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What a polyclonal antibody sees in von Willebrand factor

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Abstract

Introduction—Von Willebrand factor (VWF) plays a critical role in hemostasis by carrying factor VIII (FVIII) and binding to specific ligands on the surface of blood platelets and within the blood vessel wall.

Materials and Methods—We constructed a gene-specific phage display library containing small, random VWF fragments. Using this library, we mapped the repertoire of immune epitopes recognized by a commonly used commercial rabbit anti-human VWF polyclonal antibody.

Results—A total of eight discrete epitopes within the VWF protein were identified, including two dominant epitopes that account for 74% of immuno selected VWF fragments. Comparison with previously mapped epitopes for mouse monoclonal antibodies reveals four overlapping regions that may identify common antigenic determinants. The distribution of these epitopes was not readily predicted from primary amino acid sequence divergence among these mammalian species or standard algorithms for the prediction of antigenicity, hydrophobicity, or surface probability.

Conclusion—Taken together with previous monoclonal antibody epitope mapping studies, our results suggest that a limited number of exposed domains on the surface of the human VWF protein may be the primary determinants of immunogenicity.

Introduction

Functioning as the carrier for FVIII and a bridge between platelets and the injured blood vessel, von Willebrand factor (VWF) plays a pivotal role in both physiologic hemostasis and pathologic thrombosis. Multiple discrete functional domains have been defined within VWF that mediate its interactions with FVIII, receptors on the platelet surface, and ligands within the blood vessel wall [1,2]. The D'/D3 domain at the N-terminus of mature VWF is responsible for binding to FVIII [3]. The A1 domain binds to GPIIb, the primary receptor for VWF on the platelet surface [4,5], and the A3 domain binds to both type I and III collagen, presumably key ligands within the vessel wall [6]. An RGD sequence within the C1 domain interacts with platelet $\alpha_{IIb}\beta_3$ and potentially other integrins [7]. Finally, the A2 domain contains the cleavage site for the metalloproteinase ADAMTS13, which is critical for the regulation of VWF multimer size and function [8].

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Identification of antigenic determinants within a protein may provide clues as to the locations of functionally important domains (reviewed in [9]). For example, the epitope recognized by an anti-VWF monoclonal antibody that inhibits FVIII binding was successfully mapped to a 19 amino acid segment at the N-terminus of VWF, suggesting a location for a critical part of the FVIII binding domain [10]. Subsequent studies identified several single amino acid mutations within this same VWF segment in patients with type 2N von Willebrand disease (VWD) that resulted in markedly decreased affinity of VWF for FVIII [11,12]. A similar approach has been used to map epitopes within factor VIII recognized by factor VIII inhibitor antibodies from hemophilia A patients [13,14].

A variety of epitope mapping strategies have been developed, including the use of multiple overlapping recombinant fusion proteins, truncation mutants, and overlapping synthetic peptides. Phage display has also been used extensively for epitope mapping as well as a number of other applications (reviewed in [9,15,16]). To construct a phage display library, DNA fragments encoding part or all of the protein(s) of interest are engineered to fuse with the phage minor coat protein III or major coat protein VIII. Filamentous phage libraries can contain $>10^9$ individual phage, each displaying on its surface the specific protein fragment corresponding to its encoded DNA segment. In addition to the advantage of large library size, phage display libraries have the capacity to represent epitopes with the correct conformational fold and/or disulfide bond formation, as well as the unique potential for repeated rounds of competitive selection and amplification to identify antigenic determinants with the highest affinity/avidity. Thus, phage display provides a unique means for probing the immunogenicity of specific protein of interest.

In this study, we report the generation of a large phage display library containing random fragments of the VWF protein sequence as small peptides fused to the filamentous bacterial phage coat protein gene III. We screened this library with a commercial rabbit anti-human VWF polyclonal antibody that is widely used in assays of VWF quantity or function. We identified eight discrete epitopes within the VWF protein, including two dominant epitopes that accounted for 74% (60/81) of reactive VWF fragments. We conclude that these two narrow regions represent the primary sequences within VWF recognized by this widely used antibody.

Materials and Methods

Antibodies

The rabbit polyclonal anti-human VWF antibody was purchased from Dako Cytomation (Carpinteria, CA 93013). This antibody (Dako code number A0082) is the purified immunoglobulin fraction of rabbit antiserum raised against VWF purified from human plasma. Two different lots of this antibody reagent, purchased over 5 years apart, were used for the two independent phage "panning" experiments described below. The first experiment used lot 105 (edition 17.05.99) and the second used lot 111(101) exp 11.07.

Construction of the VWF fragment phage display library

The VWF fragment phage display library was constructed with the recombinant phage antibody system kit (RPAS, Amersham Bioscience Corp, Piscataway, NJ), according to the manufacturer's instructions. Full length VWF cDNA in the vector pBluscript [17] was partially digested using DNase I (DNase Shotgun Cleavage kit, Novagen, Inc.) in the presence of manganese following the manufacturer's instructions. The resulting random fragments between 100–700 bp in length were collected using pre-treated NA-45 DEAE paper (Schleicher and Schuell, Keene, NH) and eluted following the manufacturer's protocol. The ends of purified fragments (~ 8 μ g) were blunted using T4 DNA polymerase and treated with T4 polynucleotide kinase to prepare for ligation. The resulting fragments were ligated with two

hairpin linkers (Integrated DNA Technologies, Coralville, IA), pCAN_Sfi (5'-GGCCGGCTGGGCCCCGGTGCACGGAGAATACCTCCGTGCACCGGGCCCCAGCCGGC-3') and pCAN_Not (5'-GCGGCCGCACCTCTGGTGGTTGCGCAATACGCGGAACCACCAGAGGTGCGGCCGC-3'). After digestion with Sfi I and Not I, the fragments were purified by agarose gel electrophoresis and subcloned into the phagemid vector pCANTAB5E (Amersham Bioscience Corp, Piscataway, NJ). The ligation mixture was electroporated into TG1 cells (Stratagene) in a 0.1 cm cuvette using a Bio-Rad *E. coli* Pulser at a voltage setting of 1800 V. The transformed TG1 cells were grown on plates containing ampicillin and glucose. Resulting clones were collected into 2YTAG (2XYT containing 100 µg/ml ampicillin and 2% glucose) medium and stored as library stock at -80 °C.

Single phage colonies selected for analysis were lysed in deionized water at 95 °C for 10 minutes, and the VWF inserts were amplified by PCR from 10 µl of lysate using the S1 and S6 primers (RPAS kit, Amersham). DNA sequence analysis was performed at the University of Michigan sequencing core.

Selection of antibody-reactive phage clones

The expression module of the RPAS kit (Amersham, Buckinghamshire, UK) was used to generate fresh phage stock from the VWF fragment phage display library that had been prepared in TG1 cells and stored at -80 °C, according to the manufacturer's instruction. Briefly, library stock containing 7.7×10^6 independent colonies was grown to log phase ($OD_{600}=0.3 \sim 0.4$) and rescued with M13KO7 helper phage. The rescued library stock was grown overnight at 37 °C with vigorous shaking in the presence of ampicillin and kanamycin for phage production. After removal of bacteria by centrifugation, the fresh phage stock was prepared by precipitation overnight with 4% polyethylene glycol (PEG) and 0.5 M NaCl.

Two different lots of rabbit anti-human VWF polyclonal antibody was used for human VWF epitope mapping (see above). The first round of panning employed a solid phase panning procedure. In brief, wells of a 6 well plate (Costar 3046, Corning Inc., Corning, NY) were coated with the VWF antibody at a concentration of 10 µg/ml in phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, PH 7.4) and blocked with 2 % BSA in PBS. After washing with PBS, 5 µl of phage stock in 2ml 2% non-fat milk PBS was incubated at room temperature for 1 hour, followed by washing with 1 ml PBST (0.1% Tween-20 in PBS) 12 times and PBS 10 times. The bound phage were recovered directly by infecting mid log phase ($OD_{600}=0.3 \sim 0.5$) TG1 cells, followed by rescue with M13KO7 helper phage and overnight growth in a shaking incubator.

PEG-purified phage recovered from the first round were screened in suspension for the second round, as previously described [18]. Briefly, polyclonal anti VWF antibody (10 µg/ml in PBS) was incubated with 1 µl ($\sim 3 \times 10^9$) freshly amplified phage stock from the 1st round of panning in 500 µl PBST at room temperature for 30 minutes and then incubated with 250 µl of protein-G sepharose (Amersham) at room temperature for another 30 minutes. After 10 washes in 1 ml PBST, antibody specific binding phage were rescued and amplified as described above. This procedure was repeated for each subsequent round of panning. For a second independent panning experiment, the first round of panning was in suspension, with the consecutive rounds performed using the solid phase panning procedure described above.

Identification of VWF antibody binding clones by ELISA

A secondary screen was used to identify antibody binding phage after 3 rounds of selection. The detection module of the RPAS kit (Amersham, Buckinghamshire, UK) was used per the manufacturer's instructions with minor modifications. Individual phage clones were isolated

in colonies of infected TG1 cells and grown in 96 well plates, one clone per well. A fresh plate was coated with polyclonal Dako anti-VWF antibody (10 ug/ml in PBS) and then blocked with 2% BSA in PBS. In another fresh plate, phage prepared from each well were blocked with 2% non-fat milk in PBS at room temperature for 30 minutes. The resulting blocked phage were added to the antibody coated plate and incubated for 1 hour at room temperature. After washing, a monoclonal antibody against the M13 major coat protein, PVIII (Amersham, Buckinghamshire, UK), was used to detect the bound phage. The plate was developed with ABTS (2',2'-Azino-Bis (3-Ethylbenz-Thiazoline-6-Sulphonic Acid) and the signal was quantitated on a microplate reader (Molecular Devices, Sunnyvale, CA) at $\lambda = 410$ nm. Positive colonies, as defined by signal > 2X background, were selected for PCR amplification and DNA sequence analysis.

Results and Discussion

Construction of phage-display library and Identification of antibody-reactive colonies

The VWF fragment phage display library contained $\sim 7.7 \times 10^6$ independent clones. PCR analysis of random phage confirmed insert sizes generally ranging from 100 to 700 bp in length. To construct this library, the random VWF fragments were generated from full length human VWF cDNA cloned in the plasmid vector pBluescript [17]; 1/3 of the clones were thus expected to be derived from the vector sequences. Of the remaining 2/3 containing VWF cDNA, only 1/2 should be in the correct orientation for expression as a PIII fusion protein. Of these, only 1/3 should be in the correct 5' reading frame and 1/3 of these in the correct 3' reading frame to give a VWF/PIII coat protein fusion product. Thus, only 1 out of 27 clones ($2/3 \times 1/2 \times 1/3 \times 1/3$) in the library would be predicted to express an authentic VWF fragment on the phage surface. The bulk of the library (26/27) thus serves as an internal negative control. Therefore, an isolated phage clone with the correct orientation and reading frame is unlikely to have been selected by chance. Given the initial size (7.7×10^6 independent clones), the library is expected to contain $\sim 2.5 \times 10^5$ authentic VWF fragments, which should be sufficient for > 500 fold coverage of any specific short VWF segment. Sequence analysis of 68 randomly selected clones from the library revealed that all clones contained inserts, with 48 (71%) corresponding to VWF cDNA fragments, 21 in the correct orientation and only 1 in both the correct orientation and correct reading frames, consistent with the above predictions.

After 3 rounds of panning with the polyclonal rabbit anti human VWF antibody, we randomly selected 216 phage clones from two independent panning experiments for analysis. Of these 216 phage, 126 (53%) were confirmed as positives by ELISA. In contrast, none of 48 random phage clones without antibody selection were ELISA positive. From these 126 ELISA confirmed phage clones, 92 were subjected to DNA sequence analysis. Eighty one (88%) encoded authentic VWF fragments in the appropriate orientation and reading frames for expression as fusion proteins with the phage coat protein PIII. Of 24 control phage analyzed by DNA sequencing (12 ELISA negative and 12 randomly picked from the initial library), all contained insert, though none were in correct orientation and reading frame.

Epitope mapping of an anti-human VWF polyclonal antibody

The 81 ELISA positive and sequence confirmed phage, identified from two independent panning experiments, corresponded to 32 distinct VWF fragments (Table 1). Although additional epitopes were identified in the second panning, as indicated in Table 1 (highlighted in grey), epitopes E4 and E7 remained the predominant epitopes. In both experiments, antibody-reactive clones corresponding to these two epitopes represent 76% and 62% of total antibody-reactive clones identified, respectively. No duplicate clones were identified in the two independent panning experiments, consistent with the calculated depth of the library. Several phage clones were repeatedly identified within each panning (Table 1), suggesting a

selection advantage for these clones due to an increased avidity of binding to the antibody compared to other positive clones. Taken together, these 81 positive phage sequences, ranging in size between 11 to 97 amino acids, define 8 discrete regions within VWF, E1–E8 (Fig 1). As shown in Figure 2, 60 of the 81 total antibody-reactive phage clones localize to 2 dominant epitopes, E4 and E7, which are 35 and 11 amino acids long, respectively. An additional 6 epitopes were detected by the remaining 26% of phage clones (Table 1).

Of note the most prominent epitopes (E1, E4, and E7) are identified independently in both panning experiments, whereas the remaining 5 epitopes are each identified in only a single screening experiment. Though the set of epitopes identified by the 81 sequenced phage clones may account for a significant portion of the polyclonal antibody immunoreactivity, these results also suggest that this analysis was not saturating, and that a large number of minor epitopes may still remain to be identified.

Comparison of epitopes identified by the rabbit polyclonal antibody to previous mouse monoclonal antibody studies

Previous epitope mapping studies from our laboratory and others with panels of mouse monoclonal anti-human VWF antibodies have reported a total of 10 distinct epitopes, EM1 – 10 (Fig 1), within the VWF protein [10,19–20]. An additional study also mapped the epitopes for 5 monoclonal antibodies raised specifically against the VWF A1 domain [27] (second row of monoclonal epitopes in Fig 1). Several monoclonal antibodies from the previous studies mapped to epitopes which overlap with the polyclonal epitope E2 (1261–1313), identified in the present study. Three other previously reported monoclonal epitopes also overlap with the polyclonal epitopes E 3, E6, and E7 (Fig 1).

However, comparison of the human vs rabbit, human vs mouse, or rabbit vs mouse VWF sequences showed no obvious difference in the degree of sequence identity for the segments encompassing the shared epitopes vs. those specific for the polyclonal antibodies or monoclonal antibodies (Table 2). Analysis using standard algorithms for the prediction of antigenicity [21], surface probability [22], or hydrophilicity [23] were performed on the mature VWF protein sequence, as shown in supplemental Figure 1. No obvious correlation is evident for any of these predictions and the location of the 18 mapped epitopes.

Several of the polyclonal epitopes identified here map within important VWF functional domains. Epitope E1 (809–826) lies within the FVIII binding domain of VWF[2], immediately amino-terminal to the epitope defined by 2 monoclonal antibodies known to block FVIII binding to VWF (841–859) [10]. Indeed, 4 known type 2N VWD mutations (associated with decreased FVIII binding) are located within the E1 epitope (see the ISTH SSC VWF Database at: <http://www.vwf.group.shef.ac.uk/index.html>). Epitopes E3 and E2 lie within the VWF A3 and A1 domains associated with collagen and GPIb binding, respectively. However, the E2 epitope appears to be outside of the segment required for GPIb binding function[4,5,7,27].

Epitope mapping studies using phage display differ in several ways from the lambda phage-based epitope methods previously employed for mapping VWF monoclonal antibody epitopes. With phage display, the potential peptide epitope is folded on the surface of the phage tip in the nonreducing environment of the bacterial periplasmic space and allowed to interact with the antibody in solution, as compared to potentially partially unfolded lambda-expressed protein bound to a nitrocellular membrane. Phage display may thus have an advantage in detecting conformational epitopes, as well as a bias toward higher affinity epitopes as a result of the repeated rounds of competitive selection.

Immunodominance, defined as an immune reaction induced by only a small fraction of all possible epitopes, is a well-known phenomenon observed in both T and B cell immune

responses. The observation that 74% of all antibody-reactive clones clustered in 2 dominant epitopes could be attributed to immunodominance of these epitopes for the human VWF protein when presented in the context of the rabbit immune system. Alternatively, other features of these regions may result in particular high avidity to the antibody.

Interestingly, E7, one of the two dominant epitopes, is also contained within the single 600 bp cDNA clone previously identified by immunologic screening in one of the initial reports of the identification of VWF cDNA in 1985 by expression cloning [17]. In addition, the cDNA clones identified by 3 other groups by similar immunologic approaches also contained this epitope [24–26]. Except for one group that used a commercial rabbit antiserum [24], the other 3 groups each used an independent rabbit antiserum to human VWF prepared by their own lab. Thus, E7 appears to have been the critical epitope, recognized by 4 independent antibodies, that was essential for the original cDNA cloning of VWF in 1985. As shown in Figure 1, E7, a region of 11 residues between 2676 and 2686, overlaps with EM10, an epitope of 47 residues between 2649 and 2696 identified by mouse monoclonal antibody CM1[20].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Localization for the eight polyclonal epitopes mapped by phage display in the current study (E1–E8). Ten previously reported monoclonal antibody epitopes [10,19–20] are indicated as EM1 – EM10. An additional 5 epitopes recognized by monoclonal antibodies raised specifically against the VWF A1 domain[27] are depicted just below (labeled "VWF A1 Epitopes"). The complete VWF cDNA (8752 bp) and protein (2813 amino acids (aa)) are shown schematically, with repeated sequence domains indicated (A–D). "sp" indicates the signal peptide. The four overlapped mouse and rabbit epitopes are indicated by box outlines. The location of known functional domains within VWF[1,3–7] are indicated at the bottom of the figure.

Figure 2.

Fine mapping of two dominant epitopes E4 and E7. The 12 independent clones identifying dominant epitope E4 are shown, with a minimum overlap from S2285 to D2304 (gray bar). The minimum region for dominant epitope E7, K2676 – W2686, defined by 17 independent clones spanning nucleotide 7942 – 8209, is also indicated.

Table 1
Anti-human VWF polyclonal antibody epitope localizations

Epitope	# of Phage	Region spanned (bp)	Minimum overlap (bp)	Minimum epitope (AA)	Size(AA)
	1	2281–2478			
	1	2413–2497			
	5	2425–2478			
	1	2308–2601			
E1	1	3784–3919	2425–2478	809–826	18
E2	1	5362–5592	3784–3939	1261–1313	53
E3	1	6847–7044	5362–5592	1768–1864	97
	2	6847–7044			
	2	6847–7081			
	6	6850–7011			
	2	6853–7081			
	10	6850–7041			
	1	6733–7035			
	1	6748–6957			
	1	6754–7035			
	1	6847–7047			
E4	1	7177–7350	6853–6957	2285–2319	35
	1	7207–7341			
E5	2	7372–7509	7207–7341	2403–2447	45
	5	7384–7494			
E6	3	7942–8092	7384–7494	2462–2498	37
	1	7942–8127			
	9	7942–8181			
	5	7942–8187			
	4	7975–8058			
	2	7978–8169			
	1	8026–8160			
	5	8029–8160			
	1	8029–8209			
	1	7942–8070			
	1	7942–8209			
	1	7954–8151			
E7	2	8411–8187	8029–8058	2676–2686	11
E8			8101–8187	2701–2729	29

Column 1 lists the 8 epitopes identified; column 2 lists the number of times each independent VWF fragment clone was identified; column 3 indicates the base pair range. Nucleotide and amino acid numbering is according to the nomenclature guidelines of the International Society on Thrombosis and Haemostasis (ISTH) (<http://www.vwf.group.shef.ac.uk/nomenclature.htm>). Column 4 gives the localization of each epitope as defined by minimum base pair overlap of the corresponding independent clones; column 5 lists the minimum peptide epitope; column 6 lists the minimum epitope size in amino acids (AA); clones shaded in grey were identified in the second independent panning experiment, whereas unshaded clones are from the first panning experiment. Rows outlined in a thick black border give the minimum segment defined by the overlap of a group of phage clones. E2, E3, and E8 are each based on a single clone.

Table 2 Comparison of sequence similarity of amino acids in the region of shared or antibody specific epitopes between species

Epitope	N	AL(AA)	R:M	H:R	H:M
Shared Epitope	4	28	86.0%	85.6%	82.6%
Polyclonal Ab Only	4	32	88.1%	88.7%	86.7%
Monoclonal Ab Only	5	35	81.0%	81.9%	83.3%
Full VWF		2050	81.5%	83.8%	83.5%

Column 1 lists the classes of epitopes or the full length of mature VWF sequence; column 2 shows number (N) of epitopes in each class; column 3 indicates the average length (AL) in amino acids (AA) of each class; column 4, 5, and 6 represent the percentage of identity in sequence of amino acid in the epitopic region between rabbit and mouse (R:M), human and rabbit (H:R), and human and mouse (H:M), respectively.