Characterization of tryptophan residues of human coagulation factor V required for binding to phospholipid membranes

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Abstract

Interactions between factor Va (HFVa) and membrane phosphatidylserine (PS) regulate the activity of the prothrombinase complex. I have previously shown that two solvent exposed hydrophobic residues located in the C2-domain, Trp2063 and Trp2064, are required for binding to immobilized PS and for expression of procoagulant activity on membranes containing 5% PS. In order to fully define the functional importance of these two residues I have expressed and isolated recombinant factor Va (rHFVa) W2063A/W2064A double mutant. In contrast to the native protein the two glycoforms resulting from alternative glycosylation of Asn2181 eluted as a single peak with rHFVa1 W2063A/W2064A eluting on the leading edge and rHFVa2 W2063A/W2064A eluting on the trailing edge. The double mutant rHFVa2 W2063A/W2064A expressed little or no procoagulant activity on membranes containing 1-10% mol % PS. In contrast, the procoagulant activity of this mutant was slightly greater than the native protein on membranes containing >18 mol % PS. The binding of rHFVa2 W2063A/W2064A to immobilized phospholipid vesicles was markedly reduced compared to the native protein in a surface plasmon resonance binding assay. I conclude that Trp2063 and Trp2064 are required for high affinity binding of factor Va to PS membranes and that this interaction is necessary for assembly of the prothrombinase complex on membranes containing physiological concentrations of PS.

Key words – human coagulation factor V, membrane binding

Introduction

Thrombin-activated coagulation factor V is an essential component in the prothrombinase complex, which activates the zymogen prothrombin to thrombin. This complex consists of the enzyme factor Xa, the protein cofactor factor Va, calcium ions and a phospholipid membrane surface [6,14]. Formation of the prothrombinase complex requires the presence of a membrane surface to which the individual components will bind and subsequently assemble into a functional complex. Activated platelets, platelet microparticles, and damaged vascular cells provide this surface in vivo [29]. Factor Va binds to phospholipid vesicles containing 25% phosphatidylserine and 75% phosphatidylycholine with a Kd of approximately $10^{-9}$ M [2,8,11,23,25,28,29]. Binding to phospholipid membranes does not require cofactor activation [2,11,23] or the presence of calcium ions [2,11,27,28]. The high affinity binding of factor Va with phospholipid membranes appears to involve both hydrophobic [10,12] and electrostatic interactions [23,24,28].

The binding of factor Va to a phospholipid membrane is mediated primarily by the light chain of the cofactor [2,27,28]. A membrane binding site is located within the A3 domain of the factor Va light chain [4,5,11]. A pro-

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teolytic fragment containing the first ~220 amino acids of the A3 domain of the light chain of bovine factor Va was found to bind to phospholipid vesicles containing phosphatidylserine [11]. This region corresponds to amino acids 1546 through 1765 in human factor V. The role of the A3 domain in membrane binding is further supported by chemical crosslinking studies using the crosslinking reagent 1-azidopyrene [4]. The observation that the factor Va heavy chain binds weakly (Kd 0.3 μM) to neutral phospholipid membranes suggests that the A1 and/or A2 domains may also interact with the membrane [9].

The C2 domain of factor V (residues 2037-2196) is also required for the binding of factor V to phosphatidylserine [17,19,20]. Deletion of the C2 domain prevents the binding of factor V to phosphatidylserine [17]. Monoclonal antibodies and factor V inhibitors that bind to the amino terminus of the C2 domain block factor V binding to phosphatidylserine and inhibit procoagulant activity [19,20]. The structures of the factor V C2-domain [13] and recent alanine scanning mutagenesis study [7] provide further evidence that the C2-domain interacts with the membrane. The three-dimensional structure of the C2-domain was solved in monomeric and dimeric crystal forms [13]. The factor V C2-domain exhibits a distorted jelly-roll β-barrel motif, consisting of eight major antiparallel strands arranged in two β-sheets of five and three strands packed against one another. Three adjacent loops (Ser2058-Trp2068, Asn2076-Asn2082 and Gly2112-Tyr2121) protrude like spikes from the bottom of the β-barrel. At the apex of spike-1, the indole moieties of two consecutive tryptophan residues (Trp2063 and Trp2064) are fully exposed to solvent. Spike-1 was identified as the primary membrane-binding site of the C2 domain [21]. It has been demonstrated that monoclonal antibody HV-1 and the inhibitory antibody H1, which map to spike-1 interfere with PS-specific binding and abolish procoagulant activity [7,18]. Glycosylation of Asn2181 in the neighboring S7-S8 loop also interferes with membrane binding of FVa [7,9,15,25]. Substitution of alanine for the two tryptophan residues in spike-1 blocks binding of factor V to immobilized PS. At low concentrations of phospholipid vesicles, the W2063A/W2064A double mutant lacks procoagulant activity [7,16]. I now report the detailed characterization of purified rHFVa W2063A/W2064A double mutant and demonstrate that the tryptophan residues located in spike-1 of the factor V C2 domain are essential for high affinity membrane binding and for expression of cofactor activity on membranes containing ≤10% PS.

Materials and Methods

Materials

Human prothrombin, thrombin and factor Xa were obtained from Haematologic Technologies Inc. (Essex Junction, VT). 1-palmitoyl-2-oleoyl phosphatidylserine (POPS) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), brain L-α-phosphatidylcholine and brain L-α-phosphatidylserine were from Avanti Polar Lipids (Alabaster, AL). All other reagents were from Sigma (St. Louis, MO).

Phospholipid vesicle preparation

Phospholipid vesicles were prepared as described [8]. Appropriate amounts of POPC and POPS in chloroform/methanol solution were pipetted with glass syringes (Hamilton, Reno NV) into Corex tubes (Corning, Corning NY). The chloroform was evaporated under a stream of nitrogen gas and the dried phospholipids were resuspended in appropriate buffer. The lipid suspension was then sonicated under a mild stream of nitrogen for 10 minutes in an ice-bath. After sonication, the suspension was centrifuged at 72,000 rpm for 25 min at 15°C in a Beckman TL100 centrifuge to obtain a homogeneous suspension of vesicles.

Prothrombinase assay

Factor Va procoagulant activity was determined by
measuring the rate of thrombin generation catalyzed by prothrombinase in the reaction mixture containing 5 nM of factor Xa, 1.4 μM prothrombin, phospholipid vesicles and factor Va in 20 mM Tris, pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 10 mg/ml BSA and 5 mM CaCl₂. The reaction mixtures were incubated at 37°C for one minute and then quenched by adding 10 μl of 50 mM EDTA, 200 mM MOPS, pH 7.4. Thrombin activity was determined by incubating test samples with 50 μl of 0.5 mM S2387 (Chromogenix, Molndal, Sweden) and measuring the absorbance at 405 nm using a Vmax kinetic microtiter reader (Molecular Devices, Menlo Park, CA). Thrombin concentrations were determined using a standard curve prepared with purified human thrombin. Concentrations of factor Va were determined using the Bio-Rad assay (Bio-Rad).

**Immobilized PS binding assay**

The binding of factor Va to immobilized PS was investigated using a solid-phase ELISA, as previously described [7,17,19,20]. Detection was performed using the biotinylated monoclonal antibody 6A5.

**Surface plasmon resonance studies**

Surface plasmon resonance studies were carried out using a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). The instrument allows real-time molecular interaction analysis from which association and dissociation rate constants and equilibrium constants can be derived. Phospholipid vesicles were captured on the surface of a Pioneer Sensor Chip L1 by incubation of unilamellar POPC:POPS (75:25 mol/mol) or 100% POPC vesicles at 10 μg/ml in 20 mM Tris, pH 7.4 and 150 mM NaCl for 15 min at 25°C, which produced a signal of 5000 resonance units (RU). Binding of factor Va to coated vesicles was measured using the instrument, where 1 ng of protein bound per mm² of the sensor chip produces a resonance signal of 1000 RU. To regenerate the sensor chip, complete dissociation of bound ligands was achieved by the addition of 10 mM NaOH for 45 s. Binding and subsequent dissociation was measured in 20 mM Tris, pH 7.4, 150 mM NaCl and 5 mM CaCl₂ at 25°C.

**Results**

Purification and Characterization

In order to more precisely define the role of the two tryptophan residues in the binding of factor Va to phospholipid membranes 1 purified rHFVa W2063A/W2064A double mutant. The mutant factor Va was expressed in COS-7 cells and purified by FPLC chromatography as previously described [8]. Activation of purified human factor V by thrombin results in release of a 105 kDa heavy chain and two forms of the light chain. Rosing et al. [25] separated two forms of human factor Va, designated factor Va1 and factor Va2, that exclusively contained either the 74 or 71 kDa light chain, respectively. Chromatography of native rHFVa on Mono S results in elution of two activity peaks corresponding to factor Va1 (fraction 19) and factor Va2 (fraction 24) (Fig. 1A). In contrast, the mutant eluted as a single peak (fraction 13) following chromatography on Mono S (Fig. 1B). Fractions from the leading edge of the activity peak contained mostly rHFVa1 W2063A/W2064A (fraction 11), whereas the fractions from the trailing edge contained mostly rHFVa2 W2063A/W2064A (fractions 13-15) as shown by SDS-PAGE analysis (Fig. 2). There is also evidence for heterogeneity in the heavy chain of rHFVa W2063A/W2064A double mutant which is most likely due to differences in glycosylation.

Comparison of cofactor activities

The striking difference between the prothrombinase activity of native and mutant factor Va2 in the presence of membranes containing limiting concentrations of PS was confirmed using purified recombinant proteins (Fig. 3). Half-maximal rates of thrombin activation in the presence of 5 nM factor Xa and 0.5 μM POPC:POPS...
Fig. 1. FPLC chromatography of rHFVa (panel A) and rHFVa W2063A/W2064A double mutant (panel B) on Mono S. Factor Va was purified by FPLC chromatography using a Mono S column. Factor Va was eluted using 15 ml NH₄Cl gradient (0.05 M to 1.0 M NH₄Cl). Fractions (0.5 ml) were collected and absorbance at 280 nm (open circles) and factor V clotting activity (closed circles) were determined.

Fig. 2. SDS-PAGE analysis of rHFVa and rHFVa W2063A/W2064A double mutant. Factors Va1 and Va2 were purified using a FPLC Mono S column and analyzed by SDS-PAGE using a 7.5% acrylamide gel followed by silver staining. The numbers shown at the top of the gel correspond to the fractions shown in Fig. 1 (see text). The mobility of heavy chain (HC) and light chain (LC) are shown on the left side of the figure.

Fig. 3. Cofactor activities of rHFVa2 and rHFVa2 W2063A/W2064A double mutant in a prothrombinase assay. Rates of thrombin generation were determined in a reaction mixture containing 20 mM Tris, pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 10 mg/ml BSA, 3 mM CaCl₂, 5 mM factor Xa, 1.4 µM prothrombin, factor Va2 and either 0.5 µM phospholipid vesicles (POPC:POPS, 95:5, mol/mol, open symbols) or 5.0 µM phospholipid vesicles (POPC:POPS, 75:25, mol/mol, closed symbols). Native rHFVa (circles), rHFVa W2063A/W2064A double mutant (squares).

(95:5 mol/mol) were observed at 3.7 nM rHFVa2 and >84 nM rHFVa2 W2063A/W2064A (Fig. 3). However, the cofactor activity of rHFVa2 was similar to rHFVa2 W2063A/W2064A, when the experiment was performed using phospholipid vesicles containing a higher mole percentage of phosphatidyl serine (5 µM POPC:POPS, 75:25 mol/mol). Half-maximal rates of prothrombin activation in the presence of 5 nM factor Xa and 5 µM POPC:POPS (75:25 mol/mol) were observed at 1.3 nM rHFVa2 and 1.9 nM rHFVa2 W2063A/W2064A (Fig. 3).

Effect of phospholipid (95:5 POPC:POPS) concentration on the cofactor activity of wild type and mutant factor Va in prothrombin activation

The previous experiments have shown that mutant factor Va2 has much lower cofactor activity than wild type factor Va2 at low concentrations of phospholipid vesicles (0.5 µM) with a low phosphatidyserine content (5% PS) and mutant factor Va2 has similar cofactor activity to wild type at high concentrations of phos-
pholipid vesicles (5 μM) with a high phosphatidylserine content (25% PS). The difference of PS content between limiting and saturating phospholipid vesicles is 50-fold. In this experiment, I increased the concentration of 95:5 POPC:POPS vesicles up to 60-fold (0.5 μM to 30 μM). The data shown in Fig. 4 demonstrates that the cofactor activity of mutant factor Va2 is markedly decreased compared to the wild type protein even at highest concentrations of POPC:POPS (95:5 mol/mol) vesicles indicating that the PS content of the membrane is more important than total phospholipid concentration.

Effect of phosphatidylserine on the cofactor activity of wild type and mutant factor Va in prothrombin activation

The cofactor activities of wild type and mutant factor Va2 were determined on 5 μM phospholipid vesicles containing varying amounts of PS. Increasing the amount of PS in the membrane surface resulted in an increase of activities of both wild type and mutant factor Va2. On membranes with low PS content, wild type factor Va2 was a more effective cofactor than mutant factor Va2, however, the cofactor activities of both forms of factor Va2 were similar on 0.5 μM phospholipid vesicles containing 18 mol % PS (Fig. 5). Thus it appears that the intrinsic abilities of rHFVa2 and rHFVa2 W2063A/ W2064A double mutant to promote factor Xa catalyzed cleavage of prothrombin are similar except for the difference in sensitivity to stimulation by PS membranes.

Immobilized phosphatidylserine binding assay

I have used a solid-phase phospholipid binding ELISA to assess the binding of wild type and mutant factor Va2 to phosphatidylserine. Although apparent binding constants determined by solid phase assays do not yield real equilibrium binding constant values, these types of assays can provide useful qualitative descriptions of binding and may allow comparisons of relative binding affinities for similar proteins. Wild type factor Va2 bound tightly to immobilized PS (Fig. 6). Apparent Kd value calculated

![Fig. 4. Effect of phospholipid concentration on the cofactor activities of wild type and mutant factor Va2 in prothrombin activation. Rates of thrombin generation were determined in a reaction mixture containing 20 mM Tris, pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 10 mg/ml BSA, 3 mM CaCl₂, 5 nM factor Xa, 1.4 μM prothrombin, 1 nM factor Va2 and varying concentrations of phospholipid vesicles (POPC:POPS, 95:5, mol/mol). Native rHFVa2 (circles), rHFVa2 W2063A/W2064A double mutant (squares).](image1)

![Fig. 5. Effect of membrane PS content on the cofactor activity of wild type and mutant factor Va2 in prothrombin activation. Rates of thrombin generation were determined in a reaction mixture containing 20 mM Tris, pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 10 mg/ml BSA, 3 mM CaCl₂, 5 nM factor Xa, 1.4 μM prothrombin, 1 nM factor Va2 and 5 μM phospholipid vesicles with varying mole percentages of PS. Native rHFVa2 (circles), rHFVa2 W2063A/ W2064A double mutant (squares).](image2)
by Scatchard analysis was 3.7 nM for wild type factor Va2. In contrast, mutant factor Va2 showed only weak binding (apparent Kd > 1 μM) indicating that Trp2063 and Trp2064 are essential for high affinity binding to immobilized PS.

Surface plasmon resonance studies
I established an assay for measuring the binding of factor Va to phospholipid membranes using surface plasmon resonance. In these experiments a solution containing phospholipid vesicles was injected over the hydrophobic surface of an L1 Sensor Chip in order to create a membrane coated surface. Factor Va binding to the membrane was monitored by surface plasmon resonance using a BIACore 3000 instrument. No binding of factor Va2 was observed when the L1 Sensor Chip was coated with 100% PC phospholipid membranes (data not shown). In contrast high affinity binding of factor Va2 was observed when the L1 Sensor Chip was coated with POPC:POPS (75:25) phospholipid membranes (Fig. 7). In preliminary experiments I found that the shape of the binding isotherms was affected when the flow rate was varied (5-75 μl/min) indicating a mass transport effect. Therefore I included a correction for mass transport in analysis of the binding data. When the sensograms from the experiment shown in Fig. 7 were subjected to global analysis using the BIAnalyst software 3.0 I found a Kd of 1 nM which was similar to the affinity observed for factor Va binding to POPC:POPS (75:25) vesicles [21]. Furthermore the calculated k_on of 4.58 ± 0.08 × 10^6 M^−1s^−1 and k_off of 5.03 ± 0.08 × 10^3 s^−1 are strikingly similar to the values of 6.1 × 10^6 M^−1s^−1 and of 3.3 × 10^3 s^−1 observed by Gilbert for factor V binding to liposomes [1]. Consistent with earlier studies, I found that monoclonal antibody HV-1 blocked factor Va binding to the membrane while monoclonal antibody 6A5 had no effect (data not shown) [13]. I next used the surface plasmon resonance membrane binding assay to characterize the binding of rHFVa2 W2063A/W2064A double mutant to POPC:POPS (75:25) membranes. As shown in Fig. 8 the binding of the mutant was dramatically reduced compared to the native recombinant protein. At the concentrations of
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Fig. 8. Sensorgram depicting binding of 10 nM rHFVa2 or rHFVa2 W2063A/W2064A double mutant to immobilized POPC:POPS (75:25) membranes. Association and dissociation kinetic data was obtained at 10 nM of rHFVa2 as described in Fig. 7.

mutant factor Va used in this experiment I was not able to calculate binding constants using kinetic analysis. Thus these experiments confirm that the tryptophan residues located in spike-1 of the factor V C2 domain are essential for high affinity membrane binding.

**Discussion**

The recently solved crystal structures of the C2-domains of factor V [13] and factor VIII [22] have suggested models for the interaction of these cofactors with phospholipid membranes that involve insertion of solvent exposed hydrophobic amino acid side chains into the hydrophobic membrane core and interactions of positively charged amino acid side chains with phosphatidylserine. Consistent with this model my initial characterization of a series of factor V mutants containing alanine substitutions in the C2-domain indicated that mutation of Trp2063 and/or Trp2064 resulted in decreased binding to immobilized PS by ELISA assay and reduced cofactor activity in the presence of membranes containing <10% PS. However in their experiments, both wild-type and mutant protein preparations contained mixtures of both C2-domain glycoforms. In the present study the importance of Trp2063 and Trp2064 has been further defined by characterizing the cofactor activity and membrane-binding properties of purified native and mutant proteins. My findings indicate that Trp2063 and Trp2064 are required for expression of cofactor activity on membranes containing 1-10% PS. In contrast, on membranes containing >20% PS the cofactor activity of rHFVa2 W2063A/W2064A double mutant appeared to be enhanced compared to the native protein. The molecular basis for this observation is not yet clear. The binding of purified rHFVa2 W2063A/W2064A double mutant to immobilized PS was markedly decreased compared to the native protein using an ELISA binding assay. In order to facilitate analysis of the membrane binding properties of factor V mutants I developed a surface plasmon resonance based membrane binding assay using phospholipid vesicles immobilized on an L1 sensor chip. The validity of this assay was supported by analysis of the native factor Va2 binding to immobilized POPC:POPS (75:25) membranes. The observed values for Kd, k on and k off were similar to those obtained using more conventional fluorescence binding assays [1,11]. My failure to detect binding of rHFVa2 W2063A/W2064A double mutant to immobilized membranes using the surface plasmon resonance binding assay is strong evidence that Trp2063 and Trp2064 are required for high affinity membrane binding and supports my hypothesis that insertion of Trp2063 and Trp2064 into the membrane bilayer contributes to this interaction. At the present time I can not explain the apparent discrepancy between the similar cofactor activity observed for rHFVa2 and rHFVa2 W2063A/W2064A double mutant in the presence of POPC:POPS (75:25) membranes (Fig. 3 and 5) and the marked difference in the binding of these two proteins to immobilized POPC:POPS (75:25) membranes (Fig. 8). I speculate that these differences could be due to the
presence of factor Xa in the prothrombinase assay, the
effect of flow in the BIAcore binding assay, differences in
the effective membrane concentration or subtle differences
in the physical state of the membranes. Further studies
will be required to resolve this issue. These experiments
will be complicated by the relatively large amounts of
recombinant protein needed to obtain quantitative mem-
brane binding affinities for rHFVa2 W2063A/W2064A
double mutant. The present data provides further evi-
dence for an important role for the factor V C2-domain
in membrane binding. It has been recently found that the
isolated factor V C2-domain (rHFV-C2) binds to phos-
pholipid membranes but that the affinity for this
interaction is 30-fold lower than for the binding of factor
Va to membranes [26]. These observations are consistent
with the proposal that the A3-domain [11] and/or the C1-domain [13] contribute significantly to
factor Va binding to membranes. Thus the present data
support a complex multi-domain and likely dynamic in-
teraction between factor Va and procoagulant phospholipid
membranes [13].

The functional differences between rHFVa2 and rHFVa2
W2063A/W2064A double mutant are reminiscent of the
differences between the two glycoforms of factor Va,
factor Va1 and factor Va2. Factor Va1 and factor Va2
were originally defined based on fractionation of the two
species during chromatography on Mono S [25]. In factor
Va1, the presence of an N-linked oligosaccharide chain at
Asn2181 resulted in impaired procoagulant activity in the
presence of POPC:POPS (95:5) phospholipid mem-
branes, impaired binding to immobilized PS, and earlier
elution from Mono S [8,15]. However, mutation of
Trp2063 and Trp2064 results in a more profound func-
tional defect compared to glycosylation of factor Va at
Asn2181. Interestingly, during chromatography on Mono S,
rHFVa W2063A/W2064A double mutant is eluted
earlier than native rHFVa1 and with decreased resolution
of the two glycoforms. The significance of this obser-
vation, if any, remains to be determined. The relative
importance of factor Va1 and factor Va2 in procoagulant
and anticoagulant pathways has been investigated [3].
Paradoxically, factor Va1 has greater procoagulant activity
due to impaired inactivation by activated protein C.
Similar studies with rHFV W2063A/W2064A double
mutant may provide further insights into the regulation
of the APC pathway by phospholipid membranes.

Acknowledgements

This work was supported by Korea Research Foun-
dation Grant. (KRF-2001-003-F00013)

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초록: 인지질막 결합에 필요한 제5혈액응고인자 트립토판잔기들의 역할규명

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제5인자와 지질막 phosphatidylinerine과의 상호작용은 prothrombinase 복합체의 활성을 조절하는데 중요하다. 본 연구에서 제5인자의 지질 결합부위에 위치한 Trp2063과 Trp2064를 동시에 돌연변이 시킨 제조합 제5인자를 과발현 시키고 정제하였다. 돌연변이된 제5인자는 1-10%의 phosphatidylinerine을 포함하는 지질막에서 아주낮은 활성을 보였다. surface plasmon resonance에 의해서 지질막과의 결합을 측정한 결과 돌연 변이된 제5인자가 본래의 제5인자보다 고정된 지질막에의 결합이 현저하게 멀어지는 것을 관찰하였다. 제5인자가 phosphatidylinerine을 포함하는 지질막에 높은 친화력으로 결합하기 위해서는 Trp2063과 Trp2064가 필수적이고 이러한 상호작용은 생리적인 phosphatidylinerine 농도를 포함하는 지질막 위에서 prothrombinase 복합체의 형성에 필요하다는 결론을 내렸다.