBS, 0.5% BSA, and 25 mM CaCl₂. The clotting time was measured using an ST4 coagulometer (Diagnostica Stago, Asnières, France). APC inactivation by serpins present in plasma was measured essentially according to the protocol of Heeb et al. (20).

Inactivation of FVa was measured according to the following procedure. A mixture of 1 mM FVa with 25 μM phospholipid vesicles was made in 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% BSA, 5 mM CaCl₂, and 0.1 mM MnCl₂. Inactivation was initiated by the addition of APC. One-microliter aliquots were removed at time points and added to 40 μl containing 1.25 mM factor Xa (FXa) with 31 μM phospholipid vesicles, followed by the addition of 10 μl of 3 μM prothrombin (final concentrations: 1 mM FXa, 20 pM FXa, 25 μM phospholipid vesicles, and 0.6 μM prothrombin). After 2.5 min a 15-μl aliquot of this mixture was quenched by the addition of 55 μl of HBS containing 10 mM EDTA, 0.5% BSA, pH 8.2. Chromogenic substrate CBS 34-47 was determined in the same buffer except that the CaCl₂ was replaced with 5 mM EDTA. Negligible changes in Km and kcat for this small chromogenic substrate are taken to suggest that mutation did not result in a major structural defect in the protein that would complicate interpretation of any functional results.

Most of the mutant APCs did not significantly differ from wild type in their Km and kcat for Pefachrome PCa. Only four mutants had notable differences. First, the mutation W231A had an increased kcat both in the presence and the absence of CaCl₂, however, the Km was unchanged. The APC mutant K191A/K192A/K193A also had an increased kcat. The mutant R222A had a large change in both Km and kcat in the presence and absence of CaCl₂. Finally, the mutant D227A had significantly altered kinetic parameters for cleavage of Pefachrome PCa, but in this case the difference in Km from wild type was smaller in the presence of CaCl₂ than it was in the presence of EDTA. This suggests that this mutation had some effect on calcium binding. In fact, the backbone carbonyl of Asp 227 is proposed to be a calcium ligand (6), so it is not unexpected that mutation of this residue could affect calcium binding. Alterations in the APC amidolytic activity caused by these two mutations (Arg222 and Arg227) suggest that the conformation of

| Mutant | 5 mM EDTA | 5 mM CaCl₂ |
|--------|-----------|-----------|
|        | Km | kcat | Km | kcat |
| Wild-type | .39 | 45 | .43 | 64 |
| S190A | .40 | 66 | .68 | 102 |
| K191A | .33 | 54 | .59 | 80 |
| K192A | .40 | 59 | .45 | 90 |
| K193A | .45 | 60 | .46 | 79 |
| K191A/K192A/K193A | .45 | 60 | .46 | 79 |
| D214A/E215A | .35 | 71 | .45 | 102 |
| K217A/K218A | .45 | 53 | .40 | 63 |
| R222A | .37 | 78 | .44 | 102 |
| R229A | .45 | 53 | .40 | 63 |
| R230A | .45 | 37 | .45 | 53 |
| R229A/R230A | .40 | 60 | .46 | 79 |
| W231A | .52 | 50 | .44 | 69 |
| K233A | .45 | 53 | .40 | 63 |
| E232A/W234A | .44 | 45 | .49 | 71 |
Functional activity of select APC mutants. A, dilute prothrombin time assay of APC anticoagulant activity over a concentration range of APC. B, FVA inactivation assay. Factor Va was inactivated by APC for various times, and residual factor Va activity was assayed in a prothrombinase assay as described under "Materials and Methods." Wild type APC (+), R229A, (●); R230A (○); R229A/R230A (△). Data shown are averages of three to eight experiments.

To further confirm that the mutations with effects on FVa inactivation rates did not have significant changes in APC active site functional activity, several mutants and wild type APC were assayed for their rate of inactivation in plasma, which primarily reflects inactivation of APC by the serpins, α1-antitrypsin (AAT), and protein C inhibitor (22). Wild type APC activity had a half-life (t_1/2) for inactivation in plasma of 21.4 min. The APC mutants D214E/A215A (t_1/2 = 24.1), R229A (t_1/2 = 18.8), R230A (t_1/2 = 23.6), R229A/R230A (t_1/2 = 27.5), and K191A/K192A/K193A (t_1/2 = 21.0) had half-lives in plasma that were very similar to that of wild type APC. In contrast, the two APC mutants that appeared to have perturbations of the active site, R222A (t_1/2 = 208 min) and D227A (t_1/2 = 83.0 min), were clearly defective in their inactivation by serpins, further confirming that these proteins have global conformational changes that affect active site interactions with various substrates.

Functional Activity of Mutant APCs—All of the mutant APCs were evaluated for anticoagulant function in a dilute prothrombin time assay as described under "Materials and Methods." This assay is highly sensitive to factor V activity in plasma, but controls were done to verify that it is insensitive to factor VIII activity as well as factor IX activity (data not shown), confirming that this assay reflects APC anticoagulant activity. Fig. 2A shows the anticoagulant activity of select APC mutants. Mutation of Arg_229 or Arg_230 to Ala significantly decreased the anticoagulant activity of each mutant as shown in Fig. 3 as a percentage of wild type. Although, in the calcium-binding loop mutations at residues Arg_229 and Arg_230 markedly decreased APC activity, mutation of residues Trp_231, Glu_232, Lys_233, and Trp_234 did not have any negative effect on anticoagulant activity. Therefore, it would appear that these residues do not have a significant role in FVa recognition by APC. As seen in Fig. 3, specific Lys residues in loop 37, especially Lys_193, are essential for normal anticoagulant activity, whereas Ser_190 has no role in anticoagulant activity. In loop 60, residues Asp_214 and Glu_215 moderately contribute to anticoagulant activity, while Lys_217 and Lys_218 have no significant role in anticoagulant activity. Mutation of Arg_222 to Ala greatly reduced anticoagulant activity, but, as noted above, this mutation altered amidolytic activity, so it likely caused a global structural perturbation of APC. Although the reduction in anticoagulant activity observed for R222A may be because of loss of a specific binding interaction between APC and FVa, we cannot draw any conclusion because global structural perturbations could have the same effect. In the calcium-binding loop, mutation of Asp_237 also had a moderate effect on anticoagulant activity, but, as was the case for R222A, this mutation altered amidolytic activity and appeared to cause structural perturbations of APC. Furthermore, it is likely that this mutation affected calcium ion binding. Therefore, we cannot draw any conclusions about the role of Asp_237 in FVa inactivation.

To define effects of mutations on specific cleavages in FVa by APC, we studied FVa inactivation in a purified system. Fig. 2B shows results from experiments using normal plasma-derived FVa. Cleavage rate constants for Arg_306 and Arg_506 cleavages by each APC mutant were derived from inactivation time courses according to established equations (9, 21). Wild type APC had a value for k_506 (Arg_506 cleavage) of 93 × 10^{-6} M^{-1} sec^{-1}. Fig. 4A shows k_506 values for wild type and mutant APCs. Wild type APC had a value for k_306 (Arg_306 cleavage following Arg_506 cleavage) of 3.0 × 10^{-6} M^{-1} sec^{-1}. Fig. 4B shows k_306 values for wild type and mutant APCs. The cleavage rate for Arg_306 cleavage in the absence of Arg_506 cleavage (k_306) was...
also determined for all APC mutants by measuring cleavage of plasma-derived Gin506-FVa. The value of $k_{506}$ for wild type APC was $1.7 \times 10^5$ M$^{-1}$ sec$^{-1}$. This value was about 60% of the value of $k_{506}$, and all APC mutants gave similar values for $k_{506}$ relative to their respective $k_{306}$ values (data not shown). Consistent with previously published data (9, 21), this suggests that cleavage at Arg506 modestly enhances the rate of cleavage at Arg306.

From Fig. 2B and Fig. 4A it is clear that both the R229A and the R230A mutations had large effects in decreasing the FVa Arg506 cleavage rate constants, and the double mutation R229A/R230A had an even larger effect. Fig. 4A also shows that the mutation K193A had a large effect on the Arg506 cleavage rate constant, whereas the mutations K191A and K192A had only moderate effects. In contrast, the double mutation D214A/E215A increased the rate constant for Arg306 almost twofold, but not for Arg506 cleavage. With that exception, the effects of most of these mutations were largest for cleavage at Arg506 ($k_{506}$) and apparently much less for cleavage at Arg306 ($k_{306}$) as shown in Fig. 4C, where the ratio of the Arg506 cleavage rate constant ($k_{506}$) to the Arg306 cleavage rate constant ($k_{306}$) is shown for wild type and mutant APCs. Wild type APC cleaves Arg506 ($k_{506}$) 31× faster than it cleaves Arg306 following Arg506 cleavage ($k_{306}$). APC mutants that had reduced activity mainly lost activity for Arg506 cleavage. This is reflected in the reduced ratio ($k_{506}/k_{306}$, Fig. 4C). This reduced ratio is clear for the mutations K191A, R229A, and R230A. The triple mutation, K191A/K192A/K193A, had an even greater reduction in ratio in an apparent additive effect of the three individual mutations. The double mutation R229A/R230A also manifested an additive effect of the two individual mutations. This graph indicates that the double mutation D214A/E215A had a significant effect on the ratio of $k_{506}$ to $k_{306}$. But, as noted above, this is apparently because of an increase in the Arg506 cleavage rate rather than a decrease in the Arg306 cleavage rate.

**DISCUSSION**

Mutational analysis using conservative alanine mutations to probe protein-protein interactions is a well established method for characterizing the molecular details of the interaction (9, 24). However, it cannot be conclusively ruled out that mutations altering packing forces of the protein or exert their effects at a distance from the interaction site rather than alter a specific protein-protein interaction. To minimize this possibility, we analyzed the kinetic parameters of mutant APCs for cleavage of a small chromogenic substrate (Table I), and for selected APC mutants we also measured the rate of inactivation by plasma serpins. In both cases, kinetic parameters equivalent to those of wild type APC suggest that no gross structural changes were caused by the mutations. As exceptions, significant changes in these parameters for two mutants (R222A and D227A) caused us to remove them from consideration in our structure-function analysis. In support of this, the x-ray crystallographic structure of APC (7) illustrates that all of these mutated residues are fully surface exposed, except for Arg222 and Asp227, which are only partially exposed, so mutation of them might be expected to have structural consequences. Therefore, for the remaining mutations, it is reasonable to assume that functional changes caused by mutations are the result of alteration of specific protein-protein interactions. Accordingly, our results provides insights into APC exosite interactions with FVa. Residues Arg229, Arg230, and Lys191 play the largest role in FVa interactions, whereas residues Lys192, Lys192, Arg214, and Glu215 appear to play minor roles. The rate of cleavage at Arg506 in FVa is significantly affected by mutation of each of these residues, whereas cleavage at Arg506 is only mildly affected. Conversely, residues Lys271, Lys218,
Trp<sup>231</sup>, Glu<sup>232</sup>, Lys<sup>233</sup>, and Trp<sup>234</sup> do not appear to play any significant role in FVa interactions with APC.

Friedrich et al. (11) mutated Arg<sup>229</sup> (CHT 74) to Gln, a naturally occurring type II protein C mutation, and concluded that this residue played, at most, a minor role in the interaction of APC with the Arg<sup>506</sup> cleavage site of FVa. This is in contrast to the significant reduction in APC activity we observed here for the R229A mutant. But the mutation was not the same, so direct comparison is not possible. In fact, both naturally occurring mutations of Arg<sup>229</sup> (R229Q and R229W) are still potential hydrogen bond donors. In contrast, the mutation R229A removes all hydrogen bonding possibility at residue 229. Therefore, it is possible that these mutations (R229Q and R229W) maintain part of the functional contact provided by Arg<sup>229</sup>, which could also involve hydrogen bonding. Thus, mutation to these naturally occurring mutants, while reflecting what happens in individuals with these mutations, would not necessarily elucidate the normal functional role of the wild type Arg<sup>229</sup> in FVa interactions. We conclude that Arg<sup>229</sup> does indeed play a significant role in interactions of APC with FVa. Furthermore, Arg<sup>230</sup> clearly plays a significant role in interactions of APC with FVa. Thus, the calcium-binding loop is central in the interaction of APC with FVa.

Friedrich et al. (11) also concluded that residues Lys<sup>191</sup>, Lys<sup>192</sup> and Lys<sup>193</sup> (CHT 37–39) were involved in cleavage of Arg<sup>506</sup> in FVa by APC, whereas residues Lys<sup>217</sup> and Lys<sup>218</sup> (CHT 62 and 63) were not involved in this interaction. This agrees well with our results, but we mutated residues 191, 192, and 193 individually as well as together, whereas Friedrich et al. (11) only mutated them all at once. From these individual mutations we were able to determine that the three Lys residues did not contribute equally to this interaction. Rather, Lys<sup>193</sup> played the largest role in the interaction of APC with FVa.

In a previous study (9), we identified several residues in the autolysis loop of APC (residues 301–316, equivalent to chymotrypsin 142–153) that also play a very significant role in FVa interactions and further distinguish between cleavages at Arg<sup>506</sup> and Arg<sup>504</sup>. This surface loop is also part of the extended positive exosite on APC, and these residues are nearly contiguous in tertiary space with the residues identified in the current study as FVa binding residues. Another residue, Arg<sup>214</sup>, identified as a site for FVa interaction, is also contiguous with the positive exosite (10). Together these studies map out an extended FVa binding site on the surface of the protease domain of APC that appear to play a major role in FVa binding (Lys<sup>193</sup>, Arg<sup>229</sup>, Arg<sup>230</sup>, Lys<sup>311</sup>, Arg<sup>312</sup>, and Arg<sup>314</sup>) and at least four that appear to play a minor but significant role (Lys<sup>217</sup>, Lys<sup>218</sup>, Lys<sup>507</sup>, and Glu<sup>508</sup>), with two more at the periphery of the positive exosite that may play a minor role (Asp<sup>214</sup> and Glu<sup>215</sup>). These results serve to outline an extensive FVa binding exosite on the surface of the protease domain of APC. In future work it will be interesting to see how this FVa binding exosite compares to exosites for other APC/PC molecular partners such as thrombomodulin and FVIIIa.

Acknowledgment—We thank Cynthia Kos for technical assistance.

REFERENCES

1. Kisiel, W. (1979) J. Clin. Invest. 64, 761–769
2. Stearns-Kurosawa, D. J., Kurosawa, S., Molica, J. S., Ferrell, G. L., and Esmon, C. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10212–10216
3. Kisiel, W., Canfield, W. M., Ericsson, L. H., and Davey, E. W. (1977) Biochemistry 16, 5824–5831
4. Walker, F. J., Sexton, P. W., and Esmon, C. T. (1979) Biochim. Biophys. Acta 571, 333–342
5. Marlar, R. A., Kleiss, A. J., and Griffin, J. H. (1983) Blood 59, 1067–1072
6. Fisher, C. L., Greengard, J. S., and Griffin, J. H. (1994) Protein Sci. 3, 588–599
7. Mathur, T., Oganessian, Y., Hof, P., Huber, R., Foundling, S., Esmon, C., and Bode, W. (1996) EMBO J. 15, 6822–6831
8. Messers, R. M., Heeb, M. J., and Griffin, J. H. (1993) Protein Sci. 2, 1482–1489
9. Gallo, A. J., Heeb, M. J., and Griffin, J. H. (2000) Blood 96, 585–593
10. Rintelen, C., Yegneswaran, S., and Griffin, J. H. (2001) Thromb. Haemostasis 85, 274–279
11. Friedrich, U., Nicolaes, G. A. F., Villoutreix, B. O., and Dahlback, B. (2001) J. Biol. Chem. 276, 23105–23108
12. Knobe, K. E., Berndtsson, A., Shen, L., Morser, J., Dahlback, B., and Villoutreix, B. O. (1999) Proteins 35, 218–234
13. Friedrich, U., Blom, A. M., Dahlback, B., and Villoutreix, B. O. (2001) J. Biol. Chem. 276, 24122–24125
14. Heeb, M. J., Kojima, Y., Greengard, J., and Griffin, J. H. (1995) Blood 85, 3465–3471
15. Heeb, M. J., Messers, R. M., Tans, G., Rosing, J., and Griffin, J. H. (1993) J. Biol. Chem. 268, 2872–2877
16. Messers, R. M., Houghten, R. A., and Griffin, J. H. (1999) J. Biol. Chem. 266, 24514–24519
17. Zhang, L., and Castellino, F. J. (1990) Biochemistry 29, 10826–10834
18. Gruber, A., Harker, L. A., Hanes, S. S., Kelly, A. B., and Griffin, J. H. (1991) Circulation 84, 2454–2462
19. Chase, T., and Shaw, E. (1996) Biochem. Biophys. Res. Commun. 229, 508–514
20. Heeb, M. J., Bichoff, R., Courtneym, M., and Griffin, J. H. (1990) J. Biol. Chem. 265, 2365–2369
21. Nicolaes, G. A. F., Tans, G., Thomassen, M. C. L. G. D., Hemker, H. C., and Pabinger, I., Varadi, K., Schwarz, H. P., and Rosing, J. (1995) J. Biol. Chem. 270, 21158–21166
22. Heeb, M. J., Esparcia, E., and Griffin, J. H. (1998) Blood 93, 446–444
23. Merritt, E. A., and Bacon, D. J. (1997) Methods Enzymol. 277, 505–524
24. Wells, J. A. (1991) Methods Enzymol. 203, 389–413
25. Pellequer, J. L., Gale, A. J., Getzoff, E. D., and Griffin, J. H. (2000) Thromb. Haemostasis 84, 849–857
26. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950