The preparation and phospholipid binding property of the C2 domain of human factor VIII

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Introduction

Factor VIII is the precursor of a cofactor essential to coagulation. It circulates as a complex with polymeric von Willebrand factor (vWF) (1, 2). Factor VIII is a single-chain glycoprotein consisting of 2332 amino acid residues with six domains, A1, A2, B, A3, C1 and C2 (3, 4). Another coagulation cofactor, factor V, has a sequence and domain organization analogous to those of factor VIII (5). Thrombin activates factor VIII and its light chain, but were about 7000nM for C-C2. These results indicated C-C2 has 100-fold less binding affinity than factor VIII or the light chain. Direct binding to solidified phosphatidylserine-containing phospholipids also showed that C-C2 has ~50-fold less binding affinity than does the light chain. C-C2 poorly inhibited Xase activity. These results together clearly show that the C2 domain alone does not have full membrane binding activity, and suggest that the other light chain domains, A3 and/or C1, are also involved in the phospholipid binding activity of factor VIII.

Keywords
Factor VIII, C2 domain, phospholipid binding, annexin V

Thromb Haemost 2003; 89: 788–94

Summary

The C2 domain of human factor VIII was expressed in a yeast secretion system and its binding properties were studied. A cDNA coding the C2 domain sequence of human factor VIII with a N-terminal six amino acids extension (C-C2) was constructed, transformed into Pichia pastoris cells and expressed. The product was purified by ammonium sulfate fractionation and anion exchange chromatography. It emerged as a single peak from both ion exchange and gel filtration columns, indicating C-C2 is a homogenous monomer. The binding activity of C-C2 to phosphatidylserine-containing phospholipid vesicles was measured by competitive binding with annexin V. The values of IC50 were approximately 70nM for both factor VIII and its light chain, but were about 7000nM for C-C2. These results indicated C-C2 has 100-fold less binding affinity than factor VIII or the light chain. Direct binding to solidified phosphatidylserine-containing phospholipids also showed that C-C2 has ~50-fold less binding affinity than does the light chain. C-C2 poorly inhibited Xase activity. These results together clearly show that the C2 domain alone does not have full membrane binding activity, and suggest that the other light chain domains, A3 and/or C1, are also involved in the phospholipid binding activity of factor VIII.
surfaces. This complex has a high catalytic efficiency in converting factor X to Xa (9). Kemball-Cook first reported the light chain is exclusively involved in the PL binding activity of factor VIII (10). Subsequently, Arai et al. showed that the PL binding site is located in the C2 domain (11); human anti-factor VIII antibodies that bind to epitopes within the C2 domain can inhibit factor VIII binding to PS-containing PL membrane. Factor V has a similar binding affinity (2.7 nM) to PS-containing PL vesicles (12), and two regions in its light chain were shown to be involved in the PL binding (13, 14).

Previously, Pratt et al. determined a crystal structure of the C2 domain using a similar C2 domain construct prepared by the present method, and proposed its potential binding mode to PL membrane (15). The C2 domain of factor V was also crystallized and a similar binding mode has been proposed (16). In this report, we present the methods of the expression, purification and measuring the PL binding property of the factor VIII C2 domain.

Materials and methods

Materials

T7 DNA sequence version 2.0 DNA polymerase and sequencing reagents with 7-deaza-dGTP were obtained from U.S. Biochemical (Cleveland, OH). 5′-[a-35S]dATP (1,000 Ci/mmole) was purchased from Amersham (Arlington Heights, IL). The pichia pastoris expression system and pPIC9K vector were from Invitrogen (San Diego, CA). Human factor VIII cDNA was provided from Dr. Ezban, Novo Nordisk, Denmark. r-Factor VIII (4023U/mg) was obtained from Dr. Lundblad, Baxter Health Care Corp., Hyland Division. Human factor X was provided by Dr. Kisiel, University of New Mexico. Human factor IX was a gift from Dr. Uemura, Alpha Therapeutics, Los Angeles and it was activated by factor XIa prepared in our laboratory. Fluorescein isothiocyanate (FITC)-annexin V was prepared as described by Tait et al. (17). Yeast extract, peptone and yeast nitrogen base without amino acids were from Difco Laboratories (Detroit, MI). Diheptanoyl-phosphatidylcholine (PC), 1-palmitoyl-2-oleoyl-PC and 1-palmitoyl-2-oleoyl-PS sodium salt were obtained from Avanti Polar Lipids (Alabaster, AL). Phosphorus standard solution, biotin, goat anti-mouse IgG peroxidase conjugate and TMB liquid substrate system for ELISA were purchased from Sigma (St. Louis, MO). Geneticin (G418 sulfate) was from Gibco BRL (Gaithersburg, MD). Anti-human factor VIII monoclonal antibody, ESH8, was purchased from American Diagnostica (Greenwich, CT). ELISA plate (CovaLink) was a product of Nunc (Roskilde, Denmark). Chromogenic substrate, S-2222, was purchased from Chromogenix (Franklin, OH). BCA protein assay reagents and Tris(2-carboxyethyl)phosphine-HCl were from Pierce (Rockford, IL). Thiol and sulfide quantitation kit was from Molecular Probes (Eugene, OR).

Construction of expression plasmid for C-C2

At the beginning we designed two forms of C2 domains, V-C2 and C-C2. We found that C-C2 is expressed at a higher level than V-C2 and used solely in the present study. A cDNA of C-C2 was prepared through the construction of a V-C2 cDNA as follows. A PCR amplifier that contains the coding region of V-C2 (Cys2169-Tyr2332 with Cys2169 replaced by Val) was prepared using primers, F8NC2-5′ (5′-ATCTCTCGAGAAAAAG AGTGGATTAAATGTGACGAT-3′) and F8NC23 (5′-AGACACGGGCCCAGTAGAGGTCCTTGCTGTCGCA-3′) with human factor VIII cDNA as a template, and it was subcloned into the pPIC9 vector at the Xhol and NotI sites. Then, a DNA fragment was isolated by digestion with BamHI and NotI. This fragment was subcloned into the pUC18 plasmid to form the pUC18-C2 vector. To construct the expression vector for C-C2, complementary oligonucleotides, C2CYS5′ (5′-TCGAGAAAAAGAATTGGGCTGTATTTGAAATTCCTG CAGCATG-3′) and C2CYS3′ (5′-CTGCAAGAATCTCAAAATCACAGCCCATCTTTTC-3′) that have Xhol and Spnl sites at 5′ and 3′ ends, respectively, were synthesized and phosphorylated using T4 DNA kinase. They were annealed and subcloned into pUC18-C2. This was then digested with BamHI and NotI, and the C-C2 containing fragment was cloned into the expression vector pPIC9K. The sequence of the coding regions was confirmed by dideoxy-terminator sequencing (18). The expression vector was linearized by SacI digestion and transformed into the methylotrophic yeast Pichia pastoris strain GS115 by electroporation at 2,500 V/cm, 25 μF, 400 ohm (w). His5 transformants with geneticin resistance were screened on minimal dextrose medium plates containing 2mg/ml geneticin as described (19).

Culture conditions of yeast cells

The culture conditions were basically the same as described in the manufacturer’s instruction manual for Pichia pastoris Expression kit, version E (Invitrogen). An overnight 25 ml-culture in BMGY (0.1 M potassium phosphate buffer, pH 6.0, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 × 10−5% biotin, 1% glycerol) was inoculated into 500 ml of BMGY in a 2-liter baffled flask and cultured as described in (19).
**Purification of C-C2**

Ammonium sulfate was added to the culture supernatants at 45% saturation and the pellets were collected by centrifugation (10,000 × g, 20 min, 4°C). The precipitates were dissolved in 40 ml of 50 mM HEPES, pH 7.6/25 mM NaCl/5 mM EDTA, centrifuged (10,000 × g, 5 min) and dialyzed overnight at 4°C against 4 liters of 50 mM HEPES, pH 7.6/25 mM NaCl. The sample was then filtered through a 0.45 µm membrane and was applied to Waters Protein-Pak CM-column (2 × 10 cm) connected to Waters Advanced Protein Purification System. After the column was extensively washed with the HEPES buffer, adsorbed proteins were eluted by a linear NaCl gradient from 25 mM to 925 mM in the HEPES buffer.

**Preparation of light chain of factor VIII**

r-Factor VIII, 0.5 ml (0.67 mg/ml), was incubated overnight at 4°C with 50 µl of 0.5 M EDTA (pH 8.0). The sample was applied to Superose-6 column (1 × 30 cm) (Pharmacia). The column was run with 20 mM HEPES, pH 7.6/50 mM NaCl/0.5 mM EDTA/0.02% Tween-20/10 mM DTT. The fractions of the last peak, which gave a single 80 kDa band on SDS-PAGE, were pooled.

**Determination of phospholipid binding affinity by competition with annexin V**

Solution binding measurements were performed by a fluorescence quenching assay similar to that previously described (17). Competition assays were performed at 25°C in 0.05 M HEPES, pH 7.4, 0.1 M NaCl, 0.02% NaN₃, 1.2 mM CaCl₂, 100 µg/ml ovalbumin, in a quartz fluorescence cuvette. Variable amounts of competitor protein were added, mixing was by inversion and the background fluorescence was measured. After addition of 1 nM FITC-annexin V and mixing, total fluorescence was measured. Finally 200 nM PS/PC (20%/80%) vesicles were added, the reaction was incubated for 8 min in the dark, and fluorescence was measured again. All measurements were carried out on the SLM 8000C fluorometer (SLM/Aminco, Urbana, IL). Excitation was at 495 ± 4 nm with emission at 520 ± 10 nm. The % quenched fluorescence was determined by comparison of quenched fluorescence in the absence and presence of competitor.

**Direct binding by ELISA**

The binding activity to solid phase PL was performed by the ELISA method described by Bloom (20) with modifications. The methanol solutions (50 µl) of 50 µM PS/PC (20%/80%) or PC (100%) were placed in plate wells and air-dried overnight. The wells were blocked by incubation at 37°C for 1 hr with 300 µl of 50 mM HEPES, pH 7.40.15 M NaCl/0.5% gelatin/0.02% NaN₃ (blocking buffer). C-C2 (3.5-112.8 nM) or the light chain (0.078-1.85 nM) in 50 µl of blocking buffer were placed in the wells and incubated at 37°C for 1 hr. The wells were washed three times with 200 µl of 0.2%Tween-20/0.15M NaCl (wash buffer). The monoclonal antibody, ESH-8, and antimouse IgG peroxidase conjugate were used to measure the amounts of proteins bound. Each 50 µl sample (2,000-fold dilution in blocking buffer) was incubated at 37°C for 1 hr and washed three times with 200 µl of wash buffer. Color was developed with 125 µl of TMB substrate, and absorbance at 650 nm was recorded using a plate reader.

**Inhibition of Xase activity**

The inhibitory activity was measured as follows; 1 µl of 146 nM r-factor VIII, 38 µl of varying concentrations of C-C2 or the light chain and 70 µl of 71 mM HEPES, pH 7.5/0.21 M NaCl/14 mM CaCl₂/1.4 mM NaCl/0.71% BSA were mixed, and 10 µl of 56 µM PS/PC (20%/80%) were added. After pre-incubation at room temperature for 2 min, 1 µl of factor IXa (220 nM) was added and incubated for 5 min followed by incubation for 5 min with 10 µl of factor X (470 nM). Finally, 20 µl of 5 mM S-2222 were mixed and incubated for 5 min. The reaction was stopped by the addition of 50 µl of 0.1 M citric acid. Aliquots (100 µl) of the reactions were transferred to fresh plate wells and absorbance at 405 nm was read using the plate reader. The values of negative controls, in which the reactions were carried out in the absence of PL, were subtracted.

**Preparation and quantitation of PL vesicles**

Small unilamellar PL vesicles were prepared according to Gabriel and Roberts (21) as detailed by Tait et al. (17).

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**Figure 1:** Sequence of C-C2.
Phosphate analysis was performed after ashing with perchloric acid according to Chen et al. (22).

Other analyses
SDS-PAGE was performed according to Laemmli (23). The concentrations of proteins were determined by BCA protein assay using bovine serum albumin as a standard. For dot blot analysis 0.1% of ESH8 and anti-mouse IgG alkaline phosphatase conjugate in 20 mM Tris, pH 8.0/0.5 M NaCl/1% gelatin were used and color was development with BCIP/NBT substrate. The presence of free thiol groups was examined using thiol/sulfide quantitation kit. Partial reduction of C-C2 conjugates was carried out at room temperature for 3 hrs with a 10-fold molar excess of Tris(2-carboxyethyl)phosphine in 50 mM HEPES, pH 7.2/25 mM NaCl and the product was purified by Protein-Pak CM-column. Sequence analysis was performed using Applied Biosystems 477A Protein Sequencer. Mass spectrometry analysis was conducted in the Mass Spectrometry Facility in the Department of Biochemistry, University of Washington.

Results

Expression of C-C2
The C2 domain is located at the C-terminus of the light chain, which consists of residues from Ser2173 through Tyr2332. It is involved in the binding of factor VIII to PL. In order to study the structure and PL binding properties of the C2 domain, we have expressed C-C2 (Met2167 - Tyr2332) using the Pichia expression system. C-C2 has an extension of six amino acids at the N-terminus of the C2 domain, which contains one disulfide bond as illustrated in figure 1. The N-terminal extension contains Cys2169, which is a part of the C1 domain sequence and forms a disulfide bond with Cys2021 in the C1 domain, but constitutes an unpaired cysteine residue in the C-C2 construct. The expression plasmid was constructed as described in Methods and transformed into Pichia pastoris cells by electroporation. The cells were grown in tripled concentrations of yeast extract and peptone (BMMY - 3 x YP), because higher concentrations of yeast extract and peptone help increase the production level of proteins and prevent the products from proteolytic degradation (24). Later we found that the triple concentration of yeast extract/peptone is not necessary to produce C-C2. The higher concentrations may help to produce mutant C2 domains, which are susceptible to proteases. The cells with the highest expres-

Figure 2: CM-column chromatography of C-C2. The dialyzed ammonium sulfate fraction was applied to the Waters Protein-Pak CM-column (2 x 10 cm) equilibrated with the HEPES buffer, pH 7.6. After the column was extensively washed with the buffer, adsorbed protein was eluted by a salt gradient (25 mM-925 mM NaCl) in the HEPES buffer at a flow rate of 4 ml/min and collected in 4 ml-fractions. Inset, SDS-PAGE of C-C2 on a 7.5% gel. Bottom two markers are soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Figure 3: Gel filtration of C-C2. The fraction from the CM-column was applied to a Superdex 75 column (1 x 30 cm) and eluted with 50 mM Bis-Tris, pH 7.5/0.15 M NaCl at 0.5 ml/min. Arrows 1 and 2 show the elution positions of soybean trypsin inhibitor (20.1 kDa) and lysozyme (13.1 kDa), respectively.
sion level were selected by dot immuno tests using the monoclonal antibody, ESH8, which is specific to the C2 domain. Cysteine (5 mM) was added to the culture medium to block the sulfhydryl group of Cys2169. Without cysteine supplementation, C-C2 products were conjugated with several small compounds (as shown by mass analysis). Partial reduction of these conjugates produced a molecule with a single mass, 18,820, which matches non-conjugated C-C2.

**Purification and physical-chemical characterization of C-C2**

The culture medium was first fractionated by ammonium sulfate precipitation between (0-45%). The precipitated fraction was then applied to the CM-column and adsorbed proteins were eluted by a salt gradient. C-C2 was tightly bound to the column and eluted in a single peak at a high salt concentration with a shoulder (Fig. 2). Although the fractions in the shoulder contained C-C2, only the fractions in the major peak were pooled. The purified protein migrated as a single 19 kDa band on SDS-PAGE under reduced conditions (inset in Fig. 2). To rule out the possibility of aggregation, the molecular weight of C-C2 was examined in non-denaturing conditions. A portion of the CM-column fraction was applied to a gel filtration Superdex 75 column. It eluted as a single peak in a position between soybean trypsin inhibitor (20.1 kDa) and lysozyme (13.1 kDa) (Fig. 3), indicating C-C2 is a monomer. Sequence analysis gave the correct sequence, Met-Gly-X-Asp-Leu-Asn-Ser, indicating that the α-factor secretion signals were removed by a signal peptidase at the expected site. With cysteine supplementation to the medium, C-C2 did not contain detectable sulfhydryl groups nor form dimers, as shown by the gel filtration results above. Mass spectrometry analysis gave the mass, 18,940.8, which matches the expected mass, 18,939.8, of C-C2 conjugated with 1 cysteine. Under these conditions, 20-40 mg of protein was usually obtained from six liters of the culture.

**Competitive binding of C-C2, light chain and factor VIII with annexin V**

Annexin V is known to have a strong binding affinity to PS-containing PL (17). The affinity of C-C2 for phospholipid vesicles in solution was measured by a competition assay that uses FITC-annexin V (Fig. 4). Consistent with earlier studies (8, 25), the light chain had the same affinity for PS/PC (20%/80%) vesicles as does intact factor VIII. In contrast, the affinity of the C-C2 was approximately 100-fold weaker.

**Direct binding of C-C2 and light chain to solid PL**

The PL binding activity of C-C2 was next studied by direct binding to immobilized PL using ELISA system. In these experiments, fixed amounts of PL, 50 µl of 50 µM PS/PC (20%/80%) or 50 µM PC (100%), were coated onto plate wells and 50 µl of varying concentrations of C-C2 or the light chain were incubated. The amounts of the proteins bound were measured by immuno-detection as described in Methods. These experiments were carried out in one plate and the results were compared. The binding of C-C2 to the light chain to PL is concentration-dependent and ~50 times more C-C2 is required than the light chain for the same amounts of binding to PS-containing PL (Fig. 5). The majority of the binding is specific to PS-containing PL, though weak binding to PC is observed for both proteins. These results are consistent with the above results obtained for competitive binding with annexin V.

**Inhibition of Xase by C-C2 and light chain**

Because the binding of the C2 domain to PL membranes is a major function of the light chain that enhances the Xase activity by factor VIIIa, the inhibitory activity of C-C2 to Xase was studied. To construct Xase, we studied the concentration-dependency of factor VIII and PL on Xase activity. The factor Xa generation increased with factor VIII concentrations up to 1.2 nM and it was proportional to PL concentrations between 0.1 mM to 20 mM (data not shown), when a fixed concentration of IXa (1.8 nM) was used. We selected 1.2 nM factor VIII and 4.7 mM PL to construct the Xase complex. At these concentrations, both factor VIII and PL are limiting to the Xase activity, and the inhibitory activity of C-C2 and the light chain can be determined. The dose-dependent inhibition by these two proteins is shown in figure 6. Both proteins inhibited Xase activity. To achieve 50% inhibition 0.25 µM of the light chain is required, whereas 100 µM of C-C2 is needed. The inhibitory activity of C-C2 is ~400-fold less than that of the light chain by this method. The difference between two proteins in Xase inhi-
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Bition is larger than the difference in the PL binding activities indicated by the other two methods. These differences could be caused by the presence of the factor IXa binding site in the A3 domain of the light chain (26). The dual inhibition of the light chain to PL and IXa bindings could cause the higher inhibition observed for the light chain relative to C-C2, which can inhibit PL binding, but not affect interactions of factor IXa binding.

Discussion

In the present study, we showed that the C2 domain has a weak but measurable binding activity to PS-containing PL by three different methods. The binding activity obtained by the competition with annexin V showed two orders less binding affinity than the light chain or entire factor VIII molecule. The binding affinity of factor VIII and the light chain for PS-containing PL is reported to be 2-3 nM (8, 25). Therefore, the binding affinity of C-C2 is likely to be in a range of 200-300 nM. A similar binding affinity was observed with an analogous construct of the C2 domain of factor V that was expressed in Baculovirus-insect cells. This protein bound to soluble PS with a K_d of ~2 µM and its affinity was 10-fold higher with PS-containing membranes (27). It also had a weak inhibitory activity to prothrombinase (28), similar to the weak inhibitory activity of C-C2 to Xase. A controversial result has been reported that the C2 domain of factor VIII expressed in Baculovirus-insect cells has a full binding affinity with immobilized PS/PC monolayers, as determined by surface plasmon resonance (25). This discrepancy could be caused by the different methods used for determining the binding affinities.

The two orders less binding activity of the C2 domain relative to the light chain indicates the involvement of another region in A3 and/or C1 domains in generating full binding activity. Therefore, either a second binding site(s) is present in these domains or an extended sequence is required to maintain the conformation of the C2 domain in a membrane binding mode. Two distinct binding sites to PL vesicles has been found in the light chain of factor V. The first binding site was found to locate in the C-terminal region of the A3 domain by protection experiments, which showed that PL protected the A3 domain from proteolytic digestion (13). A second and major PL binding site was found in the C2 domain by deletion experiments (14). In our preliminary experiments, PL also protected a large 38 kDa region within the light chain extending from Asn_1977 and Met_1973 at the C-terminal region of the A3 domain to the end of the C2 domain from chymotryptic and tryptic digestions (29). This result suggests that a second PL binding site is also present in the light chain of factor VIII.

By the yeast expression system described here, C-C2 is expressed in high levels and the protein is readily isolated in a...
two-step purification. The product is a homogenous monomer and it was easily crystallized for the structural study (15). A similar C2 domain construct was also co-crystallized with a human-derived monoclonal antibody Fab fragment, identifying an epitope on the surface of the C2 domain (30). Mutants of the C2 domain as well as wild type protein can be expressed by the present method and these protocols may be useful to carry out further structure and function studies of the C2 domain of factor VIII.

Acknowledgements

We would like to express our gratitude to Dr. Earl Davie for his support and encouragements and Dr. Kathleen Pratt for careful reading and correction of the manuscript. We thank Dr. Roger Lundblad, Mirella Ezban, Walter Kisiel and Yahito Uemura for providing precious proteins. We are also indebted to Brad McMullen for sequence analysis.

References

2. Tuddenham EGD. Factor VIII in Molecular Basis of Thrombosis and Hemostasis, 1995 High KA, Robert HR eds. Marcel Dekker 147.
11. Arai M, Scandella D, Hoyer LW. Molecular basis of factor VIII inhibition by human antibod-