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Monoclonal Antibody Light Chain with Prothrombinase Activity[†]

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ABSTRACT: Prothrombin is the precursor of thrombin, a central enzyme in coagulation. Autoantibodies to prothrombin are associated with thromboembolism, but the mechanisms by which the antibodies modulate the coagulation processes are not understood. We screened a panel of 34 monoclonal antibody light chains isolated from patients with multiple myeloma for prothrombinase activity by an electrophoresis method. Two light chains with the activity were identified, and one of the light chains was characterized further. The prothrombinase activity eluted from a gel-filtration column run in denaturing solvent (6 M guanidine hydrochloride) at the characteristic positions of the light chain dimer and monomer. A constant level of catalytic activity was observed across the width of the light chain monomer peak, assessed as the cleavage of IEGR-methylcoumarinamide, a peptide substrate corresponding to residues 268–271 of prothrombin. Hydrolysis of this peptide by the light chain was saturable and consistent with Michaelis–Menten–Henri kinetics (K_m 103 μ M; k_{cat} of 2.62×10^{-2} /min). Four cleavage sites in prothrombin were identified by N-terminal sequencing of the fragments: Arg¹⁵⁵–Ser¹⁵⁶, Arg²⁷¹–Thr²⁷², Arg²⁸⁴–Thr²⁸⁵, and Arg³⁹³–Ser³⁹⁴. The light chain did not cleave radiolabeled albumin, thyroglobulin, and annexin V under conditions that readily permitted detectable prothrombin cleavage. Two prothrombin fragments (M_r 55 000 and 38 000), were isolated by anion-exchange chromatography and were observed to cleave a thrombin substrate, tosyl-GPR-nitroanilide. Conversion of fibrinogen to fibrin was accelerated by the prothrombin fragments generated by the light chain. These findings suggest a novel mechanism whereby antibodies can induce a procoagulant state, i.e., prothrombin activation via cleavage of the molecule.

Thrombin, the enzyme responsible for conversion of fibrinogen to fibrin, is derived by factor Xa-catalyzed cleavage of Arg²⁷¹–Thr²⁷² and Arg³²⁰–Ile³²¹ bonds in prothrombin (1). Autoantibodies to prothrombin found in certain autoimmune states are associated with an increased incidence of thromboembolic phenomena (2, 3), but the mechanism(s) whereby the antibodies induce the hypercoagulable state is not known. Conventional mechanisms of antibody action predict that the autoantibodies should cause bleeding by accelerated clearance of prothrombin–antibody complexes and/or by reduced generation of thrombin due to steric masking of the factor Xa-susceptible sites in prothrombin. The association of the autoantibodies with thrombosis has remained, therefore, an enigma.

Certain autoantibodies are known to be capable of antigen-specific catalysis (4–6). We hypothesized that the autoantibodies can catalyze the cleavage of prothrombin and generate thrombin-like activities. Because previous studies have indicated that the catalytic site of proteolytic antibodies is located in the light chain subunit (7–10), we chose to analyze the cleavage of prothrombin by a panel of well-

characterized human monoclonal antibody light chains (10). These light chains have been previously characterized in regard to isotype, purity, ability to catalyze the cleavage of various oligopeptide substrates, and kinetic parameters (10, 11). Two light chains with prothrombinase activity were identified. Prothrombin cleavage by one of the light chains generated a thrombin-like activity and promoted the conversion of fibrinogen to fibrin, providing proof-of-principle that catalytic autoantibodies to prothrombin might induce a prothrombotic state.

EXPERIMENTAL PROCEDURES

Purified Proteins. The panel of monoclonal light chains, purified to electrophoretic homogeneity from the urine of multiple myeloma patients, was provided by Dr. Alan Solomon, University of Tennessee, Knoxville, TN (11). Gel filtration was in 6 M guanidine hydrochloride, pH 6.5, on a Superose-12 FPLC column (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The light chain fractions were renatured by dialysis against 50 mM Tris-HCl, pH 7.7, 100 mM glycine, 0.025% Tween-20, and 0.02% sodium azide, for 2 days with four buffer changes using a Gibco multi-well dialysis device (Life Technologies Inc., Rockville, MD) to a final guanidine concentration of <1 nM, assuming equilibration across the dialysis membrane. Purity analysis of the light chains included SDS electrophoresis and immunoblotting with specific goat anti-human light chain (κ) antibodies (8, 10). Human thrombin was a gift of Dr. John Fenton, New

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York State Department of Health, Albany, NY. Prothrombin was purified from human plasma (American Red Cross, Portland, OR) by barium citrate adsorption, ammonium sulfate elution, DEAE-Sephadex chromatography and dextran sulfate–agarose chromatography (12, 13). Fibrinogen was purified from normal plasma according to Kazal et al. (14) with the addition of 0.1 M ϵ -aminocaproic acid in the buffers and the plasma (15). Separation of prothrombin fragments digested with the catalyst was performed on a Mono Q column (Amersham Pharmacia) with a linear gradient of sodium chloride (0–1 M; in 200 mL of 10 mM Tris-HCl, pH 8.1; flow rate 0.5 mL/min). Recombinant human annexin V was purified as in ref 16. Purified human thyroglobulin and bovine serum albumin (RIA grade) were from UCB Co., Belgium, and Sigma Chemical Co., St. Louis, MO, respectively.

Protein Cleavage Assays. Prothrombin was labeled with Na¹²⁵I (Amersham Life Sciences Inc., Arlington Heights, IL) using Iodogen (Pierce Chemical Co., Rockford, IL) (10 μ g of Iodogen/mg of prothrombin, 10 min at 4 °C; specific activity 900–1600 cpm/ng) (17). ¹²⁵I-prothrombin (~10 000 cpm) or unlabeled prothrombin (2 μ M) was incubated with light chains (18 h, 37 °C) in 20 μ L of 50 mM Tris-HCl, 100 mM glycine, pH 7.7, and 0.025% Tween-20. Electrophoresis of the reaction mixture was on SDS–polyacrylamide gels (8–24%, Phastgels) under reducing conditions. Autoradiography for detection of ¹²⁵I was with Cronex intensifying screens and Kodak XAR-5 film. The intensity of the intact prothrombin band was estimated by use of an image analyzer equipped with electrophoresis scanning software (Eagle Eye still video system, Stratagene, La Jolla, CA). The amounts of radioactivity loaded on the gels were always within the linear response range of the X-ray film. In experiments with unlabeled prothrombin as substrate, product protein bands were identified by silver staining. For N-terminal sequencing of the product bands, unlabeled prothrombin digests were fractionated on a larger SDS–polyacrylamide gel (8–20%; 8 \times 8 cm), the gel was electroblotted onto a poly(vinylidene difluoride) (PVDF) membrane (Immobilon P, Millipore Corp., Bedford, MA), the blot was stained with Coomassie Blue R, the product bands were carefully cut out, and their sequence was determined by use of a pulsed liquid-phase sequenator with online phenylthiohydantoin-amino acid detection (Applied Biosystems, model 477A; Foster City, CA) at the Baylor College of Medicine Protein Sequencing Facility.

Preparation of ¹²⁵I-thyroglobulin (98 \times 10³ cpm/ng) and ¹²⁵I-albumin (74 \times 10³ cpm/ng) was by the chloramine T method (18), and ¹²⁵I-annexin V (2.5 \times 10³ cpm/ng) was prepared by the Iodogen method (17). In each case, a single major radiolabeled protein band was evident by SDS gel electrophoresis and autoradiography with the expected mobility (thyroglobulin 330 kDa, albumin 67 kDa, and annexin V 34 kDa).

IEGR-MCA Cleavage. IEGR-MCA (Peptides International, Inc., Osaka, Japan) was treated with the catalyst in 96-well microplates (Microfluor W, Dynatech, Chantilly VA) in 50 μ L of 0.05 M Tris-HCl, 0.1 M glycine, pH 7.7, and 0.025%

Tween-20 (4 h, 37 °C). Cleavage of the R-MCA bond in this substrate is evident as an increase in fluorescence (λ_{ex} 370 nm, λ_{em} 460 nm; Perkin-Elmer LS-50 spectrofluorometer equipped with a plate reader). To estimate kinetic parameters, the rate of cleavage of increasing concentrations of IEGR-MCA was measured, the values were corrected for fluorescence in the absence of the catalyst (14–38 FU), and the data were fitted to the Michaelis–Menten–Henri equation ($v = V_{\text{max}} [S]/(K_m + [S])$). The fluorescence yield of standard aminomethylcoumarin analyzed at 1 μ M was 14.5 FU.

t-GPR-NA Cleavage. Thrombin-like activity present in prothrombin digests was measured with t-GPR-NA (0.4 mM, Boehringer Mannheim, Indianapolis, IN) as the thrombin substrate in 96-well microplates by use of an enzyme-linked immunosorbent assay (ELISA) reader (410 nm) in 10 mM Tris-HCl, pH 7.5, and 0.15 M NaCl (19, 20). As reference, the activity of purified thrombin (kindly supplied by Dr. John Fenton, New York State Department of Health, Albany, NY) was measured in parallel experiments.

Coagulation Assays. Conversion of purified fibrinogen to fibrin was measured by a coagulation assay. Prothrombin (3 μ M) was incubated with various concentration of the light chain in 20 μ L of 0.15 M NaCl and 10 mM Tris-HCl, pH 7.5. The mixture was diluted to 100 μ L in the same buffer and added to 0.2 mL of prewarmed fibrinogen (5 mg/mL) in a cuvette, and the clotting time recorded in a fibrometer (Becton-Dickinson, Cockysville, MD). For comparison, clotting times in the presence of purified thrombin were determined.

RESULTS

Prothrombinase Activity Screening. We screened a panel of 34 monoclonal light chains for the ability to cleave ¹²⁵I-labeled prothrombin. The light chain panel is a random collection isolated from the urine of patients with Bence Jones myeloma. Some of these light chains have previously been described to cleave model peptide substrates at varying levels (10). Of the 34 light chains, two cleaved prothrombin as detected by the appearance of radioactive products with mass smaller than the intact prothrombin (apparent M_r estimated by SDS–PAGE 74 000; Figure 1 A, lanes 1 and 12). One of the light chains (RHY) was characterized further. Concentration-dependent cleavage by RHY was evident with ¹²⁵I-prothrombin as the substrate (Figure 1B). The cleavage reaction is not a radiolysis artifact. Nonradioactive prothrombin was also cleaved in a concentration-dependent manner by the light chain (8.2–77.5% prothrombin cleavage at 0.1–2 μ M light chain; initial prothrombin concentration 2 μ M). Prothrombin products at 55, 38, and 27 kDa were evident by silver staining of the electrophoresis gels (Figure 2 A).

Several previous studies have validated the phenomenon of proteolysis by antibody light chains by demonstrating elution of the catalytic activity with light chains from gel-filtration columns run in denaturant (which removes adventitious proteases bound to the light chains) (8, 10), molecular cloning of a proteolytic light chain (21), and the loss of the activity by site-directed mutagenesis at the catalytic residues (22). In the present study, only two of 34 equivalently purified light chains displayed prothrombinase activity, arguing against a contribution of contaminant proteases. Gel

¹ Abbreviations: IEGR-MCA, Ile-Glu-Gly-Arg-methylcoumarin-amide; t-GPR-NA, tosyl-Gly-Pro-Arg-nitroanilide; FU, fluorescence unit; M_r , molecular weight.

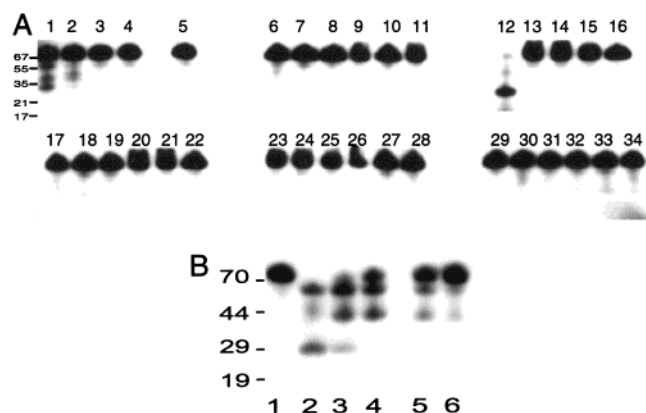


FIGURE 1: Screening for prothrombinase activity. (A) Autoradiogram of ^{125}I -prothrombin (10 000 cpm) incubated with light chains ($2\ \mu\text{M}$; 16 h, 37°C ; each lane contains a different light chain) and subjected to SDS-PAGE. Prothrombinase activity is evident in lanes 1 and 12 (light chain codes RHY and LAY, respectively). (B) Silver-stained SDS gel showing dose-dependent ^{125}I -prothrombin cleavage by increasing light chain (RHY) concentrations. Prothrombin was incubated with buffer alone (lane 1) or 280, 140, 70, 35, and 18 nM RHY (lanes 2, 3, 4, 5, and 6, respectively). Reaction conditions were as in panel A.

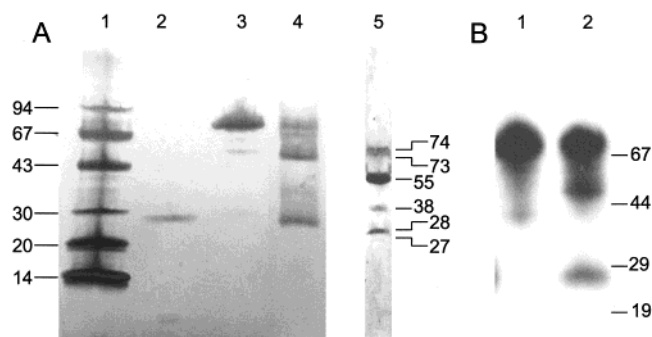


FIGURE 2: Identification of prothrombin fragments generated by the light chain and effect of hirudin on the cleavage reaction. (A) Silver-stained (lane 4) and Coomassie-stained (lane 5) SDS-polyacrylamide gels of prothrombin ($2\ \mu\text{M}$) digested with the RHY light chain ($2\ \mu\text{M}$; 37°C , 18 h). Lanes 1, 2, and 3, respectively, show silver stained M_r markers, the RHY light chain alone, and prothrombin alone. The amounts of the light chain and prothrombin loaded in lanes 2 and 3, respectively, are equivalent to the amounts of these reagents in the digest in lane 4. (B) Autoradiogram of SDS gel showing ^{125}I -prothrombin incubated with RHY (320 nM; lane 2) and buffer without light chain (lane 1). Both incubations contained 5 units/mL hirudin.

filtration in a strong denaturant (6 M guanidine hydrochloride) revealed two protein peaks. SDS electrophoresis showed that the dimer and monomer forms of the light chain were the major constituents of the two peaks (inset, Figure 3A, lanes 1 and 2). In addition to the major dimer and monomer bands, certain higher M_r bands were evident by silver staining. All of these bands were stainable with the anti-light chain (κ) antibodies following immunoblotting, suggesting that they are oligomers of the light chain (Figure 3A, inset, lanes 3 and 4). The specificity of the antibodies employed for immunoblotting was evident from the absence of staining of the M_r standards employed in the gel electrophoresis (not shown). A minor 25 kDa anti-light chain stainable band was apparent in the dimer fraction, presumably due to dissociation of the dimer during the electrophoresis procedure. The electrophoretic profile of the light chain preparation loaded on the gel-filtration column was es-

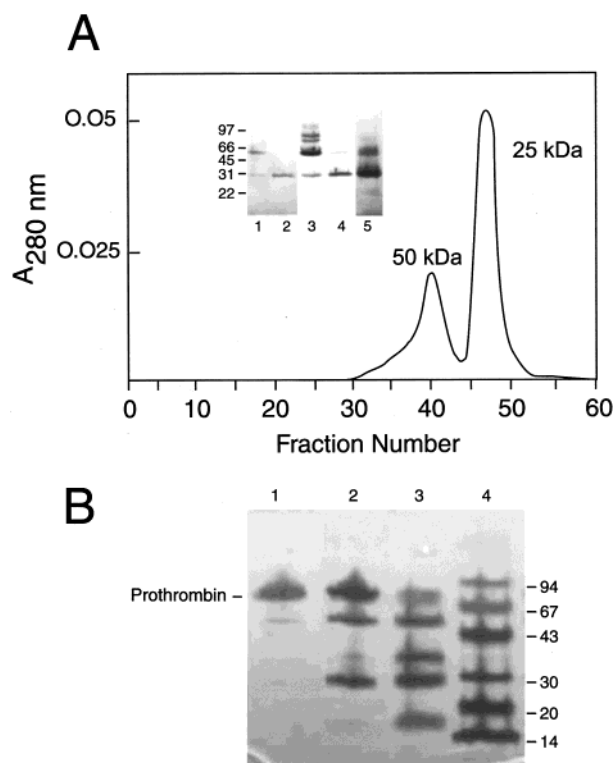


FIGURE 3: Prothrombin cleavage by the RHY light chain subjected to denaturing gel filtration (6 M guanidine hydrochloride). (A) Superose-12 fractionation of light chain ($200\ \mu\text{g}$) showing the monomer and dimer fractions (flow rate $0.25\ \text{mL/min}$). Column calibration was with thyroglobulin, IgG, albumin, ovalbumin, and ribonuclease. (Inset) Nonreducing SDS gel electrophoresis of the dimer (lane 1) and monomer fraction (lane 2) stained with silver or anti-light (κ) antibodies (lanes 3 and 4, respectively). Lane 5, silver stained, overloaded light chain prior to gel filtration. (B) Silver-stained SDS gel showing prothrombin cleavage by the dimer peak (lane 2) and monomer peak (lane 3) (fraction numbers 40 and 45, respectively, in panel A). Lane 1, prothrombin incubated in buffer without catalyst; lane 4, M_r markers. Digestion conditions: $2\ \mu\text{M}$ prothrombin, 18 h, 37°C ; $12\ \mu\text{L}$ light chain fractions. Column fractions were renatured by extensive dialysis to remove the denaturant prior to the cleavage assay.

entially a composite of the patterns observed for the dimer and monomer fractions, with minor high M_r oligomer bands visible in addition to the major 50 and 25 kDa bands. Previous studies have shown the tendency of monoclonal light chains and intact antibodies to aggregate via noncovalent interactions and disulfide exchange reactions (23, 24). N-Terminal amino acid sequencing of the monomer fraction yielded a single peptide sequence corresponding to the N-terminal framework 1 residues of κ subgroup III light chains (EIVLTQSPAT). A minor peptide sequence with two additional residues at the N terminus was also present (TGEIVLTQSPAT), presumably reflecting the failure of the leader peptidase to cleave the light chain precursor form correctly. The prothrombinase activity recovered from the gel-filtration column corresponded exactly to the retention times of the dimer and monomer fractions (Figure 3B). The monomer fraction displayed 3.5-fold greater activity than the dimer fraction, determined from the extent of depletion of the intact prothrombin band in SDS-polyacrylamide gels. To enable more rigorous quantitation, IEGR-MCA was employed as the substrate, cleavage of which can be readily measured by fluorometry in 96-well plates. This model

Table 1: Peptide Bonds in Prothrombin Cleaved by RHY Light Chain^a

<i>M_r</i>	N-terminal sequence ^b	cleavage site
73 000	ANTFL (1–5)	unidentified
55 000	SEGSS (156–160)	Arg ¹⁵⁵ –Ser ¹⁵⁶
38 000	TATES (272–276)	Arg ²⁷¹ –Thr ²⁷²
	TFGSG (284–288)	Arg ²⁸³ –Thr ²⁸⁴
27 000	NIEKI (394–398)	Arg ³⁹³ –Asn ³⁹⁴

^a Reaction conditions: light chain, 1 μ M; prothrombin, 2 μ M; 18 h, 37 °C. prothrombin fragment bands at the indicated *M_r* were electroblotted from SDS–polyacrylamide gels and sequenced by Edman degradation. ^b Corresponding prothrombin residues.

substrate corresponds to the P4–P1 residues of prothrombin recognized by factor Xa (factor Xa cleavage site Arg²⁷¹–Thr²⁷²). The specific IEGR-MCA cleaving activity observed in column fractions across the width of the monomer peak was constant (34 233, 37 447, and 36 739 fluorescence units^{–1} h (A₂₈₀ unit)^{–1} in fractions 46, 47, and 48 in Figure 3).

Cleavage Sites. To identify the scissile bonds, prothrombin product bands evident by electrophoretic separation of the prothrombin–light chain reaction mixture were electroblotted onto a PVDF membrane and subjected to N-terminal sequencing by Edman degradation. These products were absent in reaction mixtures consisting of prothrombin and a control light chain without proteolytic activity. In every case but one, the products identified were fragments of prothrombin. The exception was the band at 28 kDa (Figure 2A), the N-terminal sequence of which was identical to the Kabat database sequence of human κ light chains. The scissile bonds were identified on the basis of detection of fragments that (i) possessed a clearly identifiable amino acid sequence five or more residues in length that was identical to internal subsequences of prothrombin and (ii) sequenced at the level of 10 pmol or more. Four prothrombin fragments with apparent *M_r* 55 000, 38 000, and 27 000 that met the criteria for localization of the scissile bonds were evident (Table 1). [Note: the apparent *M_r* values determined in Figure 2A are not the true mass of the fragments—for instance, prothrombin, a 72 kDa protein, migrated as a 74 kDa band in the SDS gel.] The 38 kDa band contained two peptide fragments, as evident by the presence of two amino acids in each sequencing cycle. The 27 kDa band visible in the Coomassie-stained gel could not be discriminated from the 28 kDa light chain band in the silver-stained gels, probably because of the poorer resolving power of the latter gel (see Experimental Procedures). Residues 1–5 in the 73 kDa band were identical to the N-terminus of prothrombin, suggesting that this band is generated by an unidentified proteolytic cleavage close to the C-terminus of prothrombin. Four cleavage sites were identified from the remaining bands, all of which are Arg–X bonds.

Small amounts of thrombin are often present in prothrombin preparations and may be hypothesized to cleave prothrombin at accelerated rates following binding of the prothrombin by the light chain. Cleavage of ¹²⁵I-prothrombin by RHY was evident in the presence of excess hirudin (5 units/mL), a known inhibitor of thrombin (19, 24), evidenced by the prominent 27 kDa band and the diffuse band(s) located in the *M_r* region ~50 kDa (Figure 2B). These data indicate that the light chain is capable of directly cleaving prothrom-

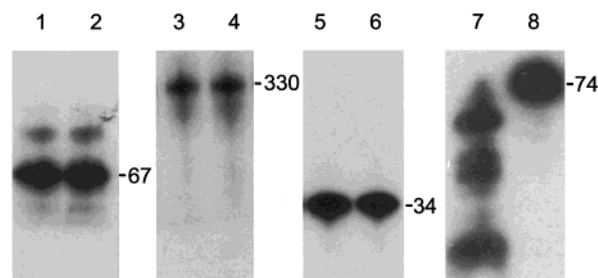


FIGURE 4: Substrate specificity of the prothrombinase light chain. Shown are autoradiograms of SDS gels. Substrates used were ¹²⁵I-albumin (lanes 1 and 2; 4–15% gels), ¹²⁵I-thyroglobulin (lanes 3 and 4; 4–15% gels), ¹²⁵I-annexin V (lanes 5 and 6; 8–25% gels), ¹²⁵I-prothrombin (lanes 7 and 8; 8–25% gels). Digestions were done in assay buffer (even-numbered lanes) or RHY light chain (odd-numbered lanes). Light chain concentrations in the albumin, thyroglobulin, annexin V, and prothrombin cleavage assays were 0.25, 2, 2, and 0.1 μ M, respectively. Incubation was for 12–18 h at 37 °C.

bin. Additional cleavage reactions may occur due to the light chain-catalyzed generation of enzymatically active prothrombin products. Hirudin decreased substantially the overall consumption of the prothrombin band, presumably because the initial light chain-catalyzed prothrombin cleavage generates a hirudin-inhibitable thrombin-like activity responsible for further prothrombin cleavage. Fine mapping of the peptide bonds sensitive to the light chain versus possible thrombin-like activity present in the reaction mixture is beyond the scope of the present studies and will require the careful temporal analysis of the reaction.

Substrate Specificity. To analyze substrate specificity, we studied the ability of the light chain to cleave albumin, thyroglobulin, and annexin V, proteins unrelated in sequence to prothrombin. ¹²⁵I-labeled albumin, ¹²⁵I-thyroglobulin, and ¹²⁵I-annexin V were treated with the light chain and the cleavage was measured by SDS–PAGE and autoradiography as for the prothrombin substrate. Undegraded albumin, thyroglobulin, and annexin V were observed after incubation with the light chain, whereas near-complete degradation of prothrombin occurred under similar conditions (Figure 4).

Kinetics. Increasing concentrations of prothrombin were incubated with a fixed concentration of the light chain, and depletion of the intact prothrombin band evident in SDS gels was measured by densitometry. The rate of prothrombin cleavage continued to increase linearly between 0.5 and 16 μ M prothrombin (not shown), suggesting an apparent *K_m* > 16 μ M (highest prothrombin concentration studied). Several prothrombin molecules were cleaved per light chain molecule over the course of the reaction [reaction rate 6.4 μ M prothrombin cleaved (μ M light chain)^{–1} (21 h)]. The *V_{max}* could not be determined because the reaction was not saturated at the prothrombin concentration studied.

Note that the prothrombin consumption rate is the sum of the rates of initial cleavage by the light chain and the subsequent cleavage reactions catalyzed by the light chain-generated thrombin-like enzymatic activity. To estimate the kinetic constants of the light chain without interference from secondary cleavage reactions, we turned to hydrolysis of IEGR-MCA, a model peptide substrate for factor Xa-catalyzed cleavage of Arg²⁷¹–Thr²⁷² in prothrombin. This substrate permits study of the kinetics of cleavage at a single bond (Arg-MCA), as opposed to the average kinetics

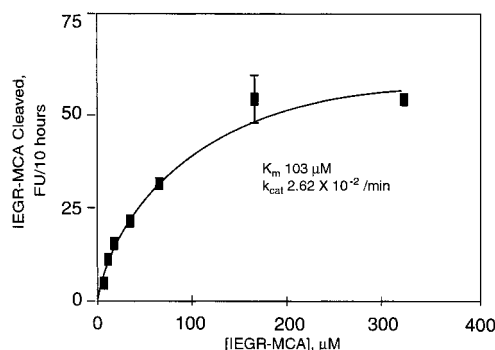


FIGURE 5: Saturable cleavage of IEGR-MCA by RHY light chain ($0.3 \mu\text{M}$). Incubation conditions, 10 h, 37°C . Values are means of triplicates \pm SD fitted to Michaelis–Menten–Henri equation by nonlinear regression. FU, fluorescence units (λ_{ex} 370 nm, λ_{em} 460 nm).

observed when measuring the cleavage of multiple bonds in large substrate such as prothrombin. The cleavage rates at various concentrations of IEGR-MCA were consistent with Michaelis–Menten–Henri kinetics, and 16 turnovers of the light chain were evident over 10 hours (K_m $103 \mu\text{M}$, k_{cat} $2.62 \times 10^{-2}/\text{min}$) (Figure 5). The light chain, therefore, is a true catalyst, in that it is capable of turnover.

Thrombin-Like Activity of Prothrombin Fragments. A known thrombin substrate, t-GPR-pNA (19) was utilized to determine the thrombin-like activity of the prothrombin cleavage products generated by the light chain. The light chain itself at concentrations up to $4 \mu\text{M}$ did not cleave this substrate, ensuring valid measurement of enzymatic activity due to prothrombin fragments. To further safeguard against interference because of the enzymatic activity of the light chain, the prothrombin–light chain reaction mixture was separated by anion-exchange FPLC. This procedure permitted identification of two A_{280} peaks (1 and 2 in Figure 6, upper panel) as t-GPR-pNA hydrolyzing species (Figure 6, lower panel; prothrombin products of 38 and 55 kDa were identified in peaks 1 and 2, respectively, see Table 1 for prothrombin cleavage site identification corresponding to these products). Peak 3 was devoid of enzymatic activity. The specific activity of peaks 1 and 2 is $\sim 20\,000$ -fold lower than of authentic thrombin (see Discussion for our assessment of the biological significance of activity level observed in the prothrombin fragments). Detailed kinetic study of the prothrombin fragments was beyond the scope of the present study, as our purpose here was limited to demonstrating that the prothrombinase activity of the light chain generates thrombin-like activity.

Procoagulant Effect of Light Chain. The ability of the light chain to generate coagulation-competent thrombin-like activity from prothrombin was studied by measuring the conversion of fibrinogen to fibrin in coagulation assays. Prothrombin incubated with the light chain was mixed with purified fibrinogen and the clotting time was recorded in a fibrometer. A progressive shortening of the clotting time by prothrombin treated with increasing concentrations of the light chain was evident (Table 2), suggesting that prothrombin cleavage by the light chain produces a fragment(s) with fibrinogen-converting enzymatic activity. The light chain itself was without effect on the clotting time in the absence of prothrombin.

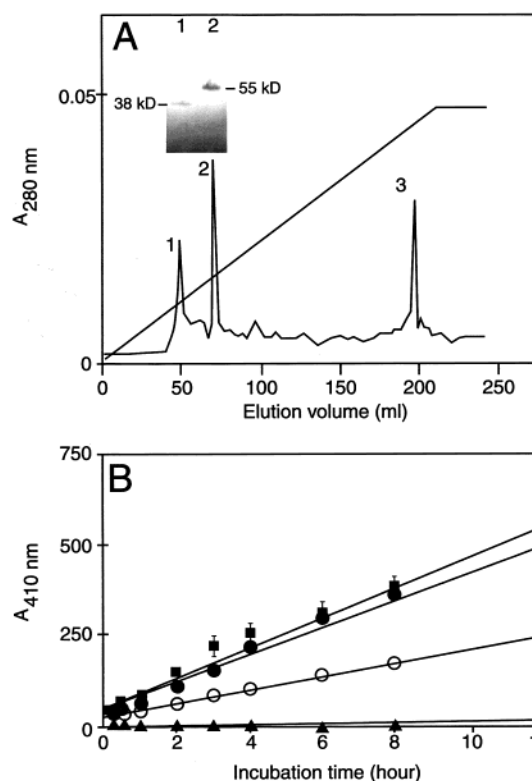


FIGURE 6: Separation of enzymatically active prothrombin fragments generated by light chain catalyzed prothrombin digestion. (A) Anion exchange separation (Mono Q) of prothrombin–light chain mixture (0–1 M NaCl linear gradient in 10 mM Tris-HCl, pH 8.1). Digestion conditions: $30 \mu\text{M}$ prothrombin, $0.5 \mu\text{M}$ RHY light chain, 18 h, 37°C . (Inset) Silver stained SDS gel of protein peak fractions labeled 1 and 2. (B) t-GPR-NA by peak 1 (○), peak 2 (●) and thrombin (■). Also shown (▲) is background esterolysis in buffer incubated with undigested prothrombin ($10 \mu\text{g}/\text{mL}$). t-GPR-NA, 0.4 mM ; peaks 1 and 2, $10 \mu\text{g}/\text{mL}$; thrombin, 4 pM .

Table 2: Procoagulant Effect of the RHY Light Chain^a

test sample	clotting time (s)
light chain ($4 \mu\text{M}$) + prothrombin	98
light chain ($3 \mu\text{M}$) + prothrombin	105
light chain ($2 \mu\text{M}$) + prothrombin	208
light chain ($1 \mu\text{M}$) + prothrombin	223
light chain ($4 \mu\text{M}$) alone (control)	>600
buffer alone (control)	>600
thrombin ($5 \text{ units}/\text{mL}$)	50.5
thrombin ($1 \text{ unit}/\text{mL}$)	118
thrombin ($0.5 \text{ unit}/\text{mL}$)	151
thrombin ($0.25 \text{ unit}/\text{mL}$)	282
thrombin ($0.125 \text{ unit}/\text{mL}$)	439

^a Prothrombin ($3 \mu\text{M}$) preincubated with various concentrations of the light chain (4 h, 37°C) was added to fibrometer cuvettes containing prewarmed fibrinogen ($5 \text{ mg}/\text{mL}$) and the clotting time was measured. Controls included fibrinogen mixed with the light chain without prothrombin, prothrombin without the light chain, and buffer without light chain or prothrombin. Thrombin was employed to validate the assay (thrombin concentrations are in NIH units; $1 \text{ unit}/\text{mL} \sim 10 \text{ nM}$).

DISCUSSION

Our studies show a model antibody light chain to be capable of cleaving prothrombin fragments and generating thrombin-like fragments that promote coagulation. The prothrombinase activity of the light chain is quite specific in that, under similar assay conditions, no cleavage of radioiodinated albumin, annexin V, thyroglobulin (present study), and VIP (10) was detected. Several other light chains

of the same isotype (κ) as the prothrombinase light chain were devoid of catalytic activity, suggesting that the activity resides in the variable domain. Active-site serine residues in certain light chains akin to those in serine proteases have previously been predicted on the basis of sequence homology studies (7) and confirmed by site-directed mutagenesis (22). In the present study, cleavage of Arg–X bonds in the prothrombin–light chain reaction mixtures was evident, similar to the scissile bond specificity of conventional coagulation proteases (most of which belong to the serine protease family) (25). Cleavage of four peptide bonds was deduced by sequencing the prothrombin fragments present in the prothrombin–light chain mixture. One of the deduced cleavage sites has not been described as a target of coagulation proteases (Arg³⁹³–Asn³⁹⁴), one is a known factor Xa cleavage site (Arg²⁷¹–Thr²⁷²), and two are known thrombin cleavage sites (Arg¹⁵⁵–Ser¹⁵⁶ and Arg²⁸³–Thr²⁸⁴). The cleavage of prothrombin was evident even in the presence of hirudin, a thrombin inhibitor, suggesting that the light chain is responsible for the initial cleavage of prothrombin. However, hirudin decreased the consumption of prothrombin. These results may be explained by the generation of a thrombin-like activity due to the light chain cleavage, which in turn may cleave prothrombin further at the thrombin-sensitive sites (Arg¹⁵⁵–Ser¹⁵⁶, Arg²⁷¹–Thr²⁷², and Arg²⁸³–Thr²⁸⁴; see Table 1).

Direct proof for the enzymatic activity of the prothrombin cleavage products was obtained by purifying the products and demonstrating their ability to hydrolyze a well-documented thrombin substrate, tosyl-GPR-NA. Moreover, the prothrombin cleavage products induced the formation of fibrin from fibrinogen, which is the terminal, thrombin-catalyzed step of blood coagulation. The procoagulant activity of the reaction products, although low compared to that of authentic thrombin, provides proof-of-principle that antibodies can activate the coagulation cascade via cleavage of prothrombin.

The obvious pitfall in these studies is that an adventitious conventional protease(s) may be responsible for the observed prothrombinase activity. Arguments against this hypothesis are as follows. Several light chains purified by procedures identical to those employed for the prothrombinase light chains were devoid of the activity. Gel filtration of the prothrombinase light chain in a solvent that precludes noncovalent protein–protein association resulted in the appearance of the proteolytic activity in the characteristic “signature” pattern of the light chain, i.e., in the 50 kDa disulfide-bonded dimer and the 25 kDa monomer fractions. The specific activity of the catalytic fractions (magnitude of the activity per unit protein) across the width of the monomer peak was constant, fulfilling a classical criterion of enzyme purity. Previously, the peptidase activity of a light chain was shown to be lost by mutagenesis at the active-site residues (22). Moreover, a recombinant Fv construct containing a peptidase VL domain linked to its natural VH domain partner displayed superior activity compared to the VL linked to an irrelevant VH domain (26). Such predictable activity changes are inconsistent with the contamination hypothesis.

Several previous studies have shown that the light chain subunit of antibodies can recognize target antigens even in the absence of the heavy chain (27–29). The prothrombinase light chains reported here were identified by random screen-

ing of a panel of monoclonal light chains from myeloma patients—the antigen(s) responsible for stimulating the synthesis of the prothrombinase light chains, as other myeloma light chains, is not known. Similarly, our data do not allow us to assess whether the prothrombinase light chains are the constituent subunits of high-affinity anti-prothrombin antibodies or whether they display the prothrombinase activity due to a cross-reactivity phenomenon.

Over the course of the reaction (21 h), about 6 molecules of prothrombin were cleaved per molecule of the light chain. The light chain, therefore, is capable of turnover, a defining feature of a catalyst. Whether the rate of cleavage is sufficiently rapid to promote coagulation *in vivo* cannot be predicted with firmness. The apparent turnover (k_{cat}) of the reaction is lower by 3 log orders compared to factor Xa-catalyzed cleavage of prothrombin (30). Note, however, that the present study was designed only to validate the hypothesis of antibody-catalyzed prothrombin cleavage and activation—additional screening for prothrombinase antibodies utilizing appropriate combinatorial libraries and mechanism-based selection reagents is likely to yield higher turnover antibodies, such as catalytic antibodies to other polypeptide targets that can display kinetic efficiencies exceeding those of conventional proteases (31). Factors that motivate the searches for antibody prothrombinases are (a) the products of prothrombin cleavage are themselves catalytic, thus, the biological efficacy of a prothrombin-cleaving antibody will depend not only on its own turnover rate but can be amplified by the enzymatically active prothrombin fragment(s); (b) the half-life of antibodies is on the order of weeks (32), compared to minutes for activated factor Xa (33), permitting repeated slow catalytic activation; (c) the antibody concentrations in blood is in the micromolar range, and antibody light chain concentration in myeloma patients is in the millimolar range, compared to trace concentrations of conventional proteases. At these concentrations, even low turnover activities might display profound biological effects.

Concerning the biological role of prothrombinase light chains, coagulation disorders in multiple myeloma patients are well-known (34). The clinical significance of the prothrombinase light chains described in the present study is not known. The light chain donors are deceased and no clinical data to clarify this possibility are available. On the other hand, it is reasonable to pursue the hypothesis that antibody-catalyzed prothrombin cleavage contributes toward thrombosis in autoimmune disorders in which prothrombin binding antibodies have been described (2, 3, 35, 36). The reasons for the statistical correlation between the presence of prothrombin binding autoantibodies and thrombosis remain a mystery. If the anti-prothrombin antibodies work by conventional mechanisms, they should cause a hypocoagulable state. For instance, the antibodies may sterically hinder the recognition of prothrombin by factor Xa, or by phospholipids and other cofactors, and thereby inhibit the proteolytic activation of prothrombin. Another conventional mechanism is the accelerated clearance of circulating prothrombin via immune complex uptake by various types of cells (35–37). In contrast, prothrombin activation by proteolytic autoantibodies provides a logical explanation for the observed association between the anti-prothrombin antibodies and thrombosis.

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REFERENCES

- Degen, S. J., and Sun, W. (1998) *Crit. Rev. Eukaryotic Gene Expression* 8, 203–24.
- Palosuo, T., Virtamo, J., Haukka, J., and Vaarala, O. (1997) *Thromb. Haemostasis* 78, 1178–1182.
- Bertolaccini, M. L., Atsumi, T., Khamashta, M. A., Amengual, O., and Hughes, G. R. (1998) *J. Rheumatol.* 25, 1104–1108.
- Paul, S., Volle, D. J., Beach, C. M., Johnson, D. R., Powell, M. J., and Massey, R. J. (1989) *Science* 244, 1158–62.
- Shuster, A. M., Gololobov, G. V., Kvashuk, O. A., Bogomolova, A. E., Smirnov, I. V., and Gabibov, A. G. (1992) *Science* 256, 665–667.
- Lacroix-Desmazes, S., Moreau, A., Sooryanarayana, Bonnemain, C., Stieltjes, N., Pashov, A., Sultan, Y., Hoebeke, J., Kazatchkine, M. D., and Kaveri, S. V. (1999) *Nature Med* 5, 1044–7.
- Erhan, S., and Greller, L. D. (1974) *Nature* 251, 353–355.
- Sun, M., Gao, Q. S., Li, L., and Paul, S. (1994) *J. Immunol* 153, 5121–6.
- Matsuura, K., Yamamoto, K., and Sinohara, H. (1994) *Biochem. Biophys. Res. Commun.* 204, 57–66.
- Paul, S., Li, L., Kalaga, R., Wilkins-Stevens, P., Stevens, F. J., and Solomon, A. (1995) *J. Biol. Chem.* 270, 15257–15261.
- Solomon, A. (1985) *Methods Enzymol.* 116, 101–121.
- Miletich, J. P., Jackson, C. M., and Majerus, P. W. (1978) *J. Biol. Chem.* 253, 6908–6916.
- Modi, G. J., Blajchman, M. A., and Ofofu, F. (1984) *Thromb. Res.* 36, 537–547.
- Kazal, L. A., Amsel, S., Miller, O. P., and Tocantins, L. M. (1963) *Proc. Soc. Exp. Biol. Med.* 112, 989.
- Martinez, J., Palascak, J. E., and Kwasniak, D. (1978) *J. Clin. Invest.* 61, 535–538.
- Thiagarajan, P., and Benedict, C. R. (1997) *Circulation* 96, 2339–2347.
- Salacinski, P. R., McLean, C., Sykes, J. E., Clement-Jones, V. V., and Lowry, P. J. (1981) *Anal. Biochem.* 117, 136–146.
- Bolton, A. E., V. Lee-Own, R. K. McLean, and G. S. Challand. (1979) *Clin. Chem.* 25, 1826–1830.
- Lottenberg, R., Hall, J. A., Fenton, J. W., 2nd, and Jackson, C. M. (1982) *Thromb. Res.* 28, 313–332.
- Fenton, J. W. 2d. (1989) *Semin. Thromb. Hemostasis* 15, 265–268.
- Gao, Q. S., Sun, M., Tyutyulkova, S., Webster, D., Rees, A., Tramontano, A., Massey, R. J., and Paul, S. (1995) *J. Biol. Chem.* 270, 20870.
- Gao, Q. S., Sun, M., Rees, A. R., and Paul, S. (1995) *J. Mol. Biol.* 253, 658–664.
- Myatt, E. A., Westholm, F. A., Weiss D. T., Solomon A., Schiffer, M. and Stevens, F. J. (1994) *Proc Natl Acad Sci U.S.A.* 91, 3034–3038.
- Li, L., Sun, M., Gao, Q.-S., and Paul, S. (1996) *Mol. Immunol.* 33, 593–600.
- Davie, E. W., Fujikawa, K., and Kisiel, W. (1991) *Biochemistry* 30, 10363–10370.
- Sun, M., Gao, Q. S., Kirnarskiy, L., Rees, A., and Paul, S. (1997) *J. Mol. Biol.* 271, 374–385.
- Pereira, B., Benedict, C. R., Le, A., Shapiro, S. S., and Thiagarajan, P. (1998) *Biochemistry* 37, 1430–1437.
- Sun, M., Li, L., Gao, Q. S., and Paul, S. (1994) *J. Biol. Chem.* 269, 734–738.
- Goni, F., Chen, P. P., Pons-Estel, B., Carson, D. A., and Frangione, (1985) *J. Immunol.* 135, 4073–9.
- Kalafatis, M., Egan, J. O., van't Veer, C., Cawthorn, K. M., and Mann, K. G. (1997) *Crit. Rev. Eukaryotic Gene Expression* 7, 241–280.
- Tyutyulkova, S., Gao, Q. S., Thompson, A., Rennard, S., and Paul, S. (1996) *Biochim Biophys Acta* 1316, 217–23.
- Spiegelberg, H. L., Fishkin, B. G., and Grey, H. M. (1968) *J. Clin. Invest.* 47, 2323–2330.
- Deykin, D., Cochios, F., DeCamp, G., and Lopez, A. (1968) *Am. J. Physiol.* 214, 414–419.
- Glaspy, J. A. (1992) *Hematol. Oncol. Clin. North Am.* 6, 1301–1314.
- Rapaport, S. I., Ames, S. B., and Duvall, B. J. (1960) *Blood* 15, 121.
- Bajaj, S. P., Rapaport, S. I., Barclay S., and Herbst, K. D. (1983) *Blood* 61, 684–692.
- Cote, H. C., Huntsman, D. G., Wu, J., Wadsworth, L. D., MacGillivray, R. T. (1997) *Am. J. Clin. Pathol.* 107, 197–205

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