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Research Article

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Characterization of a Variant Prekallikrein, Prekallikrein Long Beach, from a Family with Mixed Cross-reacting Material–Positive and Cross-reacting Material–Negative Prekallikrein Deficiency

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Abstract

Studies of plasma prekallikrein in a family with prekallikrein deficiency were made. Three children had no clotting activity but ~35% antigen levels, and the mother and five children had twice as much prekallikrein antigen as clotting activity, suggesting the presence of a dysfunctional molecule. A nonfunctional variant form of prekallikrein was purified that contained no prekallikrein clotting activity. The variant and normal molecules were both 80,000 mol wt, immunologically indistinguishable and complexed similarly with high molecular weight kininogen. Isoelectric focusing studies suggested a difference of one charged amino acid residue. The variant was cleaved by β-Factor XIIa 200 times slower than the normal molecule, and no amidolytic activity was detected for the cleaved variant. These data and other observations suggest that an amino acid was substituted in the variant near the NH_2-terminal end of the kallikrein light chain resulting in slower cleavage by β-Factor XIIa and the absence of enzymatic activity.

Introduction

Fletcher trait deficiency was first reported in 1965 by Hathaway et al. (1). The asymptomatic clotting defect was characterized by a prolonged activated partial thromboplastin time that was thought to be due to the deficiency of a previously unrecognized clotting factor named Fletcher factor. Fletcher trait plasma was also shown to possess abnormalities in the generation of kinins, in kaolin-activated fibrinolytic activity (2, 3) and in the in vitro activation of plasma prorenin (4, 5). In 1972, Wuepper (6) identified the Fletcher factor as plasma prekallikrein. Addition of purified plasma prekallikrein to Fletcher trait plasma corrected all abnormalities.

Since the original description of the Fletcher trait deficiency, several other families with a prekallikrein deficiency have been described (7–10). All patients were deficient in functional prekallikrein, but heterogeneity of prekallikrein deficiency appears to exist. Appreciable amounts of immunoreactive prekallikrein cross-reacting material (CRM^+)[1] were detected in 5 of 18 plasma samples from subjects with functional prekallikrein deficiency (11). Samples from the other subjects were totally deficient in both functional and immunoreactive prekallikrein (CRM^-). All CRM^+ samples were from persons of Mediterranean extraction, whereas most CRM^- samples were from Black Americans. The abnormal prekallikrein appeared indistinguishable from normal prekallikrein, as tested by immunologic techniques and gel filtration. Based on experiments using whole plasma, a defective activation of the abnormal prekallikrein was postulated.

In this study we report an additional family that includes three siblings whose plasma exhibits a total absence of prekallikrein functional activity. In plasma samples of these three family members, prekallikrein antigen was detected despite the absence of prekallikrein clotting activity. The abnormal prekallikrein molecule was isolated, characterized, and compared to normal prekallikrein. For this abnormal prekallikrein, we propose the name prekallikrein Long Beach (PK_{LB}) after the city where this deficiency was detected.

Methods

Patient history

A 38-yr-old Caucasian woman entered the hospital for a hysterectomy for multiple leiomyomata. She indicated to the surgeon that one year earlier when she entered another hospital for a nasoplasty an abnormal preoperative coagulation screening test was detected but the surgery was performed without bleeding. Prior history including a tonsilectomy and teeth extractions also indicated no bleeding problem. At age 19 she had an episode of swollen legs and has had protein in her urine ever since. Family history indicated no bleeding problems in her parents, nine siblings, and one child. Her mother and one brother were on chronic hemodialysis for end stage renal disease not further characterized.

Prehysterectomy screening coagulation studies were performed. A prolonged activated partial thromboplastin time and normal prothrombin time were found. Mixing of her plasma with equal amounts of normal plasma, or plasma deficient in either Factor VIII, XI, or XII completely normalized the activated partial thromboplastin time. Mixing with prekallikrein-deficient plasma did not correct the clotting time. This suggested that the plasma of the patient was deficient in prekallikrein. In agreement with this, prolongation of the preincubation phase of the activated partial thromboplastin time resulted in a marked shortening of the clotting time. A specific prekallikrein clotting test using deficient plasma later confirmed the deficiency of prekallikrein. Based on this information, she had her hysterectomy without any problem.

Materials

All chemicals obtained from commercial sources were the best grade available. pH measurements were performed at room temperature. Protein concentration was determined by the method of Lowry et al. (12) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a reference.

Coagulation tests. The clotting activities of prekallikrein, Factor XI, Factor XII, and high molecular weight kininogen were determined using

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1. *Abbreviations used in this paper:* CRM^+ or CRM^-, cross-reacting material positive or negative; PK_{LB}, prekallikrein Long Beach; PK_N, normal prekallikrein.

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activated partial thromboplastin time assays as previously described (13, 14). Prekallikrein, Factor XI and high molecular weight kininogen-deficient plasmas were obtained from George King Biomedical, Inc., Overland Park, KS. Factor XII-deficient plasma was obtained from a congenitally deficient patient. All plasmas were stored frozen at −70°C. One unit of prekallikrein, Factor XII, or high molecular weight kininogen clotting activity is defined as the amount of activity present in 1 ml of a normal plasma pool of 40 healthy donors.

Rocket immunoelectrophoresis of prekallikrein and high molecular weight kininogen were performed according to the method of Laurell (15) as described before (16). The concentration of anti-prekallikrein and of anti-high molecular weight kininogen antisera in the 0.9% agarose (SeaKem, FMC Co., Rockland, ME) solution in 25 mM sodium barbital buffer, pH 8.6, was 3 and 3.3%, respectively. Reference curves were made from the observed rockets for four twofold serial dilutions of a normal plasma pool of 40 healthy donors. One unit of prekallikrein or of high molecular weight kininogen antigen was defined as the amount present in 1 ml of the same normal plasma pool.

Antibodies directed against prekallikrein, Factor XII, and the light chain of high molecular weight kininogen were raised in goats with the use of Freund's adjuvant (Difco Laboratories, Detroit, MI). The anti-light chain of high molecular weight kininogen (17) and the anti-Factor XII antiserum (18, 19) were monospecific without absorption. The anti-prekallikrein antiserum was absorbed with a gamma globulin fraction in order to remove traces of contaminating antibodies (16). The gamma globulin fractions of the antisera were prepared using DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ) followed by passage over concanavalin A-Sepharose (Pharmacia) as described previously (18).

Purification of prekallikrein was performed according to a previously published method (20) using ion exchange chromatography on DEAE-Sephadex and SP-Sephadex columns followed by affinity chromatography on a high molecular weight kininogen-Sepharose column. Traces of contaminating gamma globulin were removed by affinity chromatography on a column containing CNBR-Sepharose 4B (Pharmacia) to which immunopurified antibodies against the contaminating gamma globulin were convalently coupled as described elsewhere (11). The prekallikrein preparation was analyzed on 10% sodium dodecyl sulfate (SDS) polyacrylamide slab gels in the presence or absence of reducing agents and gave two protein bands with apparent molecular weights of 80,000 and 82,000. The purified prekallikrein contained 20 clotting units/mg protein.

PK_{LB} was purified following the procedure used for the purification of normal prekallikrein. After the high molecular kininogen-Sepharose column, the fractions containing PK_{LB} were pooled and dialyzed against a buffer containing 0.05 M MesNa, pH 5.1, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.4 M NaCl, 1 mM benzamidine and 0.02% Na-azide. Affinity chromatography on concanavalin A-Sepharose (Pharmacia) at 4°C was then employed as described previously for Factor XI (14). PK_{LB} adhered to the concanavalin A-Sepharose and was subsequently eluted with 0.05 M Mes-Na, pH 5.1, containing 0.4 M NaCl, 1 mM benzamidine, 0.02% Na-azide, 5 mM Na_4EDTA, and 0.5 M α-D-methyl glucoside (Sigma Chemical). The fractions containing PK_{LB} were pooled and dialyzed against 4 mM sodium acetate, 2 mM acetic acid, 0.15 M NaCl, pH 5.3.

High molecular weight kininogen was purified from human plasma as a single polypeptide chain of relative molecular weight (M_r) = 110,000 as described before (14). After incubation with plasma kallikrein, the light and heavy chains of the kinin-free high molecular weight kininogen were reduced, alkylated, and isolated as described previously (14).

Human Factor XII was purified as described elsewhere (19). β-Factor XIIa (M_r = 28,000) was prepared by incubation of Factor XII with trypsin. β-Factor XIIa was then separated from trypsin and other Factor XII fragments using DEAE-Sephadex A-50 chromatography as described previously (18).

All purified proteins were stored frozen at −70°C in storage buffer containing 4 mM sodium acetate, 2 mM acetic acid, 0.15 M NaCl, pH 5.3.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out on 10% slab gels (160 mm × 70 mm × 1 mm) according to Laemmli (21). The gels were run at 6 mA for 18 h. The gels were stained for protein with Coomassie Blue R-250.

Isoelectric focusing of prekallikrein was performed on thin-layer polyacrylamide gels using LKB Ampholine PGA plates (LKB Instruments, Inc., Gaithersburg, MD) (pH range, 3.5–9.5). The procedure followed the manufacturer's instructions for the LKB 2117 Multiphor. The anode electrode solution was 1 M H_3PO_4 and the cathode electrode solution was 1 M NaOH. Samples (30–55 μl) were applied on 5 × 9 mm Whatman filter papers (Whatman, Inc., Clifton, NJ) that were deposited in the area equidistant from each electrode. A change in the constant power setting from 12 to 10 W was made after 45 min. During the 90-min experiment, the voltage increased from 340 to more than 1,000 V while the current dropped from 38 to 8 mA. As reference standards, ovalbumin, myoglobin, and chymotrypsinogen were used.

Comparison of the rate of cleavage of PK_N and PK_{LB} by β-Factor XIIa. The time course of cleavage of PK_{LB} and PK_N by β-Factor XIIa was studied. 16 μl of β-Factor XIIa (3.52 μg) in 0.1 M Tris, 0.15 M NaCl, pH 7.4 was added to a mixture of 208 μl of PK_{LB} (62.2 μg) in 4 mM Na-acetate, 2 mM acetic acid, 0.15 M NaCl, pH 5.3, and 176 μl of 0.4 M Tris, 0.15 M NaCl, pH 8.1. The reaction mixture was further incubated at 37°C. At several time points 40-μl aliquots were removed and immediately mixed with 40 μl of a 0.125 M Tris buffer, pH 6.8, containing 25 mM dithiothreitol, 25% glycerol, and 2.5% sodium dodecyl sulfate (SDS). The mixture was boiled for 5 min. 8 μl of β-Factor XIIa (1.76 μg) was added to a mixture of 112 μl of PK_N (32 μg) in 4 mM Na acetate, 2 mM acetic acid, 0.15 M NaCl, pH 5.3, and 80 μl of 0.4 M Tris, 0.15 M NaCl, pH 8.1 at 37°C. At several time points after the addition of β-Factor XIIa, 25-μl aliquots were withdrawn and mixed with 55 μl of 0.125 M Tris buffer, pH 6.8, containing 25% glycerol, 2.5% SDS, 25 mM dithiothreitol that had been prewarmed in boiling water. The mixture was boiled for 5 min. Subsequently all aliquots were analyzed on the same 10% SDS polyacrylamide slab gel.

The generation of amidolytic activity during the cleavage of PK_{LB} by β-Factor XIIa was studied using the same reaction conditions as used for the cleavage experiment described above. During the incubation at 37°C, 10-μl aliquots were withdrawn at several times and immediately mixed with 5 μl of anti-Factor XII gamma globulin fraction. After 5 min incubation at 37°C, a 7.5-μl aliquot was then added to a plastic disposable cuvette (1 cm path length) containing 0.5 ml of 0.2 mM S-2302 (Kabi, Stockholm, Sweden) in 0.15 M Tris, 1 mg/ml bovine serum albumin (BSA), pH 8.3. The initial rate of hydrolysis of this substrate was measured at 405 nm at 37°C using a Cary 210 spectrophotometer (Varian, Palo Alto, CA). At the last time point (270 min), an extra aliquot of 40 μl was taken and mixed with 40 μl of 0.125 M Tris buffer, pH 6.8 containing 25% glycerol, 2.5% SDS and 25 mM dithiothreitol, and the sample was boiled for 5 min. This sample was electrophoresed on a 10% SDS polyacrylamide slab gel to verify complete cleavage of PK_{LB} by β-Factor XIIa. Generation of amidolytic activity of PK_{LB} was compared to the amidolytic activity generated during the cleavage of PK_N by β-Factor XIIa. The anti-Factor XII antibodies blocked 90% of the amidolytic activity of β-Factor XIIa on S-2302. The amount of amidolytic activity generated by β-Factor XIIa in the absence of anti-Factor XII antibodies was 1% of the amidolytic activity present after complete activation of PK_N by β-Factor XIIa.

Results

Family studies. The family tree of the propositus (II-2) is shown in Fig. 1. Plasma samples of the parents of the propositus (II-2) and nine brothers and sisters were tested for the presence of prekallikrein, high molecular weight kininogen, Factor XI and Factor XII clotting activity and for prekallikrein (Fig. 2) and high molecular weight kininogen antigen level. The results are summarized in Table I. Three members of the family (II-2, II-6, and II-8) had undetectable prekallikrein clotting activity levels (below 1% of normal), whereas prekallikrein antigen (Fig. 2), was present in reduced amounts (35–43%). Several members, I-

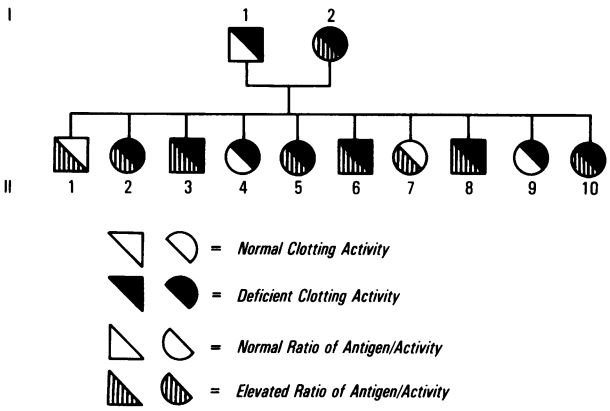

Figure 1. Family tree of prekallikrein-deficient family. Subject II-2 represents the propositus. The upper right half of the square (male) or circle (female) represents the prekallikrein clotting activity. The lower left half represents the ratio of prekallikrein antigen level to clotting activity. Open upper half indicates normal levels of clotting activity; shaded abnormal. Open lower half indicates a normal ratio of antigen to activity; shaded lower half indicates antigen in significant excess over activity (antigen to activity ratio > 1.3).

1 and II-4, have reduced amounts in both prekallikrein clotting activity and antigen. Decreased prekallikrein clotting activity but normal levels of prekallikrein antigen were present in plasma samples from patients I-2, II-1, II-3, II-5, II-7, and II-10. The ratio of prekallikrein antigen to activity in 20 normals ranged from 0.75 to 1.3. Assuming a value > 1.3 for this ratio is abnormal, these results indicate that an abnormal prekallikrein molecule is present in plasma samples of most members of the family, i.e., I-2 and II-1, 2, 3, 5, 6, 7, 8, 10 (Table I, Fig. 1). The abnormal prekallikrein molecule is called prekallikrein Long Beach (PK_{LB}) after the city where this family was detected. High molecular weight kininogen levels were within the normal range when measured by a clotting assay or by an immunological test (Table I). Factor XI clotting activity was normal in plasma samples from all members of the family.

Purification of PK_{LB}. Plasma from two members of the family (II-2 and II-6) was obtained by plasmapheresis and pooled to give 1.3 liters. PK_{LB} was purified from this plasma, following the procedure used for normal prekallikrein (PK_N).

Throughout the purification procedure, PK_{LB} was detected using radial immunodiffusion according to Mancini (22) and anti-normal prekallikrein antiserum. Ionic exchange chromatography on DEAE-Sephadex, SP-Sephadex columns and affinity chromatography on a high molecular weight kininogen-Sepharose column was performed as described before (20). The next step employed concanavalin A-Sepharose affinity chromatography to remove γ-globulin from PK_{LB}. PK_{LB} that was >95% pure on 10% SDS polyacrylamide gels was obtained in a 15% yield. On 10% SDS polyacrylamide gels purified PK_{LB} in the presence or absence of reducing agents gave two bands that comigrated with normal prekallikrein with apparent 80,000 and 82,000 mol wt (Fig. 3).

Isoelectric focusing of PK_{LB} and PK_N. PK_{LB} and PK_N were focused on LKB polyacrylamide gel plates (pH range, 3.5–10) to detect differences in charge in PK_{LB}. The isoelectric focusing pattern of PK_{LB} was compared to several different PK_N preparation obtained from different purification procedures. All PK_N preparations showed several bands of which some appeared as doublets (Fig. 4). PK_{LB} gave a similar pattern but the bands focused at a pH that was 0.10 U lower than the pH of the PK_N bands (Fig. 4). Microheterogeneity of PK_N based on isoelectric focusing studies has been previously reported (23, 24).

Titration of PK_{LB} with high molecular weight kininogen or its isolated light chain. Since affinity chromatography on high molecular weight kininogen-Sepharose was used for the purification of PK_{LB}, PK_{LB} appears to bind to high molecular weight kininogen. A quantitative study was performed using previously described techniques (17) to study the binding of PK_{LB} to high molecular weight kininogen or its light chain. This technique is based on the fact that normal prekallikrein does not migrate in agarose upon electrophoresis at pH 8.6. However, association of prekallikrein with high molecular weight kininogen or with its isolated light chain gives negatively charged complexes that are able to migrate at this pH. During Laurell rocket immunoelectrophoresis, maximum rocket heights are reached for prekallikrein when equimolar amounts of high molecular weight kininogen or of its alkylated light chain are present (17). Rocket immunoelectrophoresis experiments were carried out with PK_{LB}. PK_{LB} (1 μg) was incubated for 20 min at 37°C in plastic tubes in the absence or presence of 0 to 4.4 μg of high molecular weight kininogen or 0 to 1.7 μg of alkylated light chain in 16 μl of 25 mM sodium barbital buffer, pH 8.6. Aliquots (8 μl) were analyzed by one dimensional rocket immunoelectrophoresis. PK_{LB} alone migrated only slightly under these conditions, and it migrated much further after preincubation with either high molecular weight kininogen or its light chain (Fig. 5). The heights of the rockets increased as a function of increasing high molecular weight kininogen or light chain concentration until a maximum height was reached. The observed rocket height as a function of the molar ratio of high molecular weight kininogen or of its light chain to PK_{LB} is seen in Fig. 5. The titration data are fit using two straight lines as shown in Fig. 5 with the intersection of the two lines at molar ratios of high molecular weight kininogen or of light chain to PK_{LB} corresponding to 0.95±0.2 and 1.05±0.2, respectively. This indicates that PK_{LB}, like PK_N, forms equimolar complexes with the light chain region of high molecular weight kininogen.

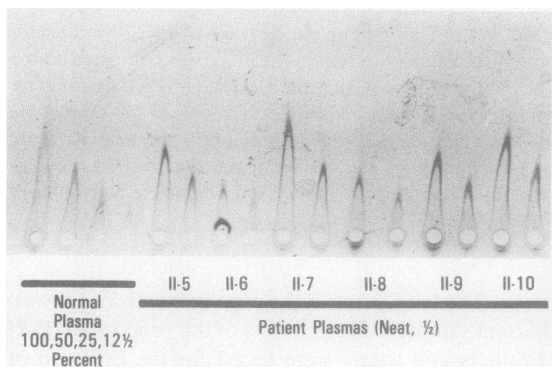

Figure 2. Rocket immunoelectrophoresis studies of prekallikrein antigen in plasma samples of several family members using antiprekallikrein (3%). From the left to the right, the wells (8 μl) contain undiluted normal plasma and three successive twofold dilutions. Six plasmas of family members of the propositus. The numbers correspond with those in Table I. All plasmas were tested undiluted and twofold diluted.

Table I. Plasma Levels of Prekallikrein, High Molecular Weight Kininogen, Factor XI, and Factor XII in Members of Prekallikrein-deficient Family

Patient no.	Prekallikrein			High molecular weight kininogen			
	Clotting activity	Antigen level	Antigen Activity	Clotting activity	Antigen level	Factor XI clotting activity	Factor XII clotting activity
	%	%		%	%	%	%
I. Parents							
1	48	50	1.04	78	79	84	95
2	51	93	1.82	98	130	130	75
II. Children							
1	74	136	1.83	95	120	110	74
2	<1	35	>35	80	63	110	59
3	35	68	1.94	88	91	125	50
4	46	60	1.30	80	91	105	84
5	42	77	1.83	100	101	100	64
6	<1	34	>34	90	78	100	68
7	63	113	1.79	90	101	94	75
8	<1	43	>43	90	81	100	76
9	54	68	1.26	76	100	105	73
10	55	97	1.76	80	108	84	68
Normal range (n = 20)	60–120	60–120	0.75–1.3	65–170	65–170	55–145	60–120

Cleavage of PK_{LB} and PK_N by β-Factor XIIa. In order to investigate the possibility that the lack of activity of PK_{LB} is caused by the absence of cleavage by β-Factor XIIa, both PK_{LB} and PK_N were incubated at 37°C with β-Factor XIIa. At several times aliquots were withdrawn and analyzed on 10% SDS polyacrylamide slab gels in the presence of reducing agents. The results are shown in Fig. 6. PK_N was cleaved by β-Factor XIIa giving rise to a heavy chain with an apparent molecular weight of 43,000 and two similar light chains of 36,000 and 33,000 molecular weight, respectively. Approximately 50% of PK_N was cleaved in 10 s. PK_{LB} was cleaved at a much slower rate. Ap-

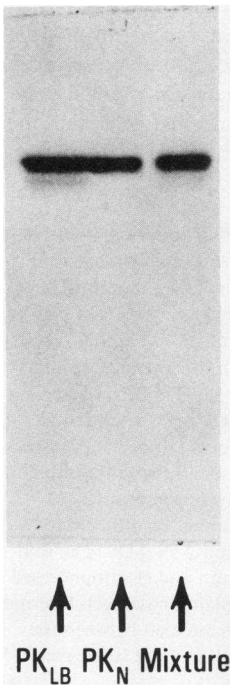

Figure 3. Reduced 10% SDS polyacrylamide electrophoresis of PK_{LB} (lane *1*), PK_N (lane *2*), and a mixture of PK_{LB} and PK_N (lane *3*). Each gel lane contained 4 μg of protein.

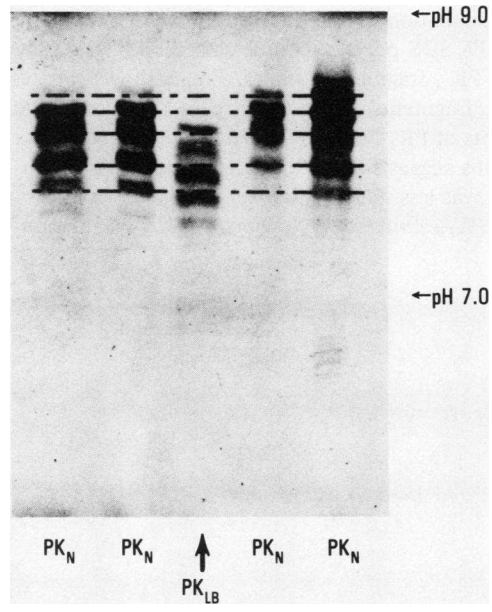

Figure 4. Isoelectric focusing of PK_{LB} and several different preparations of PK_N. Dashed lines show the alignment of different isoelectric forms of different preparations of normal prekallikrein. Isoelectric focusing was performed on LKB polyacrylamide gels as described in Methods.

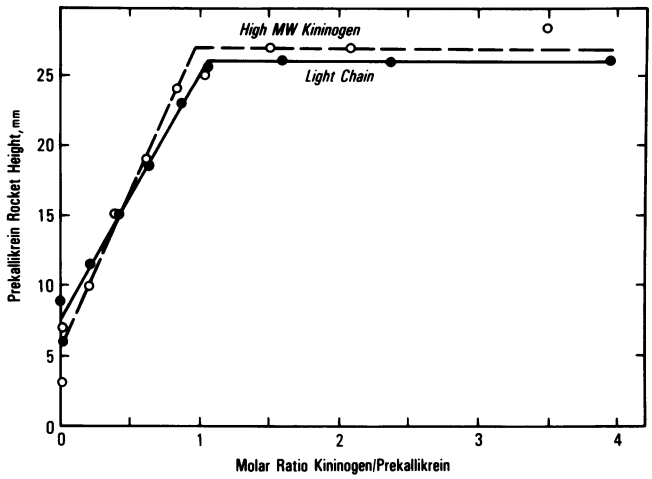

Figure 5. Titration of PK_{LB} with high molecular weight kininogen or its isolated light chain using Laurell rocket electrophoresis with anti-prekallikrein antibodies. Aliquots (8 μg) containing 0.5 μg of prekallikrein were incubated with increasing amounts of high molecular weight kininogen or its light chain, as described in the text, and were then analyzed by rocket immunoelectrophoresis. The electrophoresis was performed as described in Methods in 0.9% agarose containing 3% anti-prekallikrein γ-globulin. The rocket heights corresponding to the migration of PK_{LB} were measured. The molar ratios were calculated by using the molecular weights of 85,000 for PK_{LB}, 110,000 for high molecular weight kininogen and 40,000 for the isolated light chain.

proximately 50% was cleaved in 30–60 min. This indicates that the rate of cleavage of PK_{LB} was ~200 times slower than that of PK_N. Cleavage of PK_{LB} resulted in fragments with very similar apparent molecular weights as formed during the cleavage of PK_N. To compare the molecular weight of the fragments formed by cleavage of PK_{LB} and PK_N by β-Factor XIIa, equal amounts of the fragments of PK_{LB} and PK_N present after 270 and 1 min, respectively, of incubation with β-Factor XIIa were mixed and analyzed on a 10% SDS polyacrylamide slab gel alongside the same PK_{LB} and PK_N fragment samples. Fig. 7 shows the gel comparing these fragments. No difference in the size of the cleavage fragments of PK_{LB} and PK_N formed by β-Factor XIIa was observed. This suggests that if there is a difference in molecular weight, it was less than ~1,000.

Cleavage of PK_{LB} and PK_N by β-Factor XIIa and the appearance of amidolytic activity. Since cleavage of PK_{LB} by β-Factor XIIa takes place, albeit at a much slower rate than that of PK_N, the possibility was investigated if this cleavage was accompanied by the generation of kallikrein amidolytic activity toward the kallikrein chromogenic substrate, S2302. Using the same reaction conditions as described above for the cleavage experiment, aliquots were taken during the incubation period and mixed with anti-Factor XII antibodies. After 5 min incubation at 37°C the amidolytic activity was measured. No amidolytic activity was detected during the incubation of PK_{LB} with β-Factor XIIa although full cleavage of the molecule had taken place as verified on reduced SDS polyacrylamide gels. PK_N reached maximum activation after 1 min of incubation with β-Factor XIIa. This indicates that, although complete cleavage of PK_{LB} occurred, <1% of the amidolytic activity of PK_N was generated during cleavage of PK_{LB} by β-Factor XIIa.

Discussion

The family described in this paper with a CRM^+ deficiency of prekallikrein was detected by a preoperative coagulation screening test of one of the members of the family. Additional investigations indicated a deficiency of prekallikrein clotting activity. The presence of a nonfunctional prekallikrein molecule was detected using rocket immunoelectrophoresis. Subsequent studies using plasma samples of other members of the family indicated that the father has ~50% of normal levels of both prekallikrein antigen and activity, whereas the mother has one half of normal clotting activity but 93% of normal antigen level. This suggests that the father expresses one normal gene for prekallikrein and possesses one abnormal prekallikrein gene that is not expressed immunologically or by activity (CRM^-). The mother appears to express one normal gene and one abnormal gene (CRM^+) by producing a molecule that can be detected immunologically but not in activity measurements. This hypothesis is supported by the data for the 10 children (II-1 to II-10). Patients II-2, II-6, and II-8 exhibit undetectable (<1%) clotting activity but 34–43% antigen levels. These three patients appear to have one CRM^- gene and one CRM^+ gene. Five of the patients, II-1, II-3, II-5, II-7, and II-10, appear to have twice as much prekallikrein antigen as clotting activity. Therefore, these children presumably have one normal gene and one CRM^+ gene for prekallikrein. Patients II-4 and II-9 probably have one normal gene and one CRM^- gene.

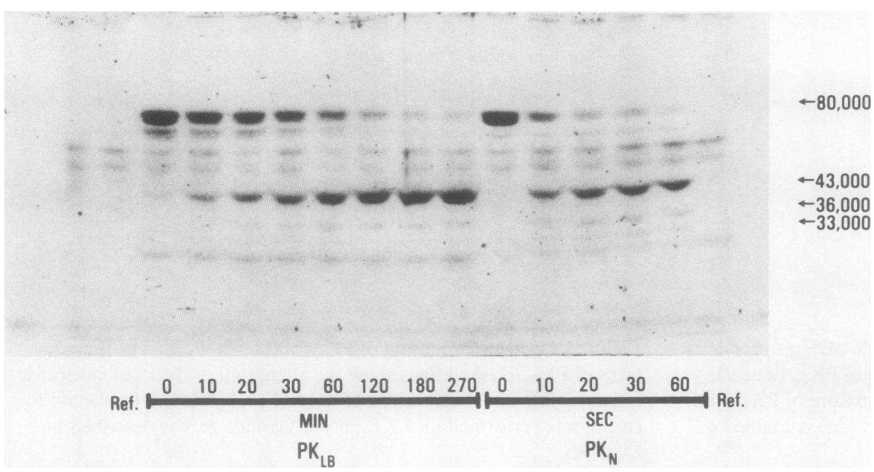

Figure 6. Cleavage of PK_N and PK_{LB} by β-Factor XIIa. PK_{LB} (156 μg/ml) was incubated in the presence of β-Factor XIIa (8.8 μg/ml) at 37°C in 0.16 M Tris HCl buffer 0.15 M NaCl, pH 8.1, in a total volume of 400 μl. PK_N (160 μg/ml) was incubated in the presence of β-Factor XIIa (8.8 μg/ml) at 37°C in 0.16 M Tris-HCl buffer, 0.15 M NaCl, pH 8.1, in a total volume of 200 μl. Aliquots (40 or 25 μl, respectively) were withdrawn as a function of time and added to a boiling solution of 0.125 M Tris-HCl, pH 6.8 containing 25 mM dithiothreitol, 25% glycerol and 2.5% SDS. The samples were boiled for 5 min and electrophoresed on a 10% SDS polyacrylamide slab gel. Internal reference protein bands are seen between the heavy chain (43,000 mol wt) and single chain (80,000) polypeptides.

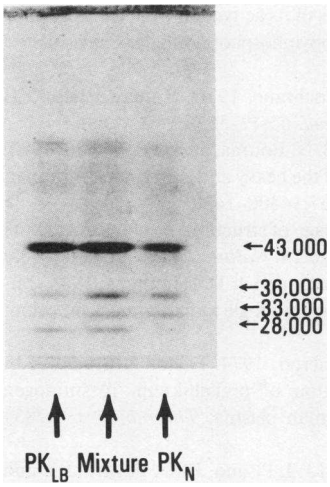

Figure 7. Reduced 10% SDS polyacrylamide slab gel of cleaved PK_{LB} (lane *1*), a mixture of cleaved PK_{LB} and PK_N (lane *2*) and cleaved PK_N (lane *3*). Each gel lane contained 6.4 μg of protein.

The prekallikrein deficiency in this family appears to be clinically asymptomatic in agreement with other reports. Three members of the family have kidney disease, but only one of these three, the original patient in this study, has total absence of functional prekallikrein. Whether this has any relationship to the recently described activation of the renin-angiotensin system by plasma kallikrein (4, 5) remains to be established.

In order to characterize the defect of the abnormal prekallikrein molecule, PK_{LB} was purified from a pool of plasma obtained from two members of the family. The purification procedure was similar to the procedure used for the purification of PK_N. PK_{LB} is indistinguishable from PK_N when analyzed on SDS polyacrylamide gels. It appeared as a doublet with apparent molecular weight of 80,000 and 82,000 in the presence or absence of reducing agents.

The observations that PK_{LB} is detected by rocket immunoelectrophoresis and that PK_{LB} binds to high molecular weight kininogen-Sepharose as used in the purification procedure indicate that PK_{LB} can bind to high molecular weight kininogen. Quantitative analysis of the complex formation of PK_{LB} with high molecular weight kininogen or its light chain using the same rocket immunoelectrophoretic technique as used before for PK_N (17) indicated that a one to one molar complex is formed and that the binding occurs via the light chain region of high molecular weight kininogen.

Saito et al. (11) were also unable to demonstrate a difference between normal prekallikrein and a nonfunctional prekallikrein using immunological techniques and gel filtration. Based on studies using plasma, they postulated a defective activation of the abnormal prekallikrein molecule. In our study the rate of cleavage of PK_{LB} by β-Factor XIIa was ~200 times slower than that of PK_N, and the cleavage fragments of PK_{LB} and PK_N were indistinguishable when analyzed on reduced SDS polyacrylamide gels, suggesting that if the cleavage occurs at a different site the molecular weight difference between the normal and the PK_{LB} fragments is <1,000. The fact that complete cleavage of PK_{LB} did not result in any detectable kallikrein amidolytic activity suggests that in the light chain region of the PK_{LB} molecule a substitution of an amino acid has taken place that hinders the cleavage of the molecule by β-Factor XIIa and the expression of enzymatic activity by the active site. The result of the isoelectric focusing studies indicated that PK_{LB} focused at a 0.1 pH unit different from PK_N. This is consistent with the substitution of one positive amino acid for a neutral amino acid or of one neutral residue for a negative one. This amino acid replacement could alter PK_{LB} such that both its proteolysis by β-Factor XIIa and its enzymatic activity are markedly altered. An alternative explanation for the different isoelectric focusing of PK_{LB} could involve a difference in carbohydrate, e.g., sialic acid. Further chemical studies are necessary to identify the amino acid substitution or difference in glycosylation of PK_{LB}. This may provide further insight in the structural requirements for a normal rate of cleavage and a normal expression of the enzymatic active site of normal prekallikrein.

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References

1. Hathaway, W. E., L. P. Belhasen, and M. S. Hathaway. 1965. Evidence for a new plasma thromboplastin Factor I. Case report, coagulation studies and physiochemical properties. *Blood.* 26:521–532.

2. Saito, H., O. D. Ratnoff, and V. H. Donaldson. 1974. Defective activation of clotting, fibrinolytic, and premeability-enhancing systems in human Fletcher trait plasma. *Circ. Res.* 34:641–651.

3. Weiss, A. S., J. I. Gallin, and A. P. Kaplan. 1974. Fletcher Factor deficiency: A diminished rate of Hageman Factor activation caused by absence of prekallikrein with abnormalities of coagulation, fibrinolysis, chemotactic activity and kinin generation. *J. Clin. Invest.* 53:622–633.

4. Derkx, F. H. M., B. N. Bouma, M. P. A. Schalekamp, and M. A. D. H. Schalekamp. 1979. An intrinsic Factor XII-prekallikrein-dependent pathway activates the human plasma renin-angiotensin system. *Nature (Lond.).* 280:315–317.

5. Sealey, J., S. A. Atlas, J. H. Laragh, M. J. Silverberg, and A. P. Kaplan. 1979. Initiation of plasma prorenin activation by Hageman Factor-dependent conversion of plasma prekallikrein to kallikrein. *Proc. Natl. Acad. Sci. USA.* 76:5914–5918.

6. Wuepper, K. D. 1973. Prekallikrein deficiency in man. *J. Exp. Med.* 138:1345–1355.

7. Hattersley, P. G., and D. Hayse. 1970. Fletcher Factor deficiency: A report of three unrelated cases. *Br. J. Haematol.* 18:411–416.

8. Essien, E. M., and M. I. Ebhota. 1977. Fletcher Factor deficiency-detection of a severe case in a population survey. *Acta Haematol. (Basel).* 58:353–358.

9. Aznar, J. A., F. Espãna, J. Aznar, A. Tascon, and C. Jiminez. 1978. Fletcher factor deficiency: report of a new family. *Scand. J. Haematol.* 21:94–98.

10. Abildgaard, C. F., and J. Harrison. 1974. Fletcher Factor deficiency. Family study and detection. *Blood.* 43:641–644.

11. Saito, H., L. T. Goodnough, J. Soria, C. Soria, J. Aznar, and F. Espãna. 1981. Heterogeneity of human prekallikrein deficiency (Fletcher trait). Evidence that five of 18 cases are positive for cross-reacting material. *N. Engl. J. Med.* 305:910–914.

12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

13. Bouma, B. N., and J. H. Griffin. 1977. Human blood coagulation Factor XI: Purification properties and mechanism of activation by activated Factor XII. *J. Biol. Chem.* 252:6432–6437.

14. Kerbiriou, D. M., and J. H. Griffin. 1979. Human high molecular weight kininogen. Studies of structure-function relationships and of proteolysis of the molecule occurring during contact activation of plasma. *J. Biol. Chem.* 254:12020–12027.

15. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* 15:45–52.

16. Bouma, B. N., D. M. Kerbiriou, H. A. A. Vlooswijk, and J. H. Griffin. 1980. Immunological studies of prekallikrein, kallikrein, and high molecular weight kininogen in normal and deficient plasmas and in normal plasma after cold dependent activation. *J. Lab. Clin. Med.* 96:693–709.

17. Kerbiriou, D. M., B. N. Bouma, and J. H. Griffin. 1980. Immunochemical studies of human high molecular weight kininogen and of its complexes with plasma prekallikrein or kallikrein. *J. Biol. Chem.* 255:3952–3958.

18. Bouma, B. N., L. A. Miles, G. Beretta, and J. H. Griffin. 1980. Human plasma prekallikrein. Studies of its activation by activated Factor XII and of its inactivation by diisopropylphosphofluoridate. *Biochemistry.* 19:1151–1160.

19. Griffin, J. H., and C. G. Cochrane. 1976. Human Factor XII (Hageman Factor). *Methods Enzymol.* 65:49–56.

20. van der Graaf, F., G. Tans, B. N. Bouma, and J. H. Griffin. 1982. Isolation and functional properties of the heavy and light chains of human plasma kallikrein. *J. Biol. Chem.* 257:14300–14305.

21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

22. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigen by single radial immunodiffusion. *Immunochemistry.* 2:235–242.

23. Laake, K., and A. M. Vennerod. 1974. Factor XII-induced fibrinolysis: Studies on the separation of prekallikrein, plasminogen proactivator and Factor XII in human plasma. *Thromb. Res.* 4:285–296.

24. Hojima, Y., J. V. Pierce, and J. J. Pisano. 1985. Purification and characterization of multiple forms of human plasma prekallikrein. *J. Biol. Chem.* 260:400–406.