Kallikrein-binding protein was purified to apparent homogeneity from rat serum by Affi-Gel Blue, DEAESepharose CL-6B, Sephacryl S-200 chromatography, and preparative gel electrophoresis or high performance liquid chromatography. The purified protein migrates as a single band of 60 kDa in a sodium dodecyl sulfate-polyacrylamide gel under reducing conditions. It is an acidic protein with isoelectric points ranging from 4.2 to 4.6. The amino-terminal 29-amino acid sequence of the binding protein is an Asp residue as determined by sequence analysis. It forms a 92-kDa sodium dodecyl sulfate-stable complex with kallikrein with a $t_{1/2}$ of 18 min. Western blot and radioimmunoassay showed a distribution of the kallikrein-binding protein in serum, urine, and various tissues with a 5–10-fold lower amount in spontaneously hypertensive rats (SHR) than in Wistar-Kyoto rats (WKY). A full length cDNA clone encoding the kallikrein-binding protein was isolated from a rat liver cDNA library by immunoscreening with human $\alpha_1$-antichymotrypsin and is identical to the amino-terminal 29-amino acid sequence of the binding protein. The cDNA sequence shares 68.8% identity with human $\alpha_1$-antichymotrypsin and is identical to that of a rat hepatic protein. Dot blot analysis shows that kallikrein-binding protein is expressed at high levels in the liver and at low levels in the lung, salivary gland, and kidney. Its mRNA level in the liver decreases by 2-fold after acute phase inflammation and is higher in male than in female rats. Genomic Southern blot analyses reveal restriction fragment length polymorphisms between SHR and WKY rats in the binding protein locus. The results indicate that rat kallikrein-binding protein belongs to the serpin superfamily and its level is significantly reduced in the spontaneously hypertensive rats.

Previous studies have shown that kallikrein-binding protein only binds to active kallikrein but not to inactive (latent) kallikrein or to active-site blocked kallikrein (Chao et al., 1986a). Kallikrein-binding protein and $\alpha_1$-antitrypsin interact differently with human tissue kallikrein in addition, neither polyclonal nor monoclonal antibodies to human $\alpha_1$-antitrypsin cross-react with kallikrein-binding protein (Chen et al., 1990). These findings indicate that there are two different potential tissue kallikrein modulators, kallikrein-binding protein and circulating autoantibodies, in addition to $\alpha_1$-antitrypsin in human serum.

Previous studies have shown that kallikrein-binding protein is involved in many important pathophysiological processes such as hypertension, diabetes mellitus, allergy, and inflammation (Pisano, 1979; Schacter, 1980; MacDonald et al., 1988). Tissue kallikrein
neously hypertensive rats (SHR) versus normotensive control Wistar-Kyoto (WKY) rats (Chao and Chao, 1988) suggest that the kallikrein-binding protein may play an important physiological role in the clearance and catabolism of kallikrein. The present studies report the purification, characterization, and distribution of rat tissue kallikrein-binding protein and cloning of a cDNA encoding this binding protein. Furthermore, the results suggest that the deficiency of kallikrein-binding protein found in spontaneously hypertensive rats may have a genetic basis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were obtained from commercial sources: DEAE-Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.); Affi-Gel Blue-agarose, Zeta-Probe (Bio-Rad); acrylamide, N,N'-methylenebisacrylamide, TEMED, porcine pancreatic elastase, bovine chymotrypsin, and Coomassie Brilliant Blue R-250 (Sigma); SDS (Boehringer Mannheim). Rat urinary kallikrein was purified to homogeneity as described previously (Chao and Margolius, 1979). Purified proteins were labeled with ¹²⁵I according to the lactoperoxidase method (Shimamoto et al., 1980).

**Assay for Binding Protein in Tissue Kallikrein**—Aliquots of rat serum or eluates from column fractions were incubated with ¹²⁵I-rat urinary kallikrein (1000 cpm, specific activity [SA] 73.2 mCi/mg) in 100 µl of PBS, pH 7.0, in a total volume of 50 µl at 37 °C for 60 min. Binding was stopped by adding 50 µl of 2 X SDS extraction buffer (0.125 M Tris-HCl, pH 6.8, 30% glycerol, 10% β-mercaptoethanol, and 5% SDS) and heating at 100 °C for 5 min. Samples were resolved by 7.5-15% linear gradient SDS-polyacrylamide gel electrophoresis (PAGE). The gels were stained with 0.2% Coomassie Brilliant Blue and destained in a solution containing 10% acetic acid and 25% methanol. The gels were dried and exposed to Kodak X-Omat films at -70 °C for 24 h. The formation of the complex of binding protein and ¹²⁵I-labeled kallikrein was monitored by densitometric scanning of the autoradiograms.

**High Performance Liquid Chromatography**—Sample was applied to a YMC G, reverse phase column. Proteins were eluted out by an acetonitrile gradient (27-63%) in 0.1% trifluoroacetic acid. The eluted peaks were neutralized, lyophilized, and reconstituted in 10 mm sodium phosphate buffer, pH 7.0. Kallikrein-binding protein was monitored by binding assay.

**Preparative Gel Electrophoresis**—A 1.5% non-SDS polyacrylamide slab gel was prepared and electrophoresed as described previously (Xiong et al., 1989). Samples of 1 µg of protein were dissolved in 0.01 M sodium phosphate buffer, pH 7.0, mixed with 2 X extraction buffer. After electrophoresis, the gels were stained briefly with Coomassie Brilliant Blue for 30 s and destained for 1 min. The stained protein bands were excised and eluted with an ISCO concentrator (model 1750) overnight. The gel eluates were collected in 0.2 ml volumes and were examined for both protein staining and binding activity to kallikrein.

**Isoelectric Focusing on Polyacrylamide Slab Gels**—Isoelectric focusing was carried out in a LKB 2117 Multiphor electrophoresis system with a pH gradient of 3.5-10.0, using an ampholine-polyacrylamide gel plate. The gel was run for 2.5 h at 4 °C with voltage increasing from 200 to 1650 V. Following completion of focusing, the edge portion of the gel was removed. The strip (6 mm width) was cut transversely at 4-mm intervals and each section was placed in a tube containing 1 ml of distilled water. After standing for 24 h, the pH values of the sections were measured and the pH gradient was obtained by plotting the sections' pH value versus their original positions on the gel. The remainder of the slab gel was fixed in 25% trichloroacetic acid overnight. The Coomassie Brilliant Blue solution for 15 min and destained in methanol/acetic acid/water (300/120/100).

**Amino-terminal Sequence Analysis**—The purified kallikrein-binding protein was first subjected to SDS-PAGE under reducing conditions and then electrotransferred to polyvinylidene difluoride membranes. The protein blotted on the membrane was stained with 0.2% (w/v) Ponceau S in 3% trichloroacetic acid for 1 min, and destained with 1 M acetic acid for 2 min. The individual bands were cut out and subjected to sequential Edman degradations performed with an Applied Biosystems Inc. model 477 gas phase sequencer equipped with an on-line narrow bore phenylthiobodyantoin derivative analyzer as described previously (Lu et al., 1989).

**Western Blot Analysis**—Samples (30 µl) were analyzed on SDS-PAGE and then electrotransferred onto nitrocellulose membranes. The immunoblotting procedures using antigen overlay were as described previously (Chao et al., 1989). Briefly, the nitrocellulose membranes were blocked with BLOTTO (5% w/v) nonfat dry milk in 0.1 M sodium phosphate, pH 7.4, 0.14 M NaCl, 1 µM paraformaldehyde, and 0.05% sodium sulfate in 0.1% Tween 20, then incubated with rabbit anti-α1-antitrypsin antisera (1:1000 in BLOTTO) for 1 h at 30 °C and then incubated with a secondary antirabbit IgG conjugated to horseradish peroxidase (20 µg/ml). The membranes were then washed three times with BLOTTO and then incubated with BLOTTO or 0.01% diammonium hydrogen phosphate (NaH₂PO₄) and 0.01% antifade A) (Johnson et al., 1984) for 1 h at 30 °C and then incubated with rabbit anti-rat kallikrein-binding protein (RKBP) (1:200 in BLOTTO) or rabbit anti-α1-antitrypsin antisera (1:500 in BLOTTO). After a 3 h incubation at 30 °C with gentle shaking, the nitrocellulose membranes were washed three times with BLOTTO and then incubated with BLOTTO or 0.01% diammonium hydrogen phosphate (NaH₂PO₄) and washed in 0.01% Triton X-100. The membranes were then washed three times with BLOTTO and once with phosphate-buffered saline (0.117 M sodium phosphate, pH 7.4, 0.14 M NaCl, air-dried, and exposed to Kodak X-Omat film.

**Tissue Extract Preparation**—Tissue was homogenized in 0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.01% sodium azide, and 0.1% Triton X-100 with a Polytron. The tissues were centrifuged at 10,000 X g for 30 min, and protein concentrations were determined by the method of Lowry et al. (1961), using bovine serum albumin as the standard.

**Immunological Screening of the Rat Liver cDNA Library**—Immuno screening procedures for the isolation of cDNAs encoding kallikrein-binding protein from a rat liver λgt11 cDNA library were similar to those described by Chao et al. (1989a). In the antibody titration curve, RKBP antisera dilutions in the assay buffer ranged from 1:1000 to 1:640,000. One hundred µl of 125I-RKBP (10,000 cpm/100 µl) and 100 µl of antibody in assay buffer was added to 0.5 ml of assay buffer containing the final volume to 400 µl. The assay mixtures were incubated at room temperature for 24 h. Anti body-bound RKBP was generated from the free protein through centrifugation in an optimum combination of 200 µl of polyethylene glycol (25%) and 100 µl of bovine γ-globulin (1%). A 1:10,000 antisera dilution was chosen for radioimmunoassay and the standard curve of the binding protein ranged from 640 pg to 80 ng.

**Immunological Screening of the Rat Liver cDNA Library**—Immunoscreening procedures for the isolation of cDNAs encoding kallikrein-binding protein from a rat liver λgt11 cDNA library were similar to those described by Chao et al. (1989a). Rabbit anti-RKBP antisera (1:500 in BLOTTO) was used for the screening. The filters were washed, blocked with BLOTTO and then incubated with 1:1 RKBP (250,000 cpm/ml). Purified RKBP at concentrations of 1.0, 0.1, and 0.02 µg was spotted on a piece of nitrocellulose and processed as positive controls.

**Immunological Screening of the Rat Liver cDNA Library**—Immunoscreening procedures for the isolation of cDNAs encoding kallikrein-binding protein from a rat liver λgt11 cDNA library were similar to those described by Chao et al. (1989a). Rabbit anti-RKBP antisera (1:500 in BLOTTO) was used for the screening. The filters were washed, blocked with BLOTTO and then incubated with 1:1 RKBP (250,000 cpm/ml). Purified RKBP at concentrations of 1.0, 0.1, and 0.02 µg was spotted on a piece of nitrocellulose and processed as above as positive controls.

**Nucleic Acid Sequencing**—The positive clones were subcloned into the M13 mp19 sequencing vector according to the method described by Messing (1983). Nucleic acid sequencing was performed using the dideoxy chain termination method (Sanger et al., 1977).
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washing, the blots were exposed at -70 °C with intensifying screens to Kodak X-Omat film.

RESULTS

Purification of Kallikrein-binding Protein—Fractions containing kallikrein-binding activity were monitored by the formation of a 92-kDa complex identified on autoradiograms following SDS-PAGE. Pooled rat sera were first fractionated with 45–80% ammonium sulfate saturation. The precipitate was dissolved in 0.02 M sodium phosphate, pH 7.0, dialyzed against the same buffer, and then passed through an Affi-Gel Blue column equilibrated with the same buffer. Kallikrein-binding protein was in the flow-through fractions. The fractions containing the active material were combined and dialyzed against 0.05 M NaCl, 0.05 M Tris-HCl buffer, pH 8.8, and then passed through a DEAE-Sepharose CL-6B column equilibrated with the same buffer. The column was eluted with a 0.05–0.2 M NaCl gradient in 0.05 M Tris-HCl, pH 8.8. The binding protein was eluted from the DEAE-Sepharose column at 0.1 M NaCl, 0.05 M Tris-HCl buffer, pH 8.8. The fractions with binding activities were combined, precipitated with 80% ammonium sulfate, dissolved in 3–5 ml of 0.05 M ammonium formate, pH 8.0, and then passed through a Sephacryl S-200 column (2.5 x 90 cm) equilibrated with 0.05 M ammonium formate, pH 8.0. The binding protein was eluted from the column in the second peak between bovine serum albumin (68 kDa) and ovalbumin (43 kDa). Kallikrein-binding protein was subsequently electrophoresed on a 7.5% non-SDS polyacrylamide gel and eluted from the gel slices using an ISCO concentrator. Alternatively, the fractions with kallikrein-binding activity eluted from the Sephacryl S-200 column were separated by HPLC using a reverse phase C4 column. The kallikrein-binding protein was eluted at 45% acetonitrile at 44 min. Table I shows the purification scheme of kallikrein-binding protein. The recovery at each step was calculated based on a radioimmunoassay developed for measuring kallikrein-binding protein as described under "Experimental Procedures."

Characterization of Kallikrein-binding Protein—Fig. 1 shows the SDS-PAGE profiles and Western blot analysis of purified RKBP and rat α1-antitrypsin. Kallikrein-binding protein migrates as a 60-kDa protein band stained with Coomassie Blue in 7.5–15% gradient SDS-PAGE under reducing conditions or electrotransferred to nitrocellulose. Middle panel, blots were incubated with anti-RKBP antiserum followed by 125I-antitrypsin. Right panel, blots were incubated with anti-α1-antitrypsin antiserum followed by 125I-antitrypsin.

FIG. 1. SDS-polyacrylamide gel electrophoresis and Western blot analysis of purified RKBP and rat α1-antitrypsin. Left panel, protein staining with Coomassie Blue. Rat serum (1 µl, lane 1), rat α1-antitrypsin (α1-AT, 5 µg, lane 2), and rat kallikrein-binding protein (RKBP, 5 µg, lane 3), were subjected to a 7.5–15% linear gradient SDS-PAGE under reducing conditions or electrotransferred to nitrocellulose. Middle panel, blots were incubated with anti-RKBP antiserum followed by 125I-RKBP. Right panel, blots were incubated with anti-α1-antitrypsin antiserum followed by 125I-antitrypsin.

FIG. 2. Isoelectric focusing of rat kallikrein-binding protein on a polyacrylamide slab gel. The details were described under "Experimental Procedures." Coomassie Brilliant Blue protein staining shows: human α1-antitrypsin (lane 1), rat kallikrein-binding protein (lane 2), and rat α1-antitrypsin (lane 3). The corresponding pH values are shown at the left axis.

panel, lane 2) and the corresponding α1-antitrypsin in rat serum (right panel, lane 1) but does not bind to the purified kallikrein-binding protein (right panel, lane 3). The results indicated that kallikrein-binding protein is immunologically distinct from α1-antitrypsin.

Analytical isoelectric focusing revealed multiple bands of RKBP, with isoelectric points (pI) ranging from 4.2 to 4.6 (lane 2), rat α1-antitrypsin with pI of 4.5–5.0 (lane 3), and human α1-antitrypsin with pI of 5.0–5.5 (lane 1) (Fig. 2). Sequential Edman degradation of the purified kallikrein-binding protein yielded a 29-amino acid sequence with Asp as the amino terminus. Tissue kallikrein (38 kDa) and the purified binding protein (60 kDa) forms an equimolar 92-kDa complex. Formation of the SDS-stable complex between kallikrein and the binding protein appears in 30 s and reaches half-maximal binding at 18 min (data not shown). When the complex formation was analyzed under non-SDS PAGE, the

TABLE I

Purification of kallikrein-binding protein from rat sera

| Step                | Total Protein | Total RKBP | Yield | Purification |
|---------------------|---------------|------------|-------|--------------|
| 1. Sera             | 6000          | 17.7       | 100   | 1            |
| 2. 45–80% ammonium sulfate fractionation | 1170 | 9.5 | 54 | 2.7 |
| 3. Affi-Gel Blue    | 350           | 8.3        | 47    | 8.0          |
| 4. DEAE-Sepharose CL-6B | 66        | 4.1        | 23    | 21.1         |
| 5. Sephacyr S-200   | 15            | 3.0        | 20    | 78.0         |
| 6. Preparative gel  | 2.2           | 2.1        | 12    | 525.6        |
rate of binding is very rapid and reaches a maximal binding within 30 s (data not shown).

**Tissue Distribution of Kallikrein-binding Protein in Normotensive and Spontaneously Hypertensive Rats**—Tissue distribution of RKBP was analyzed by both Western blot analysis and a specific radioimmunoassay. Fig. 3 shows a single protein band of 60 kDa corresponding to purified kallikrein-binding protein can be detected in serum and tissue extracts of heart, lung, kidney, salivary gland, uterus, testis, and pituitary by Western blot analysis. However, kallikrein-binding protein in the urine has a smaller molecular mass of 50 kDa, suggesting a possible cleavage of this protein before excretion. The binding protein was barely detectable in the pancreas, prostate, adrenal glands, and liver. Semiquantitation of immunoblot assays can detect rat α1-antitrypsin levels in serum (Chao et al., 1990) or the binding protein in serum or tissue extracts in a dose-dependent manner with a minimum detection of 20 ng of protein. Kallikrein-binding protein levels in the serum of spontaneously hypertensive rats (SHR) are significantly lower than those of the control normotensive Wistar-Kyoto (WKY) rats as shown by Western blot analysis (Fig. 4, left panel). Contrarily, there appears to be no difference of kallikrein-binding protein levels in the serum of Bio/Breeder (BB(C)) control versus Bio/Breeder spontaneously diabetic (BB(D)) rats (Fig. 4, left panel). When rat serum (1 μl) was incubated with purified tissue kallikrein (1 μg), the amount of the 92-kDa kallikrein complex formation is significantly lower with the serum of SHR as compared to the control WKY rats or the BB diabetic and BB control rats (Fig. 4, right panel). The results clearly demonstrate a significant reduction of both kallikrein-binding protein and complex formation in the serum of SHR.

The findings of a deficiency in RKBP levels in SHR using Western blotting were confirmed by quantitative determination based on a radioimmunoassay. A highly specific radioimmunoassay was developed for measuring RKBP. The assay incorporated a modified polyethylene glycol technique for separating free from antibody-bound forms. Optimal concentrations of 200 μl of polyethylene glycol (25%) and 100 μl of bovine γ-globulin (1%) were found from exhaustive tests, yielding low background and high specific binding. The assays can detect kallikrein-binding protein levels ranging from 0.64 to 80 ng. Serial dilutions of sera or tissue extracts from normal and hypertensive rats showed complete parallelism with the kallikrein-binding protein standard (data not shown). Fig. 5 shows the tissue distribution of RKBP in SHR versus WKY rats as determined by the radioimmunoassay. RKBP levels in the serum and all tissues of SHR are severalfold lower than those of the WKY rats.

**Isolation and Characterization of cDNA Clones Encoding Kallikrein-binding Protein**—By immunoscreening a rat liver cDNA library with the specific antiserum against the purified binding protein, a group of independent cDNA clones of various lengths were isolated and sequenced. Fig. 6 shows the amino-terminal 29-amino acid sequence of the purified kallikrein-binding protein matching with a region of the deduced amino acid sequence from the cDNA encoding kallikrein-binding protein with only two amino acid residues mismatched. The amino-terminal Asp residue of the purified RKBP can be aligned with residue 21 of the translated protein sequence from the RKBP cDNAs. The peptide bond between the Asp residue and the Cys residue amino-terminal to it, as shown by an arrow, is therefore considered to be the site of cleavage between the 20-amino acid signal peptide and the mature protein (Fig. 6). When the total cDNA sequence was searched against the GenBank™ nucleic acid sequence data base with the FASTN program by Lipman and Pearson (1985), it matches with the cDNA sequence of the rat Spi-2.3 (Yoon et al., 1987) with greater than 99% sequence identity, with the cDNA sequence of rat growth hormone-regulated protein (Le Cam et al., 1987), and with the cDNA sequence of rat thyroid hormone-regulated protein (Le Cam et al., 1987), and with the cDNA sequence of rat thyroid hormone-regulated protein with 100% sequence identity (Tecce et al., 1986). The only mismatch found between the cDNA sequence of the kallikrein-binding protein and the rat Spi-2.3 is a single base replacement which does not alter the amino acid residue encoded, suggesting that the protein encoded by the Spi-2.3 cDNA, or by the rat growth hormone-regulated protein, rat thyroid hormone-regulated protein.

**Fig. 4.** Western blot analysis of kallikrein-binding protein and its complex formation with kallikrein in the serum of normotensive, hypertensive, and diabetic rats. Left panel, rat serum (1 μl) from SHR, WKY, Bio/Breeder control (BB(C)), or Bio/Breeder diabetes (BB(D)). Right panel, rat serum (1 μl) incubated with tissue kallikrein (1 μg) at 37°C for 60 min. Proteins were subjected to 7.5-15% linear gradient SDS-PAGE under reducing conditions and electrotransferred to nitrocellulose. Blots were developed with rabbit anti-RKBP antiserum followed by 125I-kallikrein-binding protein. Arrow, kallikrein and the binding protein complex.

**Fig. 3.** Western blot analysis of RKBP in rat serum and tissue extracts. Proteins were subjected to 7.5-15% linear gradient SDS-PAGE under reducing conditions and electrotransferred to nitrocellulose. Blots were developed with rabbit anti-RKBP antiserum followed by 125I-kallikrein-binding protein. Purified kallikrein-binding protein (KBP) (1 μg), rat serum (1 μl), tissue extracts from heart (350 μg), lung (650 μg), kidney (500 μg), salivary gland (300 μg), pancreas (450 μg), uterus (250 μg), testis (200 μg), prostate gland (250 μg), pituitary (70 μg), adrenal gland (250 μg), liver (500 μg), and 10-fold concentrated urine (30 μl).
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**FIG. 5.** Quantitation of kallikrein-binding protein in rat tissues. Rat kallikrein-binding protein levels in perfused tissue extracts were measured by a direct radioimmunoassay. Values represent a mean ± S.E. (n = 4). The details were described under “Experimental Procedures.”

- **cDNA Trans.** Met Ala Phe Ile Ala Ala Leu Gly Leu Leu Met
- **A.A. Seq.** Asp Gly
- **cDNA Trans.** Ala Gly Ile Cys Pro Ala Val Leu Cys Asp Gly
- **A.A. Seq.** Ala Gly Ile Cys Pro Ala Val Leu Cys Asp Gly
- **cDNA Trans.** Ile Leu Arg Asp Thr Leu Ser His Glu Asp
- **A.A. Seq.** Ile Leu Arg Asp Thr Leu Ser His Glu Asp
- **cDNA Trans.** Glu Gly Lys Gly Arg Glu Leu His Ser X Thr
- **A.A. Seq.** X Ala Ala X Asn
- **cDNA Trans.** Leu Ala Ser Ile Asn

Fig. 6. Amino-terminal amino acid (A. A. seq.) sequence of purified rat kallikrein-binding protein and the alignment with the translated amino acid sequence of the cDNA encoding the binding protein (cDNA Trans.)

protein cDNA is the kallikrein-binding protein. The calculated molecular weight of mature RKBP based on the translated amino acid sequence is 44,587, which is less than the molecular weight of 60,000 as determined by SDS-PAGE (Fig. 1). The difference in the molecular weights is ascribed to glycosylation of the mature protein at 5 potential residues, four of which are N-linked. These potential glycosylation sites were identified by two consensus sequences: Asn-X-Ser/Thr and Ser/Thr-X-X-Pro (Marshall, 1974). The rat kallikrein-binding protein contains 2 cysteine residues in its mature form which may be involved in a potential disulfide linkage.

**Sequence Comparison of Kallikrein-Binding Protein with Members of the Serpin Superfamily**—Rat kallikrein-binding protein is most closely related to human α1-antichymotrypsin, to which it shares 68.8 and 53.3% identity in nucleotide and amino acid sequences. It also shares significant sequence identities with other serpins like human α1-antitrypsin (56.8% nucleotide, 42.8% amino acid), rat α1-antitrypsin (58.9% nucleotide, 45.5% amino acid), and human antithrombin III (53.3% nucleotide, 34.8% amino acid). Fig. 7 shows reactive center sequence comparisons of kallikrein-binding protein with other serpins including α1-antitrypsin from rat and human, human α1-antichymotrypsin, heparin cofactor II, and antithrombin III. The RKBP contains a similar reactive center sequence in its COOH-terminal region as the other serpin molecules. The sequence, starting from P1 to P5 residues, is Leu-Lys-Ser-Leu-Pro-Gln. The cleavage site for target serine protease P1-P2 (boxed) or rat kallikrein-binding protein is the bond between Leu-Lys, different from those of other serpins.

**Expression and Regulation of Rat Kallikrein-binding Protein**—Tissue-specific and regulated expression of RKBP was identified by dot blot analysis using the RKBP cDNA probe, as shown in Fig. 8. Kallikrein-binding protein mRNA was found in the liver at high levels and also in the lung, salivary glands, and kidney but at low levels (Fig. 8A). Note that the amounts of RNA from liver loaded on the filter was 10-fold less than that from other tissues. The results indicate that the major site of synthesis of kallikrein-binding protein is in the liver with much less expression in other tissues. After acute phase inflammation, kallikrein-binding protein mRNA
levels in the liver are reduced by one-half (Fig. 8B). The results indicate that in contrast to the acute-phase induced rat \(\alpha_1\)-antitrypsin (Chao et al., 1990), the binding protein is a negative acute phase reactant. Similar to \(\alpha_1\)-antitrypsin, a major sex difference in levels of kallikrein-binding protein mRNA is evident, with a severalfold higher level in male than in female rats (Fig. 8B).

**Restriction Fragment Length Polymorphism Analysis**—In genomic Southern blot analysis using the DNA from SHR and WKY rats, multiple restriction fragment length polymorphisms were detected. Using 11 different restriction endonucleases, we have identified restriction fragment length polymorphisms involving alterations in restriction fragment lengths with four restriction endonucleases: BclI, BglII, DraI, and NdeI as shown in Fig. 9. The results indicate potential mutation(s) associated with the kallikrein-binding protein gene or its flanking regions in SHR versus WKY rats.

**DISCUSSION**

In the present report, we describe the purification, characterization, and cloning of a tissue kallikrein-binding protein from rat serum. Kallikrein-binding protein is an acidic protein with molecular mass of 60 kDa and isoelectric points ranging from 4.2 to 4.6. Different isoforms may be ascribed to varying carbohydrate content since the binding protein contains five potential glycosylation sites. The amino-terminal 29-amino acid sequences of the purified kallikrein-binding protein confirm the identity of the full length cDNA clones encoding kallikrein-binding protein which were isolated using antisera against the binding protein. The cDNA encoding kallikrein-binding protein shares complete identity with the cDNA sequences of a rat hepatic protein, Spi-2.3 (Yoon et al., 1987), the rat GHRP (Le Cam et al., 1987), and the rat THRGP (Tecce et al., 1986). Therefore, rat kallikrein-binding protein belongs to the serpin superfamily.

Previous studies by Geiger et al. (1981) have shown that human \(\alpha_1\)-antitrypsin binds to human urinary kallikrein and is a slow progressive inhibitor of human tissue kallikrein. We have confirmed the studies of Geiger et al. (1981), and in addition, we have also identified a novel human kallikrein-binding protein distinguishable from human \(\alpha_1\)-antitrypsin (Chen et al., 1990). Contrarily, we found that rat tissue kallikrein cannot form a SDS-stable complex with purified rat \(\alpha_1\)-antitrypsin. It is known that human tissue kallikrein is able to cleave two peptide bonds between Met-Lys and Arg-Ala from human or bovine kininogens to produce lysyl-bradykinin, while rat tissue kallikrein lacks this ability but cleaves Arg-Arg and Arg-Ala bonds from rat kininogen to generate bradykinin (Kato et al., 1985). Since both human and rat \(\alpha_1\)-antitrypsin have a Met-Ser bond as the reactive site, it is explainable why tissue kallikrein and \(\alpha_1\)-antitrypsin of rat fail to complex. If \(\alpha_1\)-antitrypsin were indeed the sole regulator of kallikrein activity, in the case of rat there would be a lack of such regulation, unless some other protein(s) function in substitution of \(\alpha_1\)-antitrypsin.

The mechanisms by which kallikrein-binding protein acts is not understood at this moment. However, the generalized reaction mechanism for serpin-like inhibitors may apply to the interaction of kallikrein and the binding protein, which suggest a cleavage at the reactive center or the bait region of the inhibitor molecule (Carrell and Boswell, 1986). The purified binding protein (60 kDa) and tissue kallikrein (38 kDa) form a 1:1 stoichiometric complex of 92 kDa which is resistant to SDS and heat treatment, suggesting a covalent linkage. The exact cleavage site at the reactive site of the binding protein by kallikrein has not been identified. Previous studies showed that active site blocked kallikrein or latent kallikrein cannot form complexes with the binding protein (Chao and Chao, 1988). The P\(_1\)-P\(_2\) bond of the kallikrein-binding protein, Leu-Lys is not the favorite cleavage site for tissue kallikrein. Therefore, the next peptide bond, P\(_3\)-P\(_4\), Lys-Arg may serve as the cleavage site for tissue kallikrein since tissue kallikrein has a high affinity for the basic amino acid residues Arg and Lys. Such an alternative reactive site peptide bond has been observed recently in another serpin molecule, \(\alpha_2\)-antiplasmin, inactivating both plasmin and chymotrypsin using overlapping reactive sites (Potempa et al., 1988). Kallikrein-binding protein may interact with kallikrein by first
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Kallikrein-binding protein (RKBP) is a serpin-like inhibitor that binds to kallikrein. It is recognized by the antiserum to the binding protein. The reversible and SDS-dissociable kallikrein complex (referred to as a tetrahedral transition state) may play a role in maintaining the enzyme's bioavailability. Under certain physiological conditions, the reversibly bound kallikrein complex undergoes a conversion from the tetrahydroly transition state to an acyl-enzyme intermediate and a small COOH-terminal peptide from the binding protein/inhibitor is released. A conformational change of the complex occurs concomitantly with the conversion of the intermediate and the release of the small peptide and as a result, the complex becomes SDS- and heat-resistant and is referred to as irreversibly bound. Similar reaction mechanisms have been suggested for the action of other serpin-like inhibitors (Carrell and Boswell, 1986). The kallikrein complex may thus be recognized and cleared by receptors in the liver. This notion is supported by previous studies showing that kallikrein forms complexes with serum components following intravenous administration of the enzyme, and that the complexes appear to be cleared from the liver but not the kidney (Rabito et al., 1985).

The physiological target enzyme of the RKBP is not yet clear. Both rat and human tissue kallikreins can form SDS-stable complexes with RKBP. Whether kallikrein-binding protein is the physiological modulator of tissue kallikrein or whether there are other target enzymes for the binding protein is not yet known. However, in the in vitro binding assay, we found that RKBP can also form equimolar complexes with porcine pancreatic elastase and bovine chymotrypsin (data not shown). We are in the process of identifying the specific cleavage sites of kallikrein-binding protein by kallikrein and other potential target enzymes as well as carrying out kinetic analysis. The comparison of rat kallikrein-binding protein and rat α1-antitrypsin in the P1-P5 region of the reactive center shows a high degree of similarity at the PZ-Pr section and a significant difference at residues P1-P1′. This suggests two different target enzymes for these two inhibitors and coincides with our finding that rat α1-antitrypsin does not bind or inhibit rat urinary kallikrein.

Western blot analysis showed that only a single protein in whole plasma or serum, corresponding to the purified binding protein, is recognized by the antisera to the binding protein. The results demonstrate the specificity of the antisera to kallikrein-binding protein. Kallikrein-binding protein was identified in serum and in various tissue extracts by both Western blot analysis and radioimmunoassays. Although tissues were prepared from rats perfused via cardiac puncture until blood-free, it is still possible that kallikrein-binding protein is either synthesized locally or recruited from the circulation by uptake. Dot blot analysis using a cDNA probe for kallikrein-binding protein showed high expression of the kallikrein-binding protein in the liver and low levels of expression in lung, kidney and salivary gland (Fig. 8A). It is intriguing to find that although liver is the primary synthesis site of the kallikrein-binding protein, it was barely detectable in the liver extract in Western blot analysis. These results reinforce the notion that kallikrein-binding protein is a secretory protein, and is rapidly secreted into the circulation as soon as it is synthesized in the liver. We also found two other hepatic proteins, rat kininogen and α1-antitrypsin expressed at high levels in the liver as identified by Northern or dot blot analysis. However, Western blotting or radioimmunoassays only detect very low amounts of immunoreactive kininogen or α1-antitrypsin in the rat liver as compared to other tissues (Chao et al., 1988b; Chao et al., 1990).

The kallikrein-binding protein belongs to the serpin family as it shows a high degree of sequence identity with several well-characterized members of this superfamily including α1-antitrypsin, antithrombin III and plasminogen activator inhibitor. Particularly, it is closest to human α1-antichymotrypsin with 68.8% sequence identity at the nucleotide level. The degree of identity between these two proteins is almost twice that between human α1-antitrypsin and human α1-antichymotrypsin, which were previously the closest among all the serpins. This high degree of sequence identity raises the question whether the RKBP is the rat counterpart of human α1-antichymotrypsin since the latter has not been cloned. However, the reactive center sequence of kallikrein-binding protein (P1-P5 bond being Leu-Lys) differs dramatically from that of the human α1-antichymotrypsin (P1-P5 bond being Leu-Ser). Since the most important trait of a serpin molecule is its target enzyme specificity, and this is largely determined by the nature of the reactive site P1-P5 peptide bond, it is very unlikely that RKBP represents the rat counterpart of human α1-antichymotrypsin. For example, although rat α1-antitrypsin shares about 70% sequence homology with its human counterpart (Chao et al., 1990), the reactive site peptide bond in both proteins is identical, with P1-P5 bond being Met-Ser. Other evidence indicating that kallikrein-binding protein is different from α1-antichymotrypsin is that the former is a negative acute phase reactant while the latter is a major acute phase protein. This conclusion is supported by the findings that both RKBP mRNA (Fig. 8B) and protein levels as determined by semiquantitative immunoblot assay data not shown) are reduced after acute phase inflammation.

Our previous report showed that the complex formation between kallikrein and the binding protein is either absent or significantly reduced in SHR when a functional binding assay was employed (Chao and Chao, 1988). The findings of the binding assay following SDS-stable complex formation are supported by the results obtained by immunoblot (Figs. 3 and 4) and radioimmunoassay (Fig. 5). The results from three independent assays are consistent with the notion that the kallikrein-binding protein is deficient in SHR. It is possible that the reduced level of kallikrein-binding protein in SHR is caused by a reduced rate of synthesis, an accelerated rate of clearance, or by mutation(s) in the kallikrein-binding protein gene. Also the function of the kallikrein-binding protein of SHR may be impaired, since the amount of kallikrein-binding protein in SHR and BB(C) rat appears to be similar (Fig. 4), but very little complex is formed between kallikrein and the binding protein in SHR as compared to BB(C) rat. Southern blot hybridization of genomic DNA from the SHR and WKY reveals restriction fragment length polymorphisms associated with kallikrein-binding protein genes or their flanking regions (Fig. 9). The results raise the possibility that the kallikrein-binding protein may be one of the contributing factors in the hypertensive phenotype of SHR. This hypertensive animal model is ideal for the investigation of the role of the kallikrein-kinin system in the development of hypertension and could eventually lead to an understanding of the function of the system components in human cardiovascular diseases.

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