Factor VIII Arg<sub>2304</sub>→ His Binds to Phosphatidylserine and Is a Functional Cofactor in the Factor X-ase Complex

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Key words

Factor VIII, factor VIII light chain, hemophilia, phospholipid binding

Summary

Four factor VIII light chain constructs containing hemophilia A mutations at R2304 and R2307 were prepared and expressed in mammalian cells. These mutations are located in a putative phosphatidylserine binding site identified by peptide studies (spanning amino acids 2303-2332). The levels of all four mutants in conditioned medium were significantly less than wild type by immunoprecipitation and ELISA. R2304H and wild type factor VIII light chains were concentrated by cation exchange chromatography from medium. R2304H and wild type factor VIII light chains bound immobilized phosphatidylserine similarly. The reconstituted cofactor activity of R2304H factor VIII light chain was slightly greater than wild type factor VIII light chain. These results are consistent with the recently reported crystal structure of factor VIII C2 domain that suggests R2304H is not directly involved in phospholipid binding. The observed clinical phenotype is probably due to decreased circulating levels of a functional protein.

Introduction

Factor VIII is an essential component of the intrinsic pathway of blood coagulation (1). Factor VIII is synthesized as a single polypeptide with a domain structure represented as A1-A2-B-A3-C1-C2. After synthesis it is proteolyzed and circulates in the plasma as a heterodimer, consisting of a heavy chain (A1, A2, and variable portions of the B domain) linked non-covalently through a divalent metal ion to a light chain (A3, C1, and C2 domains). The cofactor is activated by thrombin, which results in a heterotrimERIC molecular complex, in which factor IXa catalyzes the proteolysis of factor X into factor Xa in the presence of calcium ions and a phospholipid membrane surface (3).

These results are consistent with the recently reported crystal structure of the C2 domains of human factors VIII and V (10, 11), it appears unlikely that the PS-binding site is located completely within the region between amino acids 2303-2332. Both C2 domain structures are similar and consist of a framework supporting three protruding spikes that appear to define the region of the domain that interacts with phospholipid membranes (10, 11). In factor VIII, hydrophobic residues that are solvent exposed and that have been proposed to interact with phospholipid membranes include M2199, F2200, W2213, V2223, L2251, and L2252 (11).

Functional deficiency of factor VIII results in hemophilia A (12). More than 250 missense mutations have been identified throughout the factor VIII gene in patients with hemophilia A (http://europium.csc.mrc.rpms.ac.uk) (13). Twenty-one mutations are located within the C2 domain, and several, including mutations at R2304 and R2307, are located within the previously identified putative PS-binding site spanning amino acids 2303-2332 (13). Missense hemophilia A mutations located at R2304 include substitutions by histidine and cysteine, and at R2307 they include substitutions with glutamine and leucine. The mutation R2307Q has been shown to result in decreased factor Xa binding, but this mutant has not been characterized functionally (14).

To investigate these hemophilia mutations directly, we expressed the factor VIII light chain and all four light chain mutant constructs in mammalian cell culture. Procoagulant activity and PS-binding properties of the wild type and the R2304H mutant light chain were characterized.

Materials and Methods

Proteins and Reagents. Purified recombinant human factor VIII was a gift from Baxter Healthcare Corp. (Duarte, CA). Murine monoclonal anti-human factor VIII antibodies ESH4 and ESH8 were from American Diagnostica Inc. (Greenwich, CT). These antibodies recognize amino acids 2303-2332 and 2248-2312 in the C2 domain, respectively (9, 15). Murine monoclonal anti-
human factor VIII antibody MAB038 was from Chemicon International, Inc., (Temecula, CA) and recognizes the amino-terminal region of factor VIII light chain (amino acids 1649-1689) (4). HZ, an IgG preparation from patient plasma containing anti-factor VIII antibodies, was kindly provided by Dr. Mirella Ezban (Novo Nordisk, Denmark) (16). Polyclonal anti-human factor VIII antibodies were prepared from rabbit serum (17). L-α-phosphatidylserine was from Avanti Polar-Lipids Inc. (Alabaster, AL).

Mutagenesis. Mutagenesis was done by a combination of PCR and splicing by overlap extension as previously described (17). The cDNA of factor VIII light chain has a ClaI site at the 5' end followed by the α1-antitrypsin signal sequence, and a SalI site at the 3' end. It was a gift from Dr. Mirella Ezban. The cDNA of mutant and wild type factor VIII light chain were subcloned into pDX (18) for transient expression and pCDNA3 (Invitrogen, Carlsbad, CA) for preparation of stable cell lines.

Transient Expression and Immunoprecipitation of Wild type and Mutant Factor VIII Light Chains. COS-7 cells were transfected with the pDX expression vector containing wild type factor VIII light chain or mutant factor VIII light chain using lipofectamine (GibcoBRL, Rockville MD). For antigen determination by ELISA the medium was switched to serum free medium (DMEM/F12 containing 2.5 mM CaCl₂, 5 mg/ml bovine serum albumin) 24 h post transfection and collected 24 h later for analysis. Immunoprecipitation of metabolically labeled [³⁵S]methionine factor VIII light chain in the medium was done as previously described except that proteins were labeled for 24 h instead of 6 h. Five anti-factor VIII antibodies were used for immunoprecipitation: rabbit polyclonal anti-factor VIII, HZ human IgG, and the three murine monoclonal antibodies MAB038, ESH4, and ESH8.

Expression of Wild type and Mutant Factor VIII Light Chains in Stable Cell Lines. Human embryonic kidney cells (293) were transfected with the pCDNA3 expression vector containing wild type or mutant factor VIII light chains using lipofectamine and 72 h later they were passaged into selective medium containing the antibiotic G418. Single G418 resistant colonies were selected by limited dilution, screened for factor VIII light chain antigen by ELISA, and expanded to 150 cm² flasks containing 30 ml of serum free medium (MCDB 302/6-MEM, 1:1 supplemented with 2 mM CaCl₂, 15 mM HEPES, 1.9 mM glucose and 1 mg/ml BSA). Conditioned medium was collected every 24 h. Wild type and R2304H factor VIII light chains were concentrated by cation exchange chromatography. Initially, a 90 ml column containing SP sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) was used. Subsequently, it was found that a 1ml SP sepharose HiTrap column (Amersham Pharmacia Biotech) was sufficient for up to 800 ml of conditioned medium. The pH of the conditioned medium was adjusted to 6.0 and 250-1020 ml was loaded onto one of the SP sepharose columns equilibrated with 50 mM MES, 0.25 M NaCl, 0.01 % Tween 20, pH 6.0. The column was washed with the same buffer until all unbound protein was removed. Factor VIII light chain was eluted with a 20 ml linear gradient from 0.25-1M NaCl in the equilibration buffer.

Pulse Chase Experiments. Pulse chase experiments were done as previously described (17). Briefly, stably transfected 293 cells expressing wild type and the four mutant factor VIII light chains were distributed onto individual wells of a six-well plate. Approximately 24 h later the medium was changed to methionine deficient medium for 2 h and then replaced with methionine deficient medium containing 150 µCi of [³⁵S]methionine. After one hour, the pulse medium was removed and replaced with serum free medium containing methionine.

Samples were removed at the indicated times (beginning with freshly added serum free medium as time 0). Cell lysates were also collected at the indicated times.

Factor VIII Light Chain ELISA. Wild type, R2304H, and R2307Q factor VIII light chain antigens were measured by a modification of an ELISA using ESH4 and biotinylated ESH8 as the capture and detection antibodies, respectively (19). Purified recombinant factor VIII was used as a standard to determine protein concentration. The values are reported as average ± standard deviation. MAB038 was also used as the capture antibody in place of ESH4 to confirm that the mutation did not effect the binding of light chain to ESH4.
limit was 0.05 and 0.5 nM factor VIII light chain when ESH4 or MAB038 was used as the capture antibody, respectively, and ESH4 was therefore routinely used as the capture antibody.

**PS-Binding Assay.** A solid-phase ELISA was used to measure the binding of wild type and R2304H factor VIII light chains, and purified recombinant factor VIII to PS (6). The plates were coated with 50 µl of PS (3 µg/ml), and factor VIII binding was detected using biotinylated ESH8.

**Separation of Heavy and Light Chains of Factor VIII.** The heavy and light chains of recombinant factor VIII were dissociated and separated by cation exchange chromatography, as described (20).

**Reconstitution of Factor VIII Cofactor Activity.** Reconstitution of the heavy chain of recombinant factor VIII with wild type and R2304H factor VIII light chains was done by a modification of previously reported methods (21, 22). Cofactor activity was measured using a Chromogenix Coatest VIII kit (Diapharma, West Chester, OH) adapted for microplates (23). Heavy chain (5 µl in 50 mM Tris pH 7.3) was combined with light chain (10 µl in 50 mM MES, 150 mM NaCl pH 6.0) in the presence of 5 mM MgCl2, in a final volume of 20 µl, incubated for 2 h at room temperature, and diluted 1:1.6 into the Coatest VIII assay. Final concentrations of factor VIII light chains in the Coatest VIII assay were 0.75 nM for both wild type and Baxter factor VIII light chains, and 0.3 nM for R2304H factor VIII light chain. Factor VIII heavy chain concentration was not determined. Factor VIII heavy chain was titrated in the assay for each light chain until maximal activity was obtained, and the baseline residual activity in the absence of added light chain was subtracted from each result.

**Results**

**Transient Expression of Wild type and Mutant Factor VIII Light Chains in Cos-7 Cells.** Immunoprecipitation of [35S]methionine metabolically labeled factor VIII light chains with five anti-factor VIII antibodies confirmed the expression and secretion of all five factor VIII light chain constructs (Fig. 1). Immunoprecipitation with polyclonal anti-human factor VIII IgG, factor VIII inhibitor IgG, and three murine monoclonal anti-human factor VIII antibodies all showed similar results, suggesting that all of the antibodies bind to wild type factor VIII light chain and each of the mutants similarly (Fig. 1). The levels of R2304H and R2307Q factor VIII light chains found in the conditioned medium were significantly less than wild type factor VIII light chain, and R2304C and R2307L factor VIII light chains were barely detectable (Fig. 1). The concentration of wild type factor VIII light chain antigen in conditioned medium was determined to be 1.05 ± 0.46 nM (N=5 separate transfections) by the ESH4/ESH8 ELISA, but none of the mutant light chains expressed transiently were detectable in conditioned medium (N=3 transfections for each mutant).

**Stable Expression of Wild type and Mutant Factor VIII Light Chains in 293 Cells.** Stable cell lines for the expression of wild type and the four mutant factor VIII light chains were prepared in 293 cells. Pulse chase experiments using the stable cell lines revealed that the appearance of the four mutants in the medium was delayed relative to wild type (Fig. 2). Wild type factor VIII light chain was detected in the medium within 1 hour after adding [35S]methionine while the four mutants were not detected in the medium in significant amounts until 4 h after addition of [35S]methionine. The levels of R2304C, R2307Q, and R2307L factor VIII light chain in the medium were also much lower than wild type factor VIII light chain, which was consistent with the immunoprecipitation results using transient expression in COS-7 cells.

Stable cell lines for wild type, R2304H, and R2307Q factor VIII light chain were also analyzed for factor VIII light chain antigen using the ESH4/ESH8 ELISA. The concentration of wild type factor VIII light chain antigen in conditioned medium was found to be 6.6 ± 1.6 nM (N=25 separate flasks). In contrast, the concentration of R2304H factor VIII light chain was 0.6 ± 0.35 nM (N=30 separate flasks) and R2307Q factor VIII light chain was not detectable in conditioned medium. The low level of R2307Q factor VIII light chain in conditioned medium precluded further experiments with this mutant.

Conditioned medium from stable cell lines expressing wild type and R2304H factor VIII light chain were concentrated by cation exchange chromatography, as described in Materials and Methods. The protein concentration of the factor VIII light chain for wild type and R2304H mutant were determined in concentrated conditioned medium by

![Fig. 1 Binding of purified recombinant factor VIII and wild type and R2304H factor VIII light chains to PS. Microtiter plate wells were coated with PS and blocked as described. Factor VIII (filled circles, solid line) or conditioned medium from wild type factor VIII light chain (open triangles, dotted line) or conditioned medium from R2304H factor VIII light chain (open circles, dashed line) were added to the wells at the indicated concentrations. Binding was detected by using biotinylated ESH8 as described.](http://www.thrombosis-online.com/2018-05-13,ThrombHaemost2001;85:260-4)

**Table 1** The R2304H mutation does not affect the ESH4/ ESH8 ELISA

<table>
<thead>
<tr>
<th>Capture Antibody</th>
<th>ESH4</th>
<th>MAB038</th>
<th>Ratio MAB038/ESH4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII Light Chain</td>
<td>2000</td>
<td>2700</td>
<td>1.3</td>
</tr>
<tr>
<td>R2304</td>
<td>6.0</td>
<td>9.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table 2** Reconstitution of cofactor activity of wild type and R2304H factor VIII light chain

<table>
<thead>
<tr>
<th>Factor VIII or Factor VIII Light Chain Concentration (nM)</th>
<th>Cofactor activity (mOD/ min)</th>
<th>Activity concentration (mOD/min/nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII (Baxter)</td>
<td>0.15</td>
<td>134</td>
</tr>
<tr>
<td>Factor VIII Heavy Chain</td>
<td>Light Chain (Baxter)</td>
<td>0.75</td>
</tr>
<tr>
<td>Wild type Light Chain</td>
<td>0.75</td>
<td>136</td>
</tr>
<tr>
<td>R2304H Light Chain</td>
<td>0.29</td>
<td>143</td>
</tr>
</tbody>
</table>

Factor VIII heavy and light chains were prepared from Baxter factor VIII as described in Materials and Methods. Wild type and R2304H factor VIII light chains were expressed in 293 cells, and conditioned medium was concentrated by cation exchange chromatography. Cofactor activity was reconstituted and measured as described in Materials and Methods. Residual cofactor activity in the heavy chain preparation was 71 mOD/min, which was subtracted from each of the reconstituted light chain experiments. The cofactor activity was divided by the light chain concentration (determined in the ESH4/ESH8 ELISA) and is expressed relative to factor VIII. Abbreviations: nM, nanomolar; mOD/min, milli-optical density units per minute, measuring the change in absorbance at 405 nm.
ELISA using two different capture antibodies (ESH4 and MAB038) to confirm that the mutation at R2304 did not significantly interfere with the binding of ESH4 (Table 1). For wild type factor VIII light chain the ratio of the antigen concentration determined using MAB038 as the capture antibody to the antigen concentration determined using ESH4 was 1.3. For R2304H factor VIII light chain, the ratio of the antigen concentration determined using MAB038 to the antigen concentration using ESH4 was 1.5. The ratios are similar, consistent with the immunoprecipitation data (Fig. 1), and confirm that the mutation at R2304 does not significantly interfere with ESH4 binding.

**Binding of Wild type and R2304H Factor VIII Light Chain to PS.** Since R2304 is putatively located in the PS-binding site of factor VIII, we compared the PS-binding properties of wild type and R2304H factor VIII light chains. Both factor VIII light chains bound to immobilized PS with approximately the same affinity (Fig. 3), suggesting that the hemophilia A mutation R2304H does not disrupt PS-binding.

**Reconstitution of Cofactor Activity.** Since R2304H factor VIII light chain bound to PS with approximately the same affinity as wild type factor VIII light chain, we next determined if this mutant light chain had similar cofactor activity as wild type after reconstitution with heavy chain. Isolated factor VIII heavy chain was incubated with wild type or R2304H light chains in the presence of 5 mM MgCl₂, and cofactor activity was measured as described in Materials and Methods. The reconstituted cofactor activity of R2304H factor VIII light chain was consistently slightly greater (range, 3-15 times greater) than that of wild type factor VIII light chain using two different preparations of both light chains and the heavy chain. Representative cofactor activity is shown in Table 2. The reconstituted cofactor activities of wild type and R2304H factor VIII were dependent on light chain as well as heavy chain concentrations (data not shown). Several factors most likely contributed to the observed variability of the relative cofactor activities, including small amounts of contaminating factor VIII light chain in the heavy chain preparations, variations in light chain concentration, and inherent variation in the assay (21, 22).

**Discussion**

Missense mutations in the factor VIII gene can produce the clinical phenotype of hemophilia A by a variety of mechanisms. We investigated the hemophilia A missense mutations involving amino acids R2304 and R2307 since previous studies using synthetic peptides (8) and factor VIII inhibitors (9) suggested that these two residues might be involved in binding to phospholipid membranes. The recently reported crystal structure and models of the factor VIII C2 domain, however, suggest that neither R2304 or R2307 are directly involved in PS-binding (10, 11, 24). Both of these amino acids are located on a common surface exposed portion of the C2 domain, which is located on the opposite end of the β-barrel structure from the proposed PS-binding region.

The arginine at position 2304 is conserved across all C domains in factor V and factor VIII, with the exception of a glutamine residue in the corresponding position in the C1 domain of canine factor VIII (Q2139) (25). Based on two models of the factor VIII C2 domain, one derived from the factor V C2 domain structure (10) and one from the amino-terminal domain of a fungal galactose oxidase (24), it was predicted that R2304 is involved in a salt bridge with amino acid D2233. This aspartic acid is conserved across all C domains, and the conserved salt bridge is consistent with the crystal structure of the factor VIII C2 domain (11) (Protein Data Bank accession code 1D7P).

Gale et al. (24), using a model of the factor VIII C2 domain, predicted that substitutions at position R2304 would potentially destabilize the C2 domain and result in a misfolded protein. Substitution of an arginine for the arginine at position 2171 in factor V, which corresponds to R2304 in the factor VIII C2 domain, resulted in significantly decreased binding to phospholipid and to two anti-factor V antibodies, and significant loss of cofactor activity (26). These results suggested an incorrectly folded domain. In contrast, we found that the histidine substitution at position R2304 in the factor VIII C2 domain did not result in significant loss of phospholipid binding, monoclonal antibody binding, or cofactor activity. Although minor decreases in phospholipid binding or cofactor activity may not have been detected in our study, the fact that both assays gave similar results suggests that the R2304H mutant is functionally comparable to the wild type protein. In contrast, Takeshima and Fujikawa (27) reported that substitution of histidine for R2304 resulted in ~70-80% lower binding activity to phospholipid than the ‘mother molecule’ (C2169–Y2332, with cysteine replaced by valine). They expressed their mutant as an isolated C2 domain construct (with 4 amino acids from the C1 domain, including the valine substitution for cysteine) in *Pichia pastoris*, however, which may explain the difference between their results and ours.

The amino acid R2307 is conserved across all C domains of factor V and factor VIII (10). This amino acid forms part of a conserved tripeptide that appears critical for proper domain folding, in which the arginine is sandwiched between tryptophan residues at positions 2229 and 2271 (10, 24). Previous reports have demonstrated that the two hemophilia A missense mutations at R2307 (R2307Q and R2307L) are associated with significantly decreased protein secretion, suggesting a defect in intracellular trafficking (14, 28). The specific activity of factor VIII R2307Q was also mildly reduced compared to the wild type cofactor, but the R2307L mutant was not functionally characterized (14). Our results with the factor VIII R2307Q and R2307L light chain mutants are consistent with these reports that mutations at position 2307 result in a profound decrease in the level of protein secretion (Fig. 2).

In summary, we report that the hemophilia A mutation R2304H does not interfere with PS-binding or with cofactor activity of reconstituted factor VIII. Our data are consistent with the crystal structure data confirming that amino acid R2304 does not contribute to binding to phospholipid. The clinical phenotype associated with this mutation is most likely due to decreased circulating levels of a functional cofactor rather than a dysfunctional protein.

**Acknowledgements**

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