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Research Article

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ABSTRACT Factor V must be converted to Factor V_a in order to bind to a high affinity platelet surface site and participate in prothrombin activation. Osterud et al. (10) presented data that suggested that human platelets contain an activated form of Factor V and a Factor V activator. We find that the Factor V released when platelets are disrupted by freezing and thawing or sonication is activated 3- to 10-fold by thrombin as determined by coagulation assay and is therefore stored as the relatively inactive procofactor rather than in the active form Factor V_a.

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The platelet Factor V activator is not inhibited by dansyl arginine-4-ethylpiperidine amide, 93 μM, indicating that it is not thrombin. When thrombin-stimulated platelets, to which dansyl arginine-4-ethylpiperidine amide had been added to inhibit the further action of thrombin, were incubated with ^{125}-labeled Factor V, there was no detectable proteolysis of the Factor V molecule. Our failure to detect activation of Factor V under these conditions suggests that <4% of the platelet protease is released by thrombin. Subcellular fractionation of platelets indicates that the platelet protease that activates Factor V is in the soluble fraction.

When Factor V_a formed by the action of platelet protease is incubated with platelets, peptides with M_r = 105,000, 87,000, and 78,000 bind to the platelet surface. All three radiolabeled peptides are displaced from platelets by unlabeled Factor V_a formed by the action of thrombin. The stoichiometry of binding suggests that the 105,000-dalton peptide is associated with either an 87,000- or a 78,000-dalton peptide. The 78,000-dalton peptide binds with greater affinity and probably accounts for the bulk of the activity of Factor V_a in coagulation assays. Whether or not the platelet protease serves to activate Factor V before thrombin formation during normal hemostasis remains to be determined.

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INTRODUCTION

Prothrombin activation requires Factors X_a, V_a, and calcium ions. Results from our laboratory indicate that prothrombin activation occurs on the platelet surface (200–300 sites/cell) (1–6). In addition to being present in plasma, coagulation Factor V is present in platelets localized in the α-granules (7–9). Platelet Factor V can be released by a variety of agents that stimulate platelet secretion (6, 8, 9) and once activated to Factor V_a, can serve to promote Factor X_a binding to platelets (6). Small amounts of thrombin are formed early in hemostasis (11, 12) and Osterud et al. (10) suggested that this might result from activation of Factor V by platelets. They performed experiments that suggested that platelets contain a Factor V activator distinct from thrombin as well as an activated form of Factor V. In contrast to these results we have found that the relatively small amounts of Factor V activity released from platelets after stimulation with arachidonic acid or calcium ionophore A23187 can be activated from 10- to 20-fold by treatment with thrombin (5). Similarly Chesney et al. (8) and Vicic et al. (9) reported that Factor V activity released by ADP or collagen was activated when exposed to thrombin. In other experiments, Giles et al. (13) concluded that the apparent "V activator-like" activity observed in frozen and thawed platelets is due to the provision of a potent source of phospholipid rather than direct activation of Factor V.

Factor V as isolated from both bovine (14–16) and

human (16–18) plasma is a relatively inactive pro-cofactor with M_r = 330,000. Treatment with catalytic amounts of thrombin or an enzyme isolated from Russell's viper venom (RVV-V)[1] results in limited and specific proteolysis of the Factor V molecule, which correlates with a 10- to 80-fold increase in activity as measured by coagulation assay. The activity of Factor V in a coagulation assay appears to be due in part to the conversion of small amounts of the pro-cofactor to the active form during the assay, but also may be due to contaminating Factor V_a (6). Thus, assessments of the exact amounts of Factor V and V_a in a sample based on coagulation assay is impossible because both Factor V and V_a contribute to the measurement to an unknown degree.

In the present study we have extended the observation of Osterud et al. (10) by comparing the effects of thrombin, RVV-V, and platelet lysates on the activity and structure of purified coagulation Factor V. We find that the soluble fraction of platelets contain a protease that can partially activate purified Factor V and that the pattern of proteolysis is different from that observed with thrombin or RVV-V. Whether or not this platelet protease serves to activate Factor V before thrombin formation during normal hemostasis remains to be determined.

METHODS

The preparation and assay of human prothrombin, thrombin, Factor X_a, Factor V, and fibrinogen have been described (2, 3, 17). Thrombin was assayed using U. S. standard thrombin lot J. Dansyl arginine-4-ethylpiperidine amide (DAPA) was synthesized from dansyl arginine (Pierce Chemical Co., Rockford, IL) and 4-ethylpiperidine (gift of Dr. Edward Sowers, Reilly Tar-Chemical, Indianapolis, IN) by the method of Nesheim et al. (19). IODO-GEN (1,3,4,6-tetrachloro-3α-6α-diphenyl glycouril) and sodium dodecyl sulfate were obtained from Pierce Chemical Co. Sodium [^{125}I]iodide was obtained from Amersham Corp., Arlington Heights, IL. Equine collagen was obtained from Hormon-Chemie Munchen GmbH, Munich, West Germany, calcium ionophore A23187 and Hepes from Calbiochem-Behring Corp., La Jolla, CA, and Apiezon oil from J. B. Biddle Co., Plymouth Meeting, PA. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO (bovine serum albumin, rabbit brain cephalin, diisopropyl fluorophosphate, benzamidine), Fisher Scientific Co., Pittsburgh, PA, or Mallinckrodt Inc., St. Louis, MO. The Factor V activator from Russell's viper venom was a gift from Craig Jackson.

Sodium dodecyl sulfate gel electrophoresis was performed (21) using 7.5% polyacrylamide with a 4% polyacrylamide stacking gel. Radioiodinated samples were detected by counting 1-mm slices in a Beckman 300 gamma counter (Beckman Instruments, Inc., Fullerton, CA) or by autoradiography (22). Factor V was iodinated by the method of Fraker and Speck (23) as previously described (6, 17). Factor V was assayed in a one-stage assay using purified human coagulation factors (17). The assay mixtures contained 50 μl each of (*a*) 6.7 mg fibrinogen/ml, (*b*) 100 μg prothrombin/ml and 20 μg rabbit brain cephalin/ml, (*c*) 25 ng Factor X_a/ml, and (*d*) sample. All of the reagents except fibrinogen were prepared in an assay buffer composed of 0.15 M NaCl, 5 mM trisodium citrate, 20 mM Tris-HCl, pH 7.4, and 10 mg bovine serum albumin/ml. Fibrinogen was prepared in the same buffer in the absence of bovine serum albumin. The assay was initiated by the addition of 50 μl of 0.1 M NaCl, 40 mM $CaCl_2$, and the time required for clot formation was measured using a fibrometer (Becton-Dickinson Co., Rutherford, NJ). Standard curves were constructed by assaying dilutions of human citrated plasma pooled from 23 donors. The assay had a functional range from 0.03 to 2 mU/ml corresponding to clotting times from 280 to 75 s.

Subcellular fractionation of platelets. Washed platelets were prepared from 500 to 1,000 ml of whole blood as described (24). The platelets were suspended in buffer containing 75 mM Tris, pH 6.4, 100 mM KCl, 12 mM sodium citrate, and were disrupted by a nitrogen decompression technique followed by fractionation on a sucrose density gradient (25). The sucrose density gradient centrifugation was adapted for use with a SW27 rotor (Beckman Instruments, Inc., Palo Alto, CA). Ultracentrifugation was for 2 h at 1.4×10^5 *g* at 4°C. Nine fractions were identified by light-scattering and collected at 4°C. Particulate fractions were diluted with buffer composed of 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5.5 mM glucose, centrifuged for 1 h at 2.0×10^5 *g* at 4°C, and then the pellets were suspended in the same buffer and stored at −80°C. The soluble fraction was stored in the same buffer used for platelet disruption and the pH was adjusted to 7.4 with 1.0 M NaOH or samples were dialyzed against 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5.5 mM glucose, and 2.5 mM $CaCl_2$ buffer before experiments.

β-Galacosidase was assayed as described by Reitman et al. (26), β-glucuronidase according to Meisler et al. (27), protein by the method of Lowry et al. (28), and results were calculated as relative specific activities (29).

Activation of Factor V. Thrombin-activated Factor V_a was prepared by incubating Factor V (100 μg/ml) in a solution containing 150 mM NaCl, 2.5 mM $CaCl_2$, 0.56 mM benzamidine, 5 mg/ml recrystallized bovine serum albumin, 20 mM Tris HCl, pH 7.4, 2 U/ml of thrombin, for 20 min at 22°C.

Activation of ^{125}I-human Factor V. ^{125}I-Factor V at a concentration of 0.4 μg/ml in buffer containing 150 mM NaCl, 20 mM Tris HCl, pH 7.4, 2.5 mM $CaCl_2$, 5 mg/ml recrystallized bovine serum albumin, was incubated for 20 min at 22°C with 2 U/ml of thrombin or with 0.6 mg/ml of platelet soluble fraction.

Binding of Factor V_a to platelets. Factor V_a formed by thrombin is designated by V_{at}. Factor V_a formed by the platelet protease is designated as V_{ap}. ^{125}I-Factor V_{at} or Factor ^{125}I-V_{ap} binding to the platelets was measured using methods similar to those previously described for measuring the binding of Factors X_a and V_a to platelets (1, 5, 6). Binding assay mixtures contained 10^8 platelets/ml, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 2.5 mM $CaCl_2$, 5.5 mM glucose, 0.1 mM DAPA, 20 ng/ml of Factor X_a, and 5 mg/ml recrystallized bovine serum albumin. DAPA was included to prevent thrombin-induced release and activation of endogenous Factor V and to block any further thrombin action due to small amounts of thrombin present in incubations containing V_{at}.

[1] *Abbreviations used in this paper*: DAPA, dansyl arginine-4-ethylpiperidine amide; PAGE, polyacrylamide gel electrophoresis; RVV-V, Factor V activating enzyme from Russell's viper venom.

Binding mixtures also contained either ^{125}I-V_{at} with or without unlabeled V_{at} or ^{125}I-V_{ap} with or without unlabeled V_{at}. Incubations were carried out for 20 min, the platelets were then sedimented through oil as previously described (1). Samples of supernatants and pellets were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21) and radioactivity was detected by autoradiography (22) or the gels were cut and radioactive spots counted in a Beckman Bio-Gamma-II γ-Counter.

Collagen aggregation of washed platelets. Washed human platelets were suspended in a buffer containing 0.135 M NaCl, 2.7 mM KCl, 11 mM $NaHCO_3$ pH 7.35, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Hepes pH 7.35, 5.5 mM glucose, 3.5 mg albumin/ml, and 0.5 mg fibrinogen/ml (33). Collagen-induced aggregation (25 μg collagen/ml) was monitored with Payton dual channel aggregometer (Payton Associates, Ltd., Buffalo, NY).

RESULTS

Thrombin activation of platelet Factor V released by sonication or freezing and thawing. Suspensions of intact washed human platelets expressed minimal amounts of Factor V activity as measured by coagulation assay (<0.001 U/10^8 platelets). When platelets were lysed by sonication or by three cycles of freezing and thawing, Factor V activity in the platelet suspensions increased to a maximum of 0.01–0.060 U/10^8 platelets after 20 min in six experiments. However, if the platelet suspensions were treated with thrombin immediately after cell lysis or 20 min after cell lysis Factor V activity rapidly increased 3- to 10-fold to 0.10–0.20 U/10^8 platelets. The total amount of Factor V activity present in thrombin-treated platelet lysates is similar to the amount released when intact platelets are treated with thrombin (5).

Activation of ^{125}I-labeled Factor V by thrombin and RVV-V. Human Factor V is a single chain high molecular weight pro-cofactor with apparent M_r = 330,000. We have previously shown that appearance of Factor V coagulation activity correlates with a specific bond cleavage in the molecule, which produces a doublet peptide with M_r = 78,000 (17). We analyzed the activation of 5 μg/ml ^{125}I-labeled Factor V by the enzymes thrombin and RVV-V using polyacrylamide gel electrophoresis with sodium dodecyl sulfate as shown in Fig. 1. The results shown here are similar to the results we obtained previously using gels stained with Coomassie Blue except that in the current experiments the M_r = 150,000 component can be visualized (17). The ^{125}I-labeled Factor V is rapidly cleaved by 0.2 U/ml thrombin at 22°C producing two transient high molecular weight intermediates and three radiolabeled final products with M_r = 150,000, 110,000, and 78,000. A fourth activation product that is weakly stained with Coomassie Blue with M_r = 30,000 (6) is not radiolabeled. The fully activated Factor V had a specific activity of 2,100 U/mg protein as measured by coagulation assay. The enzyme RVV-V cleaves Factor V at a single location to produce a high molecular weight final product and the M_r = 78,000 component. Two other peptides with apparent M_r = 160,000 and 145,000 are formed at a much slower rate than the higher molecular weight component. After 5 min at 22°C there was no longer any intact Factor V present and the preparation had the same specific activity as thrombin activated Factor V_a. Upon addition of 0.2 U/ml thrombin there was no further change in coagulation activity, however the high molecular weight product produced by cleavage with RVV-V disappeared and was replaced initially by a smaller transient high molecular weight intermediate and the M_r = 110,000 final product followed later by the appearance of the M_r = 150,000 final product.

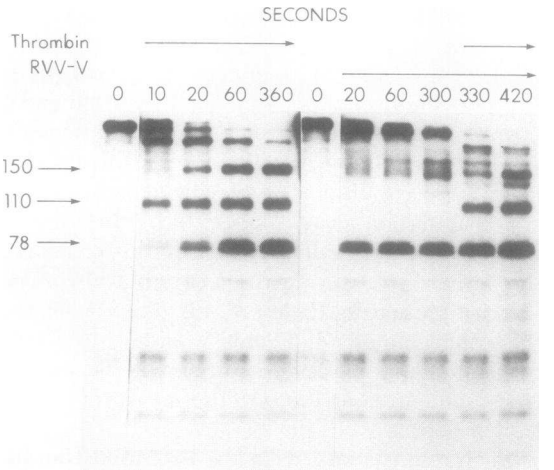

FIGURE 1 Activation of ^{125}I-labeled Factor V by thrombin and RVV-V. ^{125}I-labeled Factor V (5 μg/ml 214 cpm/ng) in a buffer containing 0.5 M NaCl, 20 mM Tris, pH 7.4, 2.5 mM $CaCl_2$, 5.5 mM glucose, recrystallized and bovine serum albumin, 5 mg/ml at 22°C, was treated with thrombin (0.2 U/ml) or RVV-V (20 μg/ml). After 300 s thrombin (0.2 U/ml) was added to the sample that was treated with RVV-V. At the indicated times samples were taken, mixed with an equal volume of buffer containing sodium dodecyl sulfate and 5% 2-mercaptoethanol, and boiled for 2 min. Samples containing 30 ng of ^{125}I-labeled protein were then electrophoresed on a 7.5% polyacrylamide slab gel containing sodium dodecyl sulfate with a 4% stacking gel according to Laemmli (21). Protein was detected by autoradiography (22).

Activation of purified Factor V by a platelet protease. We tested the ability of frozen and thawed platelets isolated from a patient with congenital Factor V deficiency to activate purified Factor V. When 5 μg/ml of purified Factor V was incubated with frozen and thawed platelets at a concentration of 10^8/ml there was only a small increase in Factor V activity during a 20-min incubation at 22°C from 0.7 to 1.1 U. When this mixture was treated with 2 U/ml of thrombin

there was a rapid ninefold increase in Factor V activity. However, when Factor V was incubated with a Factor V-deficient lysate derived from 10^9 platelets/ml the specific activity of the Factor V increased from 140±30 U/mg to a maximum of 740±240 U/mg (mean±SD of three experiments). When thrombin was added to the platelet lysate-Factor V mixture after 20 min, the activity of the Factor V increased further to 1,440±280 U/mg. When the Factor V used in this experiment was activated with thrombin in the absence of platelets it had a maximum specific activity of 1,540±60 U/mg. The platelet lysate from the Factor V-deficient patient contained no detectable Factor V activity and incubation of this lysate with thrombin activated Factor V_a did not increase the activity of Factor V_a in our coagulation assay.

In order to study the relationship between changes in Factor V activity and proteolysis of the Factor V molecule we incubated ^{125}I-labeled Factor V with platelet lysates and analyzed samples of these mixtures by SDS polyacrylamide gel electrophoresis and autoradiography. Fig. 2 shows a time course of the incubation of 5 μg/ml of ^{125}I-labeled Factor V with 3×10^9/ml of frozen and thawed platelets from a normal donor.[2] Under these conditions there is rapid cleavage of the single chain factor V molecule, which reproducibly produced radiolabeled components with apparent M_r = 105,000, 87,000, 78,000, and 65,000. The amounts of some higher molecular weight components decreased during the incubation suggesting that they are subject to further degradation. Only the component with M_r = 78,000 migrates similarly to any of the products of Factor V activation produced by thrombin or RVV-V. The peptides with molecular weights of <65,000 are not seen in any reproducible pattern. When 10^9/ml of frozen and thawed platelets isolated from a patient with Factor V deficiency were used in the same type of experiment, qualitatively similar results were obtained except that the rate of proteolysis of Factor V was slower. When 10^8/ml of sonicated platelets were used the rate of proteolysis was still slower although even under these conditions all of the single chain Factor V was degraded after 5 min of incubation (see Fig. 3). At this platelet concentration only trace quantities of the M_r = 78,000 peptide are formed, which is consistent with the observation that only minimal activation of Factor V occurs under these conditions.

When the Factor V that had been incubated with

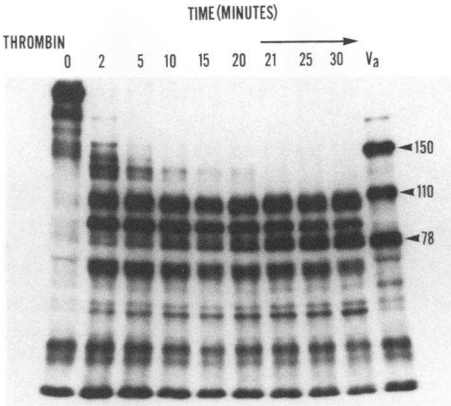

FIGURE 2 Activation of ^{125}I-labeled Factor V by a frozen and thawed platelet suspension. Platelets at a concentration of 3×10^9/ml in a buffer containing 0.15 M NaCl, 20 mM Tris-HCl pH 7.4, 2.5 mM $CaCl_2$, 5.5 mM glucose, and 5 mg/ml recrystallized bovine serum albumin were disrupted by three cycles of freezing and thawing using liquid nitrogen and a 37°C water bath. The suspension was then equilibrated at 22°C and Factor V (5 μg/ml, 1,300 cpm/ng) was added. At the indicated times samples were taken and mixed with an equal volume of buffer containing sodium dodecyl sulfate and 5% 2-mercaptoethanol and then boiled for 2 min. At 20 min thrombin (2 U/ml) was added to the reaction mixture. Samples containing 30 ng of ^{125}I-labeled protein were electrophoresed on a 7.5 polyacrylamide slab gel containing sodium dodecyl sulfate with a 4% stacking gel according to Laemmli (21). Protein was detected by autoradiography (22).

frozen and thawed platelets at a concentration of 3×10^9/ml was treated with thrombin (Fig. 2) the amount of the M_r = 87,000 peptide decreased and there was an increase in the amount of the M_r = 78,000 peptide. This product lacks the M_r = 150,000 and 110,000 peptides seen when intact Factor V is activated with thrombin. In addition the M_r = 105,000 peptide seen here does not correspond to any peptide produced by thrombin activation of intact Factor V. When ^{125}I-labeled Factor V that had been incubated with 10^8 sonicated platelets/ml (Fig. 3) was treated with thrombin all of the peptides except the M_r = 105,000 peptide disappeared and two new peptides with M_r = 110,000 and 78,000 were formed. Again, the M_r = 150,000 peptide, which is seen following activation of intact Factor V by thrombin was absent when the Factor V had been preincubated with the platelet lysates. These results suggest that the M_r = 150,000 peptide domain of the Factor V molecule is very susceptible to proteolytic cleavage by a platelet protease. This concept was reinforced in an experiment where we incubated thrombin activated Factor V_a with 10^8 sonicated platelets/ml. The M_r = 150,000 peptide was degraded while the specific activity by coagulation assay of the factor V_a remained essentially

[2] This labeled Factor V preparation is the same as that used in Fig. 2 except that it contained lower molecular weight radioactive contaminants derived from Factor V. These result from traces of proteases contained in Factor V and increase in amount during storage of Factor V.

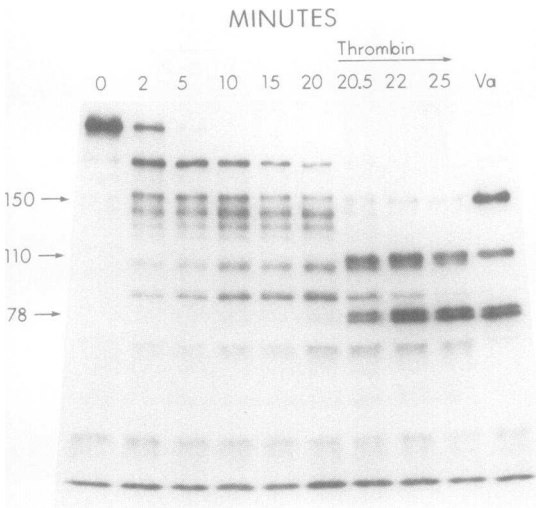

FIGURE 3 Proteolysis of ^{125}I-labeled Factor V by a sonicated platelet suspension. Platelets at a concentration of 10^8/ml in a buffer containing 0.15 M NaCl, 20 mM Tris-HCl pH 7.4, 2.5 mM $CaCl_2$, 5.5 mM glucose, and 5 mg/ml recrystallized bovine serum albumin were disrupted by sonicating twice for 15 s at 25 W at 4°C The suspension was then equilibrated to 22°C and Factor V (5 μg/ml, 1,500 cpm/ng) was added. At the indicated times samples were taken and mixed with an equal volume of buffer containing sodium dodecyl sulfate and 5% 2-mercaptoethanol and then boiled for 2 min. Samples containing 30 ng of ^{125}I-labeled protein were electrophoresed on a 7.5% polyacrylamide slab gel containing sodium dodecyl sulfate with a 4% stacking gel according to Laemmli (21). Protein was detected by autoradiography (22). At 20 min 2 U/ml of thrombin was added to the reaction mixture.

constant and the peptides of M_r = 105,000 and 78,000 remained.

Further characterization of the Factor V activator present in human platelets. We next attempted to determine whether or not the Factor V activating enzyme present in platelets is expressed when platelets are stimulated with thrombin (Fig. 4). Factor V was incubated with 3×10^9/ml of frozen and thawed platelets for 20 min, which resulted in extensive proteolysis of the Factor V molecule including production of the M_r = 78,000 peptide (Fig. 4, lane 2). The proteolysis of Factor V is not inhibited by DAPA, a specific inhibitor of thrombin (Fig. 4, lane 3). This indicates that the activation of Factor V seen in this system is not due to trace amounts of thrombin in the platelet preparation. When Factor V was incubated with intact platelets (3×10^9/ml) (lane 4) it was not degraded, indicating that the Factor V activating enzyme is not expressed in unstimulated platelets. DAPA (93 μM) is sufficient to inhibit the activation of Factor V by 2 U/ml of thrombin as shown in lane 5. When intact platelets (3×10^9/ml) were stimulated with 2 U/ml of thrombin for 5 min, followed by addition of 93 μM DAPA and then Factor V, there was no cleavage of the Factor V molecule. Therefore, the Factor V activating enzyme expressed after platelet lysis is not expressed following stimulation of platelet secretion by thrombin. We estimate that this assay system using 3×10^9/ml platelets could detect the release of the platelet protease only if 4% or more had been released since the M_r = 78,000 peptide was produced in only trace amounts when ^{125}I-labeled Factor V was incubated with 10^8/ml of frozen and thawed platelets. We carried out similar experiments where platelets were stimulated with either calcium ionophore A23187 (10 μM) or collagen (25 μg/ml) and again could detect no evidence of protease release as measured by cleavage of ^{125}I-Factor V.

Properties of platelet protease. We determined the cellular location of the platelet protease using the method of Broekman (25) (data not shown). Factor V was contained in α-granules (fractions 6–8) as described previously by Chesney (8). The protease was all found in fraction 1, which corresponds to the platelet soluble fraction. There was nearly total recovery of activity in this fraction, which produced the same pattern of cleavage of ^{125}I-Factor V as seen in crude homogenates of platelets. This suggests that the pro-

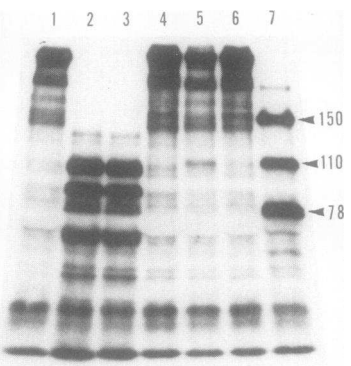

FIGURE 4 Requirements for the expression of the Factor V activator in human platelets. ^{125}I-Factor V (5 μg/ml, 1,300 cpm/ng) was incubated in a buffer containing 0.15 M NaCl, 20 mM Tris-HCl pH 7.4, 2.5 mM $CaCl_2$, 5.5 mM glucose, and recrystallized bovine serum albumin, 5 mg/ml at 22°C for 20 min in the presence of the following additional components (*a*) None. (*b*) Frozen and thawed platelets 3×10^9/ml. (*c*) Frozen and thawed platelets 3×10^9/ml, 93 μM DAPA. (*d*) Intact platelets 3×10^9/ml. (*e*) 2 U/ml thrombin and 93 μM DAPA. (*f*) Platelets at a concentration of 3×10^9/ml pretreated with thrombin for 5 min followed by addition of 93 μM DAPA and then the Factor V. After a 20-min incubation samples were taken and dissolved in an equal volume of buffer containing sodium dodecyl sulfate and 5% 2-mercaptoethanol and boiled for 2 min. Samples containing 30 ng of ^{125}I-labeled protein were electrophoresed on a 7.5% polyacrylamide slab gel containing sodium dodecyl sulfate with a 4% stacking gel according to Laemmli (21). Protein was detected by autoradiography (22).

tease is in the cytoplasm although it is possible that the protease is not truly cytosolic but is in some unique granule that is either very light or very fragile. The Factor V activator was not inhibited by DAPA thereby distinguishing it from thrombin. It cleaved Factor V at the same rate in the presence and absence of EGTA (1 mM) indicating that calcium ions are not required for activity (32). Added ATP (5 mM) neither stimulated nor inhibited proteolysis thereby distinguishing it from ATP-dependent proteases (34–35). The Factor V activator may be a serine protease since incubation of crude platelet homogenates with 0.2 mM DFP for 5 min at room temperature prior to the addition of ^{125}I-Factor V inhibited subsequent proteolysis by ~50%. Prior incubation with 2 mM DFP under the same conditions resulted in complete inactivation. The protease displays activity over a broad pH range with nearly equal activity between pH values of 6–8. Activity fell sharply at pH values <4.5 and >8.5. Cleavage of Factor V by the platelet protease depended on both time and protein concentration with complete cleavage in 10 min at 0.6 mg protein form platelet cytosol/ml. We also incubated frozen and thawed samples of other cells at a concentration of 2×10^6 cells/ml with ^{125}I-Factor V. Human monocytes, lymphocytes, cultured skin fibroblasts and umbilical vein endothelial cell extracts did not cause proteolysis of Factor V. A homogenate of neutrophils caused extensive proteolysis of Factor V but did not reproduce the cleavage pattern obtained with platelets.

Binding of platelet protease-derived Factor V_a to platelets. Factor V activated by the platelet protease is designated as V_{ap}. We incubated V_{ap} with washed intact human platelets to determine which of its components bind to platelets as shown in Fig. 5. The ^{125}I-Factor V preparation used in this experiment is shown in lane 1. It was activated with either thrombin (lane 2) or the platelet protease (lane 3). Thrombin-activated Factor V_a designated V_{at} bound to platelets as shown in lane 4. Peptides of apparent M_r = 110,000 and 78,000 were found in the platelet pellet as described previously (6). In contrast, incubation of platelet protease-activated V_a with platelets designated V_{ap} resulted in three peptides in the platelet pellet with apparent M_r = 105,000, 87,000, and 78,000. All three of these peptides are related to Factor V_a since all can be displaced from platelets with an excess of unlabeled V_{at} as shown in Fig. 6. When similar experiments were carried out in the absence of Factor X_a (not shown) V_{ap} bound less well, a result similar to that obtained with V_{at} (6). Without further studies it is impossible to determine the origin and yield of these peptides with certainty. However our data suggests that the M_r = 87,000 peptide contains the M_r = 78,000 peptide. This is most clearly seen in Fig. 3 where thrombin

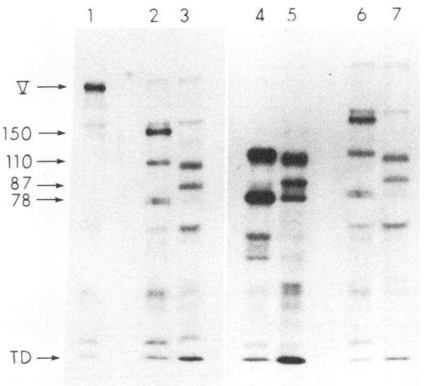

FIGURE 5 Polyacrylamide gel electrophoresis (7.5%) with stacking gel (4%) containing sodium dodecyl sulfate (21) of ^{125}I-Factor V, thrombin activated ^{125}I-V_{at}, and platelet protease-activated ^{125}I-V_{ap}. Protein was detected by autoradiography (22), lanes 1–3, 24 h of exposure and lanes 4–7, 48 h of exposure. Lane 1, 1 ng of ^{125}I-V (4,272 cpm/ng); lane 2, thrombin activated ^{125}I-V_{at} derived from 1 ng of ^{125}I-V; lane 3, platelet protease activated ^{125}I-V_{ap} derived from 1 ng of ^{125}I-V. Platelets (10^8/ml) were incubated in mixture containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 2.5 mM $CaCl_2$, 5.5 mM glucose, 5 mg/ml of bovine serum albumin, 0.1 mM DAPA, 20 ng/ml of Factor X_a and either ^{125}I-V_{at} or ^{125}I-V_{ap}, both derived from 100 ng/ml of ^{125}I-V. After 20 min of incubation platelets were sedimented through oil, and platelet pellets and 5 μl of the platelet supernatant were dissolved in buffer containing sodium dodecyl sulfate and 5% 2-mercaptoethanol. Solubilized platelet pellets were electrophoresed in lanes 4 and 5, and platelet supernatants in lanes 6 and 7. Lane 4, 0.25×10^8 platelets after incubation with ^{125}I-V_{at}; lane 5, 0.25×10^8 platelets after incubation with ^{125}I-V_{ap}; lane 6, 5 μl of platelet supernatant after incubation with ^{125}I-V_{at}; lane 7, 5 μl of platelet supernatant after incubation with ^{125}I-V_{ap}. Apparent molecular weights of V activation components are indicated $\times 10^{-3}$.

treatment of platelet-protease activated Factor V results in disappearance of the 87,000 peptide with an increase in the 78,000-dalton moiety. This apparent precursor product relationship was seen in many experiments not shown. It is also reasonable to assume that the M_r = 105,000 peptide is a slightly smaller version of the 110,000-dalton peptide found by thrombin. Using these assumptions we determined the specific activity and yield of the various peptides in the following manner. We sliced the polyacrylamide gels and counted the various peptides from the experiment, shown in Fig. 6. We assumed that thrombin cleaved Factor V completely producing 1 mol each of M_r = 110,000 peptide and M_r = 78,000 peptide. The specific activity of the peptides was found to be 296 cpm/ng, 110,000-dalton peptide, and 437 cpm/ng of 78,000-dalton peptide. The amount of each peptide bound to platelets specifically was determined by subtracting the counts bound in the presence of 14 μg/ml unlabeled V_{at} (Fig. 6). Thus, we found 2.83 ng of

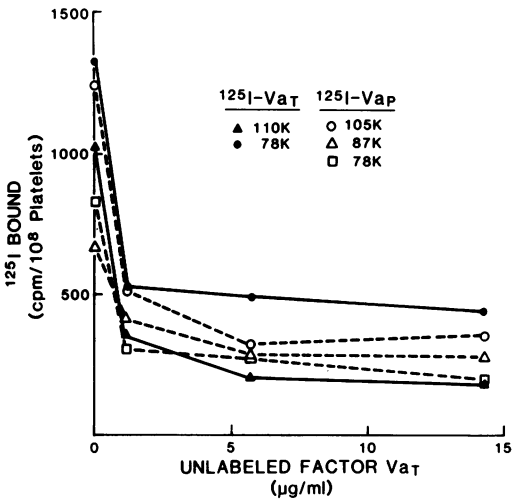

FIGURE 6 Binding of ^{125}I-V_{at} and ^{125}I-V_{ap} to platelets and displacement by V_{at} (Methods). ^{125}I-V_{at} and ^{125}I-V_{ap} were derived from 100 ng/ml of ^{125}I-V (2,405 cpm/ng).

110,000-dalton peptide bound (154 molecules/10^8 platelets) and 2.05 ng of 78,000-dalton peptide bound (156 molecules/10^8 platelets). This suggests that platelets bind the two peptides that comprise V_a after thrombin activation of Factor V in a 1:1 ratio. We determined the amounts of the various peptides bound to platelets using platelet protease-activated Factor V by assuming that the specific activity of the M_r = 105,000 peptide is the same as the M_r = 110,000 peptide and that the M_r = 87,000 peptide has the same specific activity as the M_r = 78,000 peptide contained within it, and that the platelet protease-derived peptide of M_r = 78,000 is the same as that formed by thrombin. Thus, in the experiment in Fig. 6 we calculate that the platelets bound 2.97 ng of the 105,000-dalton peptide (169 molecules/10^8 platelets), 0.86 ng of the 87,000-dalton peptide (59 molecules/10^8 platelets), and 1.3 ng of the M_r = 78,000 peptide (99 molecules/10^8 platelets). This suggests that platelet protease-activiated Factor V_a binds to platelets with 1 mol of 105,000-dalton peptide and either 1 mol of 87,000-dalton peptide or 1 mol of 78,000-dalton peptide (i.e., molecules of 105,000-dalton peptide bound = molecules of 87,000-dalton peptide plus molecules of 78,000-dalton peptide).

By the same type of analysis we estimated the yield of the three peptides in V_{ap} formed by platelet protease action. We found 0.73 mol of 105,000-dalton peptide per mole of Factor V, 0.5 mol of 87,000-dalton peptide and 0.25 mol of 78,000-dalton peptide. Thus the platelet protease results in less than a complete yield of products. The fact that the 78,000-dalton peptide is present in smaller amounts than the 87,000-dalton peptide and yet binds to platelets more efficiently (see Figs. 5 and 6) suggests that it is the active moiety. In several experiments we found that platelet protease activated Factor V_a has 0.25–0.5 the activity of thrombin produced V_a by coagulation assay and also contains 0.25–0.5 mol of 78,000-dalton peptide. Thus the 87,000-dalton peptide probably can bind to platelets in place of the 78,000-dalton peptide although with less affinity and much less or no coagulation activity.

DISCUSSION

Platelets possess Factor V in α-granules released after stimulation of platelet secretion or following platelet lysis (5, 7–10). This molecule has not been isolated although it seems likely to be similar if not identical to the Factor V molecule present in plasma, since both activities are deficient in congenital Factor V deficiency (3) and since Factor V inhibitor proteins derived from patients with acquired Factor V deficiency inactivate both molecules (3). Factor V isolated from human plasma is a relatively inactive procofactor composed of a single polypeptide chain with apparent M_r = 330,000 (16–18). Plasma Factor V is converted to the active form Factor V_a through limited and specific proteolysis of the molecule. In human Factor V the appearance of coagulation activity correlates with a cleavage that produces a peptide with M_r = 78,000 (16, 17). This peptide is a component of Factor V_a, because we have shown that when thrombin-activated Factor V_a is incubated with platelets only the M_r = 78,000 and M_r = 110,000 components bind to the platelet surface and promote prothrombin activation (6). Esmon (15) has isolated M_r = 110,000 and 73,000 components of thrombin activated bovine Factor V_a and has shown that while neither peptide had Factor V activity alone, the two peptides mixed together in the presence of calcium ions regenerated complete activity as measured in a coagulation assay. Together these results suggest that the M_r = 78,000 peptide does not itself possess Factor V_a activity, but rather that this peptide is a subunit of Factor V_a.

Osterud et al. (10) have proposed that platelets contain an enzyme that activates Factor V and that the Factor V released from platelets is already activated. Both Osterud et al. (10) and Vicic et al. (9) found that the Factor V activity released after platelet lysis was not activated upon subsequent treatment with thrombin. In contrast, we have found that the Factor V activity expressed following platelet lysis can be activated by treatment with thrombin. It is not clear why our results differ from those obtained in other laboratories, although we have excluded the possibility that the discrepancy is due to differences in buffer composition (data not shown). Our results suggest that most if not all of the Factor V present in platelets is stored

in the pro-cofactor form and that conversion to Factor V_a occurs when it is exposed to a Factor V activator.

In agreement with Osterud et al. (10), we find that platelet lysates will activate Factor V. Thus we find that purified Factor V treated with a platelet lysate (10^9 platelets/ml) has a specific activity that ranges from 25 to 50% of that of thrombin-activated Factor V_a. When this platelet-protease-activated Factor V was treated with thrombin, it increased to the same specific activity as thrombin activated Factor V_a. The products observed when ^{125}I-labeled Factor V is incubated with platelet lysates are different from those obtained when Factor V is activated by thrombin or RVV-V, although all three activators produce a M_r = 78,000 peptide. It appears that the bond cleavage that produces the M_r = 78,000 peptide is a critical event in the development of Factor V coagulation activity (16, 17). It is remarkable that under some circumstances (see Figs. 2 and 3) extensive proteolysis of the Factor V molecule can occur despite no change in its coagulation activity or activatability by thrombin. This may be explained in part by the fact that many of the cleavages appear to be made in the M_r = 150,000 peptide domain of the Factor V molecule. This peptide is released from Factor V during activation by thrombin and is not essential for coagulation activity (6). The vitamin K-dependent protease protein C_a can inactivate Factors V_a and V through limited and specific proteolysis (30, 31, 36). Apparently, the protein C_a-sensitive bonds in the Factor V molecule are not cleaved by the platelet protease. It is possible that more than one platelet protease is responsible for the observed pattern of proteolysis of the Factor V molecule. We have not investigated the possibility that the cleavages made by the platelet protease might affect the kinetics of Factor V activation by thrombin or RVV-V. The Factor V activator is distinct from the calcium dependent protease described by Phillips and Jakabova (32) since it is active in 1 mM EGTA. It appears to be a serine protease, acting at neutral pH, independent of ATP. The platelet Factor V activator is not thrombin because it is not inhibited by the specific thrombin inhibitor DAPA. This is in agreement with the results of Osterud et al. (10) who found that the Factor V activator was not inhibited by hirudin.

Three polypeptides derived from the platelet protease activated Factor V bind to platelets and are displaced by thrombin-activated Factor V_a. These peptides of M_r = 105,000, 87,000, and 78,000 therefore appear to be related to Factor V_a.

The physiological significance of the platelet protease is obscure. That it produces Factor V_a in less than complete yield might argue against specificity of the protease. It is conceivable that in the process of release of Factor V during platelet activation, the platelet protease somehow achieves access to Factor V. Since platelets contain only 200–300 potential prothrombinase receptors/cell (3), yet contain 20 times this amount of Factor V (6, 37) it is possible that limited activation of Factor V could be physiologically significant but not detected by the current experiments. Without evidence for such physiological activation of Factor V the role of the platelet protease in hemostasis is uncertain.

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