Factor V Is a Substrate for the Transamidase Factor XIIIa*

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Coagulation Factor V (M\(_{r}\) = 330,000), upon cleavage by thrombin, produces Factor Va, which is composed of two subunits with \(M\(_{r}\)\) values of 94,000 and 74,000, along with two activation fragments possessing no known function. Studies were undertaken to assess the ability of the transamidase Factor XIIIa to covalently incorporate the lysine analogs \(^{3}H\)putrescine and dansylcadaverine into the thrombin-cleaved (activated) and unactivated forms of human and bovine Factor V.

The incorporation of either probe into thrombin-activated Factor V proceeded at an initial rate approximately twice that for unactivated Factor V. The extent of the incorporation of \(^{3}H\)putrescine or dansylcadaverine into activated or unactivated human Factor V was identical; 4 mol of either probe per mol of Factor V. In the case of bovine Factor V, however, while 4 mol of probe were bound per mol of the unactivated procofactor, 5 mol of either lysine analog were covalently linked to 1 mol of thrombin-cleaved Factor V. Polyacrylamide gel fluorography, immunoadfinity chromatography, and immunoprecipitation identified the largest activation fragment of human Factor V (\(M\(_{r}\) = 150,000) and bovine Factor V (\(M\(_{r}\) = 120,000) to contain the sites of incorporation of the covalently bound probes. High molecular weight, apparently covalent polymers of Factor V were produced by the action of Factor XIIIa on activated and unactivated human or bovine Factor V. The absence of either probe in the reaction mixtures did not appear to allow an enhancement of protein polymerization.

Transamidases (transglutaminases) are calcium-dependent enzymes that catalyze an acyl transfer reaction between the \(\gamma\)-carboxamidase group of a peptide-bound glutamine residue and certain primary amines such as patrescine, cadaverine, histamine, or lysine residues of peptides (1, 2). These enzymes have been reported to cross-link keratin in hair follicles and epidermal cells (3, 4). The calcium-induced aggregation and cross-linking of erythrocyte membrane proteins have also been attributed to a transglutaminase (5, 6).

Platelet Factor XIIIa is a transamidase responsible for cross-linking aggregated fibrin monomers (7, 8), fibrin to fibronectin (9), fibrin to von Willebrand Factor, fibronecin to collagen (10), and \(\alpha_{2}\)-plasmin inhibitor to fibrin (11). Platelet Factor XIIIa has been postulated to function in cross-linking membrane proteins to cytoskeletal proteins such as actin and myosin (12). Activation of plasma Factor XIII, a tetramer composed of \(\alpha_{1}\), \(\alpha_{2}\), \(\alpha_{3}\), and \(\alpha_{4}\) subunits, produces the enzyme that catalyzes the release of a 36-amino-acid activation peptide from the NH\(_2\) terminus of each “a” chain (13). The calcium-dependent dissociation of the “b” chains from the dimer of modified “a” chains follows, unmasking each active center thiol group (14, 15). Platelet Factor XIII, existing only as an “a” chain dimer, is also activated following thrombin cleavage and a subsequent conformational change (15).

Bovine Factor V exists as a single-chain plasma and platelet protein with a relative molecular weight (\(M\(_{r}\)\) of 330,000. Upon activation with thrombin, the procofactor is proteolytically processed, producing fragments with \(M\(_{r}\)\) values of 120,000, 94,000, 74,000, and 71,000 (16). The active form of Factor V, Factor Va, is composed of the noncovalently associated \(M\(_{r}\) = 94,000 and 74,000 peptides which represent the amino and carboxyl termini (respectively) of the procofactor molecule (16). Factor Va, along with Factor Xa, platelet membrane, and/or certain phospholipids, forms the thrombin-generating prothrombinase complex (17). The remaining fragments of the thrombin-cleaved Factor V (approximately 50% of the procofactor) are activation peptides of \(M\(_{r}\) = 120,000 and 71,000 in bovine Factor V and 150,000 and 71,000 in human Factor V (18, 19). Previous studies have shown that once Factor V is cleaved by thrombin, the \(M\(_{r}\) = 94,000 and 74,000 chains of Va bind to lipid membranes, while the activation peptides fail to do so (20–22). It is also known that full prothrombinase activity can be produced in the absence of the activation peptides of Factor V (23).

The substrates of Factor XIIIa all possess elongated conformations (24–28) and are either associated with platelet membrane or are secreted by stimulated platelets (27, 29–31). Since it has been known that Factor V has an elongated shape (32, 33) and that it has been found in platelets (34), it was decided to evaluate the ability of Factor XIIIa to use Factor V as a substrate.

We present evidence which indicates that both human and bovine Factors V possess a region that contains accessible glutamine residues for the transamidase incorporation of two lysine analogs by Factor XIIIa. The incorporation of either \(^{3}H\)putrescine or dansylcadaverine identified only the large activation peptide to contain the glutamine residues accessible for transamidase cross-linking. Evidence is also presented to suggest the formation of high molecular weight stable cross-linked products of Factor V.

**EXPERIMENTAL PROCEDURES**

**Materials**

Trizma (Tris base) and monodansylcadaverine (N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide) were obtained from Sigma. Dithiothreitol was purchased from Aldrich and \(^{3}H\)putrescine (1,4-diaminobutane) was supplied by Amersham. \(\alpha\)-Phenylalanyl-l-
poly-L-arginine chloromethyl ketone (PPACK) was obtained from Calbiochem Behring. Bovine Factor V was isolated by the procedure of Nesheim et al. (35), human Factor V according to Katzmann et al. (18), and α-thrombin was purified according to Lundblad et al. (36). Human Factor XIII was isolated from plasma as previously described by Skrzynia et al. (37).

Methods

Activation of Factor XIII—Twenty five μl of a 200 μg/ml Factor XIII solution were added to 10 μl of Tris/saline buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4). Six μl of 1.0 M CaCl₂ were added, as well as 5 μl of 50 units/ml α-thrombin. The solution was heated at 37°C for 20 min in order to activate Factor XIII. Ten μl of 1 mM PPACK, a thrombin inhibitor, were added to one-half of the incubation mixtures. After 5 min at room temperature, 15 μl of [³H]putrescine (15 Ci/mmol, 2.0 × 10⁻⁴ M) or 20 μl of 2 mM dansylcadaverine, 12 μl of 0.2 M dithiothreitol, and 125 μl of Factor V (0.6 mg/ml in Tris/saline) were added. Incubations were performed in a 37°C water bath.

Incorporation of [³H]Putrescine—An analytic procedure by Lorand et al. (1) was employed to assess the incorporation of [³H]putrescine into Factor V. At various times, 30 μl of each sample were placed on discs of Whatman No. 3MM filter paper and immersed in cold 10% trichloroacetic acid for 20 min. The discs were then transferred to 5% acid, soaked for 10 min, and washed three times each in ethylene glycol (95%) and 100% acetone. They were air-dried and counted in a Beckman scintillation counter.

Incorporation of Dansylcadaverine—At the same times used for the [³H]putrescine incorporation, the samples were each transferred to a quartz cuvette and the fluorescence emission at 500 nm was measured using a Perkin-Elmer MPF-44B fluorescence spectrophotometer. Excitation was set at 360 nm. The incorporation of dansylcadaverine exhibits an overall fluorescence enhancement, as well as a blue shift of maximum emission (546 → 515 nm) (2). In order to quantitate the amount of dansylcadaverine incorporated at apparent saturation, the sample was eluted through a P6 gel filtration resin (Bio-Rad) to separate unbound probe from that bound to Factor V. The protein concentration was calculated using the extinction coefficient ε₅₃₅₆₉ of 9.6 (36) and the dansylcadaverine concentration using ε₃₄₀₅₄₃₉ of 4,300 M⁻¹ cm⁻¹ (38). For the dansylcadaverine absorbance measurement, the samples were first made to 6 M guanidine HCl. The dansylcadaverine eluting in the void volume of P6 with Factor V were identified on 5-15% polyacrylamide gels. After incubation, each sample was dissolved in 0.024 M, 10% glycerol, and 0.002% bromphenol blue and heated at 100°C for 5 min. Proteins were visualized in the gels by staining with Coomassie Blue R-250.

Immunoprecipitation—Immunoprecipitation studies, conducted in order to immunochemically identify any probe-incorporated peptides, were performed according to Foster et al. (41). After incubation, [³H]putrescine samples were diluted as necessary in radioimmunoassay buffer (0.075 M Tris-HCl, 0.075 M NaCl, 1% Triton X-100, pH 7.0, containing 10 mg/ml bovine serum albumin). Reaction mixtures were prepared by incubating 0.2 ml of antigen solution with 0.2 ml of purified murine monoclonal immunoglobulin (0.1 mg/ml in radioimmunoassay buffer) and incubated at room temperature for 30 min after which 0.05 ml of rabbit anti-mouse antiseraum was added to each tube. After 30 min, the immunoprecipitates were collected by centrifugation, washed once with radioimmunoassay buffer, and dissolved in 0.2 ml of 0.2 N acetic acid. Thirty μl of each dissolved pellet were counted for radioactivity.

RESULTS

Lysine analogs such as [³H]putrescine and dansylcadaverine are useful when attempting to identify peptides containing glutamic residues that are accessible to a transamidase such as Factor XIIIa. The [³H]putrescine and dansylcadaverine incorporation time courses are shown in Fig. 1. The incorporation of either probe into the Factor V pro-cofactor proceeded at a rate approximately one-half that of thrombin-cleaved Factor V. Since thrombin is required for Factor XIII activation (13) and Factor V is cleaved and activated by thrombin (16), the thrombin inhibitor PPACK was used to preserve the status of pro-cofactor (Factor V) in some of the samples.

At the end points of the incubation time courses, the amounts of [³H]putrescine radioactivity bound to protein on filter paper indicated that 4 mol of putrescine were incorporated into 1 mol of human pro-cofactor or thrombin-cleaved Factor V. While 4 mol of putrescine were bound per mol of bovine pro-cofactor V, the data indicated that 5 mol of probe were bound per mol of thrombin-cleaved bovine Factor V peptide mixture. It appears that the thrombin cleavage of bovine Factor V reveals 1 more glutamine residue accessible for transamidation, while the activation of human Factor V produced no additional putrescine incorporation sites.

In order to test the possibility that PPACK might somehow have caused the decreased rate of Factor XIIIa activity in the thrombin-inhibited samples (Fig. 1), parallel incubations of Factor XIIIa with dimethylcasein (Sigma) were performed. The presence of PPACK did not retard the rate of dansylcadaverine incorporation into casein (data not shown), indicating PPACK to lack influence on Factor XIIIa function.

SDS gels were used to resolve the pro-cofactor and the thrombin activation products of Factor V. The gels revealed that the presence of PPACK had prevented significant degradation of the pro-cofactor throughout the 20-h incubations (Fig. 2). Fluorographs of the gels (Fig. 2) showed that with the exception of radiolabeled material retained at the top of the 3% stacking gel, the only [³H]putrescine-labeled material existed with Mᵦ = 120,000 in thrombin-cleaved bovine Factor V, 150,000 in cleaved human Factor V, and 390,000 in PPACK-treated human and bovine Factor V samples. Fig. 2 also shows Coomassie Blue R-250-stained samples of procofactor Factor V and the thrombin-treated human and bovine proteins. As is immediately apparent, there are no detectable Coomassie Blue-stained bands at Mᵦ = 120,000 or 150,000 that correspond to the [³H]putrescine-labeled bands on the fluorograph. The large activation peptides of human and bovine Factor V have been reported to stain poorly, if at all, with Coomassie Blue, but are readily evident with silver staining or Schiff-periode indication of carbohydrate (19, 42-44).

Nesheim et al. (16) performed amino-terminal sequencing on the large activation peptide designated as Cᵦ. The Cᵦ peptide was observed to possess atypical SDS-PAGE mobility properties (16); thus, the Mᵦ values taken from SDS-gel electrophoresis are, at best, loose estimates of the true molecular weights. The Mᵦ = 120,000 and 150,000 [³H]putrescine or dansylcadaverine-labeled protein bands also corresponded in electrophoretic mobility to clear zones in SDS gels not
FIG. 1. Incorporation of $[^3]H$putrescine or dansylcadaverine into Factor V with time. Bovine Factor V is shown with $[^3]H$putrescine (A) and dansylcadaverine (C); human Factor V with $[^3]H$putrescine (B) and dansylcadaverine (D). In each case, unactivated pro-cofactor V is indicated (O—-O), as well as thrombin-activated Factor V peptide mixtures (●—●). The data show that in each situation, the rate of incorporation of probe into activated Factor V was approximately twice that of the pro-cofactor. In following dansylcadaverine incorporation, the relative fluorescence intensity was measured with an emission of 500 nm and an excitation of 360 nm. The designation Va on the right ordinate axis refers to thrombin-activated Factor V peptide mixtures.

FIG. 2. Indication of pro-cofactor V and thrombin-activated Factor V peptides resolved on SDS gels. Panel A shows the Coomassie Blue R-250 staining patterns of pro-cofactor V (lane 1), activated human Factor V (lane 2), and activated bovine Factor V (lane 3). In lanes 2 and 3, the stained components are Factor Va chains (D and E). Panel B shows SDS-PAGE profiles of dansylcadaverine-labeled pro-cofactor V (lane 1), activated human Factor V (lane 2), and activated bovine Factor V (lane 3). Panel C illustrates the electrophoretic patterns of $[^3]H$putrescine-labeled Factor V components revealed by fluorography: lane 1, human pro-cofactor V; lane 2, activated human Factor V; lane 3, bovine pro-cofactor V; lane 4, activated bovine Factor V. The probe-labeled C1 fragment bands did not stain with Coomassie Blue and the chains of Factor Va failed to reveal the presence of either $[^3]H$putrescine or dansylcadaverine. The arrows at the top of panels B and C indicate probe-labeled, covalently polymerized Factor V material. The relative PAGE mobilities of the Factor V/Va components are designated as follows: FV, pro-cofactor V ($M_r = 330,000$); C1, the large activation peptide ($M_r = 150,000$ for human and $M_r = 120,000$ for bovine); D and E, the $M_r = 94,000$ and 74,000 chains of Factor Va.

completely destained. These “ghosts” have been understood to be the carbohydrate-rich, non-Coomassie Blue-staining, large activation fragments. The addition of thrombin to dialyzed Factor V samples that had been incubated in the presence of Factor XIIIa, PPACK, $[^3]H$putrescine, or dansylcadaverine, produced SDS-PAGE patterns that were indistinguishable from those of untreated, thrombin-activated Factor V. This result demonstrated the ability of thrombin to cleave probe-incorporated Factor V in a manner very similar to, if not identical with, unmodified Factor V.

In order to further survey the distribution of incorporated $[^3]H$putrescine within Factor V, immunoaffinity chromatography and immunoprecipitation methods were employed. First, each $[^3]H$putrescine-labeled Factor V sample was eluted through the P6 gel filtration resin to separate unbound putrescine from that bound to Factor V. $[^3]H$Putrescine-Factor...
V was then eluted over a Sepharose matrix that had coupled to it a murine monoclonal antibody to human Factor V (40). This antibody has been observed to bind to the $M_r = 74,000$ chain of both bovine and human Factor Va. However, only the human Factor V pro-cofactor ($M_r = 330,000$) will bind, while that of bovine will not. For thrombin-activated Factor V (Va), the $M_r = 94,000$ chain associates with the $M_r = 74,000$ chain and the antibody-Sepharose matrix retains both chains (40). Fig. 3A shows the elution profiles of $[^3H]$putrescine-labeled human Factor V and thrombin-activated human Factor V. The elution of human Factor V from the column with 1.5 M NaCl resulted in virtually all of the radioactivity being retained on the column. However, the elution of thrombin-treated human Factor V resulted in all of the $[^3H]$putrescine-labeled protein present in the flow-through fractions (Factor V activation peptides) leaving the greater amount of 280 nm absorbing material (Factor Va) bound to the column.

In contrast to human Factor V, the antibody column binds only the two chains of bovine Va and not the bovine procofactor V molecule. Fig. 3B illustrates that appropriately all of the radiolabeled material in the bovine procofactor V samples failed to bind to the column, as did the labeled component of thrombin-cleaved bovine Factor V samples. The antibody matrix did however retain the $M_r = 94,000$ and 74,000 chains of bovine Va in the thrombin-activated samples and the bound Factor Va was shown to lack significant radioactivity. Thus, the elution of $[^3H]$putrescine-labeled bovine pro-cofactor and thrombin-activated Factor V demonstrated the absence of $[^3H]$putrescine incorporated into the Factor Va ($M_r = 94,000-74,000$) complex (Fig. 3B).

Since the SDS-PAGE fluorescence, fluorographic, and immunoprecipitation evidence indicate that the large activation fragment of human and bovine Factor V to exclusively possess the incorporated probes, immunoprecipitation employing murine monoclonal antibodies to the activation peptide itself were included in this study. Table I contains the relative amount of radioactivity precipitated by each antibody. As controls, antibodies specific to human and bovine Factor Va $M_r = 94,000$ chains were included. There was no significant amount of radiolabeled material precipitated by either of the control antibodies. The immunoprecipitation survey identified at least 90% of the $[^3H]$putrescine-labeled protein in each activated Factor V solution to be the large activation peptide.

These results indicated that the incorporation of putrescine did not significantly inhibit antibody binding to the respective epitopes and that the site of incorporation is the large, poorly staining midregion of the Factor V molecule.

**DISCUSSION**

Fig. 4 illustrates the arrangement of the Factor V peptides produced by thrombin cleavage. The $M_r = 94,000$ and 74,000 chains of Factor Va bind to phospholipid vesicles or platelets (20) and form a high affinity receptor for Factor Xa (17). The fate or function of the Factor V activation peptides has not been elucidated. Since only 51% of the Factor V molecule is employed in the expression of Factor Va in the prothrombinase complex, it would not seem unreasonable to consider there to be another role(s) for the relatively significant amount of protein represented by the activation peptides.

Factor V exists as an asymmetric molecule with a relatively large axial ratio ($a_{20,0} = 9.2$) (32) and is found in platelets, as well as plasma (34). Since most of the substrates for Factor XIIIa are also elongated in conformation (24–28) and exist in platelets (27, 29–31), Factor V was tested as a potential substrate for Factor XIIIa through the use of the lysine analogs $[^3H]$putrescine and dansylcadaverine. Factor V was demonstrated to contain glutamine residues accessible to transamidation by Factor XIIIa. When probe-incorporated, thrombin-activated human or bovine Factor V was analyzed by gel electrophoresis and fluorography, the incorporated material was observed to exist in primarily two forms. In human and bovine Factor V samples, all had probe-labeled, polymerized material that failed to enter the 3% stacker gel. In the thrombin-inhibited Factor V samples, the majority of the labeled protein existed with an $M_r = 330,000$. The thrombin-cleaved samples produced probe-incorporated material with $M_r$ values of 150,000 in human and 120,000 in bovine samples. Since the denaturing polyacrylamide gels resolved a fraction of the radiolabeled material in each sample to be too large to enter the 3% stacker gel, it seems apparent that, as is the case with fibronectin (45) and von Willebrand Factor,¹
TABLE 1

| Antibody (epitope) | Antigen | Precipitated cpm |
|-------------------|---------|-----------------|
| αBFV-7 (M, 120,000) fragment | Activated BFV | 9170 |
| αBFV-7 (M, 120,000) fragment | Unactivated BFV | 5830 |
| αBFV-5 (M, 94,000) chain | Activated BFV | 105 |
| αHFV-7 (M, 150,000) fragment | Activated HFV | 9350 |
| αHFV-7 (M, 150,000) fragment | Unactivated HFV | 8870 |
| αHFV-5 (M, 94,000) chain | Activated HFV | 135 |

* 10,000 ± 200 cpm/sample.
* αBFV, anti-bovine Factor V.
* αHFV, anti-human Factor V.

**FACTOR V**

![Factor V](image)

**REFERENCES**

1. Lorand, L., Campbell-Wilkes, L. K., and Cooperstein, L. (1972) Anal. Biochem. 50, 623-631
2. Lorand, L., Lockridge, O. M., Campbell, L. K., Myhrman, R., and Bruner-Lorand (1971) Anal. Biochem. 44, 221-231
3. Chung, S. I., and Folk, J. E. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 303-307
4. Thacher, S. M., and Rice, R. H. (1985) Cell 40, 685-696
5. Anderson, D. R., Davis, J. L., and Carraway, K. L. (1977) J. Biol. Chem. 252, 6617-6623
6. Lorand, L., Weissman, L. B., Epel, D. L., and Bruner-Lorand, J. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4479-4481
7. Lorand, L. (1972) Ann. N. Y. Acad. Sci. 202, 6-30
8. Lorand, L., Losowsky, M. S., and Milosewski, K. J. M. (1980) Prog. Hemostasis Thromb. 5, 245-290
9. Iwakawa, S., Suzuki, K., and Hashimoto, S. (1978) Ann. N. Y. Acad. Sci. 312, 56-73
10. Mooler, D. F., Schad, P. E., and Kleinman, H. K. (1979) J. Clin. Invest. 64, 781-787
11. Sakata, Y., and Aoki, N. (1980) J. Clin. Invest. 65, 290-297
12. Cohen, I., Blankenberg, T. A., Borden, D. K., and Veis, A. (1980) Biochem. Biophys. Acta 628, 365-375
13. Takagi, T., and Doilittle, R. F. (1974) Biochemistry 13, 750-756
14. Lorand, L., Gray, A. J., Brown, K., Crocri, R. B., Curtis, C. G., Domanick, R. A., and Steinberg, P. (1974) Biochem. Biophys. Res. Commun. 56, 914-922
15. Curtis, C. G., Brown, K. L., Crocri, R. B., Domanick, R. A., Gray, A., Steinberg, P., and Lorand, L. (1974) Biochemistry 13, 3774-3778
16. Nesheim, M. E., Foster, W. B., Hewick, R., and Mann, K. G. (1984) J. Biol. Chem. 259, 3187-3196
