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# Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V

(protein S/thrombosis/blood coagulation/anticoagulation)

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ABSTRACT **Recently, our laboratory described a defect in anticoagulant response to activated protein C (APC). This response, APC resistance, was shown to be inherited and associated with familial thrombophilia. As other possible mechanisms were excluded, APC resistance was hypothesized to be due to deficiency of a previously unrecognized cofactor of APC. The aim of the present study was to isolate and characterize this factor. Plasma from an individual with pronounced inherited APC resistance was used as test plasma in a biological assay which monitored APC cofactor activity during its isolation from normal plasma. A purification procedure was devised that yielded a protein which was shown to be identical to coagulation factor V. It proved impossible to separate the APC cofactor activity from factor V, even by affinity chromatography using a monoclonal antibody against factor V. The affinity-purified factor V corrected the poor anticoagulant response to APC of APC-resistant plasma in a dose-dependent manner. Because the APC-resistant plasma contained normal levels of factor V procoagulant activity, the results indicated APC resistance to be due to a selective defect in the anticoagulant function of factor V. The present results show factor V not only to express procoagulant properties after its activation by thrombin but also to play an important part in the anticoagulant system as cofactor to APC.**

Blood coagulation is a complex process involving a cascade of zymogen activations resulting in the formation of thrombin and the subsequent conversion of fibrinogen to fibrin (1, 2). The overall reaction rate is precisely regulated by cofactors and by inhibitors. In a positive feedback reaction, thrombin activates factor VIII and factor V, thereby accelerating the coagulation process (3, 4). The activated forms of factors VIII and V (factors VIIIa and Va) are phospholipid-bound cofactors involved in the activation of factor X and prothrombin, respectively. The rates of these reactions are several orders of magnitude greater in the presence of the respective cofactor than in its absence. Factor V is a single-chain high molecular weight glycoprotein ($M_r$ 330,000) present in plasma at an approximate concentration of 7 μg/ml. During its activation by thrombin, three peptide bonds are cleaved, factor Va being the resulting complex between the 105-kDa heavy chain and the 74-kDa light chain (a model of factor V is shown in Fig. 2). The function of the two large activation peptides, derived from the central portion of factor V, is unknown (3, 4).

Coagulation is inhibited through degradation of the phospholipid-bound factors VIIIa and Va by activated protein C (APC), a key component in a physiologically important anticoagulant system (5–7). The unactivated forms of factors VIII and V are poor substrates for APC. Thus, the rate of the coagulation reaction is dependent on the balance between activation of factors VIII and V and degradation of their activated forms. Another anticoagulant protein, protein S, functions as cofactor to APC (5–7). Although the importance of protein S is underscored by the relationship between thromboembolic disease and protein S deficiency, its mechanism of action is incompletely understood, as its APC cofactor activity is weak *in vitro* (8).

Recently, our laboratory (9) found a defect in anticoagulant response to activated protein C (APC resistance), showing it to be inherited and associated with familial thrombophilia. Subsequent studies showed APC resistance to be a major risk factor for venous thrombosis (10). As several possible explanations for APC resistance were excluded, it was predicted to be due to deficiency of a previously unrecognized cofactor to APC (9). This hypothesis has recently been proven experimentally, as a crude fraction obtained from normal plasma contained an activity which corrected the defect of APC-resistant plasma, whereas the corresponding fraction from APC-resistant plasma was inactive (11). We now report on the purification and characterization of this APC cofactor (referred to here as APC cofactor 2), and demonstrate its identity with coagulation factor V. The present results show that factor V, in addition to being the precursor to procoagulant factor Va, is an important component of the protein C anticoagulant system.

## MATERIALS AND METHODS

**Purification.** Freshly frozen citrate-treated plasma (2.3 liters) was thawed at 37°C and the following protease inhibitors were added (final concentrations are given in parentheses): phenylmethanesulfonyl fluoride (PMSF) (1 mM), diisopropyl fluorophosphate (DFP) (1 mM), benzamidine (10 mM), aprotinin (1.5 μg/ml), and soybean trypsin inhibitor (50 μg/ml). Most steps were performed in the cold room. The plasma (kept on an ice-bath) was subjected to barium citrate absorption and polyethylene glycol (PEG) 6000 (8%, wt/vol) precipitation as described (12). The PEG supernatant was diluted with an equal volume of 10 mM benzamidine and then mixed with 600 ml of Q-Sepharose fast flow (Pharmacia) equilibrated in 20 mM Tris·HCl, pH 7.5/0.1 M NaCl/1 mM $CaCl_2$/10 mM benzamidine. After 1 hr of gentle mixing, the gel was collected in a Büchner funnel and washed successively with 3 liters of equilibration buffer, 1 liter of equilibration buffer with 0.1% Tween 20, and 2 liters of equilibration buffer containing 0.15 M NaCl instead of 0.1 M NaCl. The gel was then packed in a column (5-cm diameter) and the adsorbed proteins were eluted with a 3-liter linear gradient of 0.15–0.5 M NaCl in 20 mM Tris·HCl, pH 7.5/1 mM $CaCl_2$/10 mM benzamidine. The flow rate was 330 ml/hr and 11-ml fractions were collected. Fractions were analyzed for APC cofactor 2 and factor V activities in 1:10 dilutions (Fig. 1).

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Abbreviations: APC, activated protein C; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; mAb, monoclonal antibody.

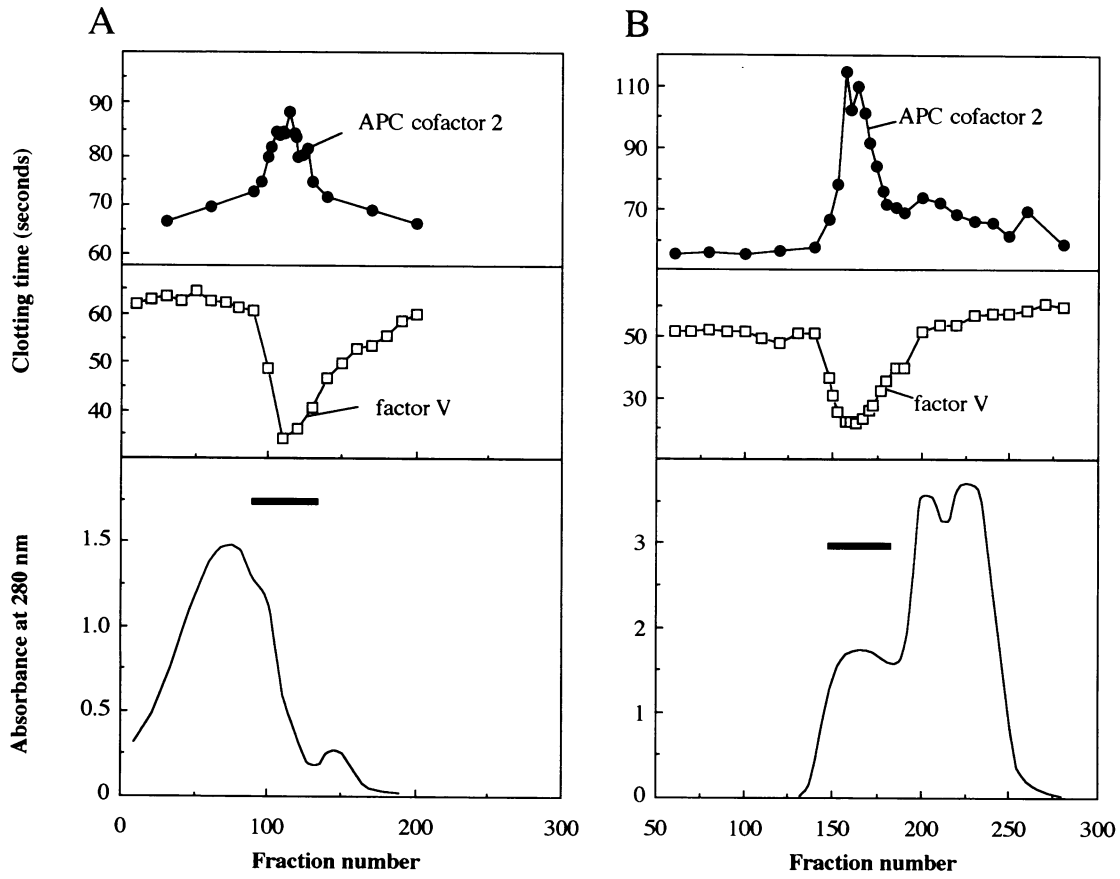


FIG. 1. Chromatography on Q-Sepharose (*A*) and Sephacryl S-300 (*B*) of factor V and APC cofactor 2. On both columns, the elution profile of APC cofactor 2 (*Top*) coincided with that of factor V (*Middle*). Factor V activity was demonstrated as a shortening of clotting time of factor V-deficient plasma, whereas APC cofactor activity was associated with an APC-dependent prolongation of clotting time of APC-resistant plasma. The fractions were pooled as shown by the horizontal bars in the $A_{280}$ profiles (*Bottom*).

Fractions were pooled as indicated by the horizontal bar and subjected to $(NH_4)_2SO_4$ precipitation (70% saturation). The precipitate was collected by centrifugation, dissolved in a minimal volume of 20 mM Tris·HCl, pH 7.5/0.15 M NaCl/1 mM $CaCl_2$/10 mM benzamidine/1 mM DFP/1 mM PMSF and applied to a column (2.5 cm × 93 cm) with Sephacryl S-300 (Pharmacia) equilibrated in the same buffer but without DFP and PMSF. The column was run at 10 ml/hr and 1.2-ml fractions were collected. The fractions were analyzed with APC cofactor 2 and factor V assays at 1:10 dilutions (Fig. 1). Fractions were pooled as indicated by the horizontal bar and stored at −70°C. The protein from an S-300 chromatography (in the illustrated run, ≈6 mg in 20 mM Tris·HCl, pH 7.5/0.1 M NaCl/2 mM $CaCl_2$, was applied to a column (0.75 cm × 7.5 cm) with immobilized monoclonal antibody (mAb) 30 (column and protein were equilibrated in 20 mM Tris·HCl, pH 7.5/0.1 M NaCl/2 mM $CaCl_2$). The column was washed until absorbance of the eluate reached zero, and then bound proteins were eluted with 50 mM diethanolamine, pH 10.6/2 mM $CaCl_2$. The pH of the eluate was immediately neutralized with 3 M Tris·HCl, (pH 7.5) (50 μl per 1-ml fraction). The fractions were analyzed (at 1:5 dilution) with APC cofactor 2 and factor V assays. Active fractions were pooled, concentrated by ultrafiltration (Amicon YM10 membranes) and stored at −70°C. The purified APC cofactor 2/factor V was activated with thrombin as described (12).

**Clotting Assays.** A biological assay for APC cofactor 2 was used, with plasma from a healthy woman (AS) with pronounced inherited APC resistance as the test plasma. AS was unrelated to the family originally described with thrombophilia and inherited APC resistance (9). As mixtures of plasma from AS and from the propositus of the family originally described were equally as APC-resistant as the two individual plasmas, they were judged to be deficient in the same anticoagulant principle. AS plasma contained normal levels of factor V, as judged by a factor V clotting assay. In the APC cofactor 2 assay, aliquots (50 μl, diluted in 20 mM Tris·HCl, pH 7.5/0.1 M NaCl) were mixed with 50 μl of AS plasma. Activated partial thromboplastin time reagent (APTT automated, Organon Teknika–Cappel) (100 μl) was added, and after 5 min of incubation at 37°C, clotting was initiated with a mixture of APC and $CaCl_2$ (APC at 1 μg/ml in 10 mM Tris·HCl, pH 7.5/0.05 M NaCl/30 mM $CaCl_2$/0.1% bovine serum albumin), and clotting time was recorded as described (9). The presence of APC-cofactor 2 activity in the tested fraction was associated with an increase in clotting time. A factor V clotting assay was performed with factor V-deficient plasma as described (12). The presence of factor V activity resulted in a shortening of clotting time of the deficient plasma. In both APC cofactor 2 and factor V assays, we chose to show the original clotting data rather than convert results into units.

**Electrophoretic, Immunological, and Other Methods.** Gradient (5–15%) polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate (SDS/PAGE) and Western blotting were performed by published techniques (13). A specific rabbit polyclonal antiserum against factor V was the gift of Dakopatts. Data demonstrating the specificity of the antiserum have been reported (14). Rabbit polyclonal antibodies were raised against the isolated heavy- and light-chain fragments of bovine factor V (13). mAbs were raised by standard methods (15). The purified protein in the Sephacryl

S-300 pool was used as antigen in the immunization of BALB/c mice. Seventeen different antibodies were obtained and their reactivities were tested with Western blotting. Approximately 20 mg of mAb 30 was coupled to 4 ml of Affi-Gel 10 (Bio-Rad) in accordance with the manufacturer's instructions. IgG fractions of the polyclonal antisera against human factor V and the bovine factor V fragments were also coupled to Affi-Gel (≈5 mg/ml).

## RESULTS

APC cofactor 2 activity was analyzed with a biological assay using plasma from an individual (AS) with APC resistance as test plasma, and a procedure was devised for purification of APC cofactor 2 from normal plasma. The first step in the procedure was barium citrate absorption, which removed the vitamin K-dependent proteins, including proteins C and S. The barium citrate eluate had no APC cofactor 2 activity. On fractionation of the supernatant plasma with PEG 6000 precipitation, APC cofactor 2 was present in the 8% PEG supernatant, whereas the dissolved 0–8% PEG 6000 precipitate had no APC cofactor 2 activity. The APC cofactor 2 in the 8% PEG supernatant was purified first by anion-exchange chromatography on a column with Q-Sepharose and then by gel filtration on Sephacryl S-300 (Fig. 1). This purification protocol was very similar to a procedure for the purification of coagulation factor V (12), and factor V was found in the same fractions as APC cofactor 2. The purification was performed at least 10 times with different modifications, and the elution profiles for factor V and APC cofactor 2 were consistently very similar. The protein in the S-300 pool expressed both factor V and APC cofactor 2 activities and manifested characteristics previously reported for factor V (12). Additional efforts to separate the two activities by use of several other chromatographic principles, such as heparin-Sepharose, Blue Sepharose, and wheat germ agglutinin-Sepharose, were unsuccessful (data not shown), and APC-cofactor 2 activity was in fact found to purify together with factor V on *every* chromatographic support we tried.

SDS/PAGE of the protein in the S-300 pool yielded a 330-kDa band (corresponding to single-chain factor V) in addition to bands 220,000 and 130,000–150,000 (Fig. 2). These bands represented cleaved factor V and, like the 330-kDa species, they reacted with a polyclonal antiserum against factor V on Western blots (Fig. 2). The 220-kDa band represented the C-terminal part of factor V, including the 74-kDa light chain of factor Va and the larger (150 kDa) of the two activation fragments, and was recognized by an antiserum against the light chain of bovine factor Va (results not shown). The 130- to 150-kDa bands comprised the N-terminal part of factor V (105-kDa heavy chain plus the smaller of the two activation fragments) and, accordingly, reacted with an antiserum against the bovine factor Va heavy chain (results not shown). Additional bands of lower molecular mass, which did not react with polyclonal factor V antiserum on Western blots, were sometimes seen, but when present, their elution profiles (as judged by SDS/PAGE) from the S-300 column did not correlate with the activity of factor V or APC cofactor 2. Incubation of the purified protein with thrombin yielded fragments characteristic for thrombin-cleaved factor V, and concomitantly the activity in the APC cofactor 2 assay was lost, suggesting that APC cofactor 2 activity is expressed by factor V and not by factor Va. On agarose gel electrophoresis, the purified protein migrated as a single species to an inter-$\alpha$ position (Fig. 2), and both factor V and APC cofactor 2 activities could be eluted from this position of the gel (data not shown).

As factor V is extremely sensitive to proteolysis, numerous protease inhibitors were included in the final protocol. When performed in the absence of protease inhibitors, the purification procedure resulted in a more degraded product lacking the 330-kDa species but containing the 220-kDa and 130- to 150-kDa bands. This purified product expressed both factor V and APC cofactor 2 activities. Factor V requires $Ca^{2+}$ for its stability, and when $Ca^{2+}$ was not included in the purification, both factor V and APC cofactor 2 activities were gradually lost.

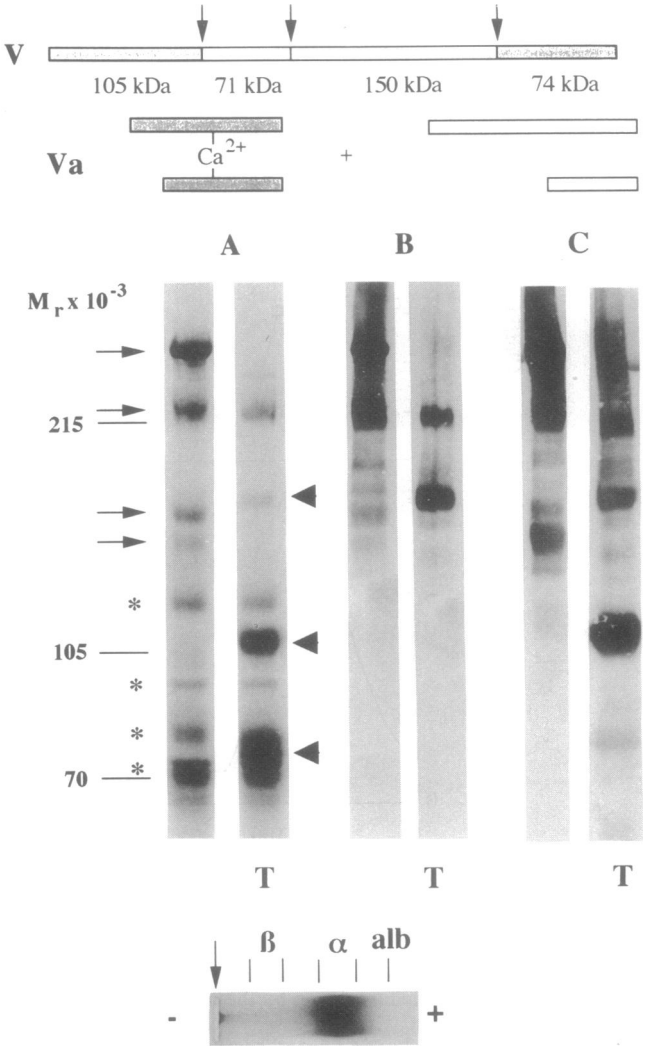


FIG. 2. Characterization of isolated APC cofactor 2/factor V by SDS/PAGE, Western blotting, and agarose gel electrophoresis. (*Top*) Schematic model of the factor V molecule (modified from ref. 16). Arrows indicate thrombin cleavage sites, and the approximate molecular masses of the fragments are given. Factor Va is a $Ca^{2+}$-dependent complex formed by the 105-kDa heavy chain and the 74-kDa light chain (shaded); the two open boxes denote activation fragments. (*Middle*) SDS/PAGE of the S-300 pool before and after incubation with thrombin. The gels were either stained with Coomassie blue (*A*) or subjected to Western blotting using mAb 30 (*B*) or polyclonal antibodies (*C*). Samples were reduced; ≈20 μg of protein was applied to each lane in the protein-stained gel, whereas ≈1 μg was applied to each of the lanes used for Western blotting. Lanes with thrombin-cleaved protein are marked T. Positions of molecular weight markers are given at left. Factor V-related polypeptides are marked with arrows, whereas fragments formed by thrombin (16) are indicated by arrowheads. The 150-kDa fragment stained poorly with Coomassie but was readily observed on Western blotting. Intermittently observed bands are denoted by asterisks. (*Bottom*) Agarose gel electrophoresis of the S-300 pool. Positions of albumin (alb), $\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$ bands of a plasma control are indicated by vertical lines.

The protein in the S-300 pool was used as antigen in the production of mAbs. Seventeen antibodies were obtained,

and they were all found to react with the 330-kDa single chain factor V as well as with the 220-kDa species, as judged by Western blotting (Fig. 2). After thrombin cleavage of factor V, all antibodies reacted with the 150-kDa activation fragment (the larger of the two activation fragments).

One of the antibodies (mAb 30) was immobilized on Affi-Gel and used for affinity chromatography (Fig. 3) of the S-300 pool. The protein that bound to the column was eluted and found to have both factor V and APC cofactor 2 activities. The elution profiles of both activities coincided, but manifested considerable trailing. Other elution conditions, such as higher or lower pH, or denaturing agents, were tried but were unsuccessful, as they resulted in loss of both activities. The S-300 pool was also applied to columns with immobilized polyclonal antibodies against human factor V or against bovine factor Va fragments. Both factor V and APC cofactor 2 activities were retained on the columns, but the denaturing conditions required to elute the bound protein resulted in loss of both biological activities (results not shown).

Increasing concentrations of affinity purified APC cofactor 2/factor V were added to AS plasma and the anticoagulant response to APC was tested. A dose-dependent increase in anticoagulant response to APC was observed (Fig. 4*A*). Approximately 25 μg/ml, which is of the same order of magnitude as the normal plasma concentration of factor V, was required to yield an APC response of AS plasma comparable to that of normal plasma (clotting times in the presence of APC, 90–110 sec). The affinity-purified protein

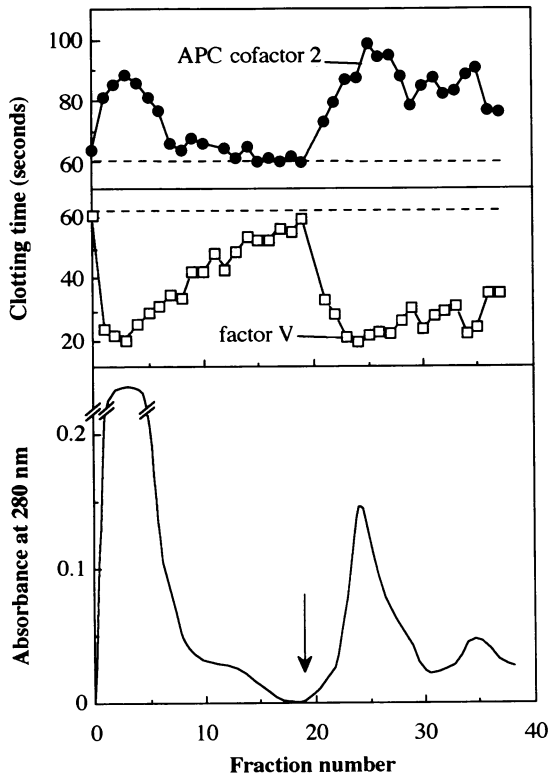


FIG. 3. Copurification of APC cofactor 2 and factor V by mAb affinity chromatography. The S-300 pool was applied to a mAb 30 affinity chromatography column. As the binding capacity of the column was exceeded, most of the protein passed through the column. After the column was washed, the bound protein was eluted with high pH (start of elution indicated by arrow). Fractions were analyzed with both APC cofactor 2 and factor V assays. Factor V activity was associated with a shortening of clotting time of factor V-deficient plasma, whereas APC cofactor activity gave an APC-dependent prolongation of clotting time of APC-resistant plasma. The two dashed lines represent clotting times of buffer controls.

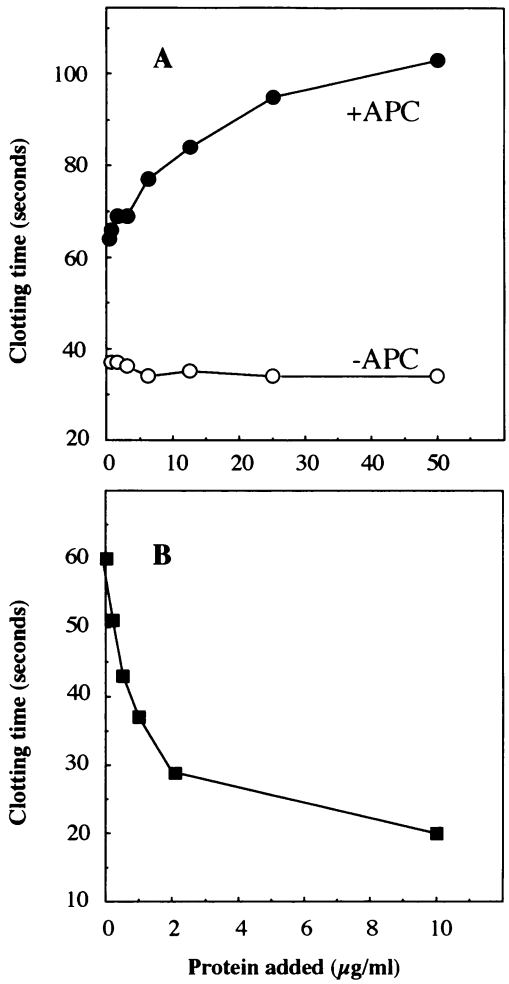


FIG. 4. Correction of APC resistance by purified APC cofactor 2/factor V. Affinity-purified APC cofactor 2/factor V (at indicated concentrations in a volume of 50 μl) was mixed with APC-resistant plasma (50 μl). The mixtures were then tested in the APC cofactor 2 assay (*A*) with (●) and without (○) APC in the $CaCl_2$ solution and in the factor V assay (*B*). Each point represents the mean of duplicate measurements.

was also active in a factor V assay, as demonstrated by a shortening of the clotting time (Fig. 4*B*).

## DISCUSSION

The results support the hypothesis that inherited resistance to APC is due to deficiency of an anticoagulant factor functioning as cofactor to APC. It appears valid to conclude that this anticoagulant activity is expressed by the factor V molecule, even though it cannot be entirely excluded that the activity is due to a molecule which is tightly associated with factor V. Characterization of the molecular defects causing APC resistance, and analysis of APC cofactor 2 activity of recombinant factor V, will help elucidate this possibility. It is noteworthy that individuals with APC resistance have normal levels of factor V procoagulant activity. The original report excluded the possibility that inherited APC resistance might be due to mutations in the APC cleavage sites of factors VIII and V, which would result in APC-resistant molecules (9). However, the possible linkage of APC resistance to the factor V gene was not investigated, as no DNA polymorphisms linked to the factor V gene were known. If APC cofactor 2 activity is assumed to be a property of the factor V molecule, our present results indicate that APC resistance is caused by

mutations in the factor V gene. Moreover, these mutations do not affect the procoagulant function of factor V.

If the assumption that different parts of the factor V molecule are important for its pro- and anticoagulant activities is right, it follows that it should be possible to modify one or the other activity by specific mAbs. In this context, it is noteworthy that we found two of our mAbs against factor V to partially inhibit the APC cofactor activity directly in plasma, whereas they did not affect the procoagulant activity of factor V (unpublished observation). Moreover, in a recent report, a patient with a severe thrombotic disease was described as having an acquired autoantibody against factor V (the epitope for the antibody was not localized) (17). Our present results provide an explanation for the apparent paradox of a patient with an antibody against factor V having a thrombotic rather than a bleeding diathesis. The patient's antibody presumably specifically inhibited the APC cofactor activity of factor V.

Why has the APC cofactor activity of factor V not been discovered previously? Factor Va is a major substrate for APC and easier to handle and more readily available than factor VIIIa. Most studies aimed at the elucidation of the function of APC and its possible cofactors have therefore used degradation of factor Va (measured with factor V assays) as a means of monitoring APC activity. This approach, which was successful for the identification of protein S as a cofactor for APC (18), is obviously inadequate to identify factor V as an APC cofactor. The present identification of factor V as an APC cofactor was dependent on the availability of plasma from an individual with inherited APC resistance.

The molecular mechanism by which factor V expresses its anticoagulant activity remains to be elucidated, although there are properties of factor V which presumably are important. Not only factor Va but also factor V bind to phospholipids on platelets and endothelial cells, and both forms interact with APC (19) as well as with protein S (20). It is conceivable that phospholipid-bound factor V forms a complex with APC and protein S which efficiently degrades factors VIIIa and Va.

At first glance, it may come as a surprise that factor V is a cofactor for APC. However, upon closer consideration, it would appear to be an appropriate, unique, and ingenious means of regulating blood coagulation. Factor V occurs free in plasma, as well as being bound to the surface of unactivated platelets and endothelial cells. Since low concentrations of protein C are known to be present in its activated form in the circulation (21), the ability of factor V to function as an APC cofactor may be instrumental in regulating the amounts of factors VIIIa and Va on the surface of these cells. As activation of factor V by thrombin results in the expression of potent procoagulant activity, and possibly in the loss of APC factor 2 activity, a new aspect of the relationship between pro- and anticoagulant mechanisms is suggested.

Thrombin was the first procoagulant enzyme also shown to express anticoagulant properties, when bound to thrombomodulin (5). We believe that factor V is the first coagulation protein demonstrated to express anticoagulant properties as well to be the precursor to a procoagulant cofactor, and our present results emphasize the importance of the balance between pro- and anticoagulant properties of the coagulation factors.

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