Roles of Low Specificity and Cofactor Interaction Sites on Thrombin during Factor XIII Activation

COMPETITION FOR COFACTOR SITES ON THROMBIN DETERMINES ITS FATE* 

Helen Philippou‡§, James Rance‡, Timothy Myles¶, Scott W. Hall¶, Robert A. Ariens¶, Peter J. Grant¶, Lawrence Leung¶, and David A. Lane‡§

From the ‡Department of Haematology, Imperial College London, United Kingdom, §Division of Hematology, Stanford University School of Medicine, Stanford, California 94305-5156, and ¶Academic Unit of Molecular Vascular Medicine, University of Leeds School of Medicine, Leeds LS1 3EX, United Kingdom

Factor XIII is activated by thrombin, and this reaction is enhanced by the presence of fibrinogen(ogen). Using a substrate-based screening assay for factor XIII activity complemented by kinetic analysis of activation peptide cleavage, we show by using thrombin mutants of surface-exposed residues that Arg-178, Arg-180, Asp-183, G1u-229, Arg-233, and Trp-380 of thrombin are necessary for direct activation of factor XIII. These residues define a low specificity site known to be important also for both protein C activation and for inhibition of thrombin by antithrombin. The enhancing effect of fibrinogen occurs as a consequence of its conversion to fibrin and subsequent polymerization. Surface residues of thrombin further involved in high specificity fibrin-enhanced factor XIII activation were identified as His-66, Tyr-71, and Asn-74. These residues represent a distinct interaction site on thrombin (within exosite I) also employed by thrombomodulin in its cofactor-enhanced activation of protein C. In competition experiments, thrombomodulin inhibited fibrin-enhanced factor XIII activation. Based upon these and prior published results, we propose that the polymerization process forms a fibrin cofactor that acts to approximate thrombin and factor XIII bound to separate and complementary domains of fibrinogen. This enables enhanced factor XIII activation to be localized around the fibrin clot. We also conclude that proximity and competition for cofactor interaction sites primarily directs the fate of thrombin.

Following initiation of coagulation, a series of carefully regulated serine proteinase reactions take place resulting in the generation of thrombin and the formation of an insoluble fibrin clot. Thrombin is a serine proteinase responsible for proteolytic cleavage of multiple substrates involved in the coagulation pathway (1, 2). One of the main roles of thrombin is the conversion of fibrinogen to an insoluble fibrin clot. This process is initiated by proteolytic cleavage of two pairs of fibrinopeptides, FPA1 and FPB, from the Aα- and Bβ-chains of fibrinogen, respectively. FPA is cleaved first, leading to spontaneous polymerization of the fibrin monomers. This is shortly followed by proteolysis of the Bβ-chain, which is associated with lateral aggregation of the fibrin protofibrils to produce thicker fiber bundles (3, 4). To ensure a more stable clot structure, activated factor XIII (factor XIIIa), a transglutaminase, covalently cross-links specific glutamine and lysine side chains of the protofibrils, resulting in increased resistance of the clot to chemical, physical, and proteolytic insults (5, 6).

Factor XIII is a heterologous tetramer with a molecular mass of 324,000 Daltons. It consists of two A-subunits that contain the active site of the transglutaminase and two B-subunits that serve a carrier function for the hydrophobic A-subunit in the aqueous environment of human plasma (5, 6). Thrombin activates factor XIII by cleavage of a 37 amino acid activation peptide from the factor XIII A-subunits (7). Consequently, the carrier B-subunits dissociate from the activated A-subunits to completely unmask the active site (8). Activation of factor XIII is closely controlled by the presence of its substrate fibrinogen. The presence of fibrinogen accelerates activation of factor XIII ~80-fold (9). This acceleration is caused by an enhancing effect of fibrin on both thrombin cleavage of the factor XIII activation peptide and dissociation of the factor XIII A- and B-subunits. It has been shown that residues 242–424 in the αC-domain of fibrinogen regulate the dissociation of the B-chains from the thrombin-cleaved A-subunits of factor XIII (10). The enhancement effect of fibrinogen on factor XIII activation can be readily inhibited by specific inhibitors of fibrin polymerization (11, 12). However, the process by which polymerizing fibrin enhances the activation of factor XIII is poorly understood.

Approximately 10% total fibrinogen exists as a variant known as γ. This fibrinogen variant occurs by alternative splicing of mRNA resulting in the deletion of four amino acids from the C terminus of γA with a substitution of an additional 20 amino acid residues (13). The γ-region is thought to bind factor XIII zymogen and thrombin (14) but the functional consequences of this for factor XIII activation are as yet uncertain (15).

In addition to activating factor XIII and proteolytic cleavage of FPA and FPB from fibrinogen, thrombin is also responsible for numerous other proteolytic interactions. It activates factors V, VIII, XI, and thrombin-activatable fibrinolysis inhibitor (2,
16). Furthermore, thrombin cleaves the protease (activated) receptors, resulting in platelet activation and aggregation (17). These functions of thrombin are all procoagulant (ultimately promoting clot formation). However, thrombin is also able to behave as an anticoagulant by activating protein C when bound to thrombomodulin. Activated protein C cleaves and thereby inhibits both activated factors V and VIII. Additionally, thrombin is directly inhibited by formation of an irreversible complex with antithrombin. As substrates of thrombin participate in either procoagulant or anticoagulant functions, thrombin is critical for regulating hemostasis. The unique specificity of thrombin toward its substrates is thought to arise by combination of insertion loops flanking the upper and lower faces of the active site (Leu-46/Asn-57 and Leu-144/Gly-155), which occlude and restrict the active site and two exosites (I and II) on opposite faces of the active cleft. Binding of substrates, cofactors, and inhibitors to either exosite is important for overcoming restricted access to the active site. The formation of the binding site defined by a cylindrical channel shaped by three β-strands appears to be important for the procoagulant (fast) form of thrombin (18).

The aim of this study was to determine how thrombin specifically recognizes factor XIII in preference to its many other substrates and how activation can be enhanced by fibrin. For this purpose, we have utilized a library of 53 thrombin mutants encompassing a total of 78 surface exposed, charged, and polar residues mutated to alanine. This library has previously been used to identify residues of thrombin involved in the interaction with many of its substrates including protein C, thrombin-activatable fibrinolysis inhibitor, fibrinogen (19), antithrombin (20), factor V (21), and factor VIII (22). Here, we have used the library to investigate which residues of thrombin are involved in direct factor XIII activation and in the fibrin-enhanced reaction. Knowledge of these residues has enabled us to draw general conclusions about how the activities of thrombin are directed.

**MATERIALS AND METHODS**

A library of 53 thrombin mutants encompassing a total of 78 surface exposed charged and polar residues mutated to alanine was prepared and checked for amodicity activity as described previously (Table I) (20, 23). These mutant thrombins were screened for their ability to activate factor XIII using a modification of a 5-biotinamido/pentylamine incorporation assay (24). Factor XIII was purified from plasma donor pools using previously described methods (11). To screen for factor XIII activation in the absence of fibrinogen, we first coated microtiter plates with 10 μg/ml N,N-dimethyl casein (Sigma) for 16 h. Factor XIII (6.7 nmol) and 36 nmol human thrombin (American Diagnostica, Greenwich, CT) were added and incubated in activation buffer (40 mM Tris-HCl, pH 8.3, 140 mM NaCl, 0.1 mM dithiothreitol, 5 mM CaCl₂, 0.02% NaN₃) containing 0.1 mM 5-biotinamido/pentylamine (Perbio Science UK Limited, Cheshire, United Kingdom) for 60 min at room temperature. The reaction was stopped by the addition of 200 mM EDTA, pH 8.3. The amount of factor XIII that was activated by thrombin was determined by measuring the amount of thrombin activated with 5-biotinamido/pentylamine cross-linked to the fibrin (Hematologic Technologies Inc.) and/or fibrin-associated alkaline phosphatase streptavidin conjugate followed by incubation with p-nitrophenol substrate (and measuring at 405 nm). When activation of factor XIII by thrombin was performed in the presence of thrombomodulin, increasing molar concentrations of rabbit thrombomodulin (Hematologic Technologies Inc.) were preincubated with 1.8 nM human factor XIII prior to the addition of 6.7 nM factor XIII (in the absence and presence of fibrin, see above).

Factor XIII activation peptide cleavage was determined by reverse-phase HPLC using a Pepmap 0.46 × 250 mm C₁₈ column (Applied Biosystems, Warrington, United Kingdom) and the AKTA basic chromatography system (Amersham Biosciences). Application and elution buffers for HPLC were phosphate- and acetonitrile-based as previously described (11) with elution using an 8.5-40% acetonitrile gradient. Purified factor XIII was dialyzed against reaction buffer (9.47 mM sodium phosphate, 137 mM NaCl, 2.5 mM KCl, 0.1% polyethylene glycol, pH 7.4) prior to use. In the absence of fibrinogen, 1.8 μM factor XIII was incubated at 37 °C with 4.1 nM of the thrombin mutant under investigation. 100-μl samples were then removed at five intervals between 1 and 220 min (dependent on the expected activity of the mutant in question). The reactions were stopped by the addition of 10 μl of 3 M perchloric acid, the precipitate was removed by centrifugation, and 100 μl of sample was loaded onto the C₁₈ column. To investigate factor XIII activation peptide release in the presence of fibrinogen, 1,8 μM factor XIII and 1.1 μM fibrinogen were incubated at 37 °C with 4.1 nM of the thrombin mutant in question. The reaction mixture was immediately aliquoted into 100-μl volumes to avoid subsampling difficulties following fibrin formation. Reactions were stopped at five intervals between 1 and 160 min by the addition of 10 μl of 3 M perchloric acid. The precipitate was removed by centrifugation, and 100 μl of the reaction was loaded onto the C₁₈ column. The relative amounts of activation peptides released were determined by measurement of the areas under the respective peaks using Unicon Analysis software (Amersham Biosciences). Catalytic efficiencies were calculated by fitting of the data from time course experiments to Equation 1 (9),

\[
V = V_{\text{max}} \times \frac{[S]}{K_m + [S]}
\]

where [S] is the concentration of substrate, \(V_{\text{max}}\) is the maximum initial rate, and \(K_m\) is the Michaelis constant.\(\times\) Data fitting was performed using Eznflitter software (Biosoft, Cambridge, United Kingdom). This equation was used to fit all of the activation peptide release curves, although it is recognized that this is an approximation in the cases of fibrinogen-enhanced factor XIII activation peptide release and for FPB release.

Purified fibrinogen fragment D was also studied as an enhancer of factor XIII activation. The D fragment preparation (Calbiochem) was characterized with respect to its ability to bind factor XIII as it is sensitive to proteolytic cleavage at its C terminus where a factor XIII binding site is located. Binding analysis of factor XIII to both fragment D and fibrinogen was carried out with surface plasmon resonance using a BIAcore 3000 system (BIAcore UK, Stevenage, United Kingdom). Fragment D at 75 μg/ml and fibrinogen at 50 μg/ml were covalently coupled to an activated carboxymethyl dextran-coated biosensor chip (CM5) using the manufacturer’s recommended protocol for kinetic analysis. The conditions employed were similar to those employed elsewhere (25) for the study of tissue-type plasminogen activator and plasminogen binding to the α2-domain of fibrinogen. However, the running buffer contained 5 mM EDTA rather than 0.1 mM phenylmethylsulfonyl fluoride and 5 mM CaCl₂, and a 1:1 (Langmuir) binding model using the BIAevaluation software (Biosoft) was fitted to the data. Enzfitter software (Biosoft, Cambridge, United Kingdom) was used (as fibrinogen contains two D-domains). Specifically, 1.8 μM factor XIII and either 2.25 μM fibrinogen or 4.5 μM fragment D were preincubated with 1.8 μM factor XIII followed by the addition of 6.7 nM factor XIII (Hematologic Technologies Inc.) were preincubated with 1.8 μM human factor XIII prior to the addition of 6.7 nM factor XIII (in the absence and presence of fibrin, see above).
RESULTS

The 53-variant thrombins were screened for their abilities to activate factor XIII in the absence of fibrinogen using the casein-coated microtiter plate assay with incorporation of pentylamine used as the reporter for factor XIIIa activity. Only three thrombin mutants showed <50% WT activity (see results marked by asterisk in Table I). These were a triple mutant of residues Arg-178/Arg-180/Asp-183 and mutants of Glu-229 and Arg-233 that had 41, 6.9, and 39% activity, respectively. Mutant W50A showed 53% activity (see Table I).

These three variants, E229A, R233A, and R178A/R180A/D183A, together with W50A were assessed further using increasing amounts of thrombin to confirm the role of these residues in the activation of factor XIII (data not shown). Each mutant required 4–8-fold increases in concentration to achieve the activity attained by WT thrombin.

The above results in Table I, obtained with the microtiter plate-screening assay, employed an arbitrary cutoff of 50% WT activity to select the thrombin mutants with reduced ability to activate factor XIII. To obtain a more reliable and quantitative assessment of activity reduction, the kinetic parameter $k_{cat}/K_m$ was determined for WT and mutant thrombins. This was achieved by determination of factor XIII activation peptide release using HPLC analysis. As factor XIII was isolated from normal pooled plasma, its activation peptide was heterogeneous, containing the polymorphic forms Val-34 and Leu-34 (see Fig. 1A). Rather than sum the relative amounts of each form, their individual relative release rates were determined for each thrombin preparation (see time courses of release in Figs. 1, C and D). The results of catalytic efficiency determinations are illustrated in Fig. 2. The $k_{cat}/K_m$ values obtained for the activation of Val-34 and Leu-34 forms of the factor XIII using WT thrombin (0.18 ± 0.008 and 0.33 ± 0.049 μM$^{-1}$ s$^{-1}$, respectively) conform to prior reports using plasma with human thrombin as an activator (11). Each thrombin mutant with <50% WT activity selected from Table I for kinetic analysis had greatly reduced $k_{cat}/K_m$ with the R233A mutant being particularly affected ($k_{cat}/K_m$ of 0.011 ± 0.0008 and 0.018 ± 0.001 μM$^{-1}$ s$^{-1}$ for cleavage of the Val-34 and Leu-34 forms of activation peptide, respectively) (Fig. 2). The position of the mutated residues on the surface of thrombin (see below) together with their limited number suggests that activation of factor XIII in the absence of cofactor is due primarily to direct (and inefficient) interactions of factor XIII within the long extended active site cleft between the S1 specificity pocket and exosite II.

Preliminary screening experiments for fibrinogen-enhanced factor XIII activation used microtiter plates coated with fibrinogen and utilizing pentylamine incorporation into the resultant fibrin by activated factor XIII as a reporter. The rate of factor XIII activation was greatly enhanced by the presence of fibrinogen, requiring only 8 min and 50% less thrombin to achieve the equivalent amount of factor XIII activity generated in 1 h in the absence of fibrinogen. More thrombin residues were found to influence fibrinogen-enhanced factor XIII activation when compared with direct fibrinogen-dependent factor XIII activation alone (Table II). Thrombin variants with 50% or less activities compared with WT were H66A, Y71A, N74A (which map to exosite I of thrombin), and R89A/R93A/E94A (which map to exosite II) in addition to the previously characterized mutants W50A, R178A/R180A/D183A, E229A, and R233A. Dose-response experiments for these mutant thrombins showed that 4–8-fold increased amounts of the mutants were required to attain WT thrombin activity, confirming the validity of the screening assay (results not shown).

All of the mutants that resulted in reduced fibrinogen-enhanced activation of factor XIII were studied further in experiments in which factor XIII activation was carried out on the surface of preformed fibrin. Fibrinogen coated onto microtiter plates was preincubated with WT thrombin to form fibrin. Thrombin was subsequently removed by washing with 40 mM Tris-HCl, 750 mM NaCl, pH 8.3. Complete removal of thrombin
was confirmed by the absence of activation of factor XIII in the blank sample. Fibrin-enhanced factor XIII activation by the mutants was then assessed. The results (Table II) show that each of the three mutants, H66A, Y71A, and N74A, had reduced ability to activate factor XIII (36\% of WT activity) more than fibrin-enhanced factor XIII activation (68\% of WT activity). This probably reflects the fibrinopeptide cleavage.

Thrombin mutants H66A, Y71A, and N74A therefore influenced both fibrinogen- and fibrin-enhanced factor XIII activation in the microtiter plate assays and were subsequently assessed further by HPLC analysis of the activation peptide release in the presence of fibrinogen. The activation experiments generated activation peptides from both factor XIII (Val-34 and Leu-34 forms of the activation peptide) and fibrinogen (FPA and FPB). These peptides were completely resolved with FPB release (Table III). These data show that residues His-66, Tyr-71, and Asn-74 affect fibrinogen-enhanced factor XIII activation with FPB release (Table III). These data confirm reduced fibrin-enhanced factor XIII activation of the three mutants. However, it is evident from Table III that the mutants also had impaired ability to convert fibrinogen to fibrin. The $k_{cat}/K_m$ for WT thrombin cleavage of fibrinogen (FPA and FPB) is 4.54 $\pm$ 0.58 $\mu M^{-1} s^{-1}$ and is lower for all of the mutants falling to 0.023 $\pm$ 0.0004 $\mu M^{-1} s^{-1}$ for Y71A. There was a parallel reduction in the catalytic efficiencies associated with FBP release (Table III). These data show that residues His-66, Tyr-71, and Asn-74 have a role in FPA and FPB cleavage.
age as well as fibrin-enhanced FXIII activation.

The residues His-66, Tyr-71, and Asn-74 form part of a known extensive surface cluster (anion-binding exosite I) used for recognition of thrombomodulin (see below) (19). If His-66, Tyr-71, and Asn-74 have a direct role in enhancing factor XIII activation, thrombomodulin would be predicted to specifically inhibit fibrin-enhanced activation on the surface of a preformed clot. This was investigated using the above microtiter plate-based functional assays for factor XIII activity in the absence and presence of thrombomodulin. In Fig. 3, the results for the addition of increasing molar concentrations of thrombomodulin to thrombin prior to the addition of factor XIII and 5-(biotinamido)pentylamine (using casein-coated microtiter plates to assess activated factor XIII activity) are illustrated (upper line). Thrombomodulin does not inhibit direct activation of factor XIII by thrombin, even at 100-fold molar excess. In contrast, fibrin-enhanced activation of factor XIII (using preformed fibrin as enhancer) clearly shows a dose-related inhibition of factor XIII activation by thrombomodulin (Fig. 3, lower line) with 10-fold excess of thrombomodulin producing maximum inhibition. At 100-fold molar excess of thrombomodulin, factor XIII activity was not completely inhibited in this assay and this presumably reflects residual non-enhanced direct activation of factor XIII by thrombin.

We then compared purified fragment D and fibrinogen as enhancers of thrombin cleavage of factor XIII activation peptides by HPLC analysis. Fragment D was studied at twice the molar concentration as fibrinogen contains two integral D-domains. The results in Fig. 4 demonstrate no enhancement of factor XIII activation by isolated fragment D, despite its retained ability to bind to factor XIII (see “Materials and Methods”). This finding confirms the importance of the polymerization reaction in the enhancement of factor XIII activation.

**DISCUSSION**

Using a library of thrombin mutants, we have been able to map the residues on thrombin involved in factor XIII activation in the absence and presence of its cofactor fibrinogen. In the absence of a cofactor, there is low efficiency activation of factor XIII (26). In accordance with a prior report (11), we find the Leu-34 peptide is more efficiently released by thrombin. Direct activation of factor XIII is shown by our data to be either mediated through or influenced by thrombin residues Arg-178, Arg-180, Asp-183, Glu-229, Arg-233, and Thrp-50. Mapping of the functional thrombin residues implicated in direct factor XIII activation shows that these are located primarily in the sodium binding site and an adjacent area (Fig. 5A). We found that a complementary area of thrombin containing His-66, Tyr-71, and Asn-74 is required for fibrin-enhanced activation of factor XIII (Fig. 5B), and this is located within exosite I. We compared our results with previously published findings of the residues involved in thrombomodulin-enhanced activation of protein C (19) by thrombin and those involved in heparin-dependent antithrombin inhibition of thrombin (20). The substrates involved in each of the respective reactions (factor XIII, protein C, and antithrombin), which have either procoagulant or anticoagulant activities, are either directly recognized or influenced by the common residues on thrombin, Glu-229, Arg-233, and Thrp-50 (Fig. 5A). Residues Glu-229 and Arg-233 are responsible for intramolecular ion pairs with residues Lys-236 and Asp-146, respectively, which are important for maintaining the structural integrity of the sodium binding site (18). Polymerization of Glu-229 or Arg-233 could lead to a conformational change of the sodium binding site altering substrate docking adjacent to the active site serine of thrombin or alternatively be involved in direct interactions. Residues of thrombin involved in enhanced activation due to cofactor binding (the cofactors here being polymerizing fibrin, thrombomodulin, and heparin)
comprise alternative subsets (Fig. 5). Although the residues used by heparin are distinct (exosite II), those utilized by fibrin and thrombomodulin overlap with His-66 and Tyr-71 common to both (exosite I). The interaction surface for thrombomodulin is significantly more extensive within exosite I, reflecting its high affinity interaction with thrombin.

Common recognition residues on thrombin for procoagulant (factor XIII) and anticoagulant (protein C and antithrombin) substrates raises the possibility of competition between the substrates for thrombin during normal hemostasis in vivo. However, it is unlikely that such a substrate competition is important in vivo because of the important enhancing role of cofactors in these reactions of thrombin. Thus, protein C can be activated directly in vitro by thrombin, but in vivo this direct activation is likely to be essentially negligible as the catalytic efficiency of the reaction is very low \( k_{\text{cat}}/K_m = 5.6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1} \) (27). It is also unlikely that thrombin directly activates factor XIII in vivo without a cofactor as there has to be a mechanism for localizing the activation onto the surface of the clot. As has been previously shown, fibrin(ogen) provides the enhancing role in factor XIII activation (9, 11, 12) and this is further supported by our data. To date, the molecular mechanism of fibrin(ogen)-enhanced factor XIII activation has been uncertain. Both thrombin and factor XIII have been reported to bind to the \( \gamma \)-chain of fibrinogen located on the fragment D-domain of fibrinogen. It is unlikely that a mechanism based on simultaneous binding of factor XIII and thrombin to the same site on fibrinogen will be dominant in enhancement, because polymerization is essential for fibrin-enhanced factor XIII activation (12). Indeed, we show that purified fragment D with a functional binding site for factor XIII is unable to enhance factor XIII activation. Rather, we propose that the fibrin polymer formed following thrombin cleavage of fibrinogen and subsequent polymerization acts in a similar way as thrombomodulin and heparin/heparan sulfate as a cofactor to initiate ternary complex formation. Under optimum conditions, ternary complex formation with fibrin, thrombin, and factor XIII will increase the catalytic efficiency ~80-fold (26). However, the precise mechanism of cofactor-enhanced activation clearly differs in a unique way for factor XIII as fibrinogen is initially

![Space-filling models of thrombin (using standard Bode orientation).](image-url)

These indicate the residues involved in interaction with substrates factor XIII (FXIII), protein C (PC), and antithrombin (AT) (A) and cofactors fibrin, thrombomodulin, and heparin (B). Catalytic serine residues (Ser-205 (S205)) are shown in red in each model. Residues illustrated in orange encompass the sodium binding and adjacent area. Purple-colored residues map to exosite I, and green-colored residues map to exosite II.

**Fig. 5.** Space-filling models of thrombin (using standard Bode orientation). These indicate the residues involved in interaction with substrates factor XIII (FXIII), protein C (PC), and antithrombin (AT) (A) and cofactors fibrin, thrombomodulin, and heparin (B). Catalytic serine residues (Ser-205 (S205)) are shown in red in each model. Residues illustrated in orange encompass the sodium binding and adjacent area. Purple-colored residues map to exosite I, and green-colored residues map to exosite II.
a substrate of thrombin. It is subsequent to the initial encounter of thrombin and fibrinogen that a cofactor surface is generated. Identification here of the residues of thrombin involved in fibrin-enhanced activation and those involved in direct factor XIII activation, together with the finding that isolated fragment D is unable to enhance factor XIII activation, enables a plausible model for this ternary complex formation and enhanced activation to be proposed. Such a model draws upon binding sites for thrombin and factor XIII on the separate and complementary domains of fibrinogen that are brought together in the polymerization reaction. One possibility, not directly tested herein, is that thrombin bound to the fragment E domain after the release of fibrinopeptides is brought together with factor XIII prebound to the fragment D domain during polymerization. This model explains much published data on the influence of polymerization on the enhanced activation of factor XIII. It also provides an explanation for localized cross-linking by factor XIIa on the surface of the newly generated clot.

If we consider that in vivo most of these reactions of thrombin are only likely to occur when their cofactor is present and that the residues of thrombin involved in substrate recognition of factor XIII, protein C, and antithrombin are common, it can be inferred that the activity of thrombin will be primarily directed by the location at which it is generated and the nature and local concentration of the cofactor. One of the first actions of thrombin in hemostasis is likely to be cleavage of fibrinopeptides with subsequent fibrin formation, and this will be followed closely by factor V and factor VIII activation (32, 33).

Factor XIIIa is then able to cross-link fibrin to form a clot on the hemostatic plug is positioned as a consequence of the clot in areas of vessel damage to intact regions of the vessel wall, it will bind to thrombomodulin and heparan sulfate, initiating the inhibition of further thrombin generation via protein C activation and its direct inhibition by antithrombin. Indeed, the present work suggests that in the undamaged vessel (on the fringe of the hemostatic plug), there will be direct competition by thrombomodulin for the cofactor interaction site of thrombin used for fibrin-enhanced factor XIII activation. As the affinity of thrombin for thrombomodulin greatly exceeds its affinity for fibrin, down-regulation of both thrombin and factor XIII activation will be favored. This model of cofactor-directed specificity and competition can also be expected to have general applicability and govern other functions of thrombin, such as platelet activation via thrombin glycoprotein 1b binding (34) and enhanced protease receptor cleavage (31).

Acknowledgments—We thank Kristina Standeven and Antonella Adami for their help with the pentylamine incorporation assay and HPLC analysis.

REFERENCES

1. Esmon, C. T. (1987) Science 235, 1314–1320
2. Mann, K. G., and Lundblad, R. L. (1987) in Haemostasis and Thrombos (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., eds) 2nd Ed, pp. 148–161, Churchill Livingstone, New York
3. Weisel, J. W. (1986) Biophys. J. 50, 1079–1093
4. Weisel, J. W., Veklich, Y., and Gorkun, O. V. (1993) J. Mol. Biol. 232, 285–297
5. Ichinose, A. (1994) in Haemostasis and Thrombosis (Bloom, A. L., Forbes, C. D., Thomas, D. P., and Tuddenham, E. G. D., eds) pp. 531–546, Churchill Livingstone, New York
6. Lorand, L. (2001) Ann. N. Y. Acad. Sci. 936, 291–311
7. Takagi, T., and Doullitte, R. P. (1974) Biochemistry 13, 750–756
8. Lorand, L., Gray, A. J., Brown, K., Credo, R. B., Curtis, C. G., Domanik, R. A., and Stenberg, P. (1974) Biochem. Biophys. Res. Commun. 56, 914–922
9. Janus, T. J., Lewis, S. D., Lorand, L., and Shafer, J. A. (1985) Biochemistry 24, 6269–6272
10. Credo, R. B., Curtis, C. G., and Lorand, L. (1981) Biochemistry 20, 3770–3778
11. Ariens, R. A. S., Philippou, H., Nagaswami, C., Weisel, J. W., Lane, D. A., and Grant, P. J. (2006) Blood 106, 958–965
12. Greenberg, C. S., and Miraglia, C. C. (1985) Blood 66, 466–469
13. Furnace, A. J., Cummings, D. E., Comeau, C. M., Cant, J. A., and Crabtree, G. R. (1984) J. Biol. Chem. 259, 12826–12830
14. Meh, D. A., Siebenlist, K. R., and Mosesson, M. W. (1996) J. Biol. Chem. 271, 23211–23215
15. Siebenlist, K. R., Meh, D. A., and Mosesson, M. W. (1996) Biochemistry 35, 10445–10453
16. Bajzar, L., Morser, J., and Nesheim, M. (1996) J. Biol. Chem. 271, 16603–16608
17. Coughlin, S. R. (2000) Nature 407, 258–264
18. Di Cera, E., Guinto, E. R., Vindigni, A., Dang, Q. D., Ayala, Y. M., Wuyi, M., and Tulinsky, A. (1995) J. Biol. Chem. 270, 22089–22092
19. Hall, S. W., Nagashima, M., Zhao, L., Morser, J., and Leung, L. L. (1999) J. Biol. Chem. 274, 25510–25516
20. Tsai, M., Jain, A. K., and Gibbs, C. S. (1997) J. Biol. Chem. 272, 12024–12029
21. Myles, T., Yun, T. H., Hall, S. W., and Leung, L. L. (2001) J. Biol. Chem. 276, 25143–25149
22. Myles, T., Yun, T. H., and Leung, L. L. (2002) Blood 100, 2820–2826
23. Tsai, M., Jain, A. K., Dunn, K. E., Rojas, M. E., Leung, L. L., and Gibbs, C. S. (1998) J. Biol. Chem. 273, 16854–16863
24. Song, Y. C., Sheng, D., Taubenfeld, S. M., and Matsueda, G. R. (1994) Anal. Biochem. 223, 88–92
25. Tserupa, G., Toonev, L., and Medved, L. (2002) Biochemistry 41, 6449–6459
26. Naski, M. C., Lorand, L., and Shafer, J. A. (1991) Biochemistry 30, 934–941
27. Esmon, C. T. (1983) Thromb. Haemost. 50, 29–35
28. Jesty, J. (1979) J. Biol. Chem. 254, 10041–10050
29. Griffith, M. J. (1982) Thromb. Res. 25, 245–253
30. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) J. Biol. Chem. 255, 10081–10090
31. De Candia, E., Hall, S. W., Rutella, S., Landolfi, R., Andrews, R. K., and De Cristofaro, R. (2001) J. Biol. Chem. 276, 4692–4698
32. Brumwell, K. E., Butenas, S., and Mann, K. G. (1999) J. Biol. Chem. 274, 22862–22870
33. Butenas, S., van’t Veer, C., and Mann, K. G. (1999) Blood 94, 2169–2178
34. De Cristofaro, R., De Candia, E., Landolfi, R., Rutella, S., and Hall, S. W. (2001) Biochemistry 40, 13268–13273
Roles of Low Specificity and Cofactor Interaction Sites on Thrombin during Factor XIII Activation: COMPETITION FOR COFACTOR SITES ON THROMBIN DETERMINES ITS FATE

Helen Philippou, James Rance, Timothy Myles, Scott W. Hall, Robert A. Ariens, Peter J. Grant, Lawrence Leung and David A. Lane

J. Biol. Chem. 2003, 278:32020-32026.
doi: 10.1074/jbc.M305364200 originally published online June 6, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305364200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 16 of which can be accessed free at http://www.jbc.org/content/278/34/32020.full.html#ref-list-1