Factor V Is Activated and Cleaved by Platelet Calpain: Comparison With Thrombin Proteolysis

By Harlan N. Bradford, Anjanayaki Annamalai, Kundan Doshi, and Robert W. Colman

Platelets are known to process human factor V during secretion and/or membrane binding. We studied the functional and structural changes produced in human factor V by purified human platelet calpain (calcium-activated thiol protease) and compared the alterations with those induced by thrombin. A maximum increase in coagulant activity of 2.5-fold was observed when factor V (1 U/mL; 33 nmol/L) was incubated with calpain (0.03 U/mL; 2.7 nmol/L) in comparison with a 8.8-fold increment for α-thrombin (0.7 U/mL; 8 nmol/L) at 25°C. Thrombin additions to reactions initiated by calpain resulted in further activation comparable to that of thrombin alone, whereas the subsequent addition of calpain had no effect on the extent or pattern of the activation of factor V by thrombin. The cleavage pattern of factor V produced by these two enzymes are distinctly different. Although thrombin activation eventually results in four final components designated C1 (150 kd), D (105 kd), E (71 kd), and F2 (71 to 74 kd), calpain yields initial components of 200 kd and 160 kd within one minute.

Further digestion of the 200 kd species by calpain gives rise first to a polypeptide of 160 kd that is converted to a 140 kd and a 120 kd species by two minutes with an increase in coagulant activity. Immunoblotting of these fragments with the monoclonal antibody (MoAb) B10 directed to factor V and the thrombin-generated C1 fragment yields results demonstrating a common epitope in these calpain-generated components of 200, 160, 140 and 120 kd. The degradation of the initial 160 kd polypeptide gives rise to polypeptides of 100 and 65 kd, both undetectable on immunoblotting with MoAb B10. The 130, 87, 58, and 48 kd components are of less certain origin. Thus, platelet calpain generates a complex but reproducible cleavage pattern different from thrombin that may explain the partial activation observed. Nevertheless, calpain processing may play a role in early hemostatic reactions involving platelets before the appearance of the first thrombin molecule.

Materials

 α-Thrombin was a generous gift of Dr John Fenton. Leupeptin, Tris (Tris), Temed, Tween 20, ammonium persulfate, 4-chloro-1-napthol, and rabbit antinmouse IgG coupled with horseradish peroxidase were obtained from Sigma Chemical Co, St Louis. Dithiothreitol (DTT) was purchased from Boehringer, Mannheim, West Germany; calcium chloride, methanol, silver nitrate, formalin, acetic acid, Coomassie brilliant blue R250, sodium carbonate, disodium ethylenediamine tetraacetate (EDTA), and citric acid were all purchased from Fisher Scientific Co, King of Prussia, PA. Glycine, sodium dodecyl sulfate (SDS), and mol wt standards were purchased from Bio-Rad Laboratories, Richmond, CA. Prestained standards were purchased from Bethesda Research Laboratories (BRL, Bethesda, MD), and nonfat dry milk (NFDM) was obtained from a local food store.

Preparation and characterization of murine monoclonal anti-

From the Hematology-Oncology Section, Department of Medicine, and the Thrombosis Research Center, Temple University School of Medicine, Philadelphia.

Submitted January 23, 1987; accepted October 1, 1987.

Presented in part at the 1987 meeting of the American Society of Biological Chemists in Philadelphia.

Address reprint requests to Robert W. Colman, MD, Hematology-Oncology Section, Department of Medicine, Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA 19140.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1988 by Grune & Stratton, Inc.
man factor V (BlO). The murine monoclonal antibody (MoAb) B10 was prepared in our laboratory as described earlier. B10, which was typed as an IgGk, recognizes the activation peptide (C) of human factor V and does not affect factor V coagulant activity.

**Purification of plasma factor V.** Factor V was purified by an immunooaffinity procedure adapted for a murine monoclonal anti-factor V (B10) instead of a human MoAb. The immunoadsorbent was prepared identically, but nonspecifically bound proteins were removed by washing with 0.02 mol/L Tris buffer, pH 7.2, containing 0.37 mol/L NaCl and then washed with the Tris buffer containing 0.15 mol/L NaCl, 1 mmol/L CaCl₂, 25% glycerol and 0.5% ethylene glycol. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the purified factor V showed a major band corresponding to a mol wt of 330 kd, a minor contaminant at 350 kd, and a partial cleavage fragment, component B, at 220 kd. The specific activity ranged between 70 and 100 U/mg and increased 17- to 20-fold upon activation with thrombin at 37°C and eight- to tenfold at 25°C.

**Purification of platelet calpain.** Platelet calcium–activated cysteine protease (calpain) was purified from human platelet cytosol by using polyethylene glycol precipitation, anion-exchange chromatography, hydrophobic chromatography on reactive red agarose, and gel filtration. The protein was a single band on nondenaturing 10% SDS-PAGE. The mol wts of hydrolysis of factor V by calpain was calculated by SDS-PAGE analysis of the eight-minute end points of a representative activation experiment by PAGE (Fig 1B) shows that, compared with the initial factor V preparation (lane 4), thrombin digestion increased extent of activation; in some cases loss of activity has previously been reported for bovine factor V. No increase was found in factor V coagulant activity when the protein was incubated in the same buffer without calpain or thrombin. When factor V (1 U/mL, 33 nmol/L) an increase of 8.0-fold at four minutes and 8.8-fold at six minutes was observed. This partial activation at 25°C (lane 2) cleaved the 330 kd intact factor V (lane 4) with a slight reduction in activity noted by six minutes (Fig 1A). Under identical conditions with thrombin (0.7 U/mL, 8 nmol/L) an increase of 8.0-fold at four minutes and 8.8-fold at six minutes was observed. This partial activation at 25°C has previously been reported for bovine factor V. No increase was found in factor V coagulant activity when the protein was incubated in the same buffer without calpain or thrombin for the same time period. When the thrombin reaction was performed at 37°C, a 17-fold increase was observed, which is in agreement with previous data (only the first four minutes of the time course is shown). Higher concentrations of calpain led to a more rapid but not an increased extent of activation; in some cases loss of activity was noted with increasing time.

**Activation of factor V by calpain: comparison with thrombin.** When factor V (1 U/mL, 33 nmol/L) was incubated with purified platelet calpain (0.03 U/mL, 2.7 nmol/L) in the presence of calcium at 25°C (to avoid heat inactivation of the enzyme at a higher temperature), an increase in activity of 2.5-fold was observed by four minutes, with a slight reduction in activity noted by six minutes (Fig 1A). Under identical conditions with thrombin (0.7 U/mL, 8 nmol/L) an increase of 8.0-fold at four minutes and 8.8-fold at six minutes was observed. This partial activation at 25°C has previously been reported for bovine factor V. No increase was found in factor V coagulant activity when the protein was incubated in the same buffer without calpain or thrombin for the same time period. When the thrombin reaction was performed at 37°C, a 17-fold increase was observed, which is in agreement with previous data (only the first four minutes of the time course is shown). Higher concentrations of calpain led to a more rapid but not an increased extent of activation; in some cases loss of activity was noted with increasing time.

**RESULTS**

**Activation of factor V by calpain: comparison with thrombin.** When factor V (1 U/mL, 33 nmol/L) was incubated with purified platelet calpain (0.03 U/mL, 2.7 nmol/L) in the presence of calcium at 25°C (to avoid heat inactivation of the enzyme at a higher temperature), an increase in activity of 2.5-fold was observed by four minutes, with a slight reduction in activity noted by six minutes (Fig 1A). Under identical conditions with thrombin (0.7 U/mL, 8 nmol/L) an increase of 8.0-fold at four minutes and 8.8-fold at six minutes was observed. This partial activation at 25°C has previously been reported for bovine factor V. No increase was found in factor V coagulant activity when the protein was incubated in the same buffer without calpain or thrombin for the same time period. When the thrombin reaction was performed at 37°C, a 17-fold increase was observed, which is in agreement with previous data (only the first four minutes of the time course is shown). Higher concentrations of calpain led to a more rapid but not an increased extent of activation; in some cases loss of activity was noted with increasing time.

Analysis of the eight-minute end points of a representative experiment by PAGE (Fig 1B) shows that, compared with the initial factor V preparation (lane 4), thrombin digestion of factor V at 37°C (lane 2) cleaved the 330 kd intact factor
Fig 1. Activation of factor V by calpain and/or thrombin at 25°C. (A) Factor V (1 U/mL, 33 nmol/L) was incubated with 0.7 U/mL (8 nmol/L) thrombin (●), with 0.03 U/mL (2.7 nmol/L) platelet calpain (□), or with buffer (▲). The factor V activation at 37°C (○) is shown for comparison. At various time intervals the incubation mixture was diluted and assayed for coagulant activity as in Methods. (B) Silver-stained 10% SDS-PAGE of the factor V preparation (lane 4), calpain (lane 3), and eight-minute digests of factor V by either thrombin at 37°C (lane 2) or by calpain at 25°C (lane 1).

V as well as the 220 kd intermediate as previously reported for bovine factor V. In contrast, highly purified calpain (lane 3) hydrolyzed factor V to different polypeptides (lane 1) than did thrombin. These experiments indicate that calpain can activate factor V, but to a lesser extent than does thrombin and with a markedly different pattern of cleavage.

Time course of hydrolysis of factor V by calpain. The sequential cleavage of factor V catalyzed by calpain at a 1:10 molar ratio of calpain to factor V is presented in more detail to better visualize the temporal changes in the activation intermediates. The experiment displayed in Fig 2 shows a 4% to 8% gradient SDS-PAGE stained with silver and uses the same molar ratio of thrombin and calpain as in Fig 1A except that the total protein was proportionally increased in the reaction mixtures to minimize the volume loaded for PAGE analysis. This change in the concentration of enzyme and substrate may have affected the reaction rate and therefore may have not directly correlated the changes on this gel with the coagulant activity. For reference, the cleavage pattern of factor V with thrombin for four minutes at 25°C is shown (Fig 2, lane 1) and includes B (220 kd), C (150 kd), D (105 kd), and F,F2 (74 to 71 kd), similar to that reported by Suzuki et al. In contrast, at 15 seconds of factor V incubation with calpain, components of 200 and 160 kd (Fig 2, lane 2) appear. By one minute the native factor V (330 kd) is completely digested to these two new components as well as a 130 kd polypeptide (lane 4). By four minutes a loss of the 200 kd component and appearance of two new fragments of 140 and 120 kd is evident (Fig 2, lane 5). Further degradation of the 140 kd fragment after eight minutes occurs without any increase in activity. Neither the 105 kd (heavy) or the 74 to 71 kd (light) chain appear. Instead, new components of 100, 87, 65, 58, and 48 kd increase in concentration (lanes 6 and 7).

Activation of factor V by calpain and thrombin sequentially. In reactions performed at 25°C, factor V coagulant activity increased 1.3-fold over control after incubation with calpain for two minutes (Fig 3A). After the addition of thrombin at two minutes and after initiation with calpain, a 7.4-fold activation was observed at four minutes and a tenfold activation after six minutes (Fig 3A, ▲). This degree of activation was similar to that seen by thrombin alone (Fig 1A). When calpain and factor V were incubated for four minutes, a 1.9-fold activation was observed. With addition of thrombin and incubation continued for an additional two
FACTOR V ACTIVATION AND CLEAVAGE BY CALPAIN

minutes, an increase to 7.6-fold was found (Fig 3A, ⑤). These results indicate that, after factor V activation by calpain, thrombin was able to activate factor V and the degree of activation was similar to that of thrombin alone. When the reciprocal experiment was performed, the addition of calpain to factor V exposed to thrombin for either two (Fig 3A, ⑤) or four (Fig 3A, ⑥) minutes gave a similar degree (9.2-fold at six minutes) as exposure to thrombin alone (8.8-fold at six minutes). When leupeptin (100 umol/L), an inhibitor of calpain, was included before calpain addition, no differences were observed from factor V incubations with buffer alone. Thus, after activation by thrombin, calpain has little if any effect on factor V activation.

To further define the molecular changes occurring with calpain action on factor V, the sequential digestion of factor V with calpain followed by thrombin and factor V with thrombin followed by calpain was monitored by SDS gels. The experiment paralleled the changes in coagulant activity seen in Fig 3A. Calpain can cleave the 220 kd (B) and 150 kd (C₁) fragments of thrombin-digested factor V but does not alter the 105 kd (D) fragment or the 74 to 71 kd (F₁,F₂) fragment (Fig 3B, lanes 2 and 5). Thrombin can cleave the 160, 140, 130, and 120 kd fragments produced by calpain to yield the 105 kd heavy chain and the 74 to 71 kd light chain (Fig 3B, lanes 3 and 4).

Fig 3. Sequential activation and cleavage of factor V by thrombin and calpain. (A) Factor V (1 U/mL, 33 nmol/L) was incubated with thrombin at 25°C for two minutes. Calpain (0.03 U/mL, 2.7 nmol/L) was then added two (①, ④) or four minutes later (②, ③). In the inverse experiment, calpain (0.03 U/mL, 2.7 nmol/L) was first added to factor V, and then thrombin (0.7 U/mL, 8 nmol/L) was added after an additional two minutes (⑤, ⑧) or at four minituces (⑥, ⑨). The control reaction consisted of factor V containing 0.5 mmol/L leupeptin before the addition of calpain and assayed for coagulant activity at various times (A). (B) SDS-PAGE was performed as in Fig 2 on representative reactions. Mol wt markers are shown (lane 1). Factor V (5 U/mL, 165 nmol/L) was incubated with thrombin (4.8 U/mL, 38 nmol/L) for four minutes, and then calpain (0.15 U/mL, 16 nmol/L) was added to this reaction, which was incubated for two more minutes in the presence of 5 mmol/L Ca²⁺ at 25°C (lane 2). Factor V and calpain, in the aforementioned concentrations, were incubated for two minutes, and then thrombin was added and digestion continued for an additional four more minutes (lane 3). Factor V and calpain were reacted for four minutes; this was followed by the addition of thrombin and digestion continued for an additional four minutes (lane 4). In lane 5 thrombin was added for four min and then calpain for four more minutes. Factor V and thrombin, incubated at 25°C for four minutes (lane 6) and calpain alone (lane 7), are presented for reference.

Fig 4. Mol wt products of calpain and thrombin: Coomassie blue analysis. Lane 1 is the same digest of factor V by thrombin (eight minutes) that is observed in Fig 1, lane 2, at 37°C. Lanes 2 and 3 are digest of factor V by calpain at 25°C for four minutes and eight minutes, respectively. Each has been electrophoresed on a 10% SDS polyacrylamide gel and stained with Coomassie blue. Mol wt markers appear in lane 4.
Further comparison of factor V digestion products by calpain and thrombin by using Coomassie stain. Calpain reactions with factor V were terminated after four or eight minutes of incubation (Fig 4, lane 2 and 3). Thrombin was incubated at 37°C for eight minutes with factor V to complete the digestion of the 220 kd fragment to its lower-mol wt end products and then analyzed on a 10% SDS-PAGE after staining with Coomassie blue (Fig 4, lane 1). Under these conditions the 150 kd (C_1) product resulting from thrombin and possibly some of the calpain cleavage derivatives resulting from products containing this piece stain poorly because of the high carbohydrate content. Thrombin also yields products of mol wt 105 kd and 74 to 71 kd, whereas calpain gives fragments of 100 and 87 kd (Fig 4, lanes 2 and 3). The smaller fragments produced by calpain below 87 kd (65, 58, and 48 kd) that are observed on silver staining (Fig 2) are below the detection limit for this Coomassie-stained gel. There is no 105 kd (D, heavy chain) or 74 to 71 kd (F,F_2, light chain) fragments in the factor V digests resulting from calpain action.

Immunoblotting to identify the domains of factor V cleaved by calpain. To define further the complex pattern of calpain cleavage, we used immunoblotting (Fig 5) with a murine MoAb, B10, that has been shown to recognize C_1, the 150 kd activation peptide from thrombin digests. The antibody recognizes the native factor V (330 kd) and the large digestion product, 220 kd (B, Fig 5, lane 1), present in this preparation of partially cleaved factor V, both of which contain the 150 kd activation peptide. In the calpain digest the 330 kd moiety is rapidly cleaved by 15 seconds (Fig 5, lane 2), with appearance of a 200 kd piece and another at 160 kd. Further cleavage (Fig 5, lanes 3 to 7) results in the

Fig 5. Immunoblot using MoAb B10 with factor V digested by calpain and thrombin. Factor V (lane 1) was digested with calpain (lanes 2 to 7) at 0.25, 0.5, 1, 2, 4, and 8 minutes, respectively, at 25°C under the same conditions as in Fig 2. The migration of the mol wt standards (BRL) are shown on the left. The calculated mol wts of the fragments of the digest reactions with B10 are shown on the right.

Fig 6. Schematic representation of the hydrolysis of factor V by calpain compared with thrombin. Intact factor V is shown by using the nomenclature of Suzuki et al., which designates the thrombin cleavage products as the domains. The scheme for thrombin digestion is also from Suzuki et al. On exposure to calpain, the digestion products of factor V by calpain are arranged according to the data resulting from the 4% to 8% gradient SDS-PAGE (Fig 2) and the 10% SDS-PAGE (Fig 4). Positive reactivity to the MoAb B10 that was determined in Fig 5 is designated as A, which is the epitope contained in the 150-kd C_1 component in the thrombin digests and in the native molecule. The lower-mol wt component reactions with MoAb B10 are presumed to derive from higher-mol wt polypeptides. The alignment is schematic because the N and C terminals of each fragment are not known.
sequential appearance of 140 and 120 kd fragments clearly
derived from the 160 kd polypeptide. None of the mol wt
species less than 120 kd are recognized by BlO on immuno-
blots, nor is the 130 kd species recognized.

DISCUSSION

Platelet proteases can cleave human factor V as first
suggested by Osterud et al. Ittyerah et al demonstrated
that plasma factor V was activated twofold by a fresh
platelet extract. The suggestion was offered that the cleavage
might be due to a calcium-activated protease. This study
provides objective evidence that platelet calpain could be the
responsible protease. The enzyme used in this study was
homogenous, and its action on factor V was inhibited by
leupeptin (Fig 1). As in the crude platelet lysate, the
maximum activation noted was 2.5-fold.

The cleavage pattern produced by calpain appears to be
different from thrombin. Figure 6 summarizes the cleavage
by thrombin as defined by Suzuki et al and contrasts it with
the pattern produced by platelet calpain that was deduced
from the SDS gels and immunoblotting experiments. Cal-
pain first attacks a bond and gives two major intermediates
of 200 kd and 160 kd (Fig 2). Even at 15 seconds we observe
smaller concentrations of 140, 100, and 65 kd components.
The 200 kd component is similar to the thrombin component
B as shown by the positive reaction with B10 (Fig 5), which
recognizes the thrombin C, domain (150 kd), and forms part
of the 220 kd component. Further cleavage by calpain of the
200 kd polypeptide liberates smaller fragments (160,
140, and 120 kd) that contain an epitope on C, as shown by
blotting with MoAb BlO (Fig 5). The 160 kd fragment seen
in Fig 2 may in fact be two different polypeptides. One of
these components, presumably not containing the B10 epi-
tope, may be further degraded to fragments of 100 and 65 kd
that are unreactive with MoAb B10. The 130 kd and other
low-mol wt fragments (87, 58, and 48 kd) do not react with
MoAb B10; however, their origin is speculative because they
could result from cleavage of the 160 kd component contain-
ing the MoAb B10 epitope and be the part of the polypeptide
not bearing the epitope. The failure of the 160 kd component
to stain well with Coomassie blue (Fig 4) is consistent with
the presence of high amounts of carbohydrate because the
thrombin-derived C, (150 kd) also stains poorly. More
recently Viskup et al reported the purification of factor V
from platelets. Despite the use of inhibitors during platelet
collection and washing, the lower-mol wt forms of factor V
reported resemble those we observe after exposure to calpain.
The inability to obtain intact factor V from platelets could be
due to the action of calpain on factor V that is associated
with activation of platelets during their isolation from whole
blood or during solubilization and subsequent purifica-
tion. In contrast, in plasma, kininogens would inhibit any
calpain escaping from the platelet cytosol.

The failure of calpain to completely cleave the light chain
of factor Va (D,F,F2) (Fig 2 and 4) is supported by the ability of thrombin to cleave factor V, which has been
previously hydrolyzed by calpain (Fig 3). Both the cleavage
and the ability of thrombin to fully activate factor V that is
digested by calpain for short times are consistent with this
construct. A more detailed analysis of the nature of the
cleavage pattern of factor V by calpain awaits the determina-
tion of the full amino acid sequence of factor V.

Platelet membrane and cytoskeletal proteins localized in
both the plasma and the platelet $\alpha$-granules, such as
fibrinogen, Willebrand factor, and H-kininogen, are
also substrates for calpain. Thus, factor V, an $\alpha$ granule
protein and a plasma procofactor, also fits well into the
group of calpain substrates. The function of activation and
cleavage of factor V by platelet calpain is not fully under-
stood. As already stressed, once thrombin is present, calpain
cannot activate factor V further; however, calpain may play
a role in the early events of hemostasis before the formation
of thrombin. Ittyerah et al found that bovine platelet factor
V was identical to that in plasma only when purified in the
presence of protease inhibitors. When whole platelets were
lysed with nonionic detergent or factor V released from
platelets after stirring with collagen, these preparations
contained cleaved bovine factor V as detected by a change
in charge on immunoelectrophoresis. Thus, factor V is cleaved
and activated, probably by platelet cytosolic enzymes includ-
ing calpain, during secretion or injury. Even a low level of
activation would facilitate the cleavage of prothrombin by
factor Xa and promote the formation of the first few
molecules of thrombin. Once thrombin was formed, the
feedback loop would rapidly produce factor Va. In this
scheme, platelet calpain might serve to allow limited activa-
tion of factor V after exposure of platelets to basement
membrane collagen in the initial phase of blood coagulation
before thrombin formation. Finally, factor V has recently
been shown to be a substrate for factor XIII. Wang et
al have demonstrated that the activation of platelets with
thrombin is associated with factor XIII catalysis of the
crosslinking of factor V to the cytoskeletal action. The
finding that calpain can activate factor XIII suggests an
additional indirect interaction between calpain and factor V
that is important for expression of this protein on the platelet
membrane.

REFERENCES

1. Colman RW: The effect of proteolytic enzymes on bovine
factor V. I. Kinetics of activation and inactivation by bovine
thrombin. Biochemistry 8:1438, 1969

2. Nesheim ME, Taswell JB, Mann KG: The contribution of
bovine factor V and factor Va to the activity of prothrombinase. J
Biol Chem 254:10952, 1979

3. Miletich JP, Jackson CM, Majerus PW: Properties of the
factor Xa-binding site on human platelets. J Biol Chem 253:6908,
1978

4. Miletich JP, Jackson CM, Majerus PW: Interaction of coagu-
lation factor Xa with human platelets. Proc Natl Acad Sci USA
74:4033, 1977
34. Ware AG, Murphy SC, Tracy PB, Mann KG: The function of Ac-globulin. Science 106:618, 1947
Factor V is activated and cleaved by platelet calpain: comparison with thrombin proteolysis

HN Bradford, A Annamalai, K Doshi and RW Colman