Epitope mapping by cDNA expression of a monoclonal antibody which inhibits the binding of von Willebrand Factor to platelet glycoprotein IIb/IIIa

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In order to study the structure–function relationship of von Willebrand Factor (vWF), we have located the epitope of a well-characterized monoclonal antibody (MAb) to vWF (MAb 9). This MAb reacts with the C-terminal portion of the vWF subunit, SPII fragment [amino acids (aa) 1366–2050], which includes an Arg-Gly-Asp (RGD) sequence at positions 1744–1746, and totally inhibits vWF and SPII binding to platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa). A recombinant DNA library was constructed by cloning small (250–500 nucleotides) vWF cDNA fragments into the λ gt11 vector and these inserts were expressed as fusion proteins with β -galactosidase. Immunological screening of the library with ^{125}I -MAb 9 identified three immunoreactive clones. vWF inserts were amplified by the PCR and their sequences demonstrated overlapping nucleotides from positions 7630 to 7855 of vWF cDNA, coding for aa residues 1698–1773 of the mature subunit, indicating that this is the epitope of MAb 9. vWF- β -galactosidase fusion protein reacted with ^{125}I -MAb 9 by Western blotting. In a solid-phase radioimmunoassay, the purified fusion proteins decreased the binding of vWF to ^{125}I -MAb 9 by 50 %, and this inhibition was dose-dependent between 3.5 and 120 nM. Therefore the epitope of MAb 9 is located within aa 1698–1773 of the vWF subunit, which includes the RGD sequence implicated in the binding of adhesive proteins of GPIIb/IIIa.

INTRODUCTION

von Willebrand factor (vWF) is a glycoprotein composed of a series of multimers (500–15000 kDa) [1]. The vWF gene spans 178 kb, contains 52 exons [2] and is located on chromosome 12. vWF mRNA is 8.8 kb in length and encodes a 2813-amino-acid (aa) precursor protein (pre-pro-vWF) composed of a 22 aa signal peptide, a 741 aa pro-polypeptide (von Willebrand antigen II; vWAgII) and the mature vWF subunit (2050 aa) [3].

vWF plays a dual role in haemostasis. First, it serves as the carrier for Factor VIII, stabilizing its activity [4]. Secondly, it promotes platelet adhesion to the subendothelium of the injured vessel wall [5] by binding to specific receptors on the platelet membrane, glycoprotein Ib (GPIb) [6,7] and glycoprotein IIb/IIIa complex (GPIIb/IIIa) [8,9].

Limited proteolytic degradation of purified vWF has allowed the identification of several functional domains [10–12]. The binding domain of vWF to GPIIb/IIIa is located on the C-terminal portion of the subunit between aa 1366 and 2050 (SPII fragment) [12] and includes an Arg-Gly-Asp (RGD) sequence (aa 1744–1746) common to adhesive proteins [13], which serves as recognition signal for the integrin GPIIb/IIIa.

Monoclonal antibodies (MAbs) specific for vWF have provided a useful tool to analyse the structure–function relationship of the protein [14]. Among a series of MAbs produced and characterized in our laboratory, MAb 9 has been shown to be a potent inhibitor of vWF binding to GPIIb/IIIa [15]. In order to demonstrate that the epitope recognized by MAb 9 is located in the C-terminal part of vWF subunit and includes the RGD sequence, we have screened with this MAb a λ gt11 expression library containing small fragments of vWF cDNA expressed as fusion proteins with β -galactosidase. The epitope of this MAb was mapped to an RGD-containing sequence corresponding to Met-1698–Val-1773 of the mature vWF subunit.

MATERIALS AND METHODS

Construction of the library of vWF cDNA fragments in λ gt11

A full-length human vWF cDNA was constructed and cloned into the plasmid vector pTZ18 (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described [16]. vWF cDNA (30 μ g) was disrupted by sonication (12 $\times$ 10 s) in ice with a Branson Sonifier B15 ultrasonic probe (Technofix, Paris, France) to generate small random DNA fragments. The size distribution of the fragments (200–600 bp) was estimated by 1 % agarose gel electrophoresis. The recovered DNA fragments were phenol-extracted, ethanol-precipitated, redissolved in 10 mM-Tris base (pH 8)/1 mM-EDTA (TE 10/1) and end-repaired using DNA polymerase I (Klenow fragment) (Boehringer–Mannheim, Meylan, France) in the presence of 2 mM-dNTPs. DNA (1 μ g) was ligated to phosphorylated *Eco*RI linkers (5' pGGAATTCGCG 3') using the Amersham λ gt11 cloning kit (Amersham International, Amersham, Bucks., U.K.). Digestion by *Eco*RI, removal of unligated linkers, ligation of DNA fragments to phosphatase-treated λ gt11 arms and packaging of the ligated material were performed according to the manufacturer's instructions. The library contained 1.6×10^6 clones and was used for screening without amplification.

Production of antibodies to vWF

A polyclonal antiserum (Ab 44) produced in rabbits by immunization with purified human vWF was prepared and rendered monospecific by immunoadsorption of contaminants with plasma from a patient with severe von Willebrand disease as reported previously [12].

MAbs to human vWF were produced and characterized as previously described [14]. MAb 9 is directed towards the C-terminal part of the vWF subunit, SPII fragment [12]. This MAb specifically inhibits the binding of vWF or SPII to GPIIb/IIIa

Abbreviations used: vWF, von Willebrand Factor; MAb, monoclonal antibody; aa, amino acid; GPIb, glycoprotein Ib; GPIIb/IIIa, glycoprotein IIb/IIIa; IPTG, isopropyl β -D-thiogalactopyranoside; PMSF, phenylmethanesulphonyl fluoride.

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[15]. MAb B200 was used as a control and is directed towards the central part of the vWF subunit. It reacts with SPI fragment (aa 911–1365) and with reduced vWF, and specifically inhibits the binding of vWF to collagen [17]. Polyclonal and monoclonal antibodies were used as purified IgG [12] (1–3 mg/ml). IgG (100 μ g) was labelled with 125 I by the Iodogen method [18] to a specific radioactivity of 2 μ Ci/ μ g.

Immunoscreening of the vWF recombinant library

The library was screened according to Young *et al.* [19]. Approx. 15×10^3 clones were plated on 80 mm-diameter Petri dishes containing a lawn of *Escherichia coli* Y1090 and incubated at 42 °C. As soon as the plaques became visible (after about 4 h), vWF- β -galactosidase fusion protein synthesis was induced by overlaying the plates with nitrocellulose filters impregnated with 10 mM-isopropyl β -D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, MO, U.S.A.) and incubated overnight at 37 °C. The filters were washed for 3 times for 10 min in TBS (50 mM-Tris/HCl, pH 7.5, 150 mM-NaCl), and stored for 6 h in TBS containing 3% BSA (Boehringer). The filters were incubated overnight at 22 °C in TBS containing the 125 I-labelled antibody (10^6 c.p.m./ml) and washed in TBS containing 0.1% Tween 100. The dried filters were autoradiographed with intensifying screens at -80 °C for 16 h. Positive phages, reacting with the labelled antibody, were picked, replated at lower density and rescreened until only immunoreactive phages were obtained.

Sequence analysis

The vWF cDNA inserts present in the vWF- β -galactosidase fusion proteins were directly amplified by the PCR [20] after elution of the phages for 30 min at 37 °C with 200 μ l of water from a single immunoreactive clone purified as described above. Commercial synthetic oligonucleotide primers (New England Biolabs, Beverly, MA, U.S.A.) were complementary to λ gt11 sequences adjacent to the *Eco*RI cloning site. The sequence of the forward primer was 5' GGTGGCGACGACTCCTGGAGCCCG 3', and that of the reverse primer was 5' TTGACACCAGACCAACTGGTAATG 3'. Plaque-eluted DNA (30 μ l) was incubated with each primer (10 pmol) and Taq polymerase (2 units) (Amersham) in a 100 μ l reaction volume containing each dNTP (0.2 mM) and PCR buffer (50 mM-KCl, 10 mM-Tris/HCl, pH 8.3, 1.5 mM-MgCl₂). PCR conditions included a 2 min denaturation step at 94 °C, a 2 min annealing step at 55 °C and a 2 min extension step at 72 °C, before a final elongation step of 10 min at 72 °C. Amplifications consisted of 25 cycles using a DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT, U.S.A.). Amplified DNA insert sizes were determined by electrophoresis on 1.5% agarose gels. PCR products were digested with *Eco*RI and ligated to sequencing vector M13mp18 (50 ng) (New England Biolabs), digested with *Eco*RI and phosphatase-treated. The DNA sequence of each insert was determined for both strands by dideoxy chain termination reactions using the Sequenase Kit (US Biochemical Co, Cleveland, OH, U.S.A.) in the presence of [α - 35 S]dATP (Amersham) [21].

Characterization and purification of the fusion proteins

vWF- β -galactosidase fusion proteins from antibody-positive clones were prepared from lysogenic cultures in *E. coli* Y1089 as described by Huynh *et al.* [22]. Following growth in L broth with ampicillin (100 μ g/ml) at 30 °C to an absorbance of 0.5 at 600 nm, λ phages lysogens were induced by incubation for 15 min at 42 °C followed by addition of 10 mM-IPTG and further incubation for 1 h at 37 °C. Cells were harvested by centrifugation (1000 g, 10 min) resuspended in 0.1 vol. of TE/0.5 M-NaCl/0.1% β -mercaptoethanol in the presence of 1 mM-phenylmethane-

sulphonyl fluoride (PMSF) (Sigma), lysed by incubation for 30 min at 0 °C with 0.2 mg of lysozyme/ml (Sigma), frozen for 30 min at -80 °C and sonicated (5 $\times$ 20 sec).

The crude bacterial lysates containing the vWF- β -galactosidase fusion proteins were analysed by electrophoresis on 0.1% SDS/7%-PAGE according to Laemmli [23]. The gels were stained with 0.05% Coomassie Blue or transferred on to nitrocellulose paper (Schleicher and Schull, Dassel, Germany [24], incubated overnight with 125 I-labelled MAb 9 (10^6 c.p.m./ml), extensively washed with 50 mM-Tris base (pH 7.5)/150 mM-NaCl, dried and autoradiographed over three to four nights.

vWF- β -galactosidase fusion proteins were purified from crude *E. coli* lysates by passage over a monoclonal anti- β -galactosidase antibody (Sigma) coupled to Sepharose 4B beads (Pharmacia), followed by elution of bound material using 0.1 M-glycine, pH 2.2. Purified fusion proteins were characterized by 0.1% SDS/7%-PAGE using standard methods [23].

Purification of vWF, SPII and SPIII fragments

Human vWF was purified from Factor VIII/vWF concentrates (donated by the Centre National de Transfusion Sanguine, Paris, France) by gel filtration on Sepharose CL4B (Pharmacia) and characterized as previously described [12]. SPII and SPIII (aa 1–1365) fragments were purified as already described [12].

Competitive binding assays

A solid-phase radioimmunoassay was used to assess competitive binding of purified vWF, SPII fragment, SPIII fragment or vWF- β -galactosidase fusion proteins to MAb 9. Purified vWF was coated overnight at 4 °C on to wells of microtitration plates at a final concentration of 10 μ g/ml in 0.1 M-sodium carbonate, pH 9.6. Residual binding sites were blocked by addition for 1 h at 37 °C of 1% BSA in TBS. Purified vWF- β -galactosidase fusion proteins, SPII or SPIII fragments (3.5–120 nM) were incubated in duplicate with 125 I-MAb 9 (15×10^3 c.p.m.) in 1% BSA. After 1 h at 37 °C, the mixtures were transferred to the vWF-coated polystyrene plates and incubated overnight at 37 °C. 125 I-MAb 9 bound to vWF was measured. No inhibition is defined as the amount of 125 I-MAb 9 bound in the absence of fusion protein or SPII and SPIII fragments.

Fig. 1. Agarose (1.5%) gel electrophoresis of amplified positive clones recognized by MAb 9

Each positive clone (lane 1, 176a; lane 2, 176b; lane 3, 178) was amplified by PCR, as described in the Materials and methods section, directly from pure immunoreactive phages with primers located in the λ gt11 phage near the *Eco*RI cloning site of the vWF cDNA fragment. $\Phi \times 174$ molecular size markers (lane M) were from Pharmacia; bp = base pair.

RESULTS

Immunological screening of the library of vWF cDNA fragments and characterization of the immunoreactive phages

Initial immunological screening of the λ gt11 library of recombinant phages containing 200–600 bp fragments of vWF cDNA was performed with a polyclonal labelled anti-vWF antibody, Ab 44. About 450 clear positive signals were identified out of 5×10^4 clones screened. Phages from the wild-type λ gt11 expressing intact β -galactosidase with no vWF cDNA insert, used as negative control, exhibited no reactivity with ^{125}I -Ab 44. When these positive clones were rescreened with ^{125}I -MAB 9, three clones (176a, 176b and 178) were immunoreactive, and

Fig. 2. MAb 9 epitope localization

The full-length vWF cDNA represented at the top spans 8.8 kb and codes for a cleavable signal peptide (SP) (22 aa), a propeptide called vWAgII (741 aa) and the native vWF subunit (2050 aa). The black rectangle at the 3' end corresponds to the portion of vWF cDNA between nt 7499 and 7979 and is enlarged in the lower part of the figure. The relative positions of cDNA inserts of the clones 176a, 176b and 178 picked from the λ gt11 library containing small random vWF cDNA fragments identified with MAB 9 are shown. The three vWF cDNA fragments contained in the clones were sequenced in both directions. The minimum overlap of the three clones is represented by the shaded area and corresponds to nt 7630–7855 coding for Met-1698–Val-1773 in the vWF subunit and represents the epitope for MAB 9. DNA sequence nt numbering is according to Bonthron *et al.* [3]. Aa numbering starts with Ser⁻¹ of the mature vWF subunit. Individual clone designation is listed at the left.

these clones were subsequently screened an additional three times to obtain purified clones. Following amplification by PCR, the vWF cDNA inserts from these three immunoreactive phages ranged in size from about 300 to 400 bp, as analysed by 1.5% agarose gel electrophoresis (Fig. 1).

An alignment of the sequences of the three clones is shown in Fig. 2. The clones had sizes of 304 bp (176a), 356 bp (176b) and 412 bp (178) respectively. The clones spanned nt 7499–7979 of vWF cDNA with a minimum overlap from nt 7630 to 7855 (Fig. 2). Therefore the antigenic site recognized by MAB 9 is included in a 75 aa sequence between residues Met-1698 and Val-1773 of the vWF subunit.

Characterization of the vWF- β -galactosidase fusion proteins

λ phage lysogens of clones 176a, 176b and 178 isolated with MAB 9 and of clone 13a identified with control MAB B200 to vWF were obtained as described in the Materials and methods section. vWF- β -galactosidase fusion proteins from the crude bacterial extracts had the expected sizes of 122, 124 and 126 kDa following 0.1% SDS/7%-PAGE (Fig. 3a). The control vWF- β -galactosidase fusion protein isolated with MAB B200 had a molecular mass of 135 kDa (Fig. 3a). Western blotting analysis with ^{125}I -MAB 9 confirmed that immunoreactivity was only observed with the fusion proteins from clones 176a, 176b and 178, and not from the control clone (Fig. 3b).

Purified vWF- β -galactosidase fusion proteins were analysed by 0.1% SDS/7%-PAGE. After elution of the bound material only one band, of molecular mass 122, 124 or 126 kDa, was observed for each fusion protein reacting with MAB 9, and one band of 135 kDa for the control fusion protein (Fig. 3c).

Competitive binding assays to MAB 9 were performed with purified vWF (10 $\mu\text{g}/\text{ml}$), vWF- β -galactosidase fusion proteins expressed from clones 176a and 178, or SPII and SPIII fragments (3.5–120 nM). Fusion protein from clone 176b was not available in sufficient amounts due to its low level of expression. Fusion proteins 176a and 178 inhibited the binding of ^{125}I -MAB 9 to vWF in a dose-dependent manner. At a concentration of 120 nM, 42 and 51% inhibition were observed respectively (Fig. 4). At the same concentration, SPII fragment demonstrated 65% inhibition of binding of ^{125}I -MAB 9 to purified vWF, whereas the control vWF- β -galactosidase fusion protein (13a) and SPIII fragment demonstrated no inhibition.

Fig. 3. Analysis and purification of vWF- β -galactosidase fusion proteins

In all cases the clones used were 13a (lane 1), 176a (lane 2), 176b (lane 3) and 178 (lane 4). (a) Coomassie Blue staining after 0.1% SDS/7%-PAGE of crude bacterial extracts prepared as described in the Materials and methods section from λ phage lysogens of immunoreactive clones 176a, 176b and 178 reacting with MAB 9 and a control clone (13a) isolated with MAB B200. Arrows indicate the positions of the expressed vWF- β -galactosidase fusion proteins. (b) Western blotting of vWF- β -galactosidase fusion proteins from clones 176a, 176b, 178 and 13a electrophoresed as in (a). The gel was electrotransferred to nitrocellulose and the filter was incubated with ^{125}I -MAB 9 (1×10^6 c.p.m./ml), dried and autoradiographed for 72 h. (c) Coomassie Blue staining after 0.1% SDS/7%-PAGE of vWF- β -galactosidase fusion proteins 176a, 176b, 178 and control 13a bound to a monoclonal anti- β -galactosidase antibody coupled to Sepharose beads as described in the Materials and methods section. After elution of the bound material with 0.1 M-glycine, pH 2.2, the eluted fusion proteins were electrophoresed as described in (a).

Fig. 4. Competitive inhibition of ^{125}I -MAB 9 binding to vWF by purified vWF- β -galactosidase fusion proteins, and SPII and SPIII fragments

Competitive binding was performed by the addition, to vWF-coated wells of microtitration plates, of ^{125}I -labelled MAB (15×10^3 c.p.m.) and preincubation for 1 h at 37°C with various amounts (3.5–120 nm) of vWF- β -galactosidase fusion protein from clones 176a, 178 and control clone 13a and SPII or SPIII fragments. The control clone 13a was a vWF- β -galactosidase fusion protein isolated with MAB B200. Inhibition (%) represents the decrease in bound c.p.m. in the presence of the added proteins relative to bound c.p.m. with purified vWF alone. Results are the means of two separate experiments in duplicate.

DISCUSSION

Epitope mapping of MABs to vWF which inhibit one of its activities is useful to clarify the importance of the corresponding functional domains. Previous studies have generally located the epitopes for these MABs by interaction with large fragments of the vWF subunit [12] or by competition between antibodies [25]. As an alternative to the use of synthetic peptides, random fragments of cDNA expressed as fusion proteins in the $\lambda\text{gt}11$ vector offer a powerful method to further define MAB epitopes. Despite the absence of post-translational processing, this recombinant DNA strategy has been successfully applied to locate antigenic determinants of proteins involved in haemostasis, and linear epitopes for Factor VIII [26, 27], Factor IX [28, 29] and vWF [30] have previously been defined as short linear sequences of about 20 aa.

MAB 9 is a well-characterized MAB to vWF which specifically inhibits binding to GPIIb/IIIa [15]. The localization of its epitope on the vWF subunit is important to improve our understanding of the structure–function relationship of this complex adhesive protein. By screening with MAB 9 a recombinant library in $\lambda\text{gt}11$ containing small fragments of the human vWF cDNA fused to β -galactosidase, three positive clones were isolated. Sequencing of the clones demonstrated that the minimal overlap of the three reactive peptides was from nucleotides 7630 to 7855, which code for Met-1698 to Val-1773 of vWF subunit, a region of 75 amino acid residues including the RGD sequence. This localization is not as well defined as that of epitopes of other MABs which have been shown to correspond to shorter sequences using the same methodology [26, 28, 30]. This may be due to the low number of positive clones or to the weak reactivity of MAB 9 with reduced vWF. Alternatively, it is possible that a longer sequence is necessary for MAB 9 to inhibit vWF binding to GPIIb/IIIa by involving residues that are juxtaposed in the folded protein but distant in terms of the primary amino acid sequence.

To confirm that the clones reacted specifically with MAB 9, the vWF inserts were expressed from the lysogenic phages as fusion

proteins with β -galactosidase. These fusion proteins specifically reacted with MAB 9 by immunoblotting and, following purification, competed with vWF for binding to MAB 9. The lack of total inhibition observed with the vWF- β -galactosidase fusion proteins may be due to: (1) the possibility that another region of vWF is involved in the MAB 9 epitope; (2) a difference in the conformation of vWF as a result of incomplete post-translational processing in *E. coli*; (3) the presence of β -galactosidase, which may interfere with the tertiary folding of the vWF fragment; or (4) the relatively low concentration of the vWF fragment as compared with that of β -galactosidase. In favour of the last hypothesis, we showed that SPII fragment at the highest concentration used for the vWF fragment in the fusion protein demonstrated only a partial inhibition (65%) of vWF binding to MAB 9, and a 10-fold higher concentration was required to block more than 85% of this binding. In addition, the presence of β -galactosidase may modify the folding of the protein and thus explain the small difference in inhibition (about 15%) by SPII and the recombinant vWF fragments.

The site of binding of vWF to GPIIb/IIIa is known to be on the C-terminal part of vWF subunit, between residues 1366 and 2050 (SPII fragment) [12] and to include at residues 1744–1746 the RGD sequence which is common to several adhesive proteins [13], including fibrinogen, fibronectin and vitronectin, and which is considered to be the recognition site of the GPIIb/IIIa receptor. The importance of this RGD sequence in the binding of vWF to GPIIb/IIIa has been demonstrated by Berliner *et al.* [31], who generated polyclonal and monoclonal antibodies, directed against synthetic peptides including the RGD sequence (Glu-1737–Ser-1750) of vWF, which inhibited its binding to GPIIb/IIIa. These authors demonstrated that the specificity of the interaction of the RGD sequence was conferred by its adjacent residues [31]. In addition to the RGD sequence, however, the possibility that, similar to the case with fibronectin [32], another specific sequence is involved in the binding of vWF to GPIIb/IIIa cannot be excluded.

In conclusion, the MAB 9 epitope has been restricted from 684 (SPII fragment) to 75 amino acids and has been shown to include the RGD sequence. Our data provide additional evidence that the domain containing this sequence is the site responsible for vWF binding to GPIIb/IIIa. These experiments demonstrate the efficient use of bacterially synthesized recombinant proteins to identify epitopes recognized by anti-vWF MABs. We have produced a number of well-characterized MABs which clearly recognize distinct epitopes on the vWF subunit and inhibit binding of vWF to GPIb, GPIIb/IIIa, collagen or Factor VIII [12, 17, 33]. Epitope mapping of these additional antibodies to vWF should contribute to the further elucidation of the structure–function relationship of this Factor.

REFERENCES

1. Meyer, D., Obert, B., Piétu, G., Lavergne, J. M. & Zimmerman, T. S. (1980) *J. Lab. Clin. Med.* **95**, 590–602.
2. Mancuso, D. J., Tuley, E., Westfield, L. A., Worrall, N. K., Shelton-Inloes, B. B., Sorace, J. M., Alevy, Y. G. & Sadler, J. E. (1989) *J. Biol. Chem.* **264**, 19514–19527.
3. Bonthron, D. T., Handin, R. I., Kaufman, R. J., Wasley, L. C., Orr, E. C., Mitscock, L. M., Ewenstein, B., Loscalzo, J., Ginsburg, D. & Orkin, S. H. (1986) *Nature (London)* **324**, 270–275.
4. Weiss, H. J., Sussman, I. I. & Hoyer, L. W. (1977) *J. Clin. Invest.* **60**, 390–404.
5. Baumgartner, H. R., Tschopp, T. B. & Meyer, D. (1980) *Br. J. Haematol.* **44**, 127–139.
6. Kao, K. J., Pizzo, S. V. & McKee, P. A. (1979) *J. Clin. Invest.* **63**, 656–664.
7. Moake, J. L., Olson, J. D., Troll, J. H., Tang, S. S., Funicella, T. & Peterson, D. M. (1980) *Thromb. Res.* **19**, 21–27.

8. Ruggeri, Z. M., Bader, R. & De Marco, L. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6038–6041
9. Piétu, G., Chérel, G., Marguerie, G. & Meyer, D. (1984) *Nature (London)* **308**, 648–649
10. Girma, J. P., Chopek, M. W., Titani, K. & Davie, E. W. (1986) *Biochemistry* **25**, 3156–3163
11. Mohri, H., Fujimura, Y., Shima, M., Yoshioka, A., Houghten, R. A., Ruggeri, Z. M. & Zimmerman, T. S. (1988) *J. Biol. Chem.* **263**, 17901–17904
12. Girma, J. P., Kalafatis, M., Piétu, G., Lavergne, J. M., Chopek, M. W., Edgington, T. S. & Meyer, D. (1986) *Blood* **67**, 1356–1366
13. Plow, E. F., Srouji, A. J., Meyer, D., Marguerie, G. & Ginsberg, M. H. (1984) *J. Biol. Chem.* **259**, 5388–5391
14. Meyer, D., Zimmerman, T. S., Obert, B. & Edgington, T. S. (1984) *Br. J. Haematol.* **57**, 597–608
15. Nokes, T. J. C., Mahmoud, N. A., Savidge, G. F., Goodall, A. H., Meyer, D., Edgington, T. S. & Hardisty, R. M. (1984) *Thromb. Res.* **34**, 361–366
16. Meulien, P., Nishino, M., Mazurier, C., Dott, K., Piétu, G., Jorieux, S., Pavirani, A., Girma, J. P., Samor, B., Courtney, M. & Meyer, D. (1992) *Thromb. Haemostasis* **67**, 154–160
17. Piétu, G., Fressinaud, E., Girma, J. P., Nieuwenhuis, K., Rothschild, C. & Meyer, D. (1987) *J. Lab. Clin. Med.* **109**, 637–646
18. Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
19. Young, R. A., Bloom, B. R., Groskinsky, C. M., Ivanyi, J. T., Thomas, D. & Davis, R. W. (1985) *Proc. Natl. Acad. Sci.* **82**, 2583–2587
20. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* **230**, 1350–1354
21. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
22. Huynh, T., Young, R. A. & Davis, R. W. (1985) *DNA Cloning Techniques: A Practical Approach*, IRL Press, Oxford
23. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
24. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
25. Meyer, D., Baumgartner, H. R. & Edgington, T. S. (1984) *Br. J. Haematol.* **57**, 609–620
26. Ware, J., Toomey, J. R. & Stafford, D. W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3165–3169
27. Ware, J., Toomey, J. R. & Stafford, D. W. (1989) *Thromb. Haemostasis* **61**, 225–229
28. Frazier, D., Smith, K. J., Cheung, W. F., Ware, J., Lin, S. W., Thompson, A. R., Reisner, H., Bajaj, S. P. & Stafford, D. W. (1989) *Blood* **74**, 971–977
29. McGraw, R., Frazier, D., De Serres, M., Reisner, H. & Stafford, D. (1986) *Blood* **67**, 1344–1348
30. Bahou, W. F., Ginsburg, D., Sikkink, R., Litwiller, R. & Fass, D. N. (1989) *J. Clin. Invest.* **84**, 56–61
31. Berliner, S., Niiya, K., Roberts, J. R., Houghten, R. A. & Ruggeri, Z. M. (1988) *J. Biol. Chem.* **263**, 7500–7505
32. Obara, M., Kang, M. S. & Yamada, K. M. (1988) *Cell* **53**, 649–657
33. Takahashi, Y., Kalafatis, M., Girma, J. P., Sewerin, K., Andersson, L. O. & Meyer, D. (1987) *Blood* **70**, 1679–1682

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