A2 Domain of Human Recombinant-Derived Factor VIII Is Required for Procoagulant Activity but not for Thrombin Cleavage

By Debra D. Pittman, Michael Millenson, Kimberly Marquette, Kenneth Bauer, and Randal J. Kaufman

Thrombin treatment of the coagulation factor VIII results in a rapid activation of procoagulant activity with a subsequent first order decay. The structural requirements for thrombin-activated factor VIII were characterized using recombinant-derived human factor VIII and site-directed DNA-mediated mutagenesis. Thrombin-activated human recombinant-derived factor VIII was isolated in an active form by passage over Mono-S fast protein liquid chromatography. The peak fractions had a specific activity of 80,000 U/mg. The subunit composition in the peak fraction contained the 50-Kd A1 domain from the heavy chain, the 73-Kd light chain fragment, and trace amounts of the 43-Kd A2 domain. The requirement of domain A2 for functional activity was shown in several ways. First, the addition of an inhibitory monoclonal antibody that recognizes domain A2 destroyed factor VIIIa activity. Second, addition of a Mono-S FPLC fraction that contained the A2 domain polypeptide back to the peak activity fraction increased activity of the factor VIIIa by 22-fold. The maximum specific activity achieved was 180,000 U/mg. Finally, expression of an A2 domain deletion mutant did not yield procoagulant activity, although the mutant was effectively secreted from the cell, exhibited appropriate heavy and light chain association, and was susceptible to thrombin cleavage. Cotransfection of this A2 domain deletion mutant with an A2 domain expression vector yielded a secreted complex and restored procoagulant activity in the conditioned medium. This result shows that the A2 domain can fold and assemble with A2-deleted factor VIIIa to yield a functional molecule. We conclude that the A2 domain is required for functional factor VIIIa activity and loss of activity in activated factor VIII may result from dissociation of A2 from the thrombin-activated heterotrimer.

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Supported by National Institutes of Health Grants No. HL07516 and HL33014.
Address reprint requests to Randal J. Kaufman, PhD, Director, Molecular and Cellular Genetics, Genetics Institute, 87 Cambridge Park Dr, Cambridge, MA 02140.

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activated recombinant-derived human factor VIII. Mono-S fast protein liquid chromatography (FPLC) was used to isolate a stable form of factor VIIIa that consists primarily of a heterodimer containing the A1 and A3-C1-C2 subunits, with a trace amount of the A2 domain subunit. The recovery of factor VIIIa activity was reproducibly low, approximately 5% of the starting activity. Addition of a Mono-S fraction containing the A2 domain subunit increased the procoagulant activity of the isolated VIIIa by 22-fold. These results indicate that the A2 subunit is required for functional activity of factor VIIIa. As an alternate approach to study the requirement for domain A2, site-directed DNA-mediated mutagenesis was performed to express an A2 domain deletion mutant of factor VIII that lacks procoagulant activity. Cotransfection of the A2 domain deletion mutant with an expression vector that expresses an A2 domain deletion molecule to yield an active complex.

**MATERIALS AND METHODS**

**Reagents.** Recombinant human factor VIII was obtained from Genetics Institute (Andover, MA). Human thrombin (8.8 mg/mL) was a gift from Hematologic Technologies Inc (Burlington, VT). Factor VIII-deficient plasma was purchased from George B. King Biomedical Inc (Overland Park, KS). Activated partial thromboplastin (APTT reagent) was purchased from General Diagnostics (Rochester, MN). Soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), aprotinin, histidine, Sigmacote, Tween-80, and rabbit brain cephalin were purchased from Sigma Chemical (St Louis, CA). Amino acid (500 1,000 1,500 2,000) chromatography resin and CL4B protein A sepharose were purchased from Pharmacia, LKB (Piscataway, NJ). d-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone (PPACK) and 2-N-morpholinethane sulfonic acid (MES) were obtained from CalBiochem (La Jolla, CA). The monoclonal antibody (MoAb) to the heavy chain of factor VIII (F8) uncoupled and coupled to CL4B sepharose was a gift from William Foster (Genetics Institute). The MoAb 60-B that recognizes the acidic region in the light chain of factor VIII was a generous gift from David Fass (Mayo Clinic, Rochester, MN).7 The rabbit polyclonal antibody 305 was a gift from Ed Alderman (Genetics Institute). The anti-light chain MoAb was purchased from Hybritech (San Diego, CA). [35S]-methionine (1,000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Methionine-free minimal essential medium (MEM) was purchased from Flow Labs, ICN (Costa Mesa, CA). Antifibrinolytic reagents and the Bradford protein assay were obtained from Biorad (Richmond, CA).

**Activation of factor VIII.** Human recombinant factor VIII at 320 μg/mL (400 to 800 U) in 50 mmol/L Tris-HCl (pH 7.2), 0.4 mol/L NaCl, 5 mmol/L CaCl₂, and 0.1% Tween-80 was incubated at room temperature with 320 μg/mL (400 to 800 U) of human thrombin. At short intervals, aliquots were removed and assayed for factor VIII clotting activity using factor VIII-deficient plasma.

**Mono-S chromatography in the presence of Tween-80.** Factor VIII (320 μg/mL) was incubated with thrombin (32 μg/mL) for 23 minutes and PPACK was added to a final concentration of 1.3 μmol/L to inhibit thrombin activity. The reaction was diluted one-half with 30 mmol/L histidine-Cl₃, pH 6.0, 5 mmol/L CaCl₂, 0.01% Tween-80, and applied to a Mono-S column equilibrated with 10 mmol/L histidine-Cl₃, pH 6.0, 5 mmol/L CaCl₂, 0.1 mol/L NaCl, and 0.01% Tween-80. The reaction was loaded via a Superloop (treated with Sigmacote) at a flow rate of 1 mL/min. The bound material was eluted first with a 1 mL/min linear gradient from 0.1 mol/L NaCl to 0.28 mol/L NaCl, followed by a 6 mL step at 0.28 mol/L NaCl. This elution was followed by a 1 mL/min linear gradient from 0.28 mol/L NaCl to 0.64 mol/L NaCl. The gradient was held at 0.64 mol/L for 5 mL where the activated factor VIII eluted. This step was followed by a linear gradient to 0.7 mol/L NaCl. The reaction was monitored for factor VIII clotting activity using factor VIII-deficient plasma.
The fractions were assayed immediately for activity in the clotting assay and peak fractions were assayed for protein according to Bradford. The fractions were then analyzed for sodium dodecyl sulfate (SDS)-polyacrylamide gels and were developed by staining with silver.

**Factor VIII assay.** Factor VIII and factor VIIIa were measured in a clotting assay using factor VIII-deficient plasma. APTT reagent (0.1 mL) was mixed with 0.1 mL of factor VIII-deficient plasma and incubated for 5 minutes at 37°C. Factor VIII and factor VIIIa were diluted into 50 mmol/L Tris-HCl, pH 7.2, 2.5 mmol/L CaCl₂, 0.15 mol/L NaCl, and 5% glycerol (buffer A) immediately before assay and 0.1 mL of the dilution was added to the plasma with APTT reagent. Prewarmed 25 mmol/L CaCl₂ (0.1 mL) was added and the clotting time was measured in an MLA Electra 750. When the 5 minutes of preincubation of plasma and APTT reagent was performed in the presence of factor VIII or factor VIIIa, the activity measured was 2.5-fold greater independent of whether factor VIII or factor VIIIa was assayed. For antibody inhibition, fraction 42 (225 µL/mL) or human recombinant factor VIII (300 µL/mL) or human recombinant factor VIIIa (0.1% human serum albumin) and the remaining dilution of the sample was added in buffer A and assayed immediately.

Increasing amounts of fraction 20 (containing 0.1% human serum albumin) were added to either factor VIIIa (fraction 2 containing 0.1% human serum albumin) or human recombinant factor VIII (containing 0.1% human serum albumin) and incubated at room temperature for 15 and 30 minutes. Samples were diluted into buffer A containing 0.1% BSA and assayed immediately for factor VIII activity by the clotting assay. One unit of factor VIII activity is that amount measured in 1 mL of normal human pooled plasma.

**Plasmid construction.** The factor VIII cDNA was previously described. The 5′ Sal I site was replaced by a unique Xho I site in the expression vector pMT2. Factor VIII mutations were introduced by site-directed mutagenesis by the heteroduplex procedure with modifications as described. The ΔA2 was constructed by introduction of a unique Mlu I restriction site at nucleotide 1,168-1,173 and 2,272-2,277 (nucleotide 1 is the A of the initiation codon ATG). This mutation corresponds to the thrombin cleavage site at arginine 371 and 740. The resulting plasmids were designated p372Mlu I and p740Mlu I, respectively. The presence of the mutations was confirmed by specific oligonucleotide hybridization, DNA sequencing, and digestion with frequent cutting restriction endonucleases. Mlu I recognizes the DNA sequence ACCCGGT that encodes for the amino acids threonine and arginine. This allows for the introduction of a unique restriction site at the thrombin cleavage site and maintains the arginine at the -1 position with respect to the cleavage site. The replacement of threonine for the amino acid at the -2 position at residue 371 or 739 does not interfere with in vitro cofactor activity (data not shown).

The A2 domain deletion was generated by ligation of the 4,973-bp Mlu I-Cla I fragment from p372Mlu I to the Mlu I-Cla I 6,139-bp fragment from p740Mlu I. The ligated DNA was used to transform Escherichia coli DH-5. The resultant plasmid, designated ΔA2, has deleted the A2 domain by juxtaposing arginine 372 to serine 741. Introduction of the Mlu I site at residue 372 changes an isoleucine at 371 to a threonine.

The A2 domain alone expression vector was derived by first constructing an expression vector, pHC, that directs synthesis of the 90-Kd heavy chain polypeptide, pHCl was constructed by introducing two stop codons (TGATGA) and a Sal I restriction site immediately after arginine 740 (nucleotide 2,278). The remainder of the factor VIII cDNA was deleted by Sal I digestion, ligation, and transformation of E. coli DH-5 to obtain pHC. Oligonucleotide encoding the factor VIII signal sequence containing a unique Mlu I site at the 3′ end and a unique Xho I site at the 5′ end were ligated into Xho I- and Mlu I-digested p372Mlu I. The resulting plasmid, designated pA1, deletes the A1 domain from amino acid residue 20 through amino acid residue 371. The A2 domain expression plasmid was then constructed by digestion of pHC with Kpn I and Xho I and ligation to Xho I- and Kpn I-digested pA1. The resultant plasmid was designated A2.

**Isolation of thrombin-activated factor VIII.** Recombinant-derived human factor VIII was reacted with thrombin for increasing periods of time and factor VIII activity determined by the ability to clot factor VIII-deficient plasma. Within the first 5 minutes there was an 80-fold activation of
factor VIII activity that declined over the next hour. Analysis of the polypeptides by SDS-polyacrylamide gel electrophoresis (PAGE) showed that after 25 minutes the majority of factor VIII was cleaved to 50-, 43-, and 73-Kd species (data not shown). To isolate and characterize the thrombin-activated complex, factor VIII was activated with thrombin for 25 minutes and the reaction stopped by addition of PPACK. The reaction mixture was applied to a Mono-S FPLC column and eluted with a salt gradient. In addition to protein recovered in the flow through fractions, two peaks of protein were detected upon elution with 0.28 mol/L and 0.64 mol/L NaCl (Fig 2A). Factor VIII activity in each fraction was measured in a clotting assay using factor VIII-deficient plasma. The results show that all activity was recovered within the high salt fractions 41 through 43 (Fig 2A, insert). The specific activity of factor VIII in this fraction was 60,000 U/mg. The recovery of factor VIII protein across the gradient was approximately 80%. However, the recovery of factor VIIIa in the peak fractions was reproducibly low. Of the 40,000 U of factor VIIIa applied to the Mono-S column in Fig 2A, 1,600 U were recovered, approximately 4% of the total starting activity. Thus, the low recovery of activity appears to result from a loss in the specific activity of the VIIIa upon Mono-S FPLC chromatography.

The ability to fractionate a stable form of thrombin-activated factor VIII was used to study the subunit composition and requirements for functional factor VIII activity. The polypeptide composition in each FPLC fraction was analyzed by SDS-PAGE. Before thrombin treatment, recombinant human factor VIII contains primarily heavy chain-derived polypeptides ranging up to 200 Kd and a light chain doublet of 80 Kd (Fig 2B, lane 1). After thrombin digestion, the heavy chain was cleaved to the 50-Kd A1 and 43-Kd A2 domain polypeptides and the light chain was cleaved to the 73-Kd A3-C1-C2 doublet (lane 2). Analysis of FPLC fractions by SDS-PAGE showed that the large heterogeneous activation peptides derived from the B-domain by thrombin cleavage are present in the flow through (lane 3). The A2 species coelutes with thrombin at approximately 0.28 mol/L NaCl (lanes 5 and 6). The fractions that exhibit factor VIII activity primarily consist of the A1 and A3-
C1-C2 polypeptides (lanes 10 through 12). In addition, a trace amount of the A2 polypeptide is detected in the fraction that exhibits peak factor VIII activity (lane 11).

When the fractionation of thrombin-activated factor VIII was repeated in the absence of Tween-80, very little activity was recovered (only 130 U/mL in the peak fraction) with a low specific activity (6,500 U/mg). The elution profile and SDS-PAGE analysis of protein from the Mono-S column in the absence of Tween-80 was very similar to that observed in the presence of Tween-80, except for the absence of detectable A2 species in the peak activity fractions (data not shown). This result suggested that the A2 domain may be required for functional factor VIIIa activity and the loss of activity in the absence of Tween-80 could result from dissociation of the A2 domain from the A1 and A3-C1-C2 heterodimer upon Mono-S chromatography. Alternatively, Tween-80 may directly enhance or stabilize the activity of factor VIIIa.

Several approaches were taken in an attempt to improve the yield by preventing the loss of activity of activated factor VIII. Thrombin-activated porcine factor VIII can be isolated in a stable form if the concentration of factor VIII is maintained greater than 25 µg/mL and at a pH of 6.0. We therefore evaluated the activity in the peak Mono-S fraction over time. After 1 month, the activity declined to 20% of its original value at a protein concentration of 25 µg/mL and at pH 6, although the concentration of protein in the tube did not change. Addition of either BSA or human serum albumin (0.1%) significantly stabilized the activity at 90% after 1 month. In contrast, addition of phospholipid resulted in a more rapid decay of factor VIII activity.

Comparison of activation and Mono-S chromatography in the presence of histidine (pH 6.0) or MES (pH 6.0) buffers showed no detectable difference in recovery or stability of the factor VIIIa (data not shown).

Domain A2 is required for activity of factor VIIIa. The requirement of the A2 domain for functional activity of isolated factor VIIIa was studied using an inhibitory antibody directed against an epitope on the A2 domain. The specificity of this MoAb, designated F8, was first determined using intrinsically [35S]-methionine–labeled factor VIII. Factor VIII-producing CHO cells were labeled with [35S]-methionine and labeled conditioned medium was immunoprecipitated with either anti-factor VIII polyclonal antibody 305 or F8 MoAb. Immunoprecipitated proteins were analyzed by SDS-PAGE. Both antibodies immunoprecipitated the heavy and light chain polypeptides of intact factor VIII (Fig 3; lanes 1 and 3). Immunoprecipitation of thrombin-digested factor VIII with anti-factor VIII polyclonal antibody 305 detects all thrombin cleavage products A1, A2, and A3-C1-C2 (lane 4). In contrast, immunoprecipitation of thrombin-activated factor VIII with F8 MoAb yields only the A2 polypeptide (lane 2). This finding shows that the inhibitory F8 MoAb reacts with an epitope within the A2 domain.

Addition of F8 antibody to Mono-S–isolated thrombin-activated factor VIII resulted in a rapid decrease in procoagulant activity similar to that observed with intact factor VIII (Table 1). The addition of another anti-factor VIII inhibitory antibody (60-B) that reacts between residues 1,674 and 1,684 in the amino terminus of the factor VIII light chain did not inhibit activity of factor VIIIa, although this antibody was capable of inhibiting activity of intact purified recombinant-derived human factor VIII (Table 1). The 60-B antibody inhibits binding of factor VIII to vWF as well as thrombin cleavage of the light chain. The result with the 60-B antibody suggests that the small 7-Kd terminal fragment of the light chain that is cleaved away by thrombin digestion is not required for activity of factor VIIIa.

Because recovery of factor VIIIa activity after Mono-S FPLC was reproducibly low and was depleted of the A2 domain polypeptide, we tested whether addition of the A2 domain polypeptide (fraction 20, Fig 2B) could restore activity of isolated factor VIIIa. At 15 or 30 minutes after a 66-fold dilution of fraction 42 (1,070 U/mL at room temperature, there was a sevenfold loss in cofactor activity to 2.5 U/mL when not corrected for the dilution (Fig 4). A similar dilution in the presence of increasing amounts of fraction 20 resulted in a 22-fold greater amount of activity (54 U/mL). Further increases in the amount of fraction 20 added did not result in further increases in activity (data not shown).
not shown). To rule out that residual active thrombin contaminating these fractions was responsible for the increase in factor VIII activity, fraction 20 was added to intact recombinant-derived unactivated factor VIII. Addition of fraction 20 did not increase the activity of unactivated factor VIII (Fig 4). Thus, it is likely that residual thrombin activity in fraction 20 was not responsible for the increase in activity observed upon its addition to fraction 42. The specific activity of the reconstituted factor VIIIa was 180,000 U/mg.

Site-directed mutagenesis identifies a functional requirement for domain A2. As an alternative method to study the requirement for domain A2 for functional factor VIII activity, residues 373 through 740 were deleted within the wild-type factor VIII cDNA and the deleted molecule was expressed in COS-1 monkey kidney cells. Conditioned medium from cells transfected with the control wild-type factor VIII cDNA expression vector exhibited 650 mU/mL activity at 60 hours posttransfection (Table 2). In contrast, no detectable activity was observed in conditioned medium from cells transfected with the A2 deletion mutant expression vector ΔA2.

To evaluate the reason for the absence of activity in the conditioned medium from ΔA2-transfected cells, the synthesis and secretion of factor VIII was studied. At 60 hours posttransfection, the cells were labeled with [35S]-methionine for 6 hours and the conditioned medium was analyzed by immunoprecipitation with either the rabbit polyclonal antibody 305 (Fig 5; lanes 1 through 8) or an anti-factor light chain MoAb (Fig 5; lanes 9 through 12). SDS-PAGE of the 305 antibody immunoprecipitated protein from wild-type factor VIII-transfected cells detects single chain factor VIII, the light chain, as well as heavy chain-derived polypeptides (Fig 5; lane 1). Similar analysis of conditioned medium from ΔA2-transfected cells shows the heavy chain and intact single chain migrating slightly faster, consistent with a small deletion within these polypeptides (lane 3). The light chain of ΔA2 comigrates the wild-type factor VIII light chain. The level of secretion of ΔA2 is similar to that observed for wild-type factor VIII. Significantly, immunoprecipitation with the factor VIII light chain MoAb results in the coprecipitation of the factor VIII heavy chain for both the wild-type (lane 9) and the ΔA2 deletion mutant (lane 11). This result shows that the A2 domain is not required for factor VIII heavy and light chain association. The results from these experiments show that ΔA2 is conformationally competent for secretion and the absence of activity in the conditioned medium of ΔA2-transfected cells does not result from a deficiency in protein synthesis, secretion, or assembly of the heavy and light chains.

The lack of activity for the ΔA2 molecule might result from inability to be cleaved and activated by thrombin. The effect of the A2 domain deletion on the susceptibility to thrombin cleavage was evaluated by thrombin digestion of immunoprecipitated factor VIII from [35S]-methionine–labeled conditioned medium obtained from wild-type factor VIII- and ΔA2-transfected cells. Thrombin cleavage of the immunoprecipitated proteins from wild-type factor VIII-transfected cells and SDS-PAGE analysis shows the appropriate polypeptides: A1, A2, and A3-C1-C2 (lanes 2 and 10). In this analysis, the presence of the anti-factor VIII light chain MoAb partially inhibits cleavage of the factor VIII light chain (lane 10). Upon thrombin digestion of immunoprecipitated ΔA2 conditioned medium, the A1 and A3-C1-C2 are detected (lanes 4 and 12). As expected, the A2 polypeptide is absent. In addition, a 30-Kd polypeptide is generated upon thrombin digestion of A2-deleted factor VIII. The 30-Kd polypeptide comigrates with a fragment generated from the A1 domain upon extensive thrombin digestion2 (data not shown). Interestingly, the A1 domain derived from thrombin-treated ΔA2 migrates slightly slower.

Table 2. Addition of A2 by Cotransfection or Mixing Conditioned Medium Restores Activity of ΔA2 Factor VIII

<table>
<thead>
<tr>
<th>Sample</th>
<th>Transfected Plasmid(s)</th>
<th>mU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>wtVIII</td>
<td>650</td>
</tr>
<tr>
<td>2</td>
<td>ΔA2VIII</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>A2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>ΔA2VIII + A2</td>
<td>85</td>
</tr>
<tr>
<td>5</td>
<td>Mixing Conditioned Medium</td>
<td>432</td>
</tr>
<tr>
<td>6</td>
<td>ΔA2VIII + A2</td>
<td>108</td>
</tr>
</tbody>
</table>

COS-1 cells were transfected with factor VIII expression plasmids encoding wild-type factor VIII (pMT2-VIII), A2 domain deletion (ΔA2), or A2 domain only (A2) as described in Materials and Methods. For cotransfection shown in sample 4, equal amounts of plasmid DNA were used. Factor VIII activity was determined at 60 hours posttransfection as described in Materials and Methods. Values shown are the concentration of factor VIII activity detected in the conditioned medium. Equal volumes of the conditioned medium from transfected cells were also mixed and incubated for 15 minutes and then factor VIII activity determined using factor VIII-deficient plasma (samples 5 and 6).
than the A1 domain derived from wild-type factor VIII. Susceptibility to endoglycosidase H digestion showed that the difference in mobility results from the presence of complex oligosaccharides on the A1 domain of ΔA2 compared with the presence of high mannose oligosaccharides on the A1 domain of wild-type factor VIII (data not shown). The results from thrombin digestion show that the ΔA2 molecule is in an appropriate conformation to serve as a substrate for thrombin cleavage. Thus, the lack of activity in the ΔA2 molecule may result from the absence of the A2 domain within the thrombin-cleaved ΔA2.

**Cotransfection of ΔA2 with A2 restores procoagulant activity.** Because A2 may be added to the A1 and A3-C1-C2 heterodimer to reconstitute factor VIII activity, we tested whether cotransfection of ΔA2 with an expression vector engineered to express a secreted A2 domain would restore functional activity in ΔA2. An expression vector, designated A2, was constructed by fusing the signal peptide of factor VIII (residues 1 to 19) to residue 372 of factor VIII. A termination codon was inserted at residue 741 and the remainder of the cDNA was deleted. Transfection of the resultant expression vector, designated A2, into COS-1 cells yielded significant levels of a 43-Kd polypeptide that was immunoprecipitated with F8 MoAb (Fig 5, lane 5). This fragment comigrated with thrombin-cleaved A2 domain expressed from wild-type factor VIII (lane 2). To determine whether coexpression of A2 with ΔA2 could generate factor VIII activity, A2 was cotransfected with ΔA2 and samples of the conditioned medium taken for factor VIII activity assay in factor VIII-deficient plasma. Although either A2 or ΔA2 alone did not exhibit any factor VIII activity, cotransfection of both plasmids yielded significant levels of factor VIII activity (Table 2). Addition of conditioned medium from ΔA2-transfected cells also yielded detectable amounts of factor VIII activity. A2 conditioned medium did not affect factor VIII activity when added to conditioned medium from wild-type factor VIII-transfected cells (Table 2). This finding shows that A2 can be expressed independently to yield a functional domain and its addition to ΔA2 can restore functional activity.

The ability of the independently expressed A2 domain polypeptide to associate with the ΔA2 polypeptide was studied by coprecipitation using an anti-factor VIII light chain MoAb. Transfected COS-1 cells were labeled with [35S]-methionine and conditioned medium harvested for immunoprecipitation analysis. Immunoprecipitation of polypeptides from wild-type factor VIII and ΔA2 factor VIII-transfected cells yields light chain, heavy chain, and single chain polypeptides (Fig 6, lanes 1 and 2). Similar immunoprecipitation from A2-alone–transfected cells yields only background polypeptides (lane 3) that are detected in the nontransfected COS-1 cells (lane 5). In contrast, immunoprecipitation from A2- and ΔA2-cotransfected cells yields in addition to the ΔA2-specific bands detectable amounts of the A2 domain polypeptide (lane 4). These results show that independent expression of A2 and ΔA2 can yield a complex that has functional activity upon exposure to thrombin.

**DISCUSSION**

Using direct biochemical isolation of thrombin-activated recombinant-derived human factor VIII as well as site-directed DNA deletion analysis we have shown that factor VIII requires domain A2 for functional activity, but not for thrombin cleavage. Thrombin-activated factor VIII was isolated by chromatography on Mono-S FPLC. Isolated factor VIIIa was predominantly a heterodimer of A1 and
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the transfection described in Table 2. Equal volumes of samples were
immunoprecipitated with anti-factor VIII light chain MoAb and immu-
oprecipitated protein analyzed by SDS-PAGE. Migration of the single
A3-CI-C2 with trace amounts of A2. The recovery of VIIIa
activity upon Mono-S FPLC chromatography was reproduc-
ibly low, approximately 5% of the original activity. The
observation that approximately 10-fold lower recovery of
activity was obtained when Tween-80 was omitted from the
activation and chromatographic buffers and that the A2
domain and the low recovery of VIIIa activity results
from dissociation of A2 from the A1/A3-C1-C2 heterodimer.
First, addition of an MoAb specific for A2
inhibited activity of factor VIIIa. In contrast, addition of an
MoAb that reacts with the amino terminus of the factor
VIII light chain between residues 1,674 and 1,684 and
inhibits factor VIII activity did not inhibit activity of factor
VIIIa. This finding shows that the small 7-kD thrombin
cleavage product from the light chain is not required for
functional activity of factor VIIIa. This result is consistent
with mutagenesis studies that show deletion of residues
1,649 to 1,689 of the light chain destroys the factor VIII
interaction with vWF, but does not alter the specific activity
of the resultant factor VIII. Second, addition of the
Mono-S FPLC fraction containing the A2 domain subunit
back to the A1/A3-C1-C2 heterodimer increased the activity
of factor VIIIa by 22-fold. The highest specific activity
observed for VIIIa was 180,000 U/mg, or approximately
100-fold greater than intact factor VIII. Recently, similar
observations were reported for human plasma-derived
factor VIII in which factor VIII was reconstituted from
the A2 domain and the A1/A3-C1-C2 heterodimer to a specific
activity greater than 40,000 U/mg. Finally, deletion of the
A2 domain in the factor VIII cDNA destroyed functional
activity in the expressed protein, although the molecule was
secreted and could serve as substrate for thrombin cleavage.
These latter observations suggest the molecule was
properly folded. It is of interest that the heavy chain was
associated with the light chain in the A2-deleted factor VIII
(Fig 5 and data not shown), suggesting that the metal ion
association of the light and heavy chain does not require the
A2 domain. Conserved cysteine and histidine residues
implicated in copper binding for ceruloplasmin are con-
served in the first and third, but not the second, A domains
within factor VIII. The association between the factor VIII
heavy and light chains may be mediated through these
cysteine and histidine residues.

Fig 6. Association of independently coexpressed A2 with ΔA2.
(35S)-methionine–radiolabeled conditioned medium was prepared from
the transfection described in Table 2. Equal volumes of samples were
immunoprecipitated with anti-factor VIII light chain MoAb and immu-
oprecipitated protein analyzed by SDS-PAGE. Migration of the single
chain (SC), heavy chain (HC), light chain (LC), and A2 domain
polypeptide (A2) are indicated.

A3-C1-C2 with trace amounts of A2. The recovery of VIIIa
activity upon Mono-S FPLC chromatography was reproduc-
ibly low, approximately 5% of the original activity. The
observation that approximately 10-fold lower recovery of
activity was obtained when Tween-80 was omitted from the
activation and chromatographic buffers and that the A2
domain was absent in the most active fractions from the
Mono-S column prompted us to evaluate the importance of the
residual levels of the A2 domain in the preparations of
thrombin-activated factor VIIIa. Our observations strongly
support the conclusion that active factor VIIIa requires the
A2 domain and the low recovery of VIIIa activity results
from dissociation of A2 from the A1 and A3-C1-C2 het-
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within factor VIII. The association between the factor VIII
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Significantly, cotransfection of an A2 domain expression
plasmid with the A2-deleted expression plasmid resulted in
the association of the A2 domain polypeptide with the
A2-deleted factor VIII and secretion of functional factor
VIII activity. Similar results were obtained by mixing the
conditioned medium from A2-transfected cells with condi-
tioned medium from A2-deleted factor VIII-transfected
cells. It was surprising that the addition of independently
expressed A2 to A2-deleted factor VIII could reconstitute
functional factor VIII activity. This finding suggests that
functional domains of factor VIII may independently fold
and association of the individual domains may occur to
yield functional activity. Because it is possible to produce
functional factor VIII by reassociation of independently
expressed subunits A1/A3-C1-C2 and A2, the coexpression
of independently expressed subunits should provide a
useful strategy to further dissect requirements for factor
VIII function.

The stability of isolated factor VIIIa has been improved
by lowering the pH that prevents dissociation of A2, as
well as by increasing the concentration of factor VIIIa. Addition
of either human serum albumin or BSA can stabilize factor VIIIa. The reported effects of reduced pH and recent studies on salt effects on VIIIa stability indicate that the A2 domain interacts with A1/A3-C1-C2 by an ionic interaction. If the A2 domain interacts with A1/A3-C1-C2 by a weak ionic interaction, dissociation may result in its
adsorption to surfaces or aggregation and thus reduce its
effective concentration. Because the localized concentra-
tion of factor VIIIa is very high at the site of injury and low
in the systemic vasculature, the dissociation of A2 may
provide an additional mechanism to the protein C anticoag-
ulant pathway to ensure localization of functional activated
factor VIII to the damaged surface and prevent systemic activation of the intrinsic coagulation cascade. It remains to be determined what effect dissociation of A2 plays under physiologic conditions. One way to approach this evaluation will be to study the stability of VIIIa upon infusion into animal models.

REFERENCES


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