รายงานการวิจัยฉบับสมบูรณ์

การเปลี่ยนแปลงโครงสร้างของวอนวิลลิแบรนด์แฟคเตอร์ของคน : การศึกษาโดยใช้รีคอมบิแนนท์เอวันโดเมน

Conformational changes in human von Willebrand factor: studies with a recombinant A1 domain

โดย นวลน้อย จูฑะพงษ์

กรกฎาคม 2551

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ผู้วิจัย

นวลน้อย จูฑะพงษ์

สังกัด

มหาวิทยาลัยเทคโนโลยีสุรนารี

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

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Conformational changes in human von Willebrand factor: studies with a recombinant A1 domain

Nuannoi Chudapongse

Suranaree University of Technology

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Von Willebrand factor (VWF) is a plasma glycoprotein that plays an important role in hemostasis and thrombosis. It promotes platelet adhesion to damaged vascular endothelium. VWF is a multimeric protein consisting of disulfide-bonded subunits, ranging from dimers to multimers extending up to 20 x 10^6 Daltons. The VWF monomer includes 13 domains, which are multiples of four domain types (A to D). The A1 domain in VWF contains multiple binding sites, including those for platelet glycoprotein Ib, heparin, and the artificial modulators ristocetin and botrocetin. This domain is thought to be a critical structural motif as several point mutations have been found within this domain in patients with type 2 von Willebrand disease. Conformational changes in the A1 domain of VWF are a topic of intense interest. In this study, the investigator has cloned A1 domain of human VWF from genomic DNA, rather than from mRNA. This approach has some advantages, which can facilitate subsequent studies of the structural and functional consequences of specific polymorphisms or mutations in the VWF gene. Moreover, in contrast to the previous reports, the A1 domain expressed in this study did not form an inclusion body, so it was in a native condition throughout the purification process without disrupting noncovalent or disulfide bonds in the protein structure. The results showed that structure of the recombinant A1 domain have changed after incubation of ristocetin. This data provides additional important evidence that changes in the structural of the A1 domain occur during the induction of platelet aggregation by ristocetin.

Keywords: von Willebrand Factor, A1 Domain, Recombinant, Ristocetin, Conformation

การเปลี่ยนแปลงโครงสร้างของวอนวิลลิแบรนด์แฟคเตอร์ของคน : การศึกษาโดยใช้รีคอมบิแนนท์เอวันโดเมน

นวลน้อย จูฑะพงษ์
มหาวิทยาลัยเทคโนโลยีสุรนารี

2551

Von Willebrand factor (VWF) เป็น glycoprotein ในพลาสมาที่มีบทบาทสำคัญในกระบวนการทำให้เลือดหยุดและการเกิดลิ่มเลือด โดยไปส่งเสริมให้เกล็ดเลือดเกาะกับ endothelium ในบริเวณเส้นเลือดที่ได้รับความเสียหาย VWF เป็นโปรตีนขนาดใหญ่ที่ประกอบด้วยหน่วยย่อยตั้งแต่ 2 หน่วยขึ้นไป เชื่อมต่อระหว่างกันด้วยพันธะ disulfide ทำให้โมเลกุลมีขนาดแตกต่างกัน โดยโมเลกุลที่มีขนาดใหญ่ที่สุดมีน้ำหนักโมเลกุลประมาณ 20 x 10^6 ดาลตัน โมโนเมอร์แต่ละโมโนเมอร์ซึ่งประกอบเป็น VWF นั้นประกอบด้วย domain 4 แบบ (A ถึง D) A1 domain ของ VWF เป็นตำแหน่งจับของทั้ง glycoprotein Ib, heparin, ยาปฏิชีวนะ ristocetin และโปรตีนจากพิษงู botrocetin เชื่อกันว่า A1 domain เป็นโครงสร้างที่มีความสำคัญมากเนื่องจากพบว่ามี point mutation หลายจุดบนยีนของผู้ป่วยที่มีความผิดปกติของการแข็งตัวของเลือดซึ่งมีชื่อโรคว่า von Willebrand disease แบบที่ 2 การเปลี่ยนแปลงโครงสร้างของ A1 domain เป็นการศึกษาที่ได้รับความสนใจเป็นอย่างมาก ในการศึกษานี้ ผู้วิจัยประสบความสำเร็จในการโคลน VWF A1 domain จาก genomic DNA ของคนแทนการ clone จาก mRNA ซึ่งวิธีที่ค้นพบนี้มีข้อได้เปรียบคือ สามารถนำไปใช้ศึกษาโครงสร้างและหน้าที่ของ A1 domain ในผู้ที่มี polymorphism ของ VWF gene ต่างกันได้ นอกจากนั้นสิ่งที่แตกต่างไปจากงานวิจัยที่มีรายงานมาก่อนหน้านี้ คือ recombinant A1 domain ที่ผลิตได้จากแบคทีเรียในการศึกษาครั้งนี้ไม่เกิดเป็น inclusion body ดังนั้นโปรตีนที่สร้างขึ้นจึงอยู่ในสภาพเดิมโดยไม่มีการทำลายพันธะ noncovalent หรือพันธะ disulfide ตั้งแต่เริ่มต้นจนกระทั่งสิ้นสุดกระบวนการสกัดแยกให้บริสุทธิ์ ผลการทดลองจากการใช้ recombinant A1 ที่สร้างได้นี้ พบว่าโครงสร้างของ A1 domain เปลี่ยนแปลงไปหลังจากมีการเติม ristocetin ข้อมูลที่ได้จากการศึกษานี้เป็นหลักฐานสำคัญเพิ่มเติมที่แสดงให้เห็นว่า ristocetin ชักนำให้เกิดการเปลี่ยนแปลงโครงสร้างของ VWF A1 domain ระหว่างที่มีการเกาะตัวกันของเกล็ดเลือด

Keywords: วอนวิลลิแบรนด์แฟคเตอร์, เอวันโดเมน, รีคอมบิแนนท์, ริสโทซีติน, โครงสร้าง

CHAPTER 1

INTRODUCTION

Rationale

VWF is an adhesive glycoprotein whose interaction with platelet surface receptors is crucial to thrombosis in narrowed arteries [1-2] or hemostasis on vascular injury [3]. Under shear stress, platelet activation is initiated by the binding of VWF to GpIb, which is the primary VWF receptor on platelets. However, VWF does not bind spontaneously to GpIb. Conformational changes are thought to expose the binding sites on VWF for GpIb after its interaction with collagen on the subendothelium [4]. VWF is a multimeric protein consisting of disulfide-bonded subunits, ranging from dimers to multimers extending up to 20 x 10^6 Daltons [5-6]. The VWF monomer includes 13 domains, which are multiples of four domain types (A to D). The A1 domain corresponds to residues V^{1212} to L^{1491} in mature VWF contains and its binding sites for GpIb and heparin [7-8]. This domain is thought to be a critical structural motif as several point mutations have been found within this domain in patients with type 2 von Willebrand disease (VWD) [9-10], thus resulting in either an enhancement (type 2B) or an impairment (type 2M) of the interaction between VWF and GpIb. Differences in VWF structure as a result of natural mutations in type 2B VWD have been examined by X-ray crystallography of recombinant A1 domain fragments [11-13]. However, direct evidence that native VWF undergoes such a conformation change is currently limited.

In vitro tests show that VWF can be stimulated to bind to platelets by various agents, such as the antibiotic ristocetin [14] and the snake venom protein botrocetin [15]. Removal of sialic acid from native VWF with neuraminidase also enhances its binding affinity for GpIb [16]. It is thought that, in the presence of ristocetin, VWF may change its conformation and expose its binding site for GpIb, in an analogous manner to what may occur under conditions of shear stress [17]. Ristocetin has been used widely to study the

mechanism of platelet aggregation. It can also provide a model for the changes in VWF conformation and function that occur under pathological conditions *in vivo*.

Although attempts have been made to demonstrate a conformational change in VWF, direct evidence of this is very limited. It has been found that the pattern of digestion of native human VWF by by trypsin and elastase, is changed in the presence of ristocetin. Whereas vancomycin, an antibiotic that is structurally very similar to ristocetin, does not affect these patterns [18]. However, since VWF has large and complicate structure, it is difficult to specify the important sites. If use is made of a recombinant A1 domain whose size is much smaller than native VWF, it will be possible to determine whether there are any conformational changes after treated with ristocetin. A detailed understanding of conformational changes in VWF will open the way to the development of new drugs for the treatment of thrombosis.

A recombinant VWF-A1 domain has been generated in several laboratories, however, the proteins are expressed in inclusion body, which making the protein precipitated and difficult to purify. This study also explored a method for optimized expression and purification of VWF-A1 which solves the problem of the insoluble expressed VWF-A1 domain in bacteria.

Literature review

Platelet

Platelet activation and inhibition

Platelets play a crucial role in hemostasis and thrombosis. The vascular endothelial cell lining is the natural barrier between the circulating blood and the underlying tissue. It prevents platelet deposition and activation. The endothelium forms a non adhesive surface for platelets, but after removal of the cells the highly reactive subendothelium becomes exposed to the circulating platelet [19-20].The interaction of platelets with subendothelium is of critical importance for normal hemostasis and it plays an important role in the pathogenesis of arterial thrombosis. The processes of platelet activation include platelet adhesion, shape change, secretion and aggregation. The glycoprotein (Gp) Ib-IX-V receptor complex on the platelet surface and von Willebrand factor (vWF) in the subendothelium and

plasma are involved in platelet adhesion to subendothelium under conditions of high shear [21-22]. The GpIb complex has been identified as a vWF receptor on platelets. The binding of vWF to GpIb complex has been shown to initiate platelet activation [23]. In addition, platelets can be activated by several aggregating agents, for example, thrombin, collagen, ADP, thromboxane A2 and platelet-activating factor (PAF). Other substances that bind to platelet receptors such as fibrinogen also contribute to platelet aggregation [24-26]. Two major signal transduction pathways in human platelet have been described. The major activating pathway is a combination of second messengers derived from enzyme-linked hydrolysis of inositol phospholipids: inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG) [27-28]. The major inhibitory signaling pathway uses the second messenger cAMP [29]. Both pathways of signal transduction are initiated by occupancy of cell surface receptor. Several intracellular events occur after platelet receptors are activated. These reponses include 1) activation of phospholipase A2, leading to formation of thromboxane A_2, 2) activation of phospholipase C, producing the second messengers, IP_3, and DG, 3) increase in cytosolic Ca^{2+} due to influx from the exterior, 4) mobilization of Ca^{2+} from internal stores by IP_3, 5) secretion of contents of α and dense granules and 6) inhibition of adenylyl cyclase [30]. Although in most cells cAMP has a positive effect on cell function, in platelets cAMP is an inhibitor. Agents that raise intracellular cAMP levels, such as prostaglandin E_1 and I_2 (prostacyclin) dampen platelet responsiveness [31].

Signaling mechanism for platelet activation by vWF

vWF plays an essential role during physiological hemostasis and pathological thrombosis in arteries, where elevated levels of shear stress occur [32-33]. The interaction of vWF with platelets is essential for platelet adhesion to subendothelium.[16] It has been shown that platelets have two potential binging sites for vWF: GpIb-IX-V and GpIIb/IIIa complex [34]. Accumulating evidence has suggested that the binding of vWF to GpIb-IX-V initiate the signal transduction cascade [35-37]. It is believed that the intraplatelet signal induces a conformational change in GpIIb/IIIa, thus resulting in the binding of GpIIb/IIIa to vWF and fibrinogen [38-39]. These events contribute to platelet activation and aggregation. It has been demonstrated that activation of human platelets by vWF is followed by an

$[Ca^{2+}]_i$ increase.Therefore, it is widely accepted that binding of vWF to GpIb-IX-V initiates platelet activation by increasing intracellular free calcium ion concentration [40-45] but the proposed mechanisms which cause $[Ca^{2+}]_i$ signal are contradictory. Some investigators have suggested that the $[Ca^{2+}]_i$ signal come from an influx of extracellular calcium through calcium channel in the platelet cell membrane and phospholipase A_2 pathway does not play a role in $[Ca^{2+}]_i$ rise [43-44]. Whereas others have proposed that release from intracellular stores is responsible for the $[Ca^{2+}]_i$ increase and this reaction is evoked by production of thromboxane A_2 from the activation of phospholipase A_2 pathway [46].

A1 domain of VWF

VWF is a large glycoprotein that plays an essential role in thrombosis and hemostasis. It is involved in platelet adhesion to the damaged vascular endothelium. In the bloodstream, native VWF normally does not bind to platelets. However, when vascular injury occurs, it contributes hemostasis by initiating platelet adhesion to exposed vascular subendothelium [39, 47-48]. VWF also plays an important role in thrombosis in narrowed arterial vessels. Passing through an areas of the circulation where blood flow create a high shear stress, platelets also promote VWF-mediated platelet adhesion and aggregation [1, 3, 49].

VWF is a multimeric plasma glycoprotein that consists of identical subunits of 2050 amino acids. The protein circulates in plasma as a series of disulfide-linked multimers ranging from 1 to 20 million Daltons [48]. Each subunit of VWF consists of four repeated domains designated A through D and arranged from amino to carboxyl terminus in the following order: D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2 [38-39, 50] (see figure 1). This mapping shows that a fragment from V^{1212}-L^{1491} encompasses two important binding domains, for the GpIb α-chain and heparin [7-8]. The A1 domain which contains binding site for GPIb, heparin, collagen, ristocetin and botrocetin corresponds to residues V^{1212} to L^{1491} [51-52]. It contains a disulfide bridge between Cys509 and Cys695 [53-54]. Several point mutations have been found within this domain in patients with type 2 von Willebrand disease (VWD) [9, 10]. Such mutations usually affect the propensity for VWF to interact with GpIb-IX-V, either enhancing such interaction (type 2B VWD) [55-56] or impairing it

(type 2M VWD) [13, 57]. It is thus important to understand both the structure of this domain and how it is regulated. Differences in VWF structure as a result of natural mutations in type 2B VWD have been examined by X-ray crystallography of recombinant A1 domain fragments [11-13]. However, direct evidence that native VWF undergoes such a conformation change is currently limited.

Native VWF in the circulation does not normally bind to platelets. However, VWF binds to platelets after it is activated by a presumed and poorly understood conformational change that occur after it interacts with exposed subendothelial elements at a site of vascular injury; the multimeric nature of VWF allows it to perform a bridging function, linking platelets to each other and to the subendothelial elements. VWF binding to GpIb-IX-V can be induced *in vitro* by various compounds, interventions and mutations. The exogenous modulator ristocetin, a glycopeptide antibiotic, mediates interaction of VWF with platelet GpIb-IX-V [14, 58]. Another exogenous modulator, the snake venom protein botrocetin, also promotes VWF binding to GpIb through a direct interaction with VWF [15, 59-60].

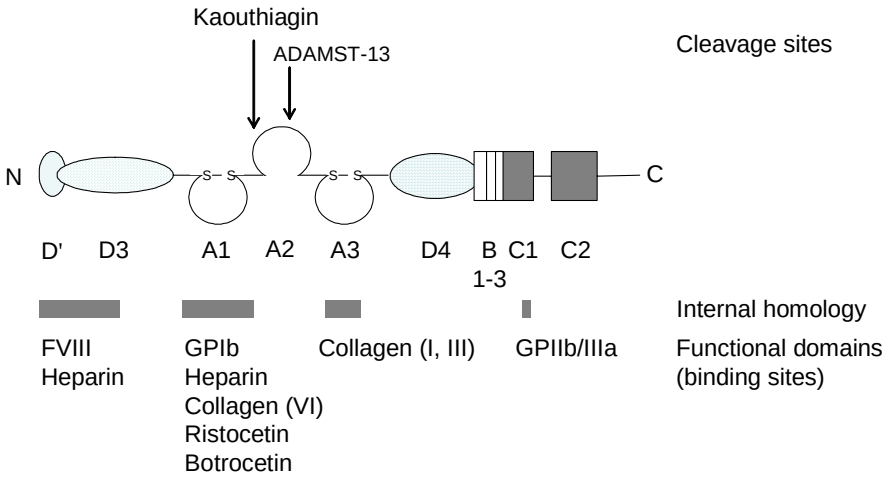

Figure 1. Schematic structure of human VWF subunit. VWF monomer consists of 2050 amino acid residue. Each subunit is comprised of multiples of four domain types, A to D. The A1 domain which contains binding site for GPIb, heparin, collagen, ristocetin and botrocetin corresponds to residues V^{1212} to L^{1491} [50].

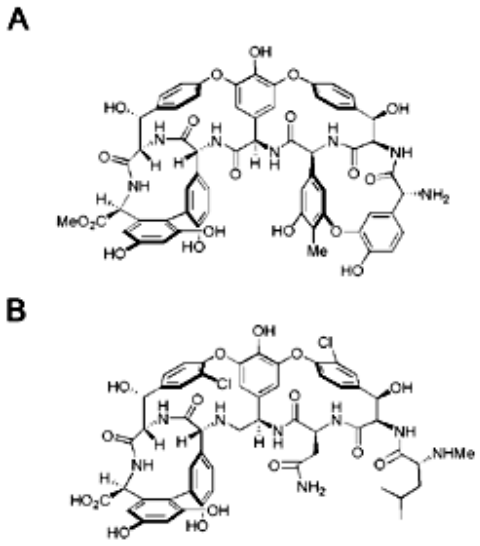

Figure 2. Chemical structure of ristocetin A and B

The glycopeptide antibiotic ristocetin is a glycopeptide antibiotic produced by Nocardia lurida, belongs to the vancomycin group of antibiotics. The structure of ristocetin which contain ristocetin A and B are shown in figure 2. The antibiotics consist of a central heptapeptide (the aglycone) and 6 sugars (mannose (2 mole), glucose, arabinose, rhamnose and ristosamin) attached to the aglycone at 3 separate sites [61]. Ristocetin A inhibits the formation of peptidoglycan moieties in the cell wall. However, the drug was withdrawal during phase III of clinical trial for antibiotic therapy because of its ability to cause aggregation of blood platelets. This effect, however, made it a useful clinical tool for diagnosing VWD. In this study, ristocetin was used to provide a model for the changes in VWF A1 domain conformation that may occur under pathological conditions *in vitro*.

Specific aims

This study comprises the following two specific aims:

1. To generate a suitable recombinant fragment for studying conformational changes in the A1 domain of human VWF.

2. To examinine the pattern of VWF-A1 domain on SDS-PAGE in the presence and absence of ristocetin.

CHAPTER 2

MATERIALS AND METHODS

1. Cell culture

COS-7cells (provided by Dr. Chanvit Leelayuwat, Department of Clinical Immunology, Khon Kaen University) were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 4 mM glutamine (Gibco BRL), 5% fetal calf serum (Gibco BRL), 25 units/ml penicillin and 25 mg/ml streptomycin (Gibco BRL) in a 5% CO_2/95% air atmosphere at 37 °C.

2. Generation of wild type recombinant A1 domain of VWF in COS-7 cells.

2.1 Amplification of the wild type gene of A1 domain from genomic DNA by the polymerase chain reaction (PCR)

The A1 domain of human VWF was amplified from genomic DNA (isolated from white blood cells from volunteers in Dr. John C. Kermode's laboratory, Department of Pharmacology and Toxicology, University of Mississippi Medical Center, U.S.A.) by PCR. The forward and reverse primers comprised 5′-acggatCCACTGTGATGTTGTCAA-3′ and 5′-aatctaGACCAAGAAGCTGTGGTCA-3′, respectively. These primers correspond to nucleotides 3985-4002 and 5086-5068 in the VWF coding sequence. The underlined nucleotides are restriction sites (for *BamH* I in the forward primer and *Xba* I in the reverse primer) that was introduced to allow subcloning.

2.2 Transient expression of the wild type recombinant A1 domain of VWF

To express the recombinant A1 domain, the PCR product was digested with *BamH* I and *Xba* I before introduction into the pcDNA3.1/His vector (Invitrogen), which includes codons for a hexahistidine tag and an enterokinase cleavage site at the N-terminal side of the expressed protein. The recombinant clone was amplified using PCR-Script^{TM} Amp Cloning Kit (Stratagene). The sequence of the recombinant A1 domain fragment was confirmed (Macrogen) that no errors occurred during the PCR amplification.

COS-7 monkey kidney cells were transfected with the pcDNA3.1/His-recombinant A1 domain using DAEA-Dextran. After culture of the transfected cells, the expression of A1 domain was checked by SDS-PAGE and immunoblotting with anti-express (Invitrogen) and anti-VWF antibody.

3. Generation of Cys/Ser mutant recombinant A1 domain of VWF by site-directed mutagenesis in COS-7 cells.

First, Cys/Ser mutant recombinant A1 domain gene at 1227 was produced by a similar procedure described in section 2.1 except for 5′-acggatCCAC**AGT**GATGTTGT CAA-3′ was used as a forward primer instead. After a recombinant plasmid, pcDNA3.1/mutant 1227, was constructed, site-directed mutagenesis at 1234 was performed followed by site-directed mutagenesis at 1237. The forward primer and reverse primer for mutagenesis at 1234 were GTTGTCAACCTCACC**AGT**GAAGCCTGCCAGG and CCTGGCAGGCTTCACTGGTGAGGTTGACAAC, respectively. Whereas, the forward primer and reverse primer for mutagenesis at 1237 were CTCACCAGTGAAGCC **AGC**CAGAGCCGGGAG and CTCCCGCTCCTGGCTGGCTTCACTGGTGAG, respectively (mutate sites are in bold, changing encoding from cysteine to serine). The expression of the mutant recombinant A1 domain was conducted in *COS-7 cells as* described earlier in section 2.2.

4. Determination of solubility of the recombinant A1 domain

After two-day expression of recombinant A1 domain, COS-7 cells were collected and resuspended in native purification buffer (50 mM NaH_2PO_4, pH 8.0, 0.5 mM NaCl). The cells were lysed by two freeze-thaw cycles in -80°C and 42°C, then sonicated 3 × 10 s with 10 s pauses at 100 W. The lysate was centrifuged at 10,000 × g at 4°C for 30 minutes. The supernatant was kept on ice and the pellet was resuspended in native purification buffer. Another experiment was done as described above, except for resuspending the pellet in the native purification buffer with 1% Triton X-100, and then centrifuged at 10,000 × g at 4°C for 30 minutes. The supernatant and pellet were separately collected. The wild type

and mutant recombinant A1 domain were checked which fraction they were by SDS-PAGE and immunoblotting with anti-VWF antibody.

5. Generation of Cys/Ser mutant recombinant A1 domain of VWF in bacteria

5.1 Construction of VWF A1 domain vector

Since, the expressed A1 domain from *COS-7* cells was inclusion body since it dissolved only in a solution containing guanidinium. The new vector was constructed again by amplifying DNA from the mutant recombinant A1 domain plasmid in section 3 using 5′-ATCCCCATGGATGTTGTCAACCTC-3′and 5′-GTCGAAGATCTCAAGAGCC CCGG-3′ as the forward and reverse primers respectively. These primers correspond to nucleotides 3985-4002 and 5086-5068 in the VWF coding sequence. The underlined nucleotides are restriction sites (for *Nco* I in the forward primer and *Bgl* II in the reverse primer).

5.2 Expression of the mutant recombinant A1 domain of VWF

After no errors during the PCR amplification was confirmed, the PCR product was digested with *Nco* I and *Bgl* II, then the DNA was introduced into the pQE60 vector (Quigen), which also includes codons for a hexahistidine tag at the C-terminal side of the expressed protein. M15 bacteria was transformed with the pQE60-recombinant A1 domain. Antibiotic resistant clone was selected, cultured and induced with isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM. The expression of the mutant recombinant A1 domain was checked by SDS-PAGE and immunoblotting with anti-VWF as described in section 2.2.

6. Determination of solubility of the recombinant A1 domains from bacteria

After seven hours of induction with IPTG, the solubility of the recombinant A1 domains from bateria was determined with the methods described in section 4 with a minor change. Cells were collected and resuspended in native purification buffer (50 mM NaH_2PO_4, pH 8.0, 0.5 mM NaCl), and then incubated on ice for 30 min with lysozyme (1 mg/ml). After two freeze-thaw cycles, cells were broken thoroughly using a sonicator, 6 × 10 s with 10 s pauses at 200 W. The lysate was centrifuged at 10,000 × g at 4°C for 30

minutes. The supernatant was kept on ice and the pellet was resuspended in the native purification buffer. The mutant recombinant A1 domain was checked which fraction they were by SDS-PAGE and immunoblotting with anti-VWF antibody.

7. Purification of the recombinant A1 domain of VWF from Bacteria

Since the recombinant A1 domain of VWF from bacteria is water soluble, purification of the protein was performed in a native condition throughout the procedure as follows. After centrifugation of cell lysate, supernatant was collected and incubated with Nickel-chelating resin (ProBond, Invitrogen) for 1 hour, then washed with washed buffer, purification buffer containing 20 mM imidazole, for 3 times. The purified protein was eluted with elution buffer, purification buffer containing 250 mM imidazole. Each fraction was subjected to SDS-PAGE and detected with anti-VWF antibody on blot. The fractions containing the recombinant A1 domain were collected and dialyzed against binding buffer (10 mM NaH_2PO_4, pH 7) and loaded onto a Heparin-Sepharose column (GE Healthcare Bio-Sciences AB). After washed with binding buffer, a stepwise elution was conducted using the binding buffer containing 0.1, 0.2, 0.4 M and 2 M NaCl, consecutively. The contamination of the purified protein was checked by SDS-PAGE. Conformation of the recombinant A1 domain of VWF was examined by comparison on reducing and non-reducing SDS-PAGE.

8. Examination of the pattern of recombinant A1 domain of VWF on SDS-PAGE in the presence and absence of ristocetin.

Ristocetin, an exogenous agent that regulates the function of VWF *in vitro*, was used to induce a conformational change in the recombinant A1 domain obtained from bacteria. The A1 domain (200 μg/ml) was incubated with ristocetion (1 μg/ml) for 5 min and then subjected to reducing and non reducing SDS-PAGE. A competitive study was also conducted using vancomycin, an antibiotics that is structurally very similar to ristocetin as a negative control.

CHAPTER 3

RESULTS

1. Recombinant A1 domain gene of VWF from PCR

Theoretically, gene that controls the synthesis of A1 domain of VWF from genomic DNA by PCR, using 5′-acggatCCACTGTGATGTTGTCAA-3′ as a forward primer and 5′-aatctaGACCAAGAAGCTGTGGTCA-3′ as a reverse primer is anticipated to have 1112 base pairs. The data from 2% agarose gel electrophoresis showed that the PCR product has corresponding size (figure 3).

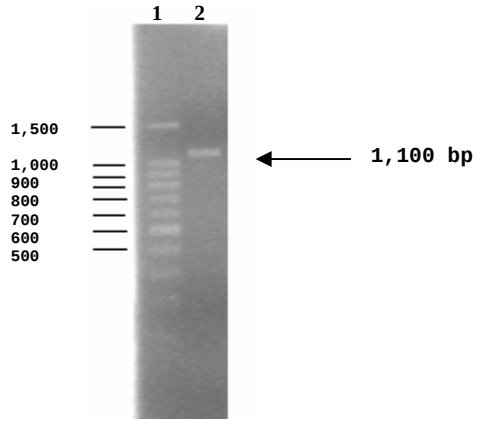

Figure 3. Detection of PCR product of VWF A1 domain on 2% agarose gel. Lane 1: standard DNA marker. The 1100 bp PCR product is indicated.

With the National Center for Biotechnology Information (NCBI) data base, the sequence of pcDNA3.1/His-recombinant A1 domain was compared and found that there is no error occurred during the PCR amplification (figure 4a and 4b)

Figure 5a and 5b are chromatograms from sequencing data. The A1 domain gene in plasmid has 3 mutation sites, changing encoding from serine to cysteine, which causing only 2 cysteines remained in the structure of the recombinant A1 domain. The two cysteines are essential for disulfide bond to construct the A1 domain.

```
Human VWF: 3985 ccactgtgatgttgtcaacctcacctgtgaagcctgccaggagccgggaggcctggtggt 4044
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 129        ccactgtgatgttgtcaacctcacctgtgaagcctgccaggagccgggaggcctggtggt 188

Human VWF: 4045 gcctcccacagatgccccggtgagccccaccactctgtatgtggaggacatctcggaacc 4104
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 189        gcctcccacagatgccccggtgagccccaccactctgtatgtggaggacatctcggaacc 248

Human VWF: 4105 gccgttgcacgatttctactgcagcaggctactggacctggtcttcctgctggatggctc 4164
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 249        gccgttgcacgatttctactgcagcaggctactggacctggtcttcctgctggatggctc 308

Human VWF: 4165 ctccaggctgtccgaggctgagtttgaagtgctgaaggcctttgtggtggacatgatgga 4224
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 309        ctccaggctgtccgaggctgagtttgaagtgctgaaggcctttgtggtggacatgatgga 368

Human VWF: 4225 gcggctgcgcatctcccagaagtgggtccgcgtggccgtggtggagtaccacgacggctc 4284
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 369        gcggctgcgcatctcccagaagtgggtccgcgtggccgtggtggagtaccacgacggctc 428

Human VWF: 4285 ccacgcctacatcgggctcaaggaccggaagcgaccgtcagagctgcggcgcattgccag 4344
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 429        ccacgcctacatcgggctcaaggaccggaagcgaccgtcagagctgcggcgcattgccag 488

Human VWF: 4345 ccaggtgaagtatgcgggcagccaggtggcctccaccagcgaggtcttgaaatacacact 4404
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 489        ccaggtgaagtatgcgggcagccaggtggcctccaccagcgaggtcttgaaatacacact 548

Human VWF: 4405 gttccaaatcttcagcaagatcgaccgccctgaagcctcccgcatcgccctgctcctgat 4464
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 549        gttccaaatcttcagcaagatcgaccgccctgaagcctcccgcatcgccctgctcctgat 608

Human VWF: 4465 ggccagccaggagccccaacggatgtcccggaactttgtccgctacgtccagggcctgaa 4524
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR:  609        ggccagccaggagccccaacggatgtcccggaactttgtccgctacgtccagggcctgaa 668

Human VWF: 4525 gaagaagaaggtcattgtgatcccggtgggcattgggccccatgccaacctcaagcagat 4584
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 669        gaagaagaaggtcattgtgatcccggtgggcattgggccccatgccaacctcaagcagat 728

Human VWF: 4585 ccgcctcatcgagaagcaggcccctgagaacaaggccttcgtgctgagcagtgtggatga 4644
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 729        ccgcctcatcgagaagcaggcccctgagaacaaggccttcgtgctgagcagtgtggatga 788

Human VWF: 4645 gctggagcagcaaagggacgagatcgttagctacctctgtgaccttgcccctgaagcccc 4704
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 789        gctggagcagcaaagggacgagatcgttagctacctctgtgaccttgcccctgaagcccc 848

Human VWF: 4705 tcctcctactctgnnnnnnnacatggcacaagtcactgtgggcccggggctcttgggggt 4764
                ||||||||||||       ||||||||||||||||||||||||||||||||||||||||
PCR: 849        tcctcctactctgccccccccacatggcacaagtcactgtgggcccggggctcttgggggt 908

Human VWF: 4765 ttcgaccctggggcccaagagga 4787
                |||||||||||||||||||||||
PCR: 909        ttcgaccctggggcccaagagga 931
```

Figure 4a. Comparison of human VWF and PCR sequences using the National Center for Biotechnology Information (NCBI) Blast2 software.T7 was used as a forward primer.

```
Human VWF: 4231 gcgcatctcccagaagtgggtccgcgtggccgtggtggagtaccacgacggctcccacgc 4290
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 877        gcgcatctcccagaagtgggtccgcgtggccgtggtggagtaccacgacggctcccacgc 818

Human VWF: 4291 ctacatcgggctcaaggaccggaagcgaccgtcagagctgcggcgcattgccagccaggt 4350
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 817        ctacatcgggctcaaggaccggaagcgaccgtcagagctgcggcgcattgccagccaggt 758

Human VWF: 4351 gaagtatgcgggcagccaggtggcctccaccagcgaggtcttgaaatacacactgttcca 4410
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 757        gaagtatgcgggcagccaggtggcctccaccagcgaggtcttgaaatacacactgttcca 698

Human VWF: 4411 aatcttcagcaagatcgaccgccctgaagcctcccgcatcgccctgctcctgatggccag 4470
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 697        aatcttcagcaagatcgaccgccctgaagcctcccgcatcgccctgctcctgatggccag 638

Human VWF: 4471 ccaggagccccaacggatgtcccggaactttgtccgctacgtccagggcctgaagaagaa 4530
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 637        ccaggagccccaacggatgtcccggaactttgtccgctacgtccagggcctgaagaagaa 578

Human VWF: 4531 gaaggtcattgtgatcccggtgggcattgggccccatgccaacctcaagcagatccgcct 4590
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 577        gaaggtcattgtgatcccggtgggcattgggccccatgccaacctcaagcagatccgcct 518

Human VWF: 4591 catcgagaagcaggcccctgagaacaaggccttcgtgctgagcagtgtggatgagctgga 4650
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 517        catcgagaagcaggcccctgagaacaaggccttcgtgctgagcagtgtggatgagctgga 458

Human VWF: 4651 gcagcaaagggacgagatcgttagctacctctgtgaccttgcccctgaagcccctcctcc 4710
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 457        gcagcaaagggacgagatcgttagctacctctgtgaccttgcccctgaagcccctcctcc 398

Human VWF: 4711 tactctgnnnnnnnacatggcacaagtcactgtgggcccggggctcttgggggtttcgac 4770
               |||||||       ||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 397        tactctgcccccccacatggcacaagtcactgtgggcccggggctcttgggggtttcgac 338

Human VWF: 4771 cctggggcccaagaggaactccatggttctggatgtggcgttcgtcctggaaggatcgga 4830
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 337        cctggggcccaagaggaactccatggttctggatgtggcgttcgtcctggaaggatcgga 278

Human VWF: 4831 caaaattggtgaagccgacttcaacaggagcaaggagttcatggaggaggtgattcagcg 4890
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 277        caaaattggtgaagccgacttcaacaggagcaaggagttcatggaggaggtgattcagcg 218

Human VWF: 4891 gatggatgtgggccaggacagcatccacgtcacggtgctgcagtactcctacatggtgac 4950
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 217        gatggatgtgggccaggacagcatccacgtcacggtgctgcagtactcctacatggtgac 158

Human VWF: 4951 cgtggagtacccttcagcgaggcacagtccaaaggggacatcctgcagcgggtgcgaga 5010
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 157        cgtggagtacccttcagcgaggcacagtccaaaggggacatcctgcagcgggtgcgaga 98

Human VWF: 5011 gatccgctaccagggcggcaacaggaccaacactgggctggccctgcggtacctctctga 5070
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR: 97         gatccgctaccagggcggcaacaggaccaacactgggctggccctgcggtacctctctga 38
```

Figure 4b. Comparison of human VWF and PCR sequences using the National Center for Biotechnology Information (NCBI)Blast2 software. BGH was used as a reverse primer.

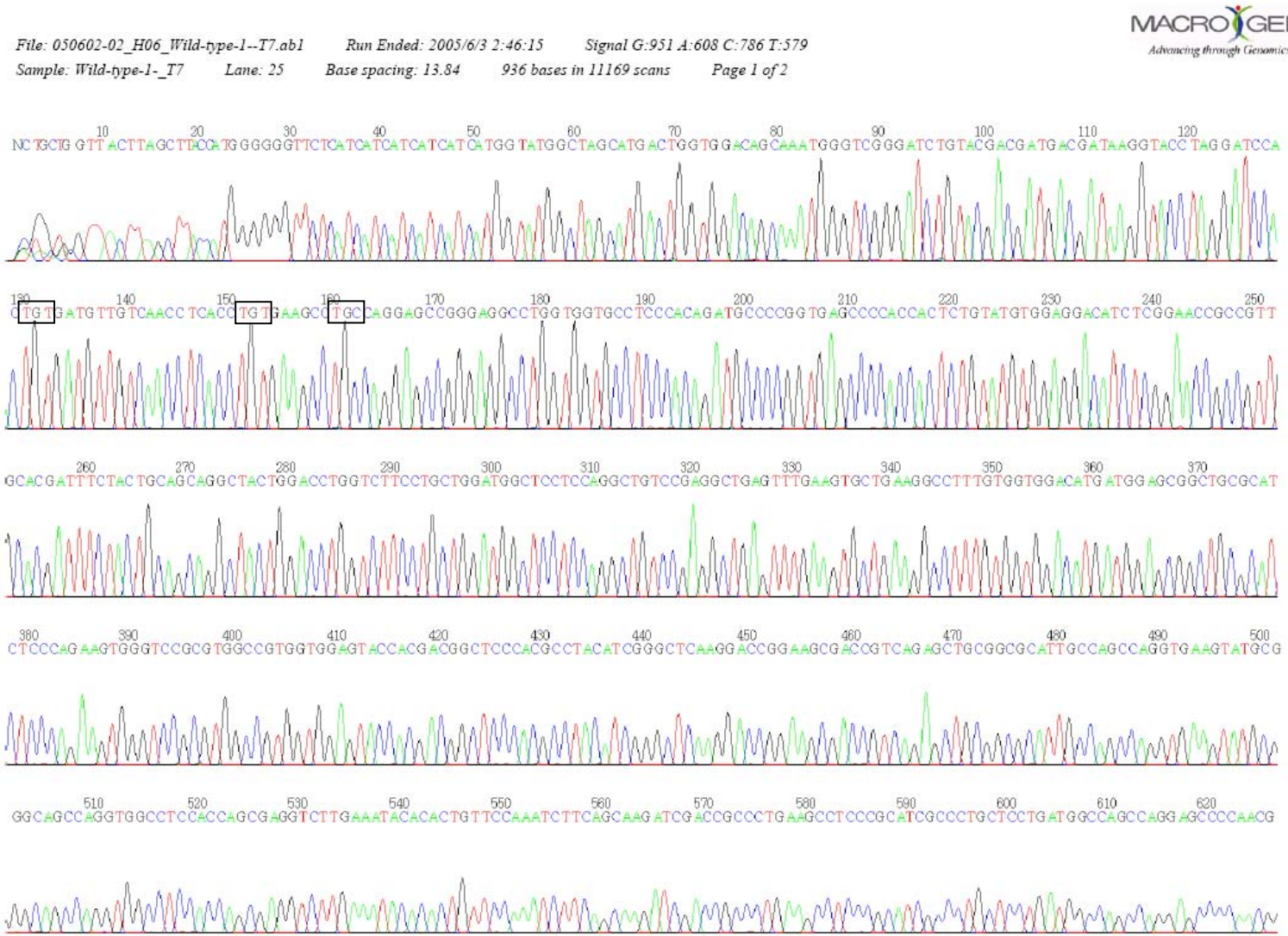

Figure 5a. Alignment of the sequences of the wild type A1 domain gene. The rectangles indicate 3 of 5 cysteines in the native structure.

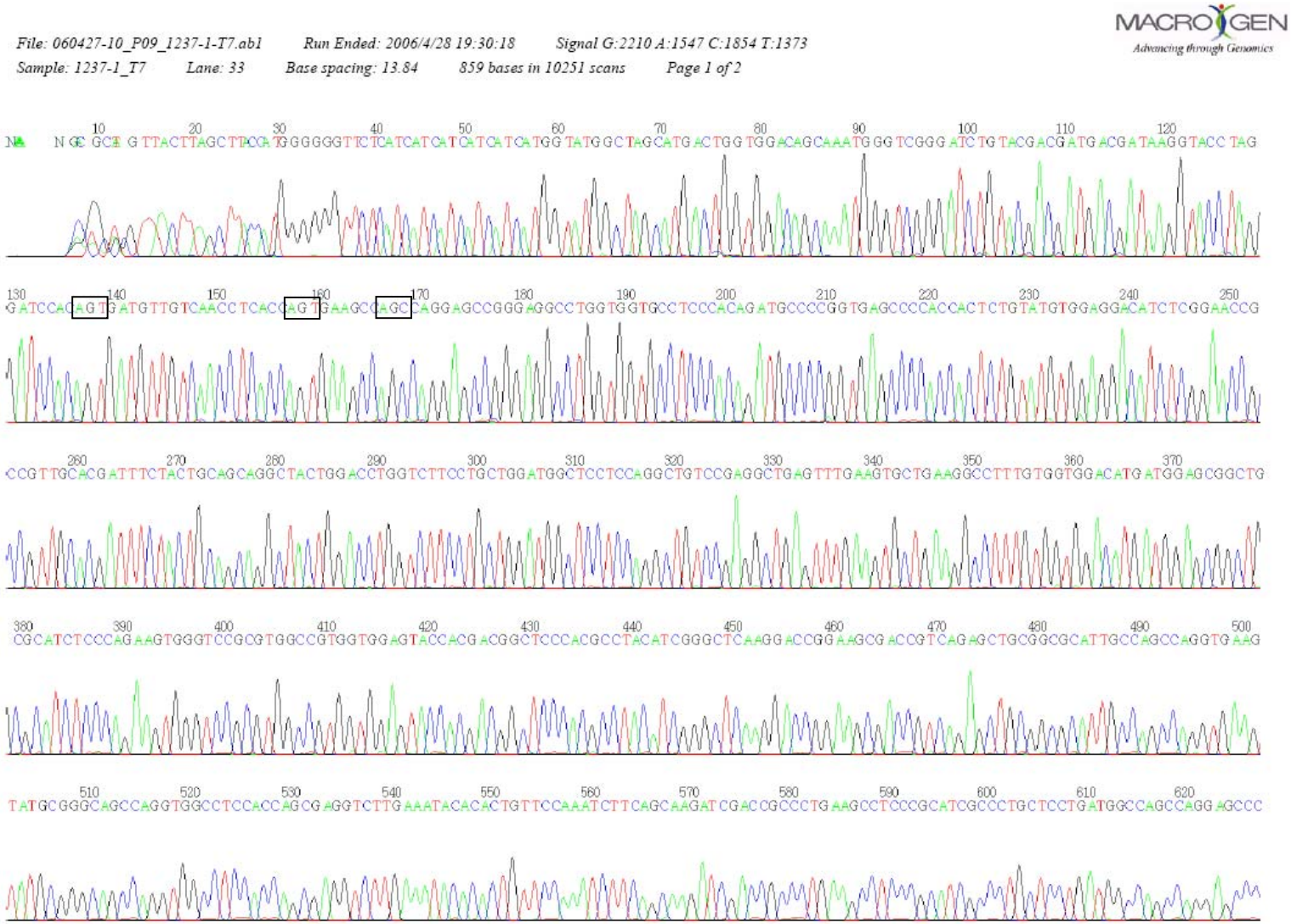

Figure 5b. Alignment of the sequences of the Cys/Ser mutant A1 domain gene. The rectangles indicate the site-directed mutagenesis from cysteine to serine.

2. Expression of the recombinant A1 domain of VWF in COS-7 cells

Figure 6 showed the expression of A1 domain gene in COS-7 cells detected with anti- VWF antibody, which binds to natural VWF, and with anti-Xpress antibody, which binds to the unnatural epitope. From the calculation (Table 1: using software from website http://proteome.gs.washington.edu), the predicted molecular weight of recombinant A1 domain is 45.1 kD. Including the molecular weight of glycosylation portion, the size of the recombinant A1 domain seems to correspond to the expected molecular weight.

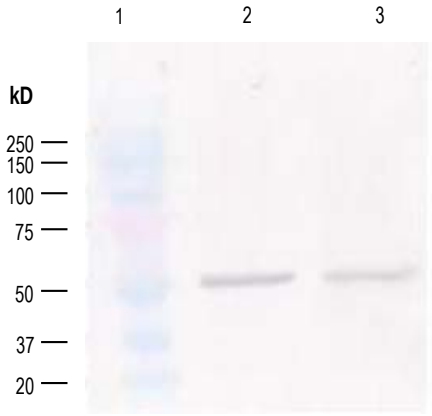

Figure 6. Expression of wild type recombinant VWF A1 domain gene in COS-7 cells. The recombinant protein from cell homogenate of the transfected cell was separated by SDS-PAGE (7.5%) and then transferred to nitrocellulose membrane by Western blotting. Lane 1: molecular weight marker; lane 2: cell homogenate detected with anti-Xpress antibody; lane 3: cell homogenate detected with anti-VWF antibody. The numbers in the left are molecular marker.

3. Solubility of the recombinant A1 domains

The recombinant proteins produced from COS-7 cells in the present study, both wild type and mutant, did not dissolve in PBS or PBS with 1% Triton-X (figure 7 and 8). This indicates that the recombinant A1 domain is not a soluble protein or membrane bound protein.

Amino acid sequence of recombinant A1 domain from COS-7 cells

M G G S H H H H H H G M A S M T G G Q Q M G R D L Y D D D D K V P R I H S D V V N L T C E A C Q E P G G L V V P P T D A P V S P T T L Y V E D I S E P P L H D F Y C S R L L D L V F L L D G S S R L S E A E F E V L K A F V V D M M E R L R I S Q K W V R V A V V E Y H D G S H A Y I G L K D R K R P S E L R R I A S Q V K Y A G S Q V A S T S E V L K Y T L F Q I F S K I D R P E A S R I A L L L M A S Q E P Q R M S R N F V R Y V Q G L K K K K V I V I P V G I G P H A N L K Q I R L I E K Q A P E N K A F V L S S V D E L E Q Q R D E I V S Y L C D L A P E A P P P T L P P H M A Q V T V G P G L L G V S T L G P K R N S M V L D V A F V L E G S D K I G E A D F N R S K E F M E E V I Q R M D V G Q D S I H V T V L Q Y S Y M V T V E Y P F S E A Q S K G D I L Q R V R E I R Y Q G G N R T N T G L A L R Y L S D H S F L V

		Average Mass: 45072.6805		**Sequence Length:** 402
Symbols	**Name**	**Mono Mass**	**Average Mass**	**Count**
Ala A	Alanine	71.03711	71.07880	23
Arg R	Arginine	156.10111	156.1876	26
Asn N	Asparginine	114.04293	114.1039	8
Asp D	Aspartic Acid	115.02694	115.0886	25
Cys C	Cysteine	103.00919	103.1448	4
Glu E	Glutamic Acid	129.04259	129.1155	27
Gln Q	Glutamine	128.05858	128.1308	21
Gly G	Glycine	57.02146	57.0520	26
His H	Histidine	137.05891	137.1412	14
Ile I	Isoleucine	113.08406	113.1595	19
Leu L	Leucine	113.08406	113.1595	39
Lys K	Lysine	128.09496	128.1742	19
Met M	Methionine	131.04049	131.1986	13
Phe F	Phenylalanine	147.06841	147.1766	13
Pro P	Proline	97.05276	97.1167	23
Ser S	Serine	87.03203	87.0782	33
Thr T	Threonine	101.04768	101.1051	14
Trp W	Tryptophan	186.07931	186.2133	1
Tyr Y	Tyrosine	163.06333	163.1760	14
Val V	Valine	99.06841	99.1326	40

Table 1. **Molecular weight calculation of the recombinant A1 domain from COS-7 cells.** The polypeptide chain of recombinant A1 domain amino acid consists of 398amino acids. Using software from website http://proteome.gs.washington.edu, predicted molecular weight of recombinant A1 domain is 44.5 kD. This does not include the molecular weight of glycosylation part.

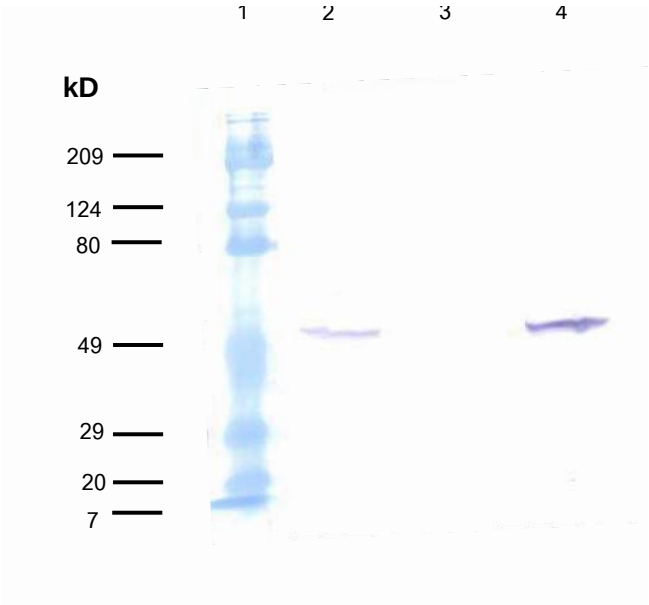

Figure 7. **Solubility of wild type and mutant VWF A1 domain protein in PBS.** A comparison of solubility of the recombinant A1 domains was performed as described in method section 4. The recombinant protein was detected with anti-VWF antibody. Lane 1: molecular weight marker; lane 2: pellet from wild type; lane 3: supernatant from wild type; lane 4: pellet from mutant; lane 5: supernatant from mutant. The numbers in the left are molecular marker.

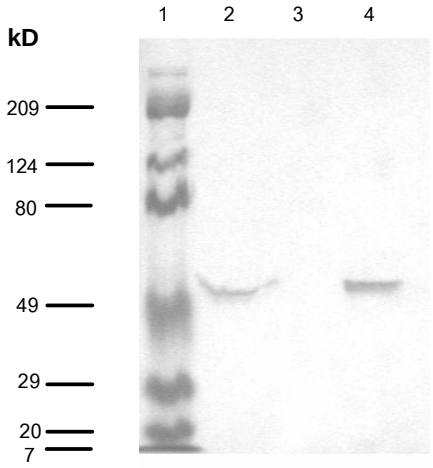

Figure 8. Solubility of mutant VWF A1 domain in 1% Triton-X. Determination of solubility of the mutant recombinant protein was done as as described in method section 4. Lane 1: molecular weight marker; lane 2: cell homogenate; lane 3 = supernatant; lane 4: pellet. The recombinant protein was detected with anti-VWF antibody. The numbers in the left are molecular marker.

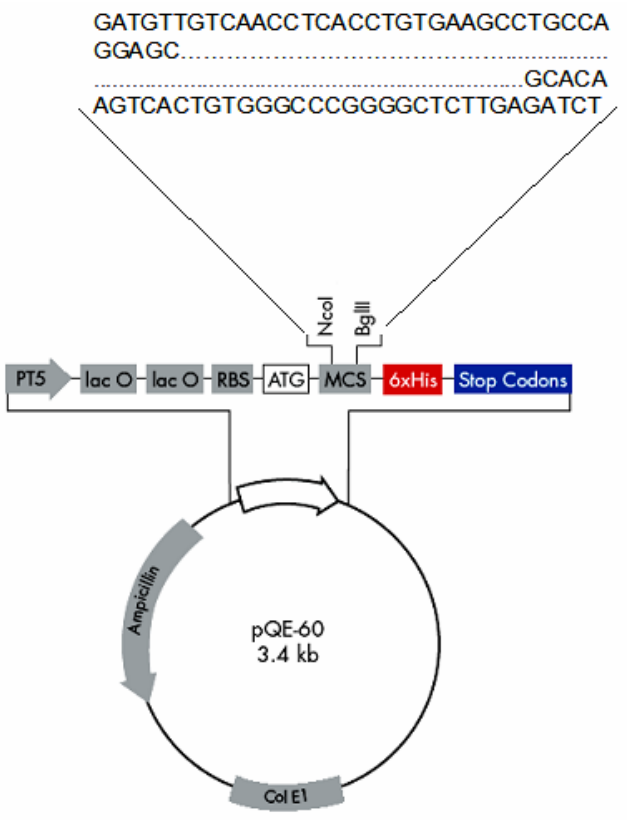

Figure 9. **Schematic map of the VWF-A1 (pQE60) plasmid.** Between *Nco* I and *Bgl* II is VWF-A1 gene insertion. For clarity, the middle part of the sequence is omitted.

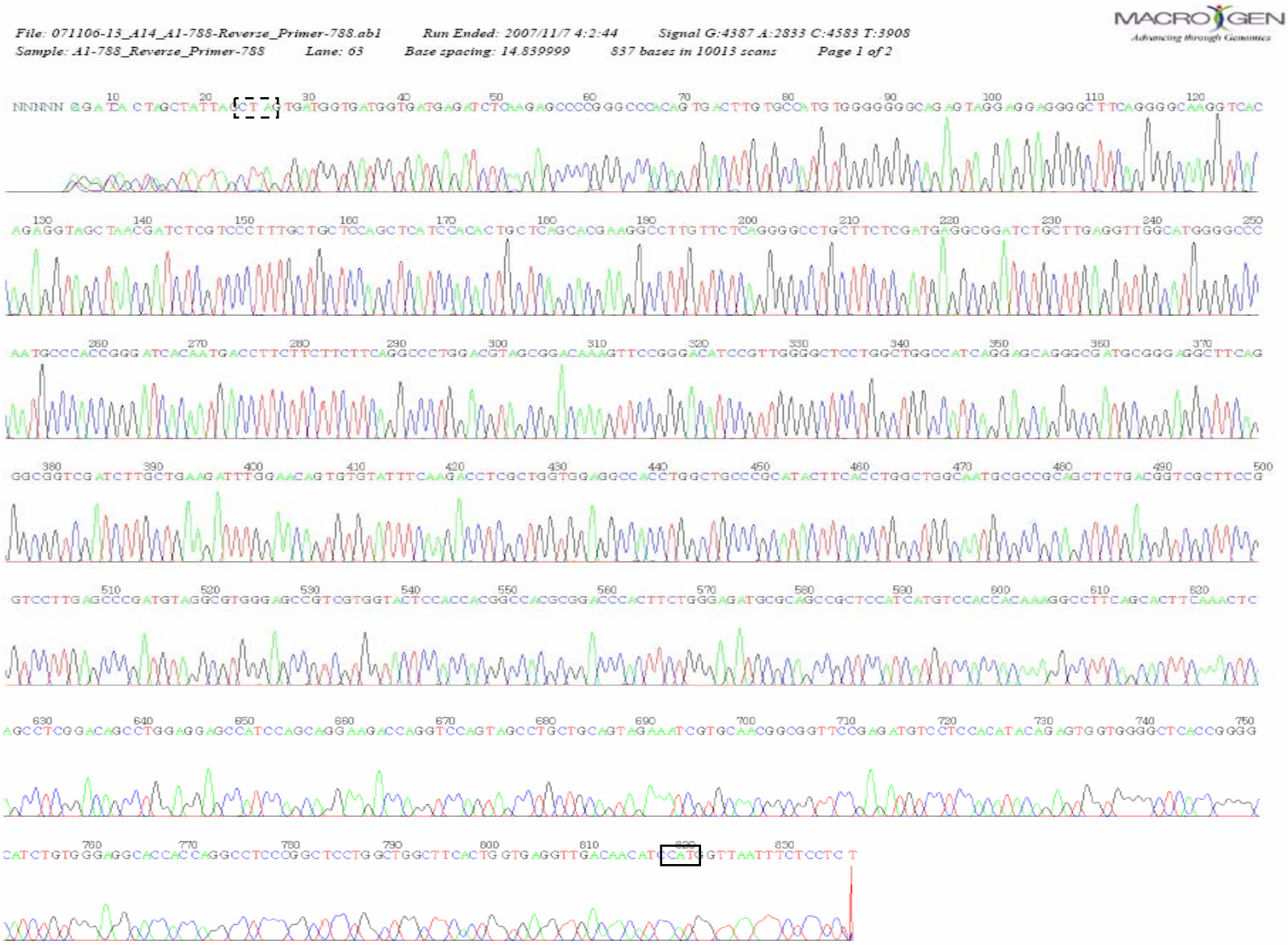

Figure 10. Alignment of the sequences of the mutant A1 domain gene in pQE60 vector. The specific sequencing reverse primer, 3′-ggtcattactggagtcttg-5′, was used**.** The solid and dash rectangles indicate stop and start codon, respectively. cysteines in the native structure.

Amino Acid Sequence

M D V V N L T S E A S Q E P G G L V V P P T D A P V S P T T L Y V E D I S E P P L H D F Y C S R L L D L V F L L D G S S R L S E A E F E V L K A F V V D M M E R L R I S Q K W V R V A V V E Y H D G S H A Y I G L K D R K R P S E L R R I A S Q V K Y A G S Q V A S T S E V L K Y T L F Q I F S K I D R P E A S R I A L L L M A S Q E P Q R M S R N F V R Y V Q G L K K K K V I V I P V G I G P H A N L K Q I R L I E K Q A P E N K A F V L S S V D E L E Q Q R D E I V S Y L C D L A P E A P P P T L P P H M A Q V T V G P G L L R S

N-Terminal Group: Hydrogen	**Mono Isotopic Mass:** 28959.29562	**Sequence Length:** 259
C-Terminal Group: Free Acid	**Average Mass:** 28977.5698	

Symbols	Name	Mono Mass	Average Mass	Count
Ala A	Alanine	71.03711	71.07880	18
Arg R	Arginine	156.10111	156.1876	17
Asn N	Asparginine	114.04293	114.1039	4
Asp D	Aspartic Acid	115.02694	115.0886	13
Cys C	Cysteine	103.00919	103.1448	2
Glu E	Glutamic Acid	129.04259	129.1155	19
Gln Q	Glutamine	128.05858	128.1308	13
Gly G	Glycine	57.02146	57.0520	11
His H	Histidine	137.05891	137.1412	5
Ile I	Isoleucine	113.08406	113.1595	13
Leu L	Leucine	113.08406	113.1595	29
Lys K	Lysine	128.09496	128.1742	14
Met M	Methionine	131.04049	131.1986	6
Phe F	Phenylalanine	147.06841	147.1766	8
Pro P	Proline	97.05276	97.1167	20
Ser S	Serine	87.03203	87.0782	23
Thr T	Threonine	101.04768	101.1051	8
Trp W	Tryptophan	186.07931	186.2133	1
Tyr Y	Tyrosine	163.06333	163.1760	8
Val V	Valine	99.06841	99.1326	27

Table 2 Molecular weight calculation of the mutant recombinant A1 domain from bacteria. The polypeptide chain of recombinant A1 domain amino acid consists of 265 amino acids. Using software from website http://proteome.gs.washington.edu, predicted molecular weight of recombinant A1 domain is 28977 Da. This protein has no glycosylation part.

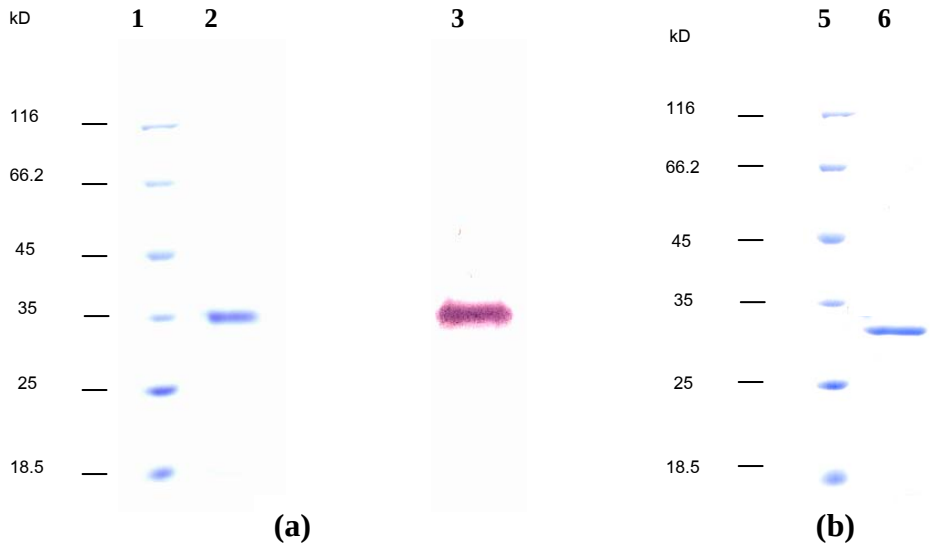

Figure 11. Purified recombinant A1 domain from bacteria. The purified recombinant A1 domain was analyzed by 10% SDS-PAGE under reducing (panel a) and nonreducing condition (panel b). From the left of panel (a), lane 1= molecular weight marker; lane 2= purified recombinant A1 protein (6 μg) stained with Coomassie blue. Lane 3 is purified recombinant A1 protein (6 μg), detected with anti-VWF antibody. From the left of panel (b), lane 1 = molecular weight marker; lane 2 = purified recombinant A1 protein (6 μg) stained with Coomassie blue.

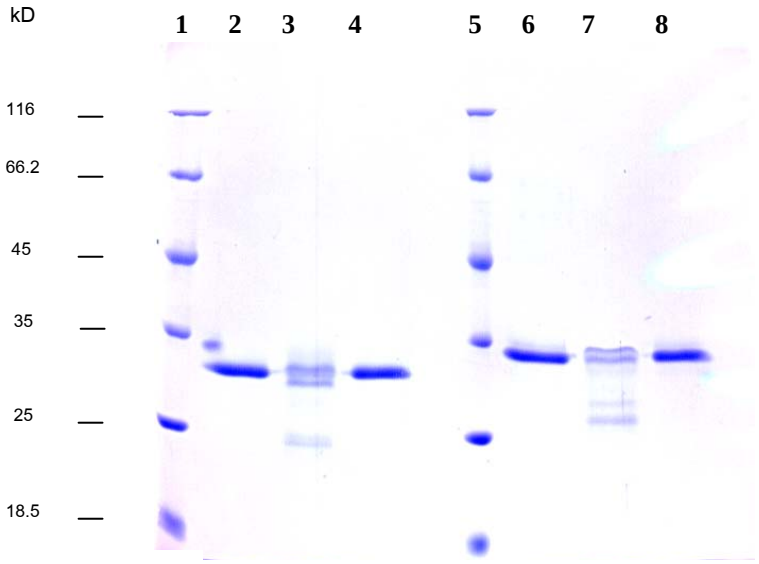

Figure 12. Pattern of purified recombinant A1 domain in the presence and absence of ristocetin. From left, lane 1 and 5: molecular weight marker; lane 2 and 6: purified recombinant A1 protein alone (6 μg); lane 3 and 7: purified recombinant A1 protein with ristocetin; lane 4 and 8: purified recombinant A1 protein with vancomycin. Lanes 1-4 are non reducing SDS-PAGE, whereas lanes 5-6 are reducing SDS-PAGE. Gel was stained with Coomassie blue.

4. Expression and purification of the mutant recombinant A1 domain of VWF from bacteria

The nucleotide sequence of mutant A1 domain gene in pQE60 vector is shown in figure 9 and 10. The insertion was in frame. The expected amino acid sequence of the mutant recombinant A1 domain from bacteria was shown in table 2. The predicted molecular weight of recombinant A1 domain is about 29 kD.

Figure 11a shows the purified A1 domain protein expressed in bacteria on SDS-PAGE and nitrocellulose membrane detected with anti-VWF. The recombinant A1 domain under nonreducing condition (figure 11b) increased electrophoretic mobility compared to that under reducing condition (figure 11a).

5. Changes of the pattern the recombinant A1 domain of VWF on SDS-PAGE in the presence and absence of ristocetin.

After 10 minute-incubation of ristocetin, the recombinant A1 domain change the pattern on SDS-PAGE from single band to several bands (figure 12) both under reducing condition and nonreducing condition**.** In addition to 3 fractions (2 fraction : approximately 30 kD and the other: approximately 26 kD), the other fraction between the lowest bands was detected under reducing condition. In contrast, vancomycin, an antibiotics used as a negative control, did not affect the pattern.

CHAPTER 4

DISCUSSION

The structure of VWF A1 domain is critical to its function as several point mutations have been found within this domain in patients with type 2 von Willebrand disease. Structural changes in the A1 domain are a topic of intense interest. In this study, the investigator has successfully cloned the A1 domain of human VWF (figure 3) from genomic DNA, rather than from mRNA. Our approach has some advantages, which can facilitate subsequent studies of the structural and functional consequences of specific polymorphisms or mutations in the VWF gene. The sequence of the recombinant A1 domain fragment was confirmed that no errors occurred during the PCR amplification (figure 4).

Recombinant proteins have been used to study structure and function of VWF A1 domain. In this study, I have cloned the A1 domain of VWF from genomic DNA, rather than mRNA. This approach has some advantages, which can facilitate subsequent studies of the structural and functional consequences of specific polymorphisms or mutations in the VWF gene. It also could be used clinically to identify patients with VWD type 2B or 2M.

It is well established that the disulfide bonded loop between C509 (1272) and C695 (1458) in the structure of VWF-A1 domain (aa 479-717 or 1242-1490) is essential for its function in terms of binding to GpIb, heparin, collagen, ristocetin and botrocetin. In addition to these two cysteine residues, there are another three cysteines which can form intermolecular bonds, causing aggregation of the expressed recombinant proteins [10-11]. To circumvent this problem, site direct mutagenesis at 1227, 1234 and 1237 was performed in order to eliminate the possibility of inclusion body formation. To circumvent this problem, site direct mutagenesis was performed in order to eliminate the possibility of inclusion body formation. The recombinant vwf-A1 plasmid was constructed using pcDNA3.1/His vector for overepression in COS-7 cells. Figure 5a and 5b show the sequences of the wild type and mutant of A1 domain that were inserted inframe. After comparison of human VWF, it shows that no errors occurred during the subcloning.

Using anti-Xpress antibody and VWF antibody, figure 6 shows that COS-7 cells expressed vwf-A1 domain. The predicted molecular weight of domain 1t Arecombinan is approximately 44.5kD which is corresponded with the detected band (approximately 50 kD), including glycosylation part. Cruz and colleagues [62] suggested that a glycosylated recombinant A1 domain is more soluble than the non-glycosylated form produced in *E. coli*. Inconsistently, the expressed A1 domain from COS-7 cells in the present study was precipitated (figure 7 and 8). One possibility is that the signal peptide for protein secretion was also inserted into the vector they used, thus making protein soluble and be able to secrete into the medium. Whereas glycosylated proteins overexpressed by COS-7 precipitated inside the cells.

The recombinant vwf-A1 plasmid was constructed using pQE60 as described earlier for overexpression in bacteria (figure 9). After comparison of human VWF, it shows that no errors occurred during the subcloning (figure 10).

From previous reports, recombinant A1 domains generated from bacteria and mammalian cell were expressed in inclusion body and precipitated [62-66]. It needed to be purified under denaturing method, and then refold. These methods could not warrant that the reducing protein would refold back into the native protein. Unlike the previous reports, the A1 domain expressed in this study did not form an inclusion body, so it was in a native condition throughout the purification process without disrupting noncovalent or disulfide bonds in the protein structure. The expressed A1 domain was soluble and was readily purified by a cation exchange chromatography, and then further cleaned up with Heparin-Sepharose column. In figure 11a, the purified protein was analyzes under reducing conditions by SDS-PAGE stained with Coomassie blue and confirmed by VWF antibody. The expressed protein migrated farther in under nonreducing condition (figure 11b), showing that it was in a native condition. Using a free on-line program, http://proteome.gs.washington.edu, the calculated molecular mass of the recombinant VWF-A1 domain is 28977. The detected bands in both reducing and nonrecducing SDS were close to the expected molecular weight. The final yield of purified protein was 2 mg/liter bacterial culture.

The investigator did not know an explanation for mechanism that what makes the protein in this study expressed in a soluble form. However, hexahistidine tag at the C-terminal side of the expressed protein may play a role. Compared to the recombinant A1

domains produced by several different laboratories [67-70], the investigator used vectors with hexahistidine tag at the C-terminal, instead of N-terminal and did not encounter a solubility problem. The recombinant A1 domain generated from a previous report [62] used the system that is similar to ours. It consists of M15[pREP4] as competent cells and pQE9 with hexahistidine tag at the N-terminal for plasmid construction. The most strikingly different physical characteristic of our recombinant A1 domain reported here was that it did not require disulfide reduction, adding urea or keeping in low pH.

The recombinant A1 domain of VWF generated in bacteria was used to study a conformational change induced by ristocetin. At first, an evaluation of digestion of A1 domain by a various proteolytic enzymes was planned. The rationale behind this technique is that a change in conformation of a protein may lead to a change in the accessibility of cleavage sites within that protein. Unexpectedly, it was found that ristocetin changed structure of the recombinant A1 domain, prior to any protease digestion on SDS-PAGE (figure 12). A group of researchers reported that ristocetin alters the patterns of digestion of human VWF by trypsin and elastase [18]. However, it is possible that VWF degradation might occur after ristocetin treatment as well since the difference in digestion pattern for VWF with and without ristocetin pre-treatment was apparent regardless of the digestion conditions used by Kang and co-worker [18]. The data from the present study provides additional evidence that changes in the structural of the A1 domain occur during the induction of platelet aggregation by ristocetin.

CHAPTER 5

CONCLSIONS

Von Willebrand factor (VWF) is a multimeric, plasma glycoprotein that plays an essential role in hemostasis and thrombosis. The A1 domain in VWF contains multiple binding sites for initiation of platelet aggregation. Structure and functions of the vwf-A1 are a topic of intense interest. Recombinant proteins have been used for studying structure and function of the A1 domain of VWF. In this study, I have cloned the A1 domain from genomic DNA, rather than from mRNA. This approach has some advantages, which can facilitate subsequent studies of the structural and functional consequences of specific polymorphisms or mutations in the VWF gene. It also could be used clinically to identify patients with VWD type 2B or 2M.

The generation of recombinant VWF-A1 domain has been encountered problems of solubility. I have achieved an optimized expression and purification of VWF-A1 domain in bacteria. Unlike from previous reports, the purified recombinant VWF-A1 domain in this study is in native conditions throughout the purification process without disrupting noncovalent or disulfide bonds in the protein structure.

The data from this study showed that structure of the recombinant A1 domain have changed after incubation of ristocetin. It provides additional evidence that changes in the structural of the A1 domain occur during the induction of platelet aggregation by ristocetin.

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