Lupus-Derived Antiprothrombin Autoantibodies from a V Gene Phage Display Library Are Specific for the Kringle 2 Domain of Prothrombin†

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ABSTRACT: Autoantibodies to prothrombin are common in patients with systemic lupus erythematosus. Although their presence is a risk factor for thrombosis, neither their origin nor their precise role in inducing the procoagulant state is known. We have developed a phage-display antibody library from patients with systemic lupus erythematosus with antiprothrombin antibodies, and we have selected two single-chain Fv antibody fragments (ScFvs) by panning on a prothrombin-coated surface. In prothrombin activation assays using purified components, these antibodies promoted prothrombin activation. These ScFvs, termed AN78 and AN129, bound to immobilized prothrombin in a concentration-dependent specific manner but not to other anionic phospholipid binding proteins such as β2-glycoprotein I or annexin V. Phosphatidylserine-bound prothrombin, but not soluble prothrombin, inhibited the binding suggesting that the epitope is available only on immobilized prothrombin. To localize the epitope, prothrombin was treated with thrombin or factor Xa and various prothrombin activation fragments were subsequently isolated and tested in ELISA with the ScFvs. Both AN78 and AN129 bound to prethrombin I (the fragment lacking the Gla domain and the first kringle domain), to fragment 1.2 (containing Gla and the two kringle domains only) and to fragment 2 but not to thrombin, thus localizing the cognate epitope to the kringle 2 domain in prothrombin. Analysis of the cDNA sequences of these antibodies show clustered mutational patterns in the complementarity determining region, suggesting that variable domains are the products of antigen-driven B cell clonal maturation.

Prothrombin is a 579 amino acid plasma glycoprotein and is the precursor of thrombin, the central enzyme in coagulation (*1*−*4*). The circulating form of prothrombin contains an amino-terminal Gla domain, two kringle domains, and a carboxy-terminal catalytic domain. The Gla domain contains 10 γ-carboxy glutamic acid residues, and it mediates the Ca^{2+}-dependent binding to anionic phospholipid vesicles. The kringle domains have two disulfide-bonded regions of internal homology of approximately 80 amino acids (kringles 1 and 2). The catalytic domain is homologous to trypsin and other serine proteases. Coagulation factor Xa cleaves two peptide bonds in prothrombin, Arg271-Thr272 and Arg320-Ile321 (*3*−*4*), releasing the enzyme thrombin and the amino-terminal activation peptide called fragment 1.2, which contains the Gla domain and the two kringle domains. Thrombin can also cleave peptide bonds Arg151-Ser152 in intact prothrombin releasing prethrombin I and fragment I, which contains the Gla domain and the first kringle domain (*4*). Thrombin cleavage of fragment 1.2, at the same site, gives rise to fragment 1 and fragment 2, and the latter contains the second kringle domain only.

The presence of antibodies to prothrombin in lupus has been known for almost 50 years, when patients with hypoprothrombinemia and circulating anticoagulants were first described (*5*−*7*). Since then these antibodies have also been described in individuals without lupus (*8*). Conventional mechanisms of antibody action predict that antiprothrombin antibodies might cause bleeding diathesis due to interference with factor Xa catalyzed prothrombin activation. Paradoxically, in the vast majority of individuals, the presence of antiprothrombin antibodies are associated with thrombosis (*8*−*11*). Neither their origin nor their precise role in inducing the procoagulant state is known.

To obtain significant quantities of these antibodies for structure−function and mechanistic studies, we have developed a phage-displayed antibody fragments library from patients with systemic lupus erythematosus with antiprothrombin antibodies. We have isolated two prothrombin-specific ScFvs[1] and localized their epitope and characterized their effect on prothrombin activation.

EXPERIMENTAL PROCEDURES

Isolation of Prothrombin and Its Activation Fragments. Human prothrombin was either purchased from Haematologic Technologies Inc. or isolated from normal plasma as

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[1] Abbreviations: ScFv, single-chain Fv antibody fragment; BSA, bovine serum albumin; TBS, Tris-buffered saline; CDR, complementarity determining region.

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described by Miletich et al (*12*) with some modifications. Following barium citrate absorption and ammonium sulfate elution prothrombin was isolated by chromatography on heparin agarose as described before (*12*). Prethrombin I was isolated from prothrombin as described before with some modifications (*13*, *14*). Briefly, prothrombin (2 mg/mL) was incubated with a 200:1 weight ratio of human thrombin at 37 °C for 90 min. The thrombin was neutralized with 5 mM diisopropylfluorophosphate (from 1 M stock in 2-propanol). After 30 min, the reaction is diluted 1:2 in 7.6% sodium citrate, and $BaCl_2$ was added to a final concentration of 100 mM. The reaction mixture was centrifuged for 20 min at 10 000*g*, and the supernatant was gel-filtered on a FPLC Superdex 200 column. Fragment 1.2 was isolated following actvation of prothrombin with the prothrombinase complex in the presence of 0.3 mM dansylarginine-*N*-(3-methyl-1,5-pentanediyl)amide as described before (*15*). Fragment 2 was isolated by thrombin digestion of fragment 1.2 as described before for the isolation of prethrombin I (*14*).

Generation of Epitope Library. Following attainment of an informed consent, 100 mL of heparinized blood sample was obtained from three patients with systemic lupus erythematosus and antiphospholipid antibodies. The mRNA from the peripheral blood leukocytes were isolated and reverse transcribed, and the VH and VL domains were amplified with *Apa*LI/*Not*I sites and *Sfi*I/*Xho*I sites, respectively, as described previously (*16*). The primers used and linker sequences were described before (*16*). The ScFv constructs were digested with *Not*1 and *Sfi*1 and cloned into the phagemid vector pHenII kindly provided by Center for Protein Engineering, Medical Research Council (MRC), Cambridge, England; GenBankTM accession number 1926701). The vector contains an IPTG-inducible lac promoter, a pelB leader sequence, a gene3 structural peptide, a poly(His) tag, a c-myc peptide tag, and a stop codon (amber) between the insert and gene3. The amber codon permits secretion of soluble V domains or their expression as p3-fusion proteins on the phage surface, depending on the host strain. *Escherichia coli* strain HB2151 cells recognize amber as a stop, while TGl cells recognize amber as glutamic acid. Host cells were transformed by electroporation, clones are selected in kanamycin, and the presence of inserts in individual colonies is confirmed by PCR using primers located in the vector upstream and downstream of the insert. Addition of helper phage (VCSM13) containing a kanamycin resistance gene permits packaging of phage particles from TG1 cell cultures. The particles in the supernatant of the culture were precipitated with 4% PEG, yielding phage ready for the selection procedures described below.

Selection of Prothrombin-Binding Phage. Nunc tubes (75 mm × 2 mm, γ-irradiated) were coated with 1 mL of prothrombin (10 μg/mL) overnight in a 0.1 M $NaHCO_3$ buffer, pH 8.6. The tubes were blocked with 5% bovine serum albumin and incubated with phages, 5×10^{12} phage in Tris-buffered saline (TBS). The nonadherent phage were removed by repeated washing in TBS containing 0.25% Tween 20 and eluted with 0.1 M glycine, pH 2.7. The eluted phage particles were treated with 1/20 volume of 2 M Tris, pH 9.0, and then were amplified by infecting 10 mL of mid-log phase *E. coli* TG1 cells and rescuing with the helper phage. The rescue-selection plating was repeated three times. After the last selection, the rescued phages were used to infect HB2151 cells and plated on ampicillin plates. Single colonies from the plates were grown in 200 μl of 2YT medium supplemented with ampicillin and 0.1% glucose for 2 h at 37 °C using 96-microwells on a rotatory shaker. The cultures were diluted 1:50, allowed to grow for 2 h, and induced with 1 mM isopropyl D-thiogalactoside (IPTG) to express soluble ScFvs for another 18 h at room temperature. Microtiter plates were centrifuged at 1800*g* for 15 min, and the supernatants were tested for prothrombin binding in an ELISA assay.

Isolation of ScFv. For large scale isolation of selected ScFvs, aliquots from glycerol stock were grown in 2× YT/1% glucose overnight at 37 °C and diluted 1:100 in a large Erlenmeyer flask containing 600 mL of 2× YT/0.1% glucose for 2 h at 37 °C and then induced with 0.1 mM IPTG for 16 h at room temperature. The periplasmic extracts were prepared (*16*), dialyzed against the loading buffer (50 mM phosphate, pH 7.5), and adsorbed to a column of Ni−NTA agarose (Qiagen, Valencia, CA). The column was washed, and the bound ScFv was eluted by lowering the pH to 5.0 with citric acid, dialyzed against TBS, and analyzed by SDS−PAGE and Coomassie blue staining.

ELISA Assay for Prothrombin. Nunc Maxisorp plates were coated with 1 μg of prothrombin in bicarbonate (100 mM $NaHCO_3$, pH 8.6) overnight at 4 °C (*9*) and blocked with 5% BSA (bovine serum albumin) in TBS for 1 h at room temperature. The wells were incubated with 200 μl of bacterial supernatants (or various concentrations of in 1% BSA) for 1 h and washed in TBS containing 05% Tween. The wells were incubated with 1 μg/mL of purified c-myc antibody (clone 9E10 from ATCC) for 1 h and washed, and bound antibody was detected using goat antimouse IgG-peroxidase and *O*-phenyldiamine and H_2O_2, and the absorbace was measured at 450λ in the ELISA plate reader. For inhibition studies, phosphatidylserine vesicles were prepared as described before (*17*) and incubated with prothrombin in the presence of 1 mM $CaCl_2$ (or 5 mM EDTA as negative control) for 30 min at room temperature. The prothrombin−phosphatidylserine complex was added to the ELISA wells along with 10 μg/mL of ScFv. To determine whether these antibodies will compete with each other, the ScFvs were labeled separately with *N*-hydroxysuccinamide−biotin (Pierce) with molar ratio of 10:1. The binding of biotin−ScFv to immobilized prothrombin and the effect of unlabeled ScFvs were determined using avidin−peroxidase.

Surface Plasmon Resonance Assay. The kinetics of interaction between purified ScFvs and prothrombin were studied by surface plasmon resonance using a BIAcore 2000 biosensor system (Biacore Inc., Piscataway, NJ) according to previously described methods (*18*). Prothrombin was immobilized on carboxymethyl sensor chips (CM 5) with a standard amine coupling reaction via *N*-hydroxysuccinimide/*N*-ethyl-*N*7-(3-diethylaminopropyl)carbodiimide (NHS/EDC) according to the instructions of the manufacturer (BIAcore). The binding of ScFvs to prothrombin was analyzed by injecting various concentrations of ScFv in hepes-buffered saline (1.6−3.07 μM) at 10 μL/min for 15 min. The chip surface was regenerated with 10 mM glycine, pH 2.5. Steady sensogram after immobilization of prothrombin was taken as baseline, and dissociation constant for ScFv binding was calculated by nonlinear fitting of sensogram data using the BIA evaluation 3.0 software (Biacore Inc).

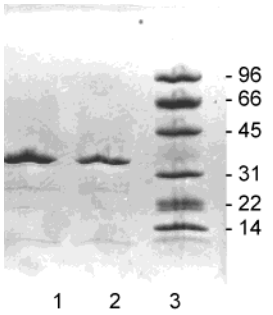

FIGURE 1: Isolation of ScFvs. The ScFvs were isolated from *E. coli* by nickel affinity chromatography as described in the methods and electrophoresed in 10−15% SDS−polyacrylamide gel electrophoresis: lane 1, AN78; lane 2, AN129; lane 3, molecular weight standards.

Prothrombinase Assay. The effect of ScFvs on prothrombin activation was measured in a prothrombinase assay using purified protein components (*19*). The reaction mixture consists of 1 μM prothrombin, 5 nM factor Va, 5 μM PC/PS, and 0−10 μg/mL ScFv. The prothrombin activation was initiated by the addition 40 pM factor Xa. An aliquot (5 μl) of the reaction mixture was removed at time intervals 0, 10, 20, 40, and 60 min and diluted in a quench buffer containing 0.175 M NaCl, 50 mM Tris, 20 mM EDTA, and 0.5 mg/mL bovine serum albumin, pH 7.9. Aliquots (10 μl) of each quenched sample were diluted 20-fold in the same buffer in wells of a 96-well plate containing 400 μM S2238. Amidolysis was determined by continuously monitoring the change in absorbance at 410 nm in a kinetic plate reader (Molecular Devices, Sunnyvale, CA). The concentration of thrombin formed as a function of time was determined by interpolation from the linear dependence of initial velocity of S2238 hydrolysis on known concentrations of thrombin performed as a control.

DNA Sequencing. V genes in the ScFv were sequenced using purified phagemid pHEN2 DNA isolated from clones using the Thermosequence dye terminator cycle sequencing. Sequence analyses of VL and VH genes with the alignments of germline V genes were performed with the online DNA sequence alignment program IgG blast (*20*) and using the Kabat database (*21*). The mutations due to primer used for PCR amplification were ignored. Regions of AN78 and AN129 VL domains employed for sequence analyses were residues 8−99 and 8−100, respectively. Regions of AN78 and AN129 VH domains employed for sequence analyses were residues 9−100.

RESULTS

Selection and Isolation of ScFvs. Recombinant phages expressing a library of ScFvs on their surface were produced by helper phage rescue and selectively enriched for prothrombin reactive clones by panning on prothrombin-coated polystyrene tubes. *E. coli* from strain HB2151 (with an amber stop codon between ScFv and M13 gene III) were infected with the phages, and the soluble ScFvs from single colonies were tested for reactivity toward prothrombin in an ELISA. We succeeded in selecting two ScFv clones, ScFv AN78 and ScFv AN129, that reacted with prothrombin. The ScFvs, containing hexa-his, were purified by metal chelate affinity chromatography, and these ScFvs had an expected apparent molecular weight of 32 kDa (Figure 1).

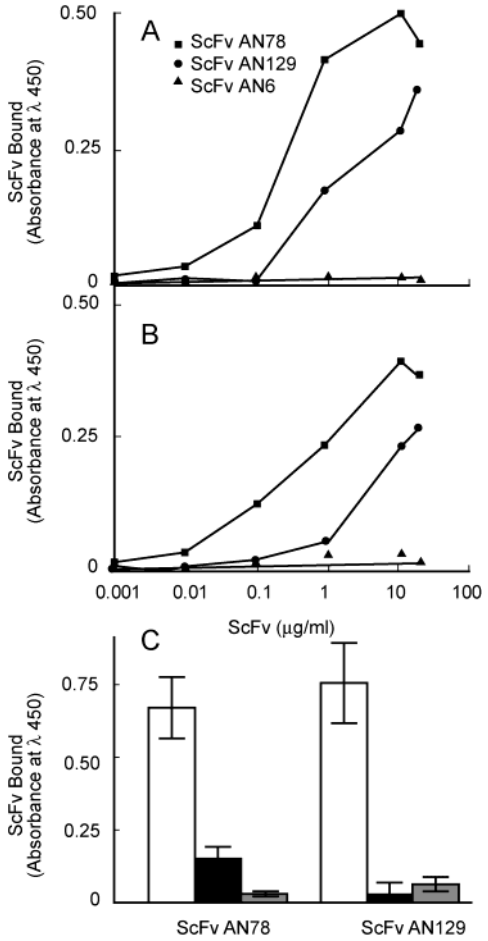

FIGURE 2: Binding of the purified ScFvs to prothrombin. In panel A, wells of ELISA plates were coated with 1 μg of prothrombin and various concentrations of purified ScFvs (0.001−10 μg/mL) were incubated for 1 h. The bound ScFvs were quantified by an ELISA using a murine monoclonal antimyc antibody and a peroxidase-labeled goat antimouse Fab. In panel B, wells were coated with 1 μg of phosphatidylserine, blocked, incubated with 1 μg of prothrombin, washed, and incubated with purified ScFvs. The bound ScFvs were measured as in panel A. In panel C, the biotin-labeled AN78 or AN129 were incubated alone (open bars) or with 50-fold excess unlabeled ScFv AN78 (gray bar) or AcFv AN129 (closed bar). The binding was measured with avidin−peroxidase.

Binding of ScFvs to Prothrombin. The purified ScFvs AN78 and AN129 showed a concentration-dependent binding to prothrombin that is immobilized on ELISA plates, while under similar conditions, a control irrelevant ScFv, AN6, did not bind significantly (Figure 2a). These ScFvs also bound to prothrombin that has been immobilized on phosphatidylserine-coated wells (Figure 2b). The binding of biotin−AN78 to prothrombin was inhibited not only by unlabeled AN78 but also by unlabeled AN129, and similarly binding of biotin−AN129 was inhibited by both unlabeled AN129 and AN78, suggesting that the binding sites were the same or spatially close (Figure 2C). Under similar conditions, these ScFvs did not bind to other phospholipid-binding proteins such as β2-glycoprotein I or annexin V or to anionic phospholipids such as phosphatidylserine or cardiolipin (Figure 3A). The binding to prothrombin was independent of cations because a similar extend of binding was seen in the presence of Ca^{2+} or EDTA (Figure 3B). Binding of the ScFvs were not inhibited by prior incubation of the ScFvs

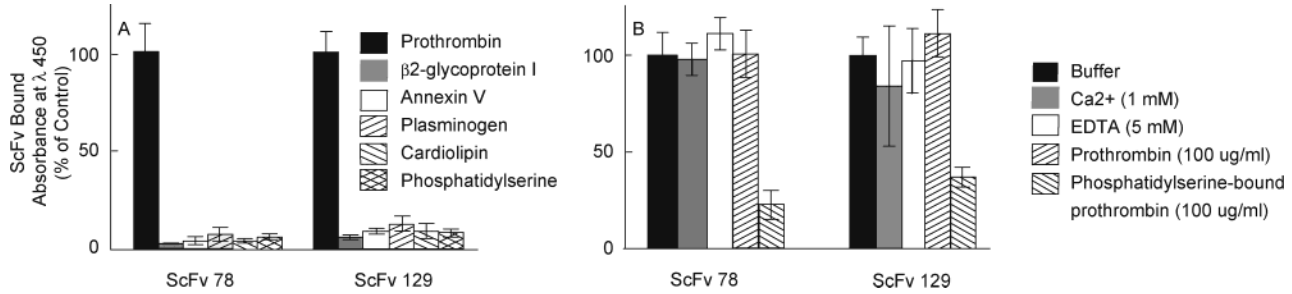

FIGURE 3: Binding specificity of ScFvs. In panel A, wells of ELISA plates were coated with 1 μg/mL of prothrombin, β2-glycoprotein I, annexin V, plasminogen, phosphatidylserine, or cardiolipin. ELISAs were performed with 10 μg/mL ScFv as in Figure 2. To compare the binding of two ScFvs, absorbance associated with binding to prothrombin was expressed as 100%. In panel B, wells of ELISA plates were coated with 1 μg prothrombin, and ScFv (10 μg/mL) was incubated in the presence of various agents, and the bound ScFvs were measured as above. Binding to prothrombin in the presence of buffer alone is considered 100%.

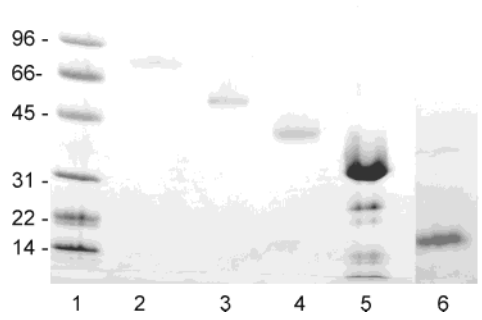

FIGURE 4: Isolation of prothrombin activation fragments. Prothrombin fragments were isolated as described in the methods, and their purity was analyzed in SDS−PAGE: lane 1, molecular weight standards; lane 2, prothrombin; lane 3, prethrombin I; lane 4, fragment 1.2; lane 5, thrombin; lane 6, fragment 2.

with soluble prothrombin up to a concentration of 100 μg/mL. However, when the ScFvs were incubated with a preformed complex of prothrombin (100 μg/mL) and phosphatidylserine (1 mM) vesicles in the presence of 1 mM $CaCl_2$, there was inhibition (Figure 3A). In contrast, when EDTA was substituted for Ca^{2+}, which would prevent association of prothrombin with phosphatidylserine, there was no inhibition of prothrombin binding. These findings suggest that the epitopes for the ScFvs are conformational epitopes available following immobilization of prothrombin on phospholipid or on an appropriate surface. Interestingly, these antibodies did not bind prothrombin immobilized on nitrocellulose or PVDF membranes (data not shown). To assess the affinity of ScFvs, we used surface plasmon resonance, in which prothrombin was immobilized covalently. Analysis of the kinetics assays yielded a dissociation constant (K_d) of 2.07 $\pm$ 0.23 and 3.35 $\pm$ 0.59 μM for AN78 and AN129, respectively.

Immunological Specificity of ScFvs. We isolated prothrombin activation products fragments from normal plasma (Figure 4) and tested in ELISA. As shown in Figure 5, these two ScFvs reacted with prothrombin fragment 1.2 but not with thrombin under similar conditions, suggesting that the epitope is in the amino-terminal half of prothrombin and not in the catalytic domain. To localize the epitope further, we also tested against prethrombin I, the fragment lacking the Gla domain and the kringle 1 domain. Both AN78 and AN129 bound to prethrombin I to a similar extent as to intact prothrombin (Figure 5), suggesting that the cognate epitope for these ScFvs is in the kringle 2 domain in prothrombin, because this domain is present in prothrombin, prethrombin

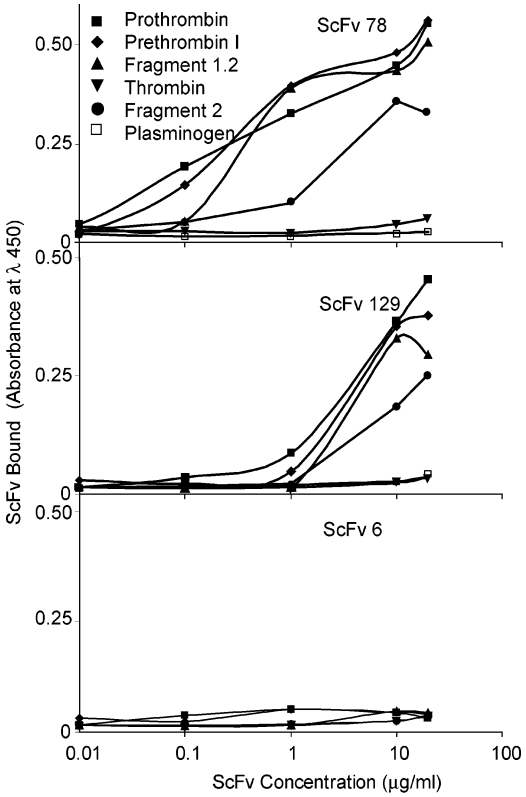

FIGURE 5: Binding of ScFvs to prothrombin activation fragments. Prothrombin activation fragments were isolated from normal plasma as described and tested on ELISA. Wells of ELISA plates were coated 1 μg of each protein and incubated with various concentrations of ScFvs, and the bound ScFvs were measured as in Figure 2: ■, prothrombin; ◆, prothrombin I; ▲ fragment 1.2; ▼, thrombin; ●, fragment 2; □, plasminogen.

I, and fragment 1.2 but is not present in thrombin (Figure 6). To confirm this, we isolated fragment 2 by thrombin digestion of fragment 1.2 and tested it in the ELISA, and as expected, these two ScFvs bound fragment 2 in a concentration-dependent manner (Figure 5).

Effect of ScFvs on Prothrombin Activation. The effects of ScFvs on prothrombin activation by the physiological prothrombinase, phospholipid, and factor Va-bound factor Xa were determined in the thrombin generation assay with purified components. Both ScFvs, AN78 and AN129, promoted prothrombin activation in a concentration-dependent manner (Figure 7). This effect is not due to possible phospholipid contamination in the ScFv preparations because no activation was observed in the absence of phospholipid.

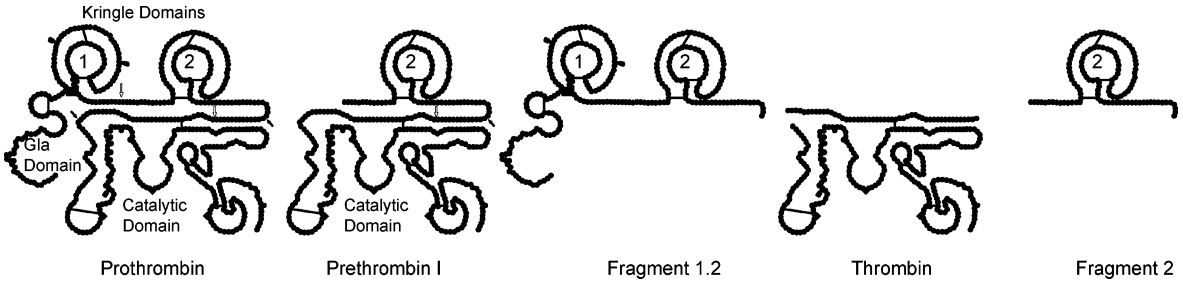

FIGURE 6: Schematic diagram of prothrombin and its activation fragments. The prothrombin is modeled after that of Degan et al. (*2*), showing the Gla domain, two kringle domains, and the catalytic domain. The thrombin cleavage sites are shown with open arrows, and the factor Xa cleavage sites are shown with closed arrows. The ScFvs bound to prothrombin, prothrombin 1, fragment 1.2, and fragment 2 but not to thrombin.

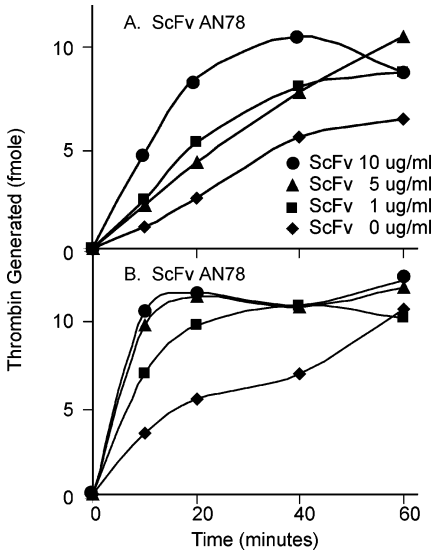

FIGURE 7: Effect of ScFvs on prothrombin activation. The reaction mixtures consist of prothrombin (100 μM), factor Va (5 nM), phospholipid (10 μM) and ScFvs (10 μg/mL), and various concentrations of ScFv. The reaction was started by the addition of 50 pM factor Xa. The thrombin formed was measured by the rate of amidolysis of the thrombin-specific chromogenic substrate S2237.

V Gene Usage of Antiprothrombin ScFvs. The deduced amino acid sequences of VH and VL domains of these two ScFvs are shown in Figure 8. Comparison of the cDNA sequences and the corresponding germline V gene counterparts revealed their closest germline counterparts as follows: AN78 and AN129 VL domains are derived from germline genes VL-13 and JL3, and AN78 and AN129 VH domains are derived from germline genes VH3-30, D3-22/D3-10, and JH6 and VH3-9, D6-19, and JH6, respectively (Figure 8). The VH domains of the clones are derived from different heavy chain families despite similar antigenic target. However, the VL domains are derived from same V gene family, V1-13. AN78 VL domain CDRs displayed R/S values (nucleotide replacement/silent ratios) greater than the frameworks (Table 1). Minimal nucleotide replacements are evident in the CDRs of AN78 VH domain and AN129 VL and VH domains. However, CDRH3s of both clones are highly diversified. AN78 CDRH3 contains one amino acid deletion, two amino acid additions, and five replacements. AN129 CDRH3 contains eight amino acid deletions, two amino acid additions, and one replacement. These results suggest that the V domains have adaptively matured via antigen-induced classical B-cell selection process (*22*).

Table 1: Sequence Analyses of VL and VH Genes[a]

	AN78		AN129	
	heavy chain	light chain	heavy chain	light chain
family	XIII	X	VI	X
subgroup	III	I	III	I
length (amino acid)	138	112	134	113
CDR1	5	14	5	14
CDR2	17	7	17	7
CDR3	15	12	14	12
germline counterpart	VH-3-9, D6-19, JH6	VI-13, JL3	VH3-30, D4-23, JH6	VI-13, JL3
V Gene Mutation				
replacements (R)	5	15	2	2
substitutions (S)	7	7	2	1
R/S CDRs	0/0	11/3	1/2	1/0
R/S FRs	5/7	4/4	1/0	1/1
Junctional Mutation Amino Acid				
deletion	1	1	8	0
insertion	2	0	2	0
replacement	5	1	1	0
silent	2	0	1	0
Nucleotide				
deletion	4	3	23	0
insertion	4	0	3	0
replacement	12	1	4	2
silent	1	0	1	0

[a] CDR, complementarity determining region; FR, framework region.

DISCUSSION

In this study, we show that the antiprothrombin ScFvs, selected from a phage-display antibody library from patients with systemic lupus erythematosus with antiprothrombin antibodies, react with the kringle 2 domain in prothrombin. The epitope in kringle 2 is accessible following immobilization of prothrombin on an anionic phospholipid surface.

The antiprothrombin antibodies belong to the larger group of so-called "antiphospholipid antibodies" (reviewed in refs *23* and *24*). The antiphospholipid antibodies are so named because of their reactivity in ELISA in which anionic phospholipids such as cardiolipin or phosphatidylserine is used to coat the wells. The antigenic targets of the majority of these "antiphospholipid antibodies" are in fact conformational epitopes in phospholipid-binding proteins present in the assay conditions. Immobilization on anionic phospholipid surface induces these epitopes on prothrombin, β2-glycoprotein I, or possibly many other proteins (*25*). The significance of these antibodies arises from the fact that the

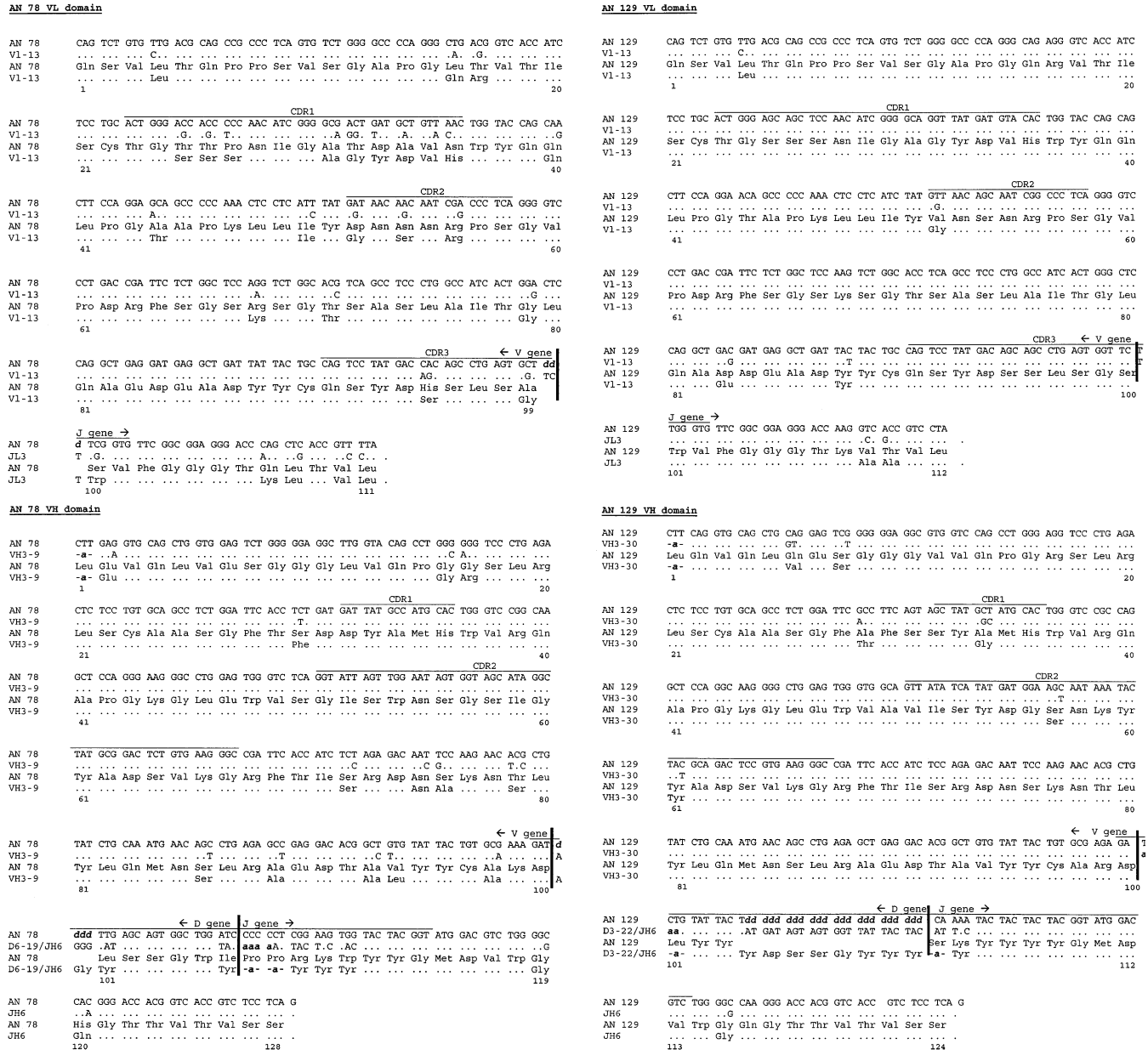

FIGURE 8: cDNA and deduced amino acid sequences of ScFvs aligned with their germline V, (D), and J counterparts. Germline counterparts and deduced amino acid were obtained using, respectively, http://www.ncbi.nlm.nih.gov/igblast/ and http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html. Identities are indicated by dots. Overlined residues indicate the complementarity determining regions (CDRs). Addition and deletion at V/J and D/J junctions are indicated by a and d, respectively. Solid vertical lines demarcate V, D, and J genes. D gene assignment for AN129 VH is ambiguous because equivalent matches were evident with D3-22 and D3-10. The first amino acid (leucine) observed in AN78 and AN129 VH is a product of the VH back primer design for the ScFv CDR sequences.

presence of these antibodies is a risk factor for thrombosis—arterial, venous, microvascular, and embolic (reviewed in refs *23* and *24*). The precise subset of antibodies that is causally related to the thrombotic event is not known. The presence of several different antibodies with various specificities in a given patient limits identifying the precise subset of antibodies responsible for the prothrombotic state (*26*). Nevertheless, the presence antiprothrombin autoantibodies is a significant risk factor for thrombosis. In the Helsinki Heart study, Vaarala et al. have found that elevated levels of antiprothrombin antibodies is an independent risk factor for myocardial infarction in initially healthy middle aged men (*9*−*11*). High levels of prothrombin antibodies were also associated with increased risk of thrombotic complications in patients with systemic lupus erythematosus (*11*).

Under physiological conditions, prothrombin activation catalyzed by the fully assembled prothrombinase complex (factor Xa, its cofactor factor Va, Ca^{2+}, and the anionic phospholipid) proceeds via an ordered, sequential reaction with primary cleavage at Arg320-Ile321 forming meizothrombin as the sole intermediate, followed by the second cleavage at Arg271-Thr272, forming fragment 1.2 and thrombin. While prothrombin is at "zymogen" conformation without any enzymatic activity, meizothrombin exhibits certain characteristics of both prothrombin and thrombin. Relative to thrombin in solution meizothrombin exhibits only 1−2% fibrinogen clotting activity despite having 100% esterase activity (*27*−*29*). The kringle domains, which remain associated, act as an activation energy barrier for the full exposure of substrate binding site for fibrinogen, especially

the kringle 2. The kringle 2 domain interacts intramolecularly with exosite II in the thrombin catalytic domain (*30*−*34*). When kringle 2 is removed proteolytically, the zymogen conformation is further destabilized and thrombin becomes fully active. When kringle 2 domain is added back to thrombin, it binds thrombin with high affinity. This interaction evokes conformational changes in the active site with inhibition of clotting activity (*31*). The complex of thrombin and kringle 2 domain have been crystallized (*32*). The crystallographic studies show that the acidic residues in the inner loop of the kringle 2 make direct contact within 4 Å with the exosite II residues (*32*). Meizothrombin rapidly loses the kringle 1 domain by autolysis to become meizothrombin desF1 (mzTBN-F1), and the crystal structure of bovine mzTBN-F1 has also been determined (*33*). The kringle 2 in mzTBN-F1 has significantly more interdomain contacts compared to a noncovalent complex of free kringle 2 and free thrombin including novel hydrogen bonds to the carbohydrate chain of thrombin. During prothrombin activation, the mass of anionic phospholipid-bound prothrombin is replaced mole for mole by fragment 1.2 and thrombin during the course of the reaction. The high-affinity, noncovalent association between thrombin and fragment 1.2 can not only down regulate its activity but also localize the initial thrombin to the anionic phospholipid on the platelet surface. Inhibition of this association will lead to an enhanced thrombin generation. In the purified system of prothrombin activation assay, the two ScFvs increase the rate of prothrombin activation only to a modest degree. However, in vivo the intact IgG antibodies have a long half-life of about 3−4 weeks in the circulation (*35*) compared to prothrombin (48 h) and may allow slow and continuous activation (*36*). Furthermore, the Fc component may also play a role in inducing the procoagulant activity. Prothrombin has binding sites on platelets (*37*), and the prothrombin-bound antibodies may activate further the platelets via the Fc receptor-mediated mechanism.

Cote et al studied a patient with antiprothrombin antibodies and hypoprothrombinemia using mutated recombinant fragments of prothrombin and localized the epitope to the kringle 2 region and the adjoining thrombin A chain (*38*). This antibody was associated with hypoprothrombinemia and bleeding but had no effect on prothrombin activation. Testing the sera from patients in the Helsinki Heart Study, Purrunen et al found reactivity of antiprothrombin antibodies associated with myocardial infarction, which were inhibited by soluble prothrombin and plasminogen in ELISA (*39*). A peptide sequence derived from a homologous region in the in the kringle 5 of plasminogen and kringle 2 of prothrombin inhibited antibody binding to prothrombin (*39*). In contrast the ScFvs described in this study do not cross-react with plasminogen and furthermore are not inhibited by soluble prothrombin up to 100 μg/mL. These findings underscore the heterogeneity of the anti-kringle 2 domain antibodies. The sequences of antiprothrombin ScFvs from another lupus phage display library described before by Chukwuocha et al are also different from AN78 and AN129 (*40*).

In summary, we have isolated and localized the epitope of antiprothrombin antibodies by phage display, and the availability of a large amount of cloned antibodies with defined specificity will allow studies to define the pathological significance in relevant animal models. Future studies will also provide essential information to develop strategies to inhibit pathogenic autoantibody formation by antigen-specific immunosuppression.

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