NEW DRUGS

遺伝子組換え型血液凝固第Ⅷ因子製剤（血液製剤）

ルリオクトコグ アルファ（遺伝子組換え）

rurioctocog alfa（genetical recombination）

2006年10月承認　2007年2月発売

アドベイト注射用250・500・1000（Advate）　バクスター

【原則禁忌】
次の患者には投与しないことを原則とするが、特に必要とする場合には慎重に投与すること：本剤の成分に対し、過敏症の既往歴のある患者

【効能・効果】
血液凝固第Ⅷ因子欠乏患者に対し、血漿中の血液凝固第Ⅷ因子を補い、その出血傾向を抑制する

【用法・用量】
本剤を添付の溶解液5mLで溶解し、緩徐に静脈内注射または点滴注入する。なお、10mL/分を超えない速度で注入すること。用量は、通常、1回体重1kg当たり10～30単位を投与するが、症状に応じて適宜増減する

【重大な副作用】
アナフィラキシー様症状（頻度不明）：呼吸困難、チアノーゼ、蒼白などのアナフィラキシー様症状を起こすことがあるので、観察を十分に行い、異常が認められた場合には投与を中止し、適切な処置を行うこと

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血友病Aは、X染色体上に存在する血液凝固第Ⅷ因子遺伝子の異常により、血液中の第Ⅷ因子活性の欠損あるいは低下を来す疾患である。その結果、打撲や切創で血管が傷ついたとき、その部位に止血栓を形成して出血をくい止める能力が低下し、関節内、筋肉内、皮下などに出血を繰り返す。ルリオクトコグ アルファ（遺伝子組換え）（アドベイト注射用250・500・1000）は、ヒト血液凝固第Ⅷ因子の遺伝子を組み込んだチャイニーズハムスター卵巣（CHO）細胞により産生された血液凝固第Ⅷ因子を、抗第Ⅷ因子マウスモノクローナル抗体を用いたイムノアフィニティークロマトグラフィーと、イオン交換樹脂クロマトグラフィーを用いて高度に精製した遺伝子組換え型製剤で、血友病A患者に対して血漿中の第Ⅷ因子を補い、その出血傾向を抑制する[1,2]。すでに海外では30か国以上で承認されており、2007年1月までに20億単位以上が使用され、優れた臨床効果と高い安全性が証明されている。わが国での臨床試験においても、関節内と筋肉内を中心とする170回の出血に対して本

剤が投与され、97.1％という高い有効率が観察されている[3]。

既存類似薬との比較

わが国で現在市販されている血液凝固第Ⅷ因子製剤には、本剤のほか、オクトコグ アルファ（コージネイトFS）、ルリオクトコグ アルファ（リコネイト）、乾燥濃縮人血液凝固第Ⅷ因子（クロスエイトM、コンファクトF、コンコエイトHT）がある。このうち、コージネイトFSとリコネイトがアドベイトと同じ遺伝子組換え型製剤で、残りの3製剤は、血漿中の第Ⅷ因子をさまざまな手法で純化精製した製剤である。

血液製剤によるB型肝炎ウイルス（HBV）、C型肝炎ウイルス（HCV）、ヒト免疫不全ウイルス（HIV）などへの感染という過去の不幸な出来事に対する反省の上に立ち、さまざまなウイルス不活化対策が講じられた結果、現在では血漿由来製剤においても既知の病原体の感染については安全性が担保されている。しかし、近年新たな感染症として注目されている伝達性海綿状脳症をはじめ、新興感染症に対しては必ずしも安全とは言い切れない。

一方、第Ⅷ因子を血漿から分離するのではなく、遺伝子組換え技術を用いて動物に産生させる製剤の開発研究が進み、1992年から欧米で使用されるようになり、1996年にはわが国にも導入された。しかし、初期の遺伝子組換え型製剤は、製造工程と最終工程でヒトあるいは動物由来の蛋白成分が使用されていて、病原体への感染リスクを完全に排除し得るものではなかった。

そこで、さらに安全性を高めるために、大量に添加されていたアルブミンをショ糖に置き換えた製剤（コージネイトFS）が開発され、追って製造工程中からもヒトおよびウシ由来成分を除去したプラズマ/アルブミンフリー製法による製剤が開発された。これがアドベイトである。

アドベイトの有効成分である第Ⅷ因子〔ルリオクトコグ アルファ（遺伝子組換え）〕のアミノ酸配列や物理化学的性質は、これまで使用されてきたリコネイトと同様で、両製剤を同一患者に投与したクロスオーバー試験では、体内薬物動態パラメーターに差が見られていない。止血効果に関しては、わが国で使用されている5製剤との間で比較対照試験が実施されていないので厳密には言及できないが、これまでの臨床試験と市販後調査成績を見ると、製剤間には差は見られない。なお、6製剤のなかでコンファクトFとコンコエイトHTは、有効成分として第Ⅷ因子以外にvon Willebrand因子も含有しており、von Willebrand病への適応を有している。

注意点

国内の臨床試験では、15例に対して本剤が計815回投与され、3例に4件の副作用（ほてり、頭痛、異常感、単球数増加、各1件）が認められたが、いずれも重篤なものではなく、本剤の継続に支障を来さなかった。また、海外の臨床試験（4試験）では、安全性評価対象例193例中17例に37件の副作用が認められた。主なものは、頭痛3例3件、浮動性めまい3例3件、瘙痒症2例2件、ほてり1例2件であり、国内外を通じて重篤な副作用の報告はない。

一方、すでに第Ⅷ因子製剤の投与歴がある患者（previously treated patients：PTPs）を対象とした国内外の臨床試験で、アドベイトを投与された208例中、実投与日で最長598日の投与により第Ⅷ因子インヒビターが発生したのは、海外主要試験における1例のみであった。さらに、海外におけるアドベイトの市販後安全性調査プログラムには、2006年11月時点で436例が登録され、このうち5例（2例は過去に製剤投与歴のない患者、3例はPTPs）にインヒビター発生が報告されている。以上の成績から、本剤でのインヒビター発生率は、既存の製剤のインヒビター発生率を超えるものではないと考えてよかろう。ただし、過去に第Ⅷ因子製剤投与歴がない患者については、今後は症例を積み重ねるなかで注意深く観察する必要がある。

まとめ

純度が高い濃縮製剤の開発により、血友病患者は出血の苦痛から早期に解放されるようになっただけではなく、定期的にこれらの製剤を補充することによって、スポーツを楽しむこともできるようになった。一方、血液製剤を介して多くの血友病患者がさまざまなウイルスへ感染した。この不幸な出来事を二度と繰り返さないために、血液製剤による病原体の感染を回避するための方策が講じられた。その集大成ともいうべき製剤がアドベイトであろう。

これから、利便性の改善に向けたさらに新しいタイプの第Ⅷ因子製剤の開発研究が進行していく。その先頭を切っているのが半減期を長くした製剤の研究で、半減期の長い製剤が登場すれば、患者の輸注回数が減り、さらにQOLが向上すると期待される。

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Tracing patients with lipodystrophy on a bubble chart of anti-retroviral drug usage generated by categorical principal component analysis

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1. Introduction

Since the introduction of protease inhibitors (PIs) into the field of HIV therapy, highly active antiretroviral therapy (HAART) using three or more drugs has spread rapidly around the world. In the early stages of HAART, one PI was combined with two nucleoside reverse transcriptase inhibitors (NRTIs), but with the increase of the number of new drugs, a large variety of drug combinations have been used.
The efficacy of HAART in patients with HIV is clear as summarized by Simon et al (2006). However, the incidence of lipodystrophy among these patients is increasing. It has been suspected that lipodystrophy may arise from the administration of NRTIs. Moreover, since presently there are various kinds of antiretroviral drugs as listed in the guideline by the Department of Health and Human Service of The United State (2006), the number of possible drug combination patterns is considerable. Therefore, we had analyzed the drug combination patterns in antiretroviral therapy in relation to lipodystrophy occurrence using procedures in multivariate analysis.

2. Subjects and Methods

We used the data of drugs obtained from 556 Japanese patients with coagulation disorders treated between 2000 and 2004. A brief summary of HIV-infected Japanese patients with coagulation disorders has

been given by Tatsunami et al (2002). The lipodystrophy status of these patients was obtained from surveillance data dated May 31, 2005. We used the numbers of nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) taken by each patient as the input variables. Three dichotomous variables, usage/non-usage of combined drugs, usage of only RTIs, and usage of only two drugs, were also included.
Therefore, the input data x_i for the i-th patient is as follows:

$$x_i=(n_{i1}, n_{i2}, n_{i3}, d_{i1}, d_{i2},d_{i3}) \qquad (1)$$

where n_{i1}, n_{i2}, n_{i3}, d_{i1}, d_{i2},d_{i3} represent numbers of NRTI, NNRTI, and PI taken orally by each patent. In addition, d_{i1}, d_{i2}, d_{i3} are dichotomous variables as described above.
The variables were subjected to categorical principal component analysis (CATPCA), and the patterns of drug combinations were expressed by the plotting of the object plot resulted from CATPCA. Then, in order to express the number of patents corresponding to a specific point in the plot, we expressed the result of the plotting with a bubble chart that was separated by the year of the report. The radian r_k of the k-th bubble was set to be proportional to the number of the patients included in the corresponding bubble as follows:

$$r_k=c\sqrt{\frac{n_k}{N}} \qquad (2)$$

where n_k is the number of patients in the bubble, N the total number of patients with drug report in a particular year, and c an appropriate constant.

3. Results

A total of 40 combination patterns were identified, from which we extracted four major patterns by CATPCA.
Eigen values and percentages of variance in the present model are summarized in Table 1. Dimensions 1 and 2 explained for more than 60% of the variance.

Table 1. Eigen value and percentage of variance with respect to six dimensions

Dimension	Variance Accounted	
	Total (Eigen value)	Percentage of Variance
1	2.538	42.3
2	1.242	20.7
3	1.137	18.9
4	0.822	13.7
5	0.184	3.1
6	0.078	1.3
Sum	6	100

The bubble chart resulted from the present procedure is illustrated in Figure 1 for 2000 and 2004, where the four major combination patterns are identified by symbols A, B, C and D.
We summarized the number of patients included in the four major bubbles and their percentages in Table 2.
A noticeable time-series change was the increased usage of a combined drug with two NRTIs. Use of this combination termed as D in Figure 1 and Table 2 was not observed in 2000; however, its annual fraction of use from 2001 to 2004 was 2%, 8%, 13% and 14%, respectively.
Patients with lipodystrophy in 2005 were found primarily in the largest bubble termed as A, which consisted of patients under therapy with two NRTIs and one PI. However, the number of these patients gradually shifted to the second largest bubble termed as C that included patients treated with two NRTIs and one NNRTI.
The remaining large bubble termed as B included patient treated with two NRTIs. Although the fraction of this combination dwindled from 2000 (19%) to 2004 (9%), its fraction was still not negligible in 2004.

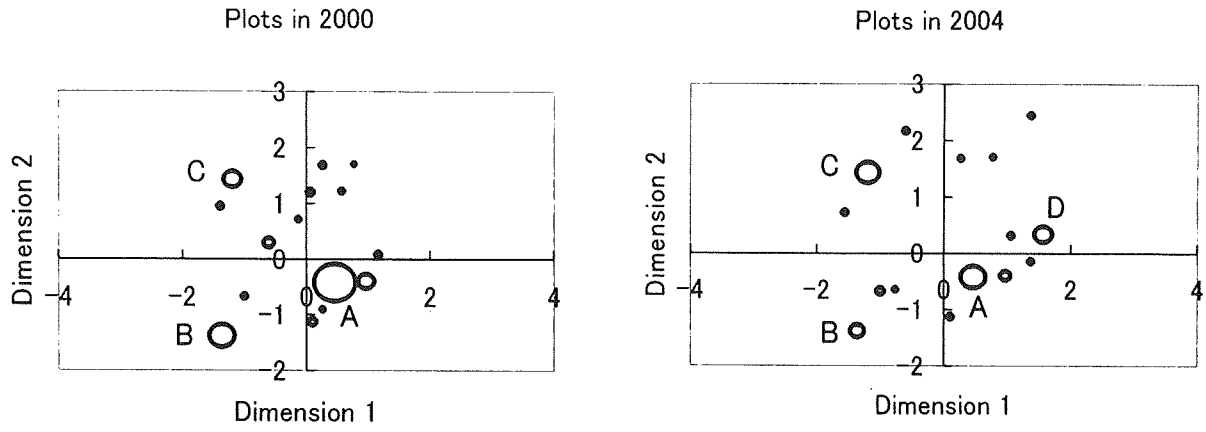

Figure 1. Plots of object points by bubble charts for 2000 (left) and 2004 (right)

Table 2. Number of patients included in the four major bubbles termed as A, B, C and D

Bubble	2000		2001		2002		2003		2004	
	Number	(%)	Number	(%)	Number	(%)	Number	(%)	Number	(%)
A	282	(50.8)	226	(44.1)	188	(38.1)	155	(31.7)	97	(28.0)
B	103	(18.6)	69	(13.5)	71	(14.4)	56	(11.5)	32	(9.2)
C	51	(9.2)	83	(16.2)	96	(19.4)	93	(19.0)	84	(24.2)
D	0	(0)	12	(2.3)	38	(7.7)	62	(12.7)	50	(14.4)

(%): Percentage divided by the total number of patients with drug report in each year from 2000 to 2004

4. Discussion

The present input variable is composed of both numbers and dichotomous variables; therefore an analytical method that can handle such mixed data as discussed by Saporta (1990) is necessary. The present application of CATPCA is meaningful as illustrated in Figures 1 and 2.

As shown in Table 1, the dimensions 1 and 2 could explain for more than 60% of the variance. Therefore, consideration of only dimensions 1 and 2 should be able to yield sufficient information. In fact, we could extract four major patterns termed as A, B, C and D from a total of 40 combination patterns.

The pattern termed as bubble A was the largest from 2000 to 2004 and it included patients treated with two NRTIs and one PI. This combination pattern is the most fundamental in HAART, therefore its high frequency of usage among Japanese patients is natural. However, its percentage decreased gradually in the course of the years surveyed. On the contrary, with the appearance of new combined drugs, the combination patterns included in the bubble D became more frequent.

The therapy with two NRTIs corresponding to bubble B is not preferred on the present point of view. However, the history of antiretroviral therapy in Japanese patients with coagulation disorders began with the era of mono-therapy using a single NRTI. Thus the size of bubble B was considerable in 2000, but has since been decreasing as shown in Table 2.

Patients with lipodystrophy in 2005 were mainly included in the largest bubble termed as A. However, over the years they had gradually moved to the second largest bubble that represented patients treated with two NRTIs and one NNRTI. The cause of this movement is not clear, although the emergence of drug resistant mutant viruses may be a factor.

There have been only three types of antiretroviral drugs so far in Japanese clinical field: NRTI, NNRTI and PI. However, a new drug based on the mechanism of HIV coreceptor inhibition had been approved in 2003 and are already in use in the United States as described by Loutfy et al (2007). In addition, the efficacy of another novel drug that inhibits HIV integrase has been verified by Markowitz et al (2006). With the elongation of the therapeutic period and the increase of the number of applicable drugs, the numerical classification of drug combination patterns will be helpful in decision making for HIV therapy.

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RESUME

The efficacy of antiretroviral therapy in patients with HIV is clear. However, the incidence of lipodystrophy among these patients is increasing. Therefore, we have analyzed the combinations of drugs used in antiretroviral therapy in relation to the occurrence of patients with lipodystrophy. Data were obtained from 556 Japanese patients with coagulation disorders treated between 2000 and 2004. The lipodystrophy status of these patients was obtained from surveillance data dated May 31, 2005. A total of 40 combination patterns were identified, and categorical principal component analysis extracted four major patterns. Patients with lipodystrophy in 2005 were found primarily in the largest bubble, which consisted of patients under therapy with two nucleoside reverstrascriptase inhibitors (NRTIs) and one protease inhibitor. However, these patients gradually moved to the second largest bubble that included patients treated with two NRTIs and one non nucleoside reverse transcriptase inhibitor. With the elongation of the therapeutic period and the increase of the number of applicable drugs, a numerical classification of drug combination patterns will be helpful in decision making for HIV therapy.

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Relationship between the Binding Sites for von Willebrand Factor, Phospholipid, and Human Factor VIII C2 Inhibitor Alloantibodies within the Factor VIII C2 Domain

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Abstract

Some factor VIII (FVIII) inhibitor alloantibodies block FVIII binding to von Willebrand factor (VWF) and phospholipid (PL) and recognize a C2 domain epitope that overlaps both binding sites. We previously showed that FVIII peptide 2315-2330 neutralized FVIII inhibitors and that Cys^{2326} and Glu^{2327} contributed to the maximum neutralizing effect. In the present study, we investigated the relationship between the essential binding sites for VWF, PL, and anti-C2 inhibitors by means of competitive-inhibition assays with overlapping synthetic peptides that span the C terminus of the C2 domain (residues 2288-2332). We identified 2 peptides (residues 2303-2317 and 2315-2330) that specifically blocked FVIII binding to VWF or PL by approximately 80% (50%-inhibitory concentration [IC_{50}], 9.0 μM) and 95% (IC_{50}, 0.12 μM), respectively. To examine in detail the residues responsible for PL binding, we prepared mutants of peptide 2315-2330 in which we sequentially substituted each residue with Gly. Two residues, Ile^{2317} and Met^{2321}, were shown to be essential for PL binding. Their substitution with Gly reduced the inhibitory effect by >90%. The data suggest that the binding sites for VWF, PL, and anti-C2 inhibitors in the C2 domain are in very close proximity but are not identical.

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Key words: Factor VIII; von Willebrand factor; Phospholipid; Binding; C2 domain; Inhibitors

1. Introduction

Factor VIII (FVIII), a plasma glycoprotein deficient or defective in individuals with the severe congenital bleeding disorder hemophilia A, functions as a cofactor in the tenase complex, which is responsible for the anionic phospholipid (PL) surface-dependent conversion of factor X to factor Xa by factor IXa [1]. FVIII is synthesized as a multidomain, single-chain molecule (A1-A2-B-A3-C1-C2) consisting of 2332 amino acid residues with a molecular mass of approximately 300 kd [2,3]. It is processed into a series of metal ion—dependent heterodimers by cleavage at the B-A3 junction, which generates a heavy chain (consisting of the A1 and A2 domains plus heterogeneous fragments of a partially proteolyzed B domain) that is linked to a light chain consisting of the A3, C1, and C2 domains [2-4].

Prior to thrombin- or factor Xa—catalyzed activation, FVIII circulates as a complex with von Willebrand factor (VWF), which protects and stabilizes FVIII [5,6]. Activation of FVIII by thrombin or factor Xa is associated with proteolytic cleavages at Arg^{372} and Arg^{740} in the heavy chain and at Arg^{1689} in the light chain [7]. The active form of FVIII (FVIIIa) is a heterotrimer consisting of the A1, A2, and A3-C1-C2 domains. FVIIIa dissociates from VWF and markedly enhances the catalytic efficiency of the tenase complex on the PL surface [8]. Thus, both VWF and PL govern the physiological function of FVIII.

Andersson and Brown [9] demonstrated that VWF interfered with FVIII binding to PL. Subsequently, investigators used preparations of recombinant C2 domain and anti-C2 monoclonal antibodies to show that VWF and PL bound directly to the C2 domain of FVIII [10,11]. Further studies using synthetic peptides in competitive-inhibition assays

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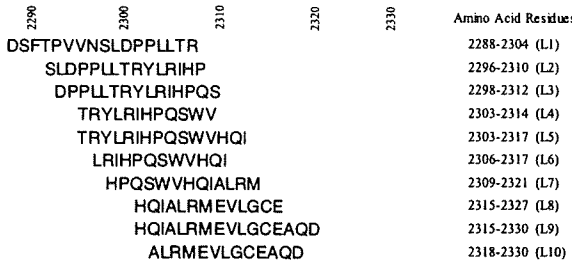

Figure 1. Schematic representation of the synthetic factor VIII (FVIII) peptides (L1-L10). The sequences of the FVIII peptides in the C terminus (residues 2288-2332) of the C2 domain are represented by their 1-letter abbreviations.

indicated that the C-terminal C2 region (residues 2303-2332) bound to VWF and PL [10,12]. All of these findings suggested that the sites of VWF and PL binding are in close proximity within the C2 domain.

The crystal structure of the C2 domain has been determined at an x-ray resolution of 1.5 Å [13]. A group of solvent-exposed hydrophobic "feet" consisting of Met^{2199}/Phe^{2220}, Val^{2223}, and Leu^{2251}/Leu^{2252}, and a ring of positively charged amino acid residues (Arg^{2215}, Arg^{2220}, Lys^{2227}, and Arg^{2320}) appear to be located behind the hydrophobic surface. These exposed hydrophobic residues in the C2 domain appear likely to contribute to PL binding [14].

FVIII inhibitors develop as alloantibodies in 20% to 30% of multitransfused patients with hemophilia A; they may also arise as autoantibodies in nonhemophilic individuals, resulting in acquired hemophilia A and a tendency for severe bleeding [15]. Inactivation of FVIII activity by these inhibitors is associated with impairment of FVIII cofactor function due to the binding of these antibodies to functionally or conformationally important epitopes in FVIII. Epitopes of this nature have been localized to one or more of the A2, C2, and A3-C1 domains [16-18]. In particular, anti-C2 inhibitors were found to prevent the interaction of FVIII with PL and VWF [11]. We previously demonstrated that C2 inhibitor epitopes, residues 2315-2330, were responsible for FVIII-neutralizing activity and that Cys^{2326} and Glu^{2327} were especially critical in this mechanism [19]. It appeared, therefore, that the epitopic region of anti-C2 inhibitors might overlap or juxtapose the binding site for VWF or PL in the C2 domain.

In the present study, we have more precisely localized the VWF- and PL-binding sites within the C2 domain and have investigated the relationship between the binding sites for VWF, PL, and anti-C2 inhibitors.

2. Materials and Methods

2.1. Reagents

Purified recombinant FVIII preparations were generous gifts from Bayer HealthCare (Berkeley, CA, USA). VWF was purified from plasma-derived FVIII/VWF concentrates (Confact F; The Chemo-Sero-Therapeutic Research Institute. Kumamoto, Japan) by gel filtration on a Sepharose CL-4B column (GE Healthcare, Uppsala, Sweden) and immunebeads coated with immobilized anti-FVIII monoclonal antibody, as has previously been reported [20]. An enzyme-linked immunosorbent assay (ELISA) did not detect FVIII antigen in the purified VWF preparation. JR8, a monoclonal antibody recognizing the A2 domain, was obtained from JR Scientific (Woodland, CA, USA). (α-Phosphatidyl-L-serine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Synthetic peptides consisting of overlapping sequences of 12 to 17 residues corresponding to the C-terminal FVIII sequence (amino acid residues 2288-2332) were prepared by Bio-Synthesis (Lewisville, TX, USA) (Figure 1).

2.2. ELISA for the Binding of FVIII to Immobilized VWF or PL

Binding of FVIII to VWF or PL was examined as previously reported [11]. In brief, VWF (40 nM) or (α-phosphatidyl-L-serine (5 μM) was immobilized onto wells of microtiter plates. After blocking with 5% human serum albumin, FVIII (1 nM) was added to the immobilized VWF or PL. Bound FVIII was detected with a biotinylated anti-A2 monoclonal antibody (JR8) and horseradish peroxidase—labeled streptavidin. To determine the inhibitory effects of the synthetic peptides, we mixed various concentrations of peptides with FVIII prior to adding it to immobilized VWF or PL. The amount of nonspecific immunoglobulin G binding in the absence of FVIII was <5% of the total signal. Specific binding was estimated by subtracting the amount of nonspecific binding.

2.3. Hydropathy Analysis

The C-terminal end of the C2 domain (residues 2288-2332) was subjected to hydropathy analysis with the ProtScale program available from the ExPASy Web site (http://expasy.org/tools/protscale.html). In brief, the program performs a Kyte-Doolittle analysis with a moving 7-residue window that continuously determines the average hydropathy as the window is advanced through the sequence. Values that are more positive represent a greater probability of hydrophilicity and surface exposure.

3. Results

3.1. Effects of C2 Synthetic Peptides on FVIII Binding to VWF

Earlier studies reported that the C terminus of the C2 domain (residues 2303-2332) contained a VWF-binding site [10]. To further localize the VWF-binding site within this region, we used ELISA to examine the inhibitory effects of 10 overlapping synthetic peptides that encompass the C terminus of the C2 domain (residues 2288-2332) on FVIII binding to VWF (Figure 2). The L5 peptide (residues 2303-2317) blocked FVIII binding to VWF by approximately 80% at the maximum concentration employed (500 μM). This inhibition was dose dependent, and the 50%-inhibitory concentration (IC_{50}) was 9.0 ± 1.7 μM (mean ± SD). However, 2 peptides (L4, residues 2303-2314; L6, residues 2306-2317)

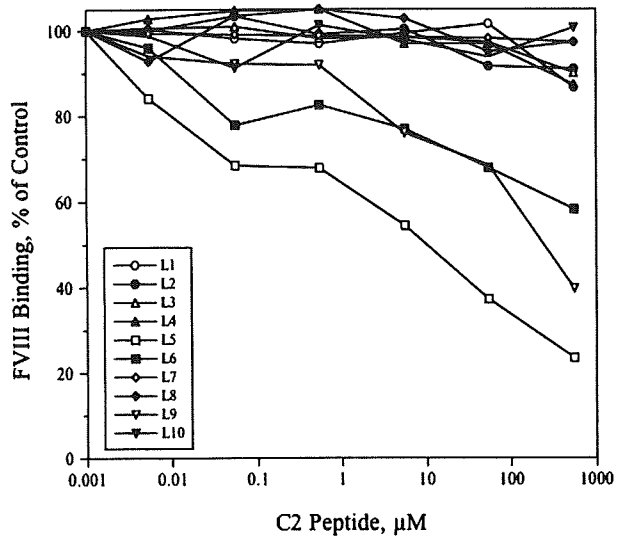

Figure 2. Effect of the C2 synthetic peptides on factor VIII (FVIII) binding to immobilized von Willebrand factor (VWF). FVIII (1 nM) was mixed with various concentrations of C2 synthetic peptide and then incubated with VWF (40 nM) immobilized onto wells of microtiter plates, as described in "Materials and Methods." Bound FVIII was detected with biotinylated anti-A2 immunoglobulin G (JR8). The absorbance values for FVIII binding to VWF in the absence of peptide represent the 100% level. The percentage of FVIII binding was plotted as a function of the C2 peptide concentration. Experiments were performed at least 3 separate times, and mean values are shown.

that had 3 residues removed from the C or the N terminus of the L5 peptide exhibited poor inhibition of this binding (approximately 10% and 40%, respectively). The L9 peptide (residues 2315-2330) also inhibited FVIII binding to VWF (by approximately 60%), but this effect was very weak, with an IC_{50} of approximately 250 μM. The other 6 peptides did not significantly inhibit binding. These data suggest that the 2303-2317 region in the C2 domain contained a VWF-interactive site.

3.2. Effects of C2 Synthetic Peptides on FVIII Binding to PL

Previous studies with synthetic peptides [12] demonstrated that the 2303-2332 region in the C terminus of the C2 domain inhibited FVIII binding to PL. To further localize the PL-interactive site within this region, we used ELISA to examine the inhibitory effects of these peptides on FVIII binding to PL (Figure 3). The L9 peptide completely blocked FVIII binding to PL at a concentration of 50 μM. This inhibition was dose dependent, and the IC_{50} value was 0.12 ± 0.02 μM. The L8 peptide (residues 2315-2327), in which 3 residues were removed from the C terminus of the L9 peptide, also completely blocked this binding in a dose-dependent manner, with an IC_{50} of 15 ± 4 μM. In contrast, the L10 peptide (residues 2318-2330), in which 3 residues were removed from the N terminus of the L9 peptide, poorly inhibited binding (approximately 45%), even at the maximum concentration. The L5 peptide, which inhibited FVIII and VWF interaction, also blocked FVIII binding to PL (by approximately 65%); however, the IC_{50} value (approximately 250 μM) was high compared with that obtained with VWF (approximately 9 μM). The other peptides did not significantly inhibit PL binding. These data suggest that the region of residues 2315 to 2330 in the C2 domain contains a PL-interactive site.

3.3. Hydropathy Analysis of the C terminus of the C2 Domain

To compare the properties of the interactive sites for VWF and PL in the C terminus of the C2 domain, we performed a hydropathy analysis of this region, as described in "Materials and Methods" (Figure 4). The results showed that the 2303-2317 sequence, which includes the VWF-interactive site, was hydrophilic and positioned on the exposed surface. In contrast, the 2315-2330 sequence, which includes the PL-interactive site, was hydrophobic and not exposed to the surface.

3.4. Effects of Individual Amino Acid Substitutions on the Ability of the L9 Peptide to Inhibit FVIII Binding to PL

Our previous findings showed that the L9 peptide (residues 2315-2330) neutralized the anti-FVIII activity of C2 inhibitor antibodies and that Cys^{2326} and Glu^{2327} in

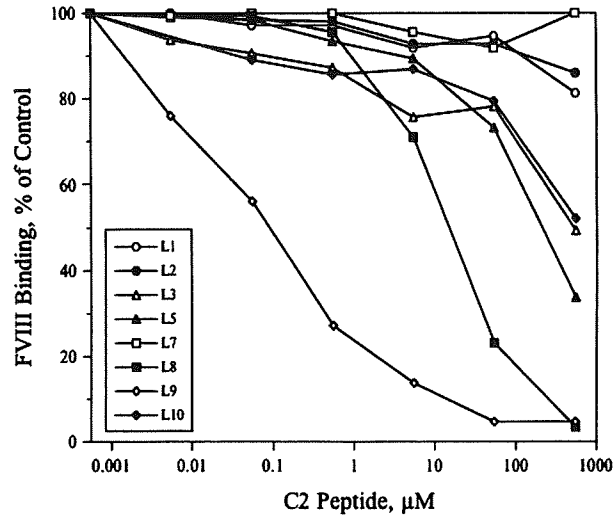

Figure 3. Effect of the C2 synthetic peptides on factor VIII (FVIII) binding to immobilized phospholipid (PL). FVIII (1 nM) was mixed with various concentrations of C2 synthetic peptides and then incubated with PL (5 μM) immobilized onto microtiter plate wells, as described in "Materials and Methods." Bound FVIII was detected with biotinylated anti-A2 immunoglobulin G (JR8). The absorbance values for FVIII binding to von Willebrand factor in the absence of peptide represent the 100% level. The percentage of FVIII binding was plotted as a function of the C2 peptide concentration. Experiments were performed at least 3 separate times, and mean values are shown.

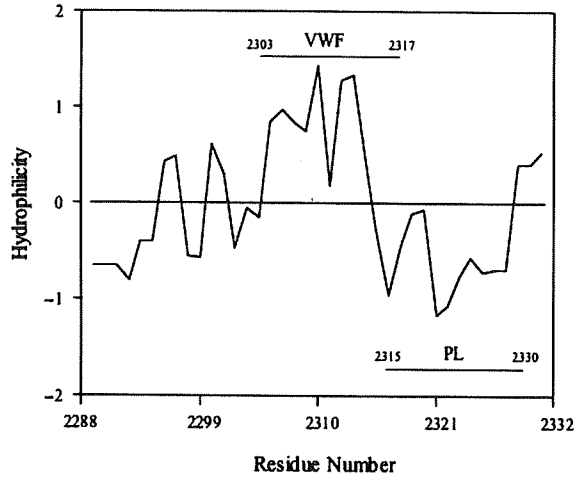

Figure 4. Hydropathy plot of the C terminus of the C2 domain. Residues 2288 to 2332 of the FVIII C2 domain were analyzed with the ProtScale program available from the ExPASy Web site (see "Materials and Methods"). A hydropathy scale from -2 (hydrophobic) to 2 (hydrophilic) was used to estimate the probability of a segment being exposed to the surface. The locations of the von Willebrand factor (VWF)- and phospholipid (PL)-binding regions obtained from the experiments with synthetic peptides are indicated by horizontal bars and residue numbers.

this sequence were required for the maximum effect [19]. Therefore, to compare the individual amino acid residues responsible for the inhibitory effect of the L9 peptide on FVIII and PL interaction, we prepared mutant peptides in which each residue in the 2315-2330 peptide (except for Gly^{2325}) was sequentially substituted with Gly. The ability of each mutant peptide (50 μM) to inhibit FVIII binding to PL was evaluated by ELISA (Figure 5). Substitution of Ile^{2317}, Leu^{2319}, Arg^{2320}, or Met^{2321} significantly reduced the capacity of the mutant peptide to inhibit FVIII binding to PL. The effect of substitution was most prominent when Ile^{2317} or Met^{2321} was replaced by Gly. In these instances, the inhibitory ability was reduced to <5%. Substitution of Leu^{2319} or Arg^{2320} reduced the inhibitory ability of the L9 peptide by approximately 40% and 50%, respectively. Increasing the concentrations of the mutant peptides to 500 μM did not affect their relative ability to inhibit FVIII binding to PL. These data suggest that the residues in the C terminus of the C2 domain responsible for PL binding are not identical to those responsible for binding of anti-C2 inhibitors.

4. Discussion

The C2 domain contains common antigenic determinants for most FVIII inhibitors. Furthermore, this domain contains the binding sites for VWF [10], PL [12], thrombin [21], and factor Xa [22]. In addition, our laboratory has demonstrated that binding of the metal ion Ca^{2+} to this domain supports the conformational structure of the light chain [23]. Thus, the C2 domain is essential for FVIII cofactor function and structure. Previous findings have also demonstrated that VWF, PL, and anti-C2 inhibitor antibodies compete for binding to the C2 domain [11], suggesting that their binding regions are in close proximity. Few reports have described their precise localizations, however. Using a panel of overlapping synthetic peptides, we previously localized the common C2 epitope for FVIII alloantibodies and monoclonal antibodies within 16 amino acid residues (2315-2330) in the C terminus of this domain [19]. We have now extended this study to examine the relationship between the VWF-binding site, the PL-binding site, and the C2 epitope.

Two major regions for VWF binding have been proposed on the basis of epitope analyses with monoclonal and polyclonal FVIII antibodies. One site appears to be located in the N-terminal highly acidic region of the A3 domain [24,25], and the other is in the C2 domain [9]. More recently, the C terminus of the A3 domain was also reported to be involved in the binding of VWF and activated protein C [26]. The precise mechanisms of these binding reactions remain unknown, however. Because the FVIII-neutralizing activity of FVIII alloantibodies with C2 epitopes was inhibited in the presence of VWF, it is possible that a region for

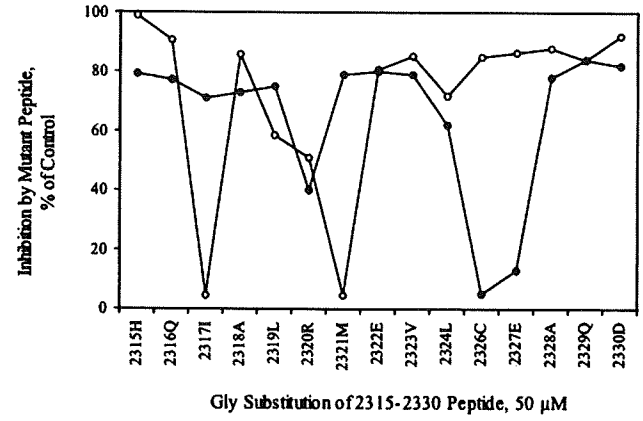

Figure 5. Effects of modified peptides on the ability of the L9 peptide to inhibit factor VIII (FVIII) binding to phospholipid (PL). Mutant L9 peptides were compared with the wild-type L9 peptide (control) for their ability to inhibit FVIII binding to PL (open circles). The horizontal axis shows the individual amino acid substituted in each mutant. The vertical axis shows the percentage of the control value: (percent inhibition of FVIII binding to PL by 50 μM mutant L9 peptide)/(percent inhibition of FVIII binding to PL by 50 μM wild-type L9 peptide) × 100. The control value for the wild-type L9 peptide was considered to be 100%. Experiments were performed at least 3 separate times, and mean values are shown. Previously published data (closed circles; see Figure 5 in [19]) are also shown to compare the effects of the substituted peptides on the ability of L9 to neutralize anti-FVIII antibody activity. The vertical axis shows the percentage of the control value: (percent residual FVIII activity in the presence of inhibitor antibody and 50 μM mutant L9 peptide)/(percent residual FVIII activity in the presence of inhibitor antibody and 50 μM wild-type L9 peptide) × 100. The mean values of 4 inhibitor antibodies are shown.

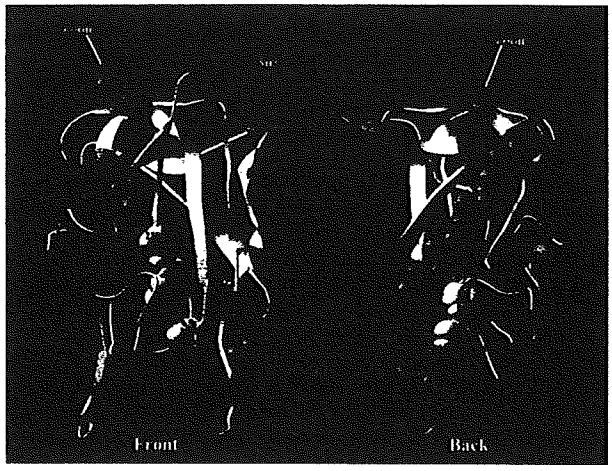

Figure 6. Binding epitopes for von Willebrand factor (VWF), phospholipid (PL), and anti-C2 inhibitor antibody on the factor VIII (FVIII) C2 domain. The FVIII C2 domain is shown in a ribbon format with β-sheets (yellow). The C2 residues responsible for VWF interaction (Thr^{2303}-Ile^{2317}) are shown in a space-filling format (green). The residues responsible for PL interaction (Ile^{2317}, Leu^{2319}, Arg^{2320}, and Met^{2321}) are shown in blue. The residues responsible for neutralizing the inhibitory activity of anti-C2 inhibitor antibody (Cys^{2326} and Glu^{2327}) are shown in pink.

VWF binding within the C2 domain is conformationally associated with the C2 epitope. Our binding experiments with overlapping synthetic peptides demonstrated that the L5 peptide (residues 2303-2317) inhibited FVIII binding to VWF (IC_{50}, 9 μM), suggesting that this region contained residues essential for VWF binding. Another 2 peptides, L4 (residues 2303-2314) and L6 (residues 2306-2317), in which the L5 peptide was truncated by 3 amino acid residues at the C terminus and the N terminus, respectively, failed to inhibit this binding (IC_{50}, >500 μM). Furthermore, peptides L2, L3, and L7, in which Thr-Arg-Tyr (residues 2303-2305) or His-Gln-Ile (residues 2315-2317) were deleted, did not inhibit this binding effectively. These data support the view that the interaction of VWF with the C terminus of the FVIII C2 domain requires conformationally stable 2303-2317 sequences. The inhibitory effect of the 2303-2317 peptide was not complete, however, suggesting the presence of an alternative VWF binding site. In this context, a reference to point mutations in the hemophilia A database and studies using mutagenesis [27] indicated that the C1-C2 marginal region containing Arg^{2150} is required for VWF binding. It seems likely, therefore, that both terminal portions of the C2 domain are juxtaposed spatially, that this juxtaposition is mediated by a disulfide bond between Cys^{2174} and Cys^{2326} [13,28], and that VWF binds to distinct sites in both terminal portions of the C2 domain.

The association of FVIII and VWF is based on a noncovalent electrostatic interaction. Hence, FVIII is readily dissociated from VWF at high cation concentrations [29]. Hydropathy analysis showed that the peptide region of VWF binding contains hydrophilic residues, and considering that the N-terminal A3 domain also contains highly acidic amino acid residues [24,25], VWF binding may require hydrophilic residues exposed on the surface of the FVIII molecule. The association of FVIII and VWF is restricted in the presence of PL [9], and the assembly of the tenase complex on membrane surfaces is enhanced in these circumstances. Foster et al [12] previously demonstrated that a peptide corresponding to residues 2303 to 2332 competes with FVIII for binding to PL. In our studies, the L9 peptide (residues 2315-2330) inhibited FVIII binding to PL (IC_{50}, 0.12 μM), confirming that this region contains a PL-binding site. Similarly, the L10 peptide (residues 2318-2330) completely inhibited PL binding, although the IC_{50} value was higher (15 μM). In addition, the L5 peptide (residues 2303-2317), which inhibited VWF binding, also weakly inhibited PL binding (IC_{50}, approximately 250 μM). In contrast, the L8 peptide (residues 2315-2327) failed to inhibit PL binding (IC_{50}, >500 μM). These data suggested that residues 2315 to 2317 (His-Gln-Ile) contributed significantly to PL interactions. Furthermore, the studies with mutant peptides in which individual amino acids were substituted with Gly revealed that Ile^{2317}, Leu^{2319}, Arg^{2320}, and Met^{2321} in the C terminus of the C2 domain were closely associated with PL binding.

The crystal structure of the C2 domain has been shown to consist of a β-sandwich core with 2 β-sheet turns and an adjacent loop, which display a group of solvent-exposed hydrophobic "feet" consisting of Met^{2199}/Phe^{2220}, Val^{2223}, and Leu^{2251}/Leu^{2252} [13]. A ring of positively charged amino acid residues (Arg^{2215}, Arg^{2220}, Lys^{2227}, and Arg^{2320}) was located behind the hydrophobic surface. These exposed hydrophobic and surrounding positively charged residues appeared likely to contribute to membrane binding. Our current data are in keeping with these proposals and suggest that Arg^{2320} and the surrounding residues (Ile^{2317}, Leu^{2319}, and Met^{2321}) located in the C-terminal portion of the C2 domain are essential for PL binding. Our data further indicate that the VWF-binding site and the PL-binding site are in very close proximity in the C2 domain but are not identical. Interestingly, the binding sites for VWF and PL are hydrophilic and hydrophobic, respectively, and comparisons of the amino acid sequences for human, porcine, murine, and canine FVIII molecules (http://europium.csc.mrc.ac.uk) indicate that the residues of both the 2303-2317 and 2315-2330 sequences are well conserved.

In summary, we have successfully identified precise regions of PL and VWF binding within the C2 domain of FVIII. Given the crystal structure of the C2 domain [13], the results of our peptide experiments demonstrate that the binding sites for PL, VWF, and anti-C2 inhibitors are in very close proximity (Figure 6). The results of competitive-inhibition studies can be explained by this close relationship within the C-terminal portion of the C2 domain.

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bjh research paper

Identification of a thrombin-interactive site within the FVIII A2 domain that is responsible for the cleavage at Arg^{372}

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Summary

FVIII is activated by cleavage at Arg^{372}, Arg^{740}, and Arg^{1689} by thrombin. This study showed that an anti-A2 monoclonal antibody, with a specific epitope for residues 484–509, and anti-FVIII inhibitor alloantibodies with similar A2 epitopes, inhibited thrombin-catalyzed FVIII activation. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis showed that cleavage at Arg^{372} but not at Arg^{740} occurred at approximately fourfold decreased rate in the presence of anti-A2 antibody. Peptide 484–509 also inhibited co-factor activation, consistent with inhibition of cleavage at Arg^{372}. Direct binding studies using active-site modified thrombin showed that a 484–509 peptide as well as the anti-A2 antibodies blocked the A2-thrombin binding. Furthermore, covalent cross-linking was observed between the 484–509 peptide and thrombin following reaction with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Mutant A2 molecules in which the clustered basic residues in this sequence were converted to alanine were used to assess the binding reactions in a surface plasmon resonance-based assay. Mutants R484A, R489A, R490A, H497A and K499A possessed two to fivefold lower affinity than wild-type A2. These findings demonstrate that clustered basic residues within the 484–509 region of the A2 domain play a part of key role in thrombin-binding, which is responsible for thrombin-catalyzed FVIII activation by cleavage at Arg^{372}.

Keywords: FVIII, A2 domain, thrombin, binding-site, cleavage.

FVIII, a plasma protein deficient or defective in individuals with the severe congenital bleeding disorder, haemophilia A, functions as a co-factor in the tenase complex responsible for anionic phospholipids surface-dependent conversion of FX to activated FX (FXa) by activated FIX (FIXa) (Mann *et al*, 1990). FVIII circulates as a complex with von Willebrand factor (VWF), which protects and stabilizes the co-factor. FVIII is synthesized as a multidomain, single chain molecule (A1-A2-B-A3-C1-C2) consisting of 2332 amino acid residues with a molecular mass of approximately 300 kDa (Vehar *et al*, 1984; Wood *et al*, 1984). It is processed into a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain consisting of the A1 and A2 domains, plus heterogeneous fragments of a partially proteolyzed B domain, linked to a light chain consisting of the A3, C1, and C2 domains.

The serine proteinases thrombin and FXa function as positive feedback amplifiers of the coagulation cascade through the specific cleavage of FVIII (Eaton *et al*, 1986). Both enzymes convert the pro-co-factor to the active co-factor, FVIIIa, by limited proteolysis. Cleavages occur in the heavy chain at Arg^{372}, separating the A1-A2 domains, and at Arg^{740} at the A2-B domain junction, generating 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa FVIII light chain is cleaved near its N-terminus at Arg^{1689} to release a 40-acidic residue-rich fragment generating a 70-kDa A3C1C2 subunit (Eaton *et al*, 1986). Mutational analysis and examination of the haemophilia A database indicate that proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa activity (Fay, 2004). However, the requirement for cleavage at Arg^{740} for co-factor activity is controversial. Cleavage at Arg^{372} site exposes a functional FIXa-interactive site within the A2 subunit that is cryptic in the unactivated molecule (Fay *et al*, 2001). Cleavage at the Arg^{1689} site liberates the co-factor from its carrier protein, VWF (Lollar *et al*, 1988), and contributes

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to the overall specific activity of the co-factor (Regan & Fay, 1995).

Thrombin recognizes macromolecular substrates and co-factors through either or both of two anion-binding exosites (ABE), exosites I (ABE-I) and II (ABE-II), characterized by a high density of solvent-exposed basic residues that are more sterically remote from the catalytic site. ABE-I binds to fibrinogen (Binnie & Lord, 1993), the acidic residue-rich tail of the potent thrombin inhibitor, hirudin (Stone *et al*, 1987), and many other proteins. ABE-II is primarily the heparin-binding exosite (Sheehan & Sadler, 1994). Both exosites are also involved in the interaction with FVIII. ABE-I appears to be essential for FVIII activation through cleavage at Arg^{372} and Arg^{1689}. ABE-II seems to have a more limited role in facilitating cleavage at Arg^{372} and Arg^{740} (Myles *et al*, 2002).

Limited information is available on thrombin-interactive sites in FVIII, however. We have previously demonstrated that one such site promotes cleavage at Arg^{1689} and is located within the C2 domain in the FVIII light chain, and that the A2 domain also contains a thrombin-interactive site (Nogami *et al*, 2000). More recently, it has been demonstrated that the acidic region, comprising residues 389–394 in the A2 domain, interacts with thrombin via the heparin-binding exosite (ABE-II) and supports cleavage at Arg^{740} during pro-co-factor activation (Nogami *et al*, 2005a). However, a thrombin-interactive site responsible for cleavage at Arg^{372} has not been previously identified. This study examined the interaction of thrombin with the FVIII A2 domain using a combination of anti-FVIII antibodies, a synthetic peptide, and recombinant A2 mutants. Our results indicate for the first time that clustered basic residues within the 484–509 region in the A2 domain, are responsible, at least in part, for interaction with thrombin and cleavage at Arg^{372}.

Materials and methods

Reagents

Purified recombinant FVIII preparations were generous gifts from Bayer Corp. Japan (Osaka, Japan). The heavy chain subunit was isolated following chromatography on SP- and Q-Sepharose columns (Amersham BioScience, Uppsala, Sweden). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the isolated subunits, followed by staining with GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA), showed >95% purity. A monoclonal antibody 413 (mAb413), which is specific for the 484–509 residue in the A2 domain, was obtained as previously described (Fay *et al*, 2001). A second anti-A2 mAb, mAbJR8, was obtained from JR Scientific Inc. (Woodland, CA, USA). Two anti-FVIII inhibitor alloantibodies (alloAbs; cases 1 and 2) were obtained from multitransfused Japanese patients with severe haemophilia A. Antibody epitopes were localized within the A2 domain by immunoblot analysis using isolated FVIII fragments. The inhibitor titres of these two cases were 57 and 118 Bethesda U/mg respectively. IgG fractions were prepared using protein A Sepharose (Amersham). $F(ab')_2$ fragments were prepared using immobilized pepsin-Sepharose (Pierce). Human alpha-thrombin, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Sigma, St Louis, MO, USA), hirudin (Calbiochem, SanDiego, CA, USA), horseradish peroxidase-labelled streptavidin (Chemicon, Borona, Victoria, Australia), anti-thrombin mAb (Antibodyshop A/S, Gentofte, Denmark), and goat antimouse peroxidase-linked secondary antibody (MP Biomedicals, Aurora, OH, USA) were purchased commercially. An active-site modified, anhydro-thrombin (Ah-thrombin), was prepared as previously described (Nogami *et al*, 2000). Biotinylated mAbJR8 was prepared using *N*-hydroxysuccinimido-biotin reagent (Pierce). The synthetic peptides corresponding to FVIII A2 residues 484–509 and 373–395 without or with biotin was prepared by BioSynthesis (Lewisville, TX, USA).

Recombinant FVIII A2 molecules

Recombinant wild-type A2 domain (wt-A2) and a set of mutants were constructed and expressed using the Bac-to-Bac baculovirus systems as previously described (Sarafanov *et al*, 2006). The mutated residues, predominantly targeting positively charged residues located on the surface of the A2 domain, were Arg^{484}, Tyr^{487}, Ser^{488}, Arg^{489}, Arg^{490}, Leu^{491}, Lys^{493}, Lys^{496}, His^{497}, Lys^{499} and Lys^{510}. Each selected residue was replaced by alanine. The A2 expression cassette was assembled on the basis of an MHGX vector and subcloned into a pFastBac1 vector. The chimeric gene encoded a polypeptide, six His tag and FXa cleavage site at the N-terminus. The protein was expressed in Sf9 cells and purified by affinity chromatography using CNBr-activated Sepharose 4B with immobilized anti-A2 mAb. The eluates were mixed with FXa and treated with Xarrest agarose (Novagen, Madison, WI, USA). The resultant A2 was >90% pure as judged by SDS-PAGE and Western blotting. The A2 proteins were quickly frozen at −80°C.

Clotting assay

FVIIIa activity was measured at 37°C in a one-stage clotting assay using FVIII-deficient plasma. Thrombin-catalyzed activation of FVIII was assessed in HEPES-buffered saline (HBS) buffer (20 mmol/l HEPES, pH 7·2, 0·1 mol/l NaCl, 5 mmol/l $CaCl_2$, and 0·01% Tween 20) containing 0·01% bovine serum albumin (BSA). Samples were removed from the mixtures at the indicated times, and thrombin reaction was rapidly terminated by the addition of hirudin (1 U/ml) and approximately 10 000-fold dilution. This was followed immediately by the assay for FVIII activity. The presence of thrombin and hirudin in the diluted samples did not affect measurements of FVIII in this coagulation assay.

Cleavage of isolated heavy chain subunit by thrombin

Human thrombin was added to the isolated heavy chain subunit in a 1:50 molar ratio in HBS buffer at 37°C. Samples

were taken at indicated times and the reactions were immediately terminated and prepared for SDS-PAGE by adding SDS and boiling for 3 min.

Cross-linking with EDC

Variable concentrations of biotinylated 484–509 peptides were mixed with a fixed concentration of thrombin (200 nmol/l) and incubated with 300 μmol/l EDC at pH 7·2 at 22°C for 45 min. Following this cross-linking protocol, the samples were subjected to SDS-PAGE and immunoblotting. Reactions were terminated by addition of SDS electrophoresis sample buffer and boiling.

Electrophoresis and Western blotting

SDS-PAGE was performed using 8% gels at 150 V for 1 h. For Western blotting, the proteins were transferred to a polyvinylidene difluoride membrane at 50 V for 2 h. Proteins were probed using biotinylated anti-A2 mAbJR8 or anti-thrombin mAb, followed by horseradish peroxidase-labelled streptavidin or peroxidase-linked secondary antibody respectively. The signals were detected using enhanced chemiluminescence (Perkin-Elmer Life Science, Boston, MA, USA). Densitometry scans were quantitated using Image J 1.34 (National Institute of Health, Bethesda, MD, USA).

Kinetics measurement using real-time biomolecular interaction analysis

The kinetics of A2 subunit-thrombin interactions were determined by a surface resonance plasmon (SPR)-based assay using a BIAcore X instrument (Biacore AB, Uppsala, Sweden; Nogami *et al*, 2000). Ah-thrombin was covalently coupled to a CM5 chip surface at a density of 6 ng/mm^2. Association of the ligand was monitored in 10 mmol/l HEPES, pH 7·4, 0·15 mol/l NaCl, 0·005% polysorbate 20, at a flow rate of 15 μl/min for 5 min. Dissociation of bound ligand was recorded over 5 min by replacing the ligand-containing buffer with buffer alone. Nonspecific binding corresponding to ligand binding to the uncoated chip was subtracted from the signal. Reactions were run at 37°C. The rate constants for association (k_{ass}) and dissociation (k_{diss}) were determined by nonlinear regression analysis using the evaluation software provided by Biacore AB. Dissociation constants (K_d) were calculated as k_{diss}/k_{ass}.

Binding assay by enzyme-linked immunosorbent assay (ELISA) using immobilized Ah-thrombin

Microtitre wells were coated with 50 μl of Ah-thrombin (100 nmol/l) in 20 mmol/l Tris and 0·15 mol/l NaCl, pH 7·4, at 4°C overnight. The wells were washed with phosphate-buffered saline (PBS) containing 0·02% Tween 20, followed by blocking with PBS containing 5% BSA for 2 h at 37°C. A2 subunit was added in HBS buffer containing 1% BSA and incubated for 2 h at 37°C. Biotinylated anti-A2 mAbJR8 (1 μg) was added and bound IgG was detected by the addition of horseradish peroxidase-labelled streptavidin using the substrate *O*-phenylenediamine dihydrochloride. Reactions were stopped by the addition of 2 mol/l H_2SO_4, and the absorbance was measured at 492 nm. The amount of nonspecific binding of biotinylated IgG observed in the absence of the A2 subunit was <5% of the total signal, and the amount of specific binding was obtained by subtracting the amount of nonspecific binding of biotinylated IgG.

Data analysis

All experiments were performed on at least three separate occasions, and the average values are shown. Nonlinear least squares regression analysis was performed by Kaleidagraph (Synergy, Reading, PA, USA). The interactions between the A2 subunit and thrombin in the ELISA were analysed using a single-site binding model equation 1:

$$\text{Absorbance} = \frac{A_{max}[S]}{K_d + [S]} \quad (1)$$

where [S] is the A2 subunit, K_d is the dissociation constant, and A_{max} represents maximum absorbance signal when the site is saturated by A2.

Data from experiments assessing the A2 peptide-dependent inhibition of Ah-thrombin interaction with A2 were fitted by nonlinear least squares regression using equation 2:

$$\%\ \text{binding} = \frac{B_{max}[A2]}{K_d[1 + (L/K_i)] + [A2]} + C \quad (2)$$

where L represents the concentration of Ah-thrombin or A2 peptide; B_{max} represents maximum binding; K_d is the dissociation constant for the A2 and Ah-thrombin interaction; K_i is the apparent inhibition constant for L; and C is a constant for A2-Ah-thrombin binging that was unaffected by L.

Results

Effect of anti-FVIII alloAbs with A2 epitopes on thrombin-catalyzed activation of FVIII and cleavage of heavy chain

We have previously reported that the A2 domain as well as the C2 domain in FVIII contains thrombin-interactive sites (Nogami *et al*, 2000). The present study investigated whether this thrombin interaction in the A2 domain was functionally responsible for FVIII activation by the protease. In initial tests, we examined the effects of two anti-FVIII alloAbs on thrombin-catalyzed FVIII activation. In general, the recognizing domains (epitopes) of anti-FVIII alloAbs are localized to one or more of the A2, C2, or A3C1 domains. In the current study, however, immunoblotting and ELISA demonstrated that the crucial epitopes of two alloAbs were localized within the A2 domain, not within the A1 or A3C1C2 domains (data not shown). Activation of FVIII (100 nmol/l) by thrombin

(2 nmol/l) in the presence of two alloAbs was evaluated by measurement of FVIIIa activity using a one-stage clotting assay. Control experiments showed that the presence of alloAbs, thrombin, or hirudin did not affect the assay, probably the results of the approximately 10 000-fold dilution of the reaction mixture prior to FVIIIa activity determination. Thrombin-catalyzed activation of FVIII was inhibited by the addition of anti-A2 alloAbs in dose-dependent manners (Fig 1A). The peak values of FVIIIa activity in cases 1 and 2 (at 250 nmol/l) were approximately 70% and approximately 50%, respectively, of that obtained in the presence of control IgG.

Upregulation of FVIII activity is predominantly related to proteolytic cleavage at Arg^{372} of the A1-A2 domain junction (Fay *et al*, 2001; Nogami *et al*, 2005b). We hypothesized, therefore, that the inhibitory effects of two anti-A2 alloAbs on FVIII activity might be due to inhibition of the thrombin-catalyzed heavy chain cleavage at Arg^{372}. To investigate this, SDS-PAGE was employed to visualize the effects of alloAbs on thrombin-catalyzed cleavage of FVIII heavy chain. Isolated heavy chain was used as a substrate to eliminate any contribution of light chain to the association with thrombin. Heavy chain (100 nmol/l), free of detectable light chain (data not shown), was preincubated with anti-A2 alloAbs (250 nmol/l) for 2 h, followed by reaction with thrombin (2 nmol/l). Figure 1B shows the time-course of heavy chain cleavage, analysed by Western blotting using biotinylated anti-A2 mAbJR8. Compared with the heavy chain cleavage in the presence of normal IgG (panel a), the alloAb from case 1 (panel b) did not affect the cleavage at the A2-B junction (Arg^{740}), whilst cleavage at the A1-A2 junction (Arg^{372}) was mildly diminished. The alloAb from case 2 (panel c) slightly diminished the cleavage at Arg^{740}, whilst cleavage at Arg^{372} was significantly delayed. Examination of the ratio of the A2 product/A1-A2 substrate by scanning densitometry showed that the rate of cleavage at Arg^{372} in both instances were

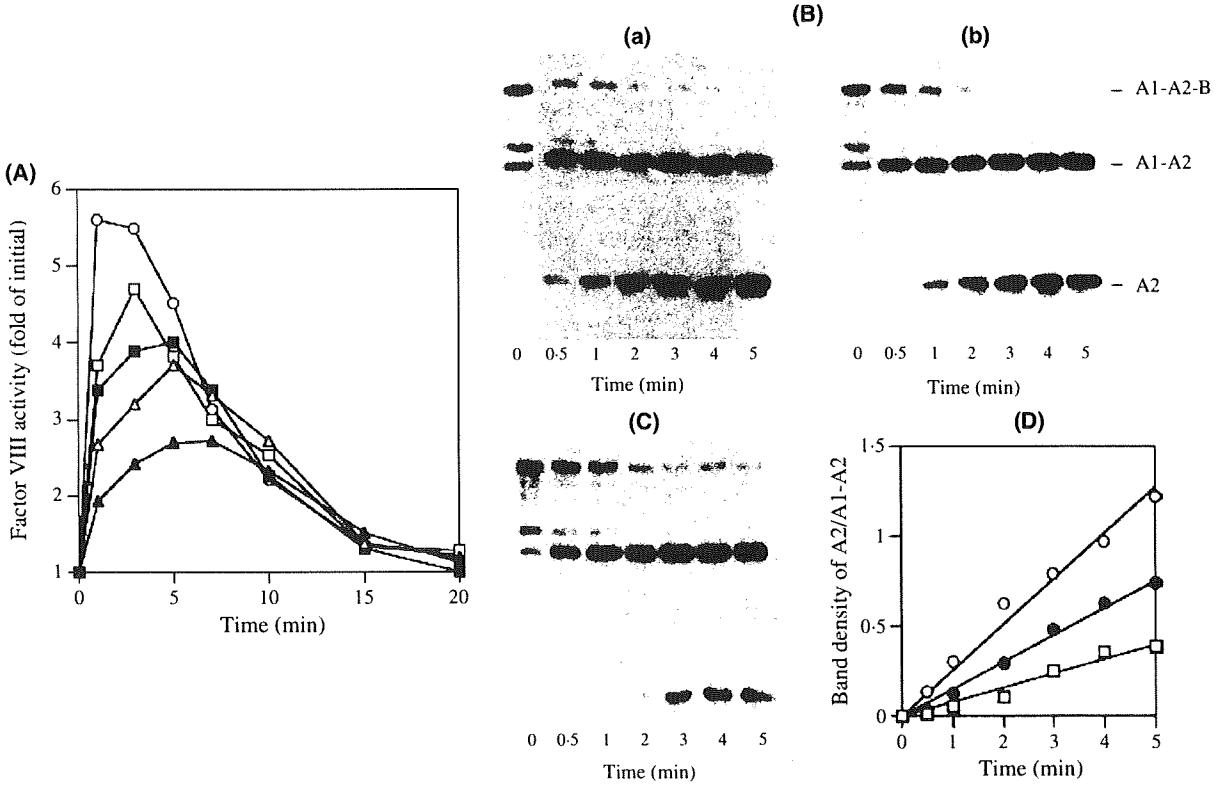

Fig 1. Effect of anti-A2 inhibitor alloAbs on thrombin-catalyzed activation and cleavage of FVIII – (A) *Activation of FVIII*: FVIII (100 nmol/l) preincubated with the anti-A2 inhibitor alloAb IgG $F(ab')_2$ for case 1 (100 nmol/l; *open squares*, 250 nmol/l; *closed squares*) and for case 2 (100 nmol/l; *open triangles*, 250 nmol/l; *closed triangles*) or normal IgG $F(ab')_2$ (control; *open circles*) for 2 h, was reacted with thrombin (2 nmol/l). FVIIIa activity was measured at the indicated times using a one-stage clotting assay. The initial activity of FVIII at time zero was approximately 30 U/ml. The values of FVIIIa activity were plotted as a function of incubation time. (B) *Cleavage of the heavy chain of FVIII*: Isolated heavy chain (100 nmol/l) was preincubated with the anti-A2 alloAbs $F(ab')_2$ [250 nmol/l; control (panel a), case 1 (panel b), or case 2 (panel c)] for 2 h and was mixed with thrombin (2 nmol/l) at the indicated time. Samples were analysed by 8% SDS-PAGE followed by Western blotting using biotinylated anti-A2 mAbJR8. Panel d shows the ratio of A2/A1-A2 subunit derived from the Western blotting data (panels a–c) by quantitative densitometry. The symbol used are; *open circles*: normal IgG, *closed circles*: case 1, and *open squares*: case 2. The data were fitted to a straight line.

approximately 60% and approximately 35%, respectively, of that obtained by the control IgG (panel d). The difference in cleavage velocity at Arg^{372} between two alloAbs appeared to reflect the results of thrombin-catalyzed activation of the pro-co-factor observed above (see Fig 1A). These results support that anti-A2 alloAbs inhibit thrombin-catalyzed FVIII activation because of inhibition of cleavage at Arg^{372} in the heavy chain.

Effect of anti-A2 mAb413 on thrombin-catalyzed cleavage of the FVIII heavy chain

Epitopes of polyclonal anti-A2 alloAbs are generally localized within residues 484–509 of FVIII (Healey *et al*, 1995). We therefore examined the thrombin-catalyzed activation of FVIII in the presence of mAb413 sharing the same epitope. Activation of FVIII (100 nmol/l) by thrombin (2 nmol/l) was evaluated in the presence of increasing concentrations of mAb413. Although it is known that mAb413 inhibits the FVIII(a)–FIXa interaction (Fay & Scandella, 1999), the presence of this antibody in the diluted samples did not affect this assay (data not shown). The mAb413 demonstrated a dose-dependent inhibition of thrombin-catalyzed activation of FVIII (Fig 2A). At the maximum concentration employed (240 nmol/l), the peak value of FVIIIa activity was reduced by approximately 50%, and the time increment to reach this value was delayed compared with that of control. In contrast, the other anti-A2 mAbJR8, which does not recognize the same epitope, did not inhibit the thrombin-catalyzed activation at the maximum concentration employed (240 nmol/l).

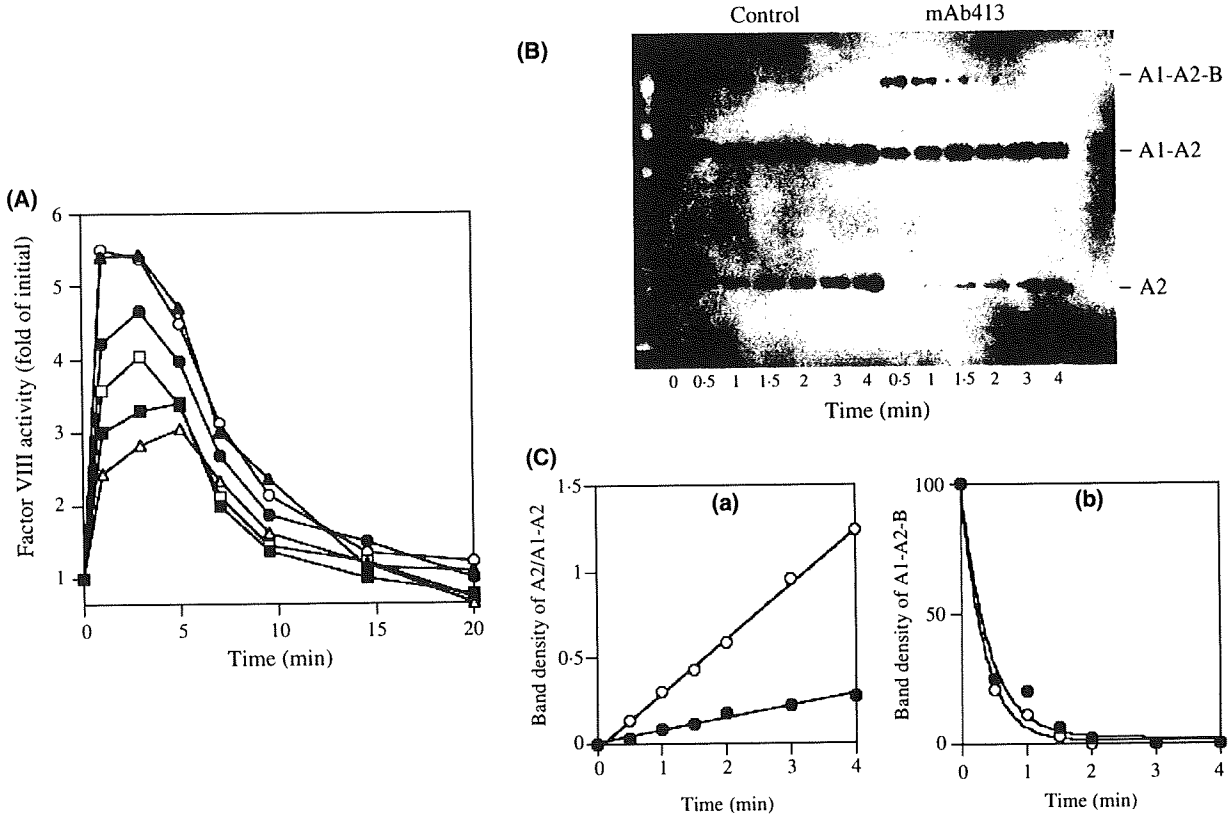

Fig 2. Effect of anti-A2 mAb413 on the activation and cleavage of FVIII by thrombin – (A) *Activation of FVIII*: FVIII (100 nmol/l) was preincubated with various concentrations of mAb413 $F(ab')_2$ (0 nmol/l, *open circles*; 30 nmol/l, *closed circles*; 60 nmol/l, *open squares*; 120 nmol/l, *closed squares*; 240 nmol/l, *open triangles*) or mAbJR8 (240 nmol/l, *closed triangles*) for 2 h, and was mixed with thrombin (2 nmol/l). FVIIIa activity was measured at the indicated times using a one-stage clotting assay. The initial activity of FVIII at time zero was approximately 30 U/ml. The values of FVIIIa activity were plotted as a function of incubation time. (B) *Cleavage of the heavy chain of FVIII*: Isolated heavy chain (100 nmol/l) was preincubated with mAb413 $F(ab')_2$ or normal IgG $F(ab')_2$ (250 nmol/l) for 2 h and mixed with thrombin (2 nmol/l) at the indicated times. Samples were analysed by 8% SDS-PAGE followed by Western blotting using biotinylated anti-A2 mAbJR8. *Panel* C shows (panel a) the ratio of A2/A1-A2 and (panel b) the change of the A1-A2-B subunit derived from the Western blotting data by quantitative densitometry. Density values of the A1-A2-B subunit without thrombin were used to represent the 100% level. The symbols used are; *open circles*: normal IgG, and *closed circles*: mAb413. The data were fitted to a straight line (panel a) and to an exponential decay (panel b).

These effects of mAb413 were further examined by SDS-PAGE. Figure 2B shows the time-course of heavy chain cleavage analysed by Western blotting using a biotinylated mAbJR8. The mAb413 did not significantly affect the cleavage at Arg^{740}, but cleavage at Arg^{372} was markedly diminished. This observation was in keeping with those of anti-A2 alloAbs. Examination of the ratio of the A2/A1-A2 subunit by scanning densitometry suggested that the rate of cleavage at Arg^{372} in the presence of mAb413 was reduced by approximately fourfold compared with the control (Fig 2C, panel a), consistent with the effect of mAb413 on thrombin-catalyzed activation of the pro-co-factor. In contrast, the rate of cleavage at Arg^{740} in the presence of antibody was not significantly different from the control ($2{\cdot}5 \pm 0{\cdot}4$ and $2{\cdot}9 \pm 0{\cdot}3$ min respectively). These results indicate that the region comprising the anti-A2 mAb epitope is likely to contain with the thrombin-interactive site(s) that affects the cleavage at Arg^{372} in the heavy chain.

Effect of synthetic peptide 484–509 on thrombin-catalyzed cleavage of FVIII

To exclude the possibility that this inhibition of thrombin-catalyzed activation resulted from the conformational steric hindrance of mAb413, a same competitive assay was developed using a synthetic peptide corresponding to residues 484–509, the recognizing epitope for mAb413. Using reaction conditions of FVIII (100 nmol/l) and thrombin (2 nmol/l), maximal FVIIIa activity was evaluated in the presence of increasing concentrations of A2 peptide. Control experiments showed that the presence of peptide did not affect this assay (data not shown). The 484–509 peptide showed a dose-dependent inhibition of thrombin-catalyzed activation of FVIII with up to approximately 65% inhibition observed (400 μmol/l; Fig 3A). Furthermore, the time to reach maximal FVIIIa activity in the presence of peptide was delayed dose-dependently compared with that of absence of peptide (data not shown). The inhibitory effect by the 484–509 peptide was mild compared to that by 373–395 peptide, a thrombin-binding site responsible for the cleavage at Arg^{740}.

The effect of this peptide on the cleavage of heavy chain by thrombin was further examined by Western blotting. Isolated heavy chain (100 nmol/l) mixed with various concentrations of the peptide was reacted with thrombin (2 nmol/l) for 2 min. Figure 3B shows the results from Western blotting of the cleavage reaction (upper panels) and from band densitometry of A2 product (lower panel). The 484–509 peptide blocked the cleavage at Arg^{372} by approximately 70% at the

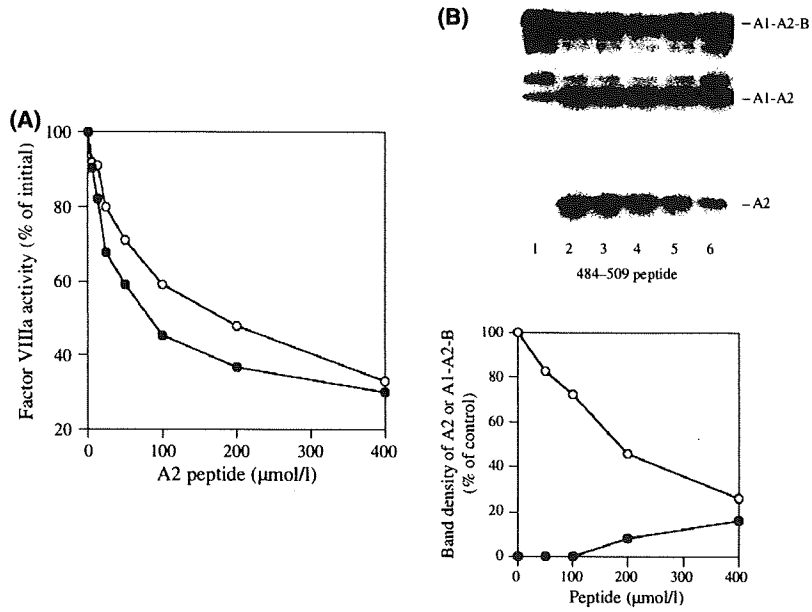

Fig 3. Effect of 484–509 peptide on FVIII activation and cleavage by thrombin – (A) *Activation of FVIII*: FVIII (100 nmol/l) was mixed with various concentrations of 484–509 peptide (*open circles*) or 373–395 peptide (*closed circles*) (0, 6, 12·5, 25, 50, 100, 200, and 400 μmol/l respectively), followed by incubation with thrombin (2 nmol/l). FVIIIa activity was measured at the indicated times using a one-stage clotting assay. The peak level of FVIIIa activity in the absence of peptide (100% level) was approximately 160 U/ml, representing an approximately sixfold increase of initial activity. The percentage of peak level of FVIIIa activity was plotted as a function of peptide concentration. (B) *Cleavage of the heavy chain of FVIII*: (*upper panel*) Isolated heavy chain (100 nmol/l) mixed with various concentrations of 484–509 peptide was reacted with thrombin (2 nmol/l) for 2 min. Samples were analysed by 8% SDS-PAGE followed by Western blotting using anti-A2 mAbJR8. *Lane 1* shows the intact heavy chain. *Lanes 2–6* show the cleavage of heavy chain in the presence of 484–509 peptide (0, 50, 100, 200, and 400 μmol/l respectively). Lower panel shows quantitative densitometry of the A2 (*open circles*) and A1-A2-B (*closed circles*) subunit derived from the Western blotting data (upper panel). Density value of A2 subunit generated by thrombin cleavage in the absence of peptide or the A1-A2-B without thrombin was used to represent the 100% level.

maximum concentration employed (400 μmol/l) and the inhibition was observed in a dose-dependent manner, consistent with that of inhibition of thrombin-catalyzed activation. However, the cleavage at Arg^{740} by thrombin was not significantly affected (by approximately 20%), compared with that at Arg^{372}. Furthermore, thrombin-catalyzed cleavage of the heavy chain using intact FVIII as a substrate was similarly blocked by this peptide, whilst the cleavage of the light chain was little affected (data not shown). These data support that the 484–509 region in A2 subunit contributes to a specific interactive-site for thrombin necessary to facilitate cleavage of the heavy chain.

Inhibitory effect of mAb413 or the 484–509 peptide on the A2 interaction with Ah-thrombin

The above data supported the hypothesis that residues 484–509 within the A2 contributed to thrombin interactions. To confirm that the inhibitory activity of mAb413 resulted from its blocking the region involved in thrombin binding, an ELISA-based assay was employed to examine the effect of the antibody on the binding of A2 to immobilized Ah-thrombin, a catalytically inactive derivative of thrombin. Binding assays using inactivated serine proteases, prepared by converting serine residues in the active site to dehydroalanine, have been already established (Nogami *et al*, 1999, 2000). Recombinant wt-A2 domain was prepared using a baculovirus expression system (Sarafanov *et al*, 2006) to completely eliminate any contribution of other subunits in the interaction with thrombin. The wt-A2 domain bound to immobilized Ah-thrombin in a dose-dependent manner (Fig 4A). The data could be comparatively well fitted by a single-site binding model, with a modest apparent K_d value (57·4 ± 11·0 nmol/l). To confirm the specificity of this binding, various concentrations of Ah-thrombin were preincubated with the wt-A2 (80 nmol/l) in the fluid phase, prior to addition to the immobilized Ah-thrombin. Ah-thrombin completely blocked the A2 binding to Ah-thrombin (Fig 4A, inset). The apparent K_i value (62·1 ± 10·5 nmol/l) was consistent with the value obtained from direct binding, confirming the specificity of this assay.

To evaluate the inhibitory effect of mAb413, the wt-A2 domain (80 nmol/l) was preincubated with increasing concentrations of antibody, prior to addition to the immobilized Ah-thrombin (Fig 4B). The mAb413 blocked A2 binding to Ah-thrombin in a dose-dependent manner by approximately 45% at the maximum concentration employed (500 nmol/l), similar to its inhibitory effect on thrombin activation of FVIII. The results showed that mAb413 blocked thrombin-catalyzed activation of FVIII in a similar manner to the anti-A2 alloAbs, by directly inhibiting A2 binding to the protease, and that the epitope of mAb413 (residues 484–509) shared sequences with the thrombin-interactive site in A2. To assess the specificity of the 484–509 region for the thrombin interaction, we examined the effect of the 484–509 peptide on A2-thrombin interaction (Fig 4B). The 484–509 peptide inhibited this binding in a dose-dependent manner. Inhibition was approximately 35% at the maximum concentration employed (400 μmol/l), and the apparent K_i value was 105 ± 22 μmol/l. A control experiment

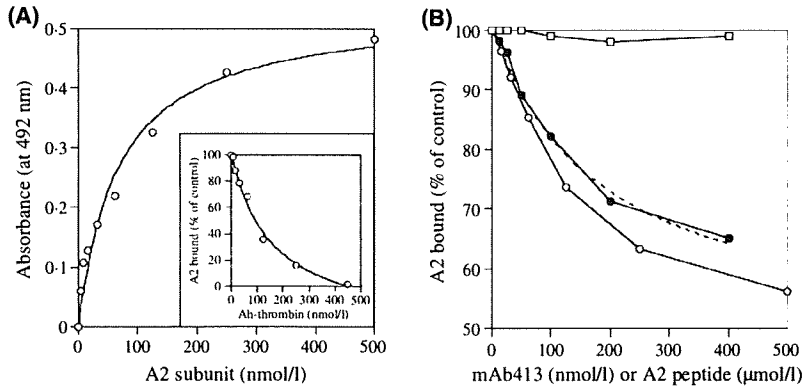

Fig 4. ELISA for the A2 subunit binding to immobilized Ah-thrombin – (A) *Recombinant wt-A2 binding to Ah-thrombin*: Various concentrations of recombinant wt-A2 domain were reacted with Ah-thrombin (100 nmol/l) that had been immobilized onto microtitre wells. Bound A2 was detected using biotinylated anti-A2 mAbJR8. Absorbance values were plotted as a function of the concentration of A2 domain, and data were fitted using equation 1 according to a single-site binding model described in Materials and methods. (inset) The mixtures of various concentrations of Ah-thrombin and wt-A2 (80 nmol/l) were incubated with immobilized Ah-thrombin (100 nmol/l). Bound A2 was detected using biotinylated mAbJR8. The absorbance value corresponding to A2 binding to Ah-thrombin in the absence of competitor was defined as 100%. The percentage of A2 binding was plotted as a function of Ah-thrombin concentration, and the plotted data were fitted by nonlinear least squares regression according to equation 2. (B) Inhibition *of anti-A2 mAb413 and the 484–509 peptide on the A2 and Ah-thrombin binding*: Recombinant wt-A2 (80 nmol/l) was preincubated with various concentrations of mAb413 $F(ab')_2$ (*open circles*), A2 peptide 484–509 (*closed circles*), or peptide $484/489/490/497/499Ala^{484-509}$ (*open squares*) for 1 h and then incubated with immobilized Ah-thrombin (100 nmol/l). Bound A2 was detected using biotinylated mAbJR8. The absorbance value corresponding to A2 binding to Ah-thrombin in the absence of competitor was defined as 100%. The percentage of A2 binding was plotted as a function of the mAb413 or A2 peptide concentration, and the plotted data were fitted (dashed line) by nonlinear least squares regression according to equation 2.

