Expression and characterization of rat kallikrein-binding protein in *Escherichia coli*

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Rat kallikrein-binding protein is a novel serine-proteinase inhibitor that forms a covalent complex with tissue kallikrein. We have purified rat kallikrein-binding protein and cloned the cDNA and the gene encoding rat kallikrein-binding protein [Chao, Chai, Chen, Xiong, Chao, Woodley-Miller, Wang, Lu and Chao (1990) J. Biol. Chem. **265**, 16394–16401; Chai, Ma, Murray, Chao and Chao (1991) J. Biol. Chem. **266**, 16029–16036]. In the present study, we have expressed rat kallikrein-binding protein in *Escherichia coli* with a T7-polymerase/promoter expression system. A high level of expression was detected by an e.l.i.s.a. with an average of 24.2 mg of recombinant rat kallikrein-binding protein per l of culture. The recombinant protein appeared as a major protein in a crude extract of *Escherichia coli* on SDS/PAGE. It showed a molecular mass of 43 kDa and was recognized by polyclonal antibody to the native rat kallikrein-binding protein in Western-blot analysis. The recombinant rat kallikrein-binding protein has been purified to apparent homogeneity by DEAE-Sepharose CL-6B, hydroxyapatite Bio-Gel HPHT and Mono P 5/5 column chromatography. The purified recombinant rat kallikrein-binding protein showed immunological identity with the native rat kallikrein-binding protein purified from rat serum, in a specific e.l.i.s.a. To confirm the fidelity of the expression, the N-terminal ten amino acids of the recombinant rat kallikrein-binding protein were sequenced and were shown to match perfectly with those of the native rat kallikrein-binding protein. The purified recombinant rat kallikrein-binding protein formed SDS- and heat-stable complexes with rat tissue kallikrein (rK1) and T-kininogenase (rK10) *in vitro*, but not with other enzymes in the rat kallikrein gene family, such as tonin (rK2) and S3 protein (rK9), which indicates enzyme-specific binding. The properties of the recombinant rat kallikrein-binding protein including its size, charge, complex formation with target enzymes and immunological characteristics were compared with those of the native protein. This expression system provides a simple way to obtain a large amount of the biologically active recombinant protein, to study structure–function relationships of the rat kallikrein-binding protein and its interaction with its target enzymes.

INTRODUCTION

Tissue kallikrein (EC 3.4.21.35) is known to process kininogens and release vasoactive kinins by limited proteolysis (Fielder, 1979). The kallikrein–kinin system is involved in many physiological and pathophysiological processes, including the regulation of blood pressure and local blood flow, and smooth muscle contraction and relaxation (Cuthbert and Margolius, 1982; Schachter, 1980; Bhoola et al., 1992). The activity of tissue kallikrein is regulated both post-translationally and at the level of gene expression (Murray et al., 1990). For many years, human α_1-antitrypsin was the only known potential inhibitor of human tissue kallikrein present in serum (Geiger et al., 1981). However human α_1-antitrypsin is a slow progressive inhibitor of tissue kallikrein and therefore is not considered to be a physiological regulator of tissue kallikrein.

In a previous study, we purified rat kallikrein-binding protein (RKBP) to homogeneity. It is an acidic glycoprotein with a molecular mass of 60 kDa (Chao et al., 1990). Its cDNA and gene have been cloned and sequenced and this demonstrated that RKBP is distinct from the known serine-proteinase inhibitors, although it shares a high degree of sequence similarity with α_1-antichymotrypsin, α_1-antitrypsin and antithrombin III (Chao et al., 1990; Chai et al., 1991). It forms a complex with rat tissue kallikrein at a molar ratio of 1:1 and the complex is resistant to SDS and to heat treatment, indicating a covalent linkage between rat tissue kallikrein and RKBP. Recently we have identified and purified a novel kallikrein inhibitor in human serum that forms a complex with human tissue kallikrein (Chao et al., 1986, 1990; Chen et al., 1990; Zhou et al., 1992). *In vivo* clearance studies in rats showed that binding to human kallikrein-binding protein prolongs the half life of kallikrein and may play a role in regulating the bioavailability of tissue kallikrein (Xiong et al., 1992). Therefore kallikrein-binding protein is regarded as a potential tissue-kallikrein inhibitor that modulates the activity of tissue kallikrein at the post-translational level (Murray et al., 1990).

Although the physiological function of kallikrein-binding protein has not been elucidated, the evidence accumulated so far suggests that RKBP may be important for pathophysiological processes. For example, spontaneously hypertensive rats have lower mRNA and protein levels of RKBP compared with Wistar–Kyoto (WKY) normotensive rats (Chao and Chao, 1988; Chao et al., 1992). Restriction-fragment-length polymorphisms (RFLP) have been observed in the RKBP gene between spontaneously hypertensive and Wistar–Kyoto rats (Chao et al., 1990). These findings indicate that RKBP may contribute to the hypertensive phenotype in spontaneously hypertensive rats. In

Abbreviations used: BLOTTO, 5% (w/v) nonfat dry milk in 10 mM sodium phosphate, pH 7.4, 0.14 M NaCl, 1 μM *p*-phenylmethanesulphonyl fluoride, 1 mg/l thimerosal, 200 mg/l NaN_3 and 0.01% (w/v) antifoam A; RKBP, rat kallikrein-binding protein.

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The nomenclature of the genes and proteins in the kallikrein gene family is according to the new nomenclature proposed by the participants at the Kinin 1991 Congress in Munich, Germany (Berg et al., 1992).

addition, the expression of RKBP gene is induced markedly by growth-hormone treatment and the RKBP mRNA levels are correlated with the growth rate of the rats, suggesting that RKBP may play a role in growth and development (Berry and Seelig, 1984; Yoon et al., 1987, 1990). During acute-phase inflammation, RKBP expression levels are reduced, indicating a potential involvement of RKBP in inflammatory responses (Le Cam and Le Cam, 1987; Schwarzenberg et al., 1989; Chao et al., 1990).

In order to elucidate the biological functions of kallikrein-binding protein and its interaction with tissue kallikrein, it is necessary to find a simple way to obtain kallikrein-binding protein in large quantities and to establish a heterologous-expression system for structure–function studies by site-directed mutagenesis. In the present study we have expressed RKBP in an *Escherichia coli* strain with a T7-polymerase-promoter system and have obtained a high yield of active recombinant RKBP which has been characterized.

EXPERIMENTAL

Materials

E. coli HB101/pGP1-2 is a strain of HB101 harbouring a vector containing gene 1 of T7 phage under the control of the inducible λ P_L and the gene for the heat-sensitive λ repressor. Plasmid pET3b is a derivative of pBR322 that contains the T7 promoter (ϕ 10) and the gene 10 translation-start site (s10) and transcription terminator (Tϕ) (Studier and Moffatt, 1986). Rat tissue kallikrein (rK1) and rat T-kininogenase (rK10*), S3 protein (rK9) and tonin (rK2) (for nomenclature see Berg et al., 1992) were purified to homogeneity in this laboratory (Xiong et al., 1990; Wang et al., 1992) and were labelled with ^{125}I by the lactoperoxidase method (Shimamoto et al., 1980). RKBP antibody was purified by a Protein A-affinity column as described previously (Chao et al., 1985). The following materials were obtained from commercial sources: DEAE-Sepharose CL-6B, Protein A–Sepharose (Pharmacia); *Taq* DNA polymerase (BRL); polyethylenimine, iminodiacetic acid, peroxidase avidin, Coomassie Blue, rifampicin (Sigma); Ampoline (LKB) and molecular mass protein standards (Bio-Rad).

Construction of the RKBP expression vector

The scheme for constructing the RKBP expression vector is shown in Figure 1. A full-length cDNA encoding RKBP had been screened from a rat-liver cDNA library and its nucleotide sequence had been determined previously (Chai et al., 1991). To delete the nucleotide sequence that encoded the signal peptide, a polymerase chain reaction (PCR) was performed using the full-length RKBP cDNA as the template. The 5′ PCR primer (CATATGGATGGCATACTGGGAAGG) starts at the first codon of the mature protein of RKBP. Adjacent to the 5′ end of the first codon is an unannealed *Nde*I site (underlined). The imported *Nde*I site in the PCR product not only served as a restriction site to facilitate cloning, but also as a translational initiation codon (ATG). The 3′ primer (GGATCCTGGGCAGAATTGTCAGATTTTGGGG) is complementary to the 3′ untranslated sequence of the cDNA and contains a *Bam*HI site. PCR products were synthesized with 30 cycles of PCR using *Taq* DNA polymerase. The single band of PCR product (1250 bp) was cloned into M13mp19 by blunt-ended ligation and was sequenced to eliminate any potential mismatch that had been generated by the *Taq* DNA polymerase (Sanger et al., 1977). No mismatch was found in the PCR-synthesized cDNA. It was then subcloned into the pET3b vector at the *Nde*I and *Bam*HI sites, immediately downstream from the T7 promoter (Figure 1).

Expression of the recombinant RKBP in *E. coli* and preparation of the cell extract

The expression procedure was as described by Studier and Moffatt (1986) with some modifications. The pET3b vector containing RKBP cDNA was transformed into HB101/pGP1-2 cells, which harbour the pGP1-2 plasmid, expressing T7 polymerase. The transformed cells were grown in 2× YT at 30 °C with vigorous shaking overnight until the A_{590} reached 1.2. Expression was then induced by heating the culture to 42 °C for 30 min, followed by adding rifampicin to a final concentration of 100 μg/ml to block the transcription driven by the *E. coli* polymerase. The exclusive expression driven by the T7 polymerase was carried out at 37 °C for an additional 2 h. The cells were harvested by centrifugation at 4000 ***g*** for 30 min. The cell-pellet was washed with 10 mM Tris/HCl, pH 8.0 and was re-suspended in a solution containing 10 mM Tris/HCl, pH 8.0, 1 mM EDTA and 1 μg/ml DNAase I. The cell lysate was prepared by three passages through a French pressure cell (SLM Instruments) at 1.1×10^5 kPa. Insoluble proteins and cell debris were removed by centrifugation at 10000 ***g*** for 2 h. The soluble *E. coli* extract was frozen at −20 °C for further characterization.

SDS/PAGE

SDS/PAGE was performed on a 7.5–15% linear polyacrylamide gel, using the buffer system described by Laemmli (1970). The gel was stained with 0.2% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid and 45% (v/v) ethanol and was destained in 10% (v/v) acetic acid and 25% (v/v) methanol.

Isoelectric focusing

Isoelectric focusing was performed essentially as previously described (Xiong et al., 1990) in an LKB-2117 Multiphor electrophoresis system with a pH gradient of 3.5–10.0, formed by Ampholine. The gel was run for 2.5 h at 4 °C with a voltage increasing from 150–1450 V. After completion of focusing, the edge of the gel was removed and strips of 6 mm width were cut transversely at 4 mm intervals. Each section was placed in a tube containing 1 ml of distilled water and was incubated for 24 h, when the pH values of the sections were measured and the pH gradient was obtained by plotting the pH value of the sections against their original positions on the gel. The remaining gel was fixed in 25% (v/v) trichloroacetic acid overnight, stained in 0.2% (w/v) Coomassie Blue solution for 15 min and destained in acetic acid/methanol/water (2:5:13, by vol.).

Western-blot analysis

The cell extract or purified RKBP was resolved on SDS/PAGE and was electrotransferred onto Immobilon-P membranes. The antigen-overlay method for immunoblotting has been described previously (Chao et al., 1989). In outline the membranes were blocked with BLOTTO solution [5% (w/v) nonfat dry milk in 10 mM sodium phosphate, pH 7.4, 0.14 M NaCl, 1 μM *p*-phenylmethanesulphonyl fluoride, 1 mg/l thimerosal, 200 mg/l NaN_3 and 0.01% (w/v) antifoam A] for 1 h and were then incubated with rabbit anti-RKBP antiserum (1:250 in BLOTTO solution) for 3 h with gentle shaking. The membranes were washed three times with BLOTTO, followed by incubation with

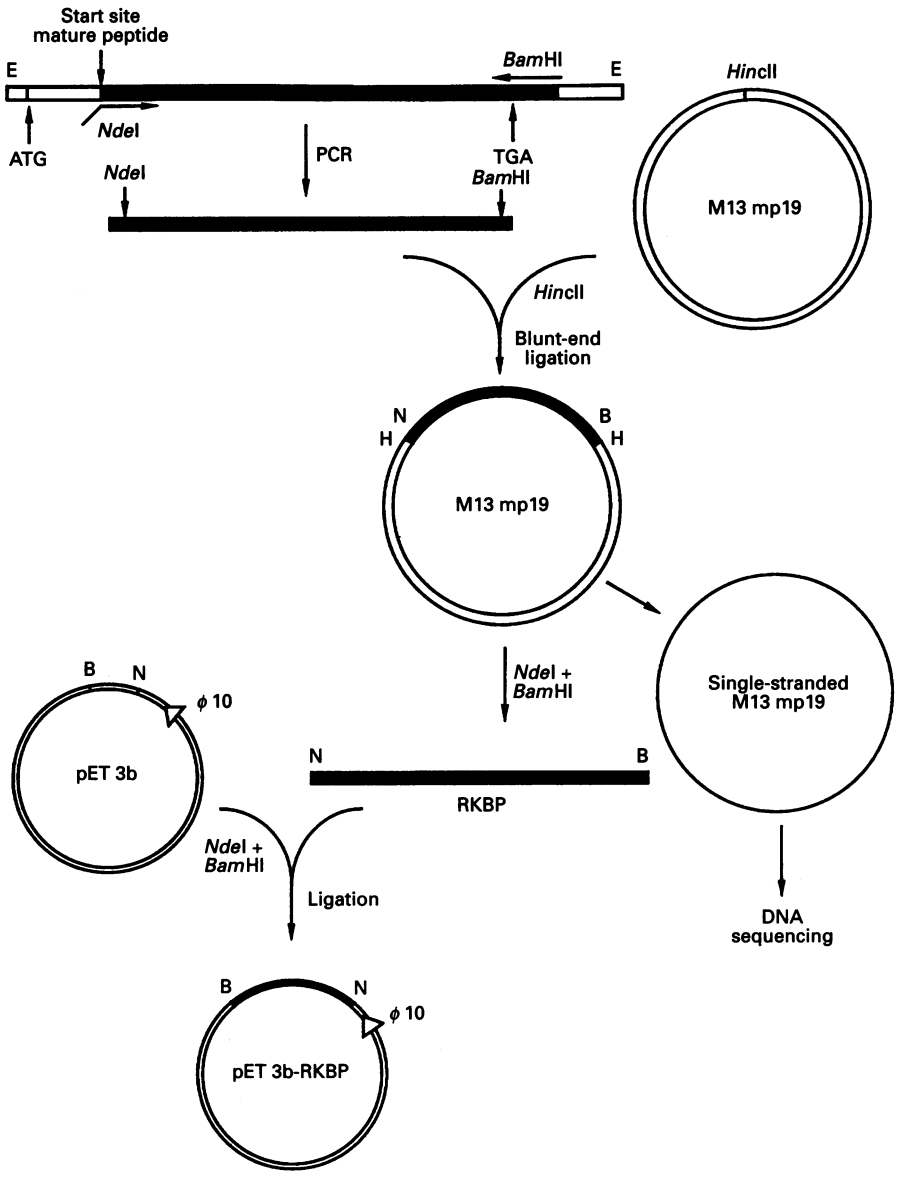

Figure 1 Scheme for constructing the RKBP expression vector

The cDNA encoding the mature protein of RKBP was amplified by PCR and an *Nde*I (N) site was added at the 5′ terminus. The PCR product was cloned into M13mp19 for sequence verification and then was subcloned into the pET3b vector at the *Nde*I and *Bam*HI (B) sites, immediately downstream from the T7 promoter. Abbreviations used: E, *Eco*RI; H, *Hin*dII.

^{125}I-labelled RKBP for 1.5 h. The membranes were then washed three times with BLOTTO and once with phosphate-buffered saline (10 mM sodium phosphate, pH 7.4 and 0.14 M NaCl), were air-dried and were exposed to Kodak X-Omat film. All procedures were carried out at room temperature.

RKBP e.l.i.s.a.

The 96-well plates, coated with Protein A affinity-purified rabbit anti-RKBP antibody (1 μg/ml), were blocked with 1% (w/v) bovine serum albumin. An RKBP standard (0.3–25 ng/ml) or the samples to be determined were incubated in the wells at 37 °C for 90 min. After the wells had been washed three times, biotinylated rabbit anti-RKBP antibody (1 μg/ml) was added to the wells and they were incubated at 37 °C for 60 min. The wells were washed and 100 μl of peroxidase–avidin (1 μg/ml) was incubated in the wells at 37 °C for 30 min. Unbound peroxidase–avidin was removed by washing five times, the substrate was added and was incubated at room temperature for 30 min. The concentration of RKBP was determined by measuring the A_{414}.

Purification of RKBP from rat serum

RKBP was purified from rat serum, as described previously, with some modifications (Chao et al., 1990). All steps were carried out at 4 °C unless otherwise specified. Aliquots of 10 ml of rat sera were dialysed against 20 mM sodium-phosphate buffer, pH 7.0 and were then centrifuged at 10000 *g* for 10 min. The dialysed sera were applied onto an AffiGel-Blue column (2.5 cm × 20 cm) that had been equilibrated previously with 20 mM sodium-

phosphate buffer, pH 7.0, containing 0.02% (w/v) NaN_3 at a flow rate of 10 ml/h. The column was washed with the same buffer until the absorbance at 280 nm reached the baseline. Fractions of 8 ml were collected. Flow-through fractions with kallikrein-binding activity were combined and were loaded directly onto a DEAE-Sepharose CL-6B column (1.5 cm × 20 cm) that had been equilibrated with 20 mM sodium-phosphate buffer, pH 7.0. The column was then washed with the same buffer and was eluted with 400 ml of gradient from 0 to 0.3 M NaCl, in 20 mM sodium-phosphate buffer, pH 7.0. Fractions of 8 ml were collected and fractions with kallikrein-binding activity were pooled, concentrated and were then dialysed against 20 mM sodium phosphate, pH 7.0. The dialysate was loaded slowly onto a hydroxyapatite Bio-Gel HT column (1.5 cm × 20 cm) that had been equilibrated with 20 mM sodium-phosphate buffer, pH 7.0. RKBP was in the flow-through fractions, which were combined, concentrated and were dialysed against 1.7 M $(NH_4)_2SO_4$ in 40 mM sodium-phosphate buffer, pH 7.0. The sample was applied at room temperature at a flow rate of 0.5 ml/min onto a phenyl-Superose 5/5 column on a Pharmacia LKB f.p.l.c. system, with starting conditions of 1.7 M $(NH_4)_2SO_4$ in 40 mM sodium-phosphate buffer, pH 7.0 (eluent A). RKBP was separated from the protein contamination in a 30 ml gradient from 0 to 100% (v/v) of eluent B (0.01 M sodium-phosphate buffer, pH 7.0). RKBP fractions were pooled, concentrated and were dialysed through a Centricon-30 with several changes of phosphate buffer and then were kept at −20 °C before characterization.

Purification of the recombinant RKBP from *E. coli* extract

A sample of 60 ml of crude *E. coli* extract was precipitated with 0.1% (w/v) polyethylenimine. The pellet was removed by centrifugation at 10000 *g* for 10 min. The supernatant was dialysed against 20 mM sodium-phosphate buffer, pH 7.0, containing 0.02% (w/v) NaN_3 and then was applied onto a DEAE-Sepharose CL-6B column (2.5 cm × 20 cm) that had been equilibrated with the same buffer. Unbound proteins were washed off with the same buffer until the absorbance at 280 nm reached the baseline. Bound proteins were eluted with 500 ml of gradient from 0 to 0.2 M NaCl, in 20 mM sodium-phosphate buffer, pH 7.0, containing 0.02% (w/v) NaN_3. Fractions of 8 ml were collected. Fractions containing kallikrein-binding activity were eluted at about 0.1 M NaCl. These fractions were pooled, concentrated and were dialysed against 20 mM sodium phosphate, pH 7.0. The dialysate was loaded onto a hydroxyapatite Bio-Gel HPHT column (1 cm × 5 cm) that had been equilibrated with the same buffer on a Pharmacia LKB f.p.l.c. system. After washing, the column was eluted with 60 ml of gradient from 20 mM sodium phosphate, pH 7.0 to 1 M NaCl in 0.3 M sodium-phosphate buffer, pH 7.0. Fractions with kallikrein-binding activity were pooled, dialysed against 25 mM Tris base (adjusted with iminodiacetic acid to pH 7.0) and applied onto a Mono P 5/5 column on an f.p.l.c. system. Recombinant RKBP was eluted at pH 4.8 as a single peak with 10% (w/v) polybuffer 74.

N-terminal amino-acid sequence analysis

The purified RKBP was subjected to SDS/PAGE under reducing conditions and was electrotransferred onto an Immobilon-P membrane. The protein blotted onto the membrane was stained with 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid for 1 min and destained with 1 M acetic acid for 2 min. RKBP was cut out and was subjected to Edman degradations performed with an Applied Biosystems Inc. model 477 gas-phase sequencer equipped with an on-line narrow-bore phenylthiohydantoin derivative analyser (Lu et al., 1989).

Kallikrein–RKBP complex formation

Aliquots of the cell extract or of purified RKBP were incubated with ^{125}I-labelled kallikrein (10000 c.p.m.) in 10 mM sodium phosphate, pH 7.0, at 37 °C for 1 h, as described previously (Chao and Chao, 1988). The binding was stopped by adding one-third volume of 3× SDS sample buffer (0.125 M Tris/HCl, pH 6.8, 30% (v/v) glycerol and 5% (w/v) SDS) and boiling for 5 min. The mixture was then resolved in SDS/PAGE under non-reducing conditions and was stained with Coomassie Blue, as described above. The gel was dried and was exposed to Kodak X-Omat film.

RESULTS

Expression and purification of recombinant RKBP from *E. coli*

RKBP was expressed with the T7 polymerase/promoter system and an *E. coli* lysate was prepared as described in the Experimental section. Soluble proteins were separated from the cell debris and from insoluble protein by ultracentrifugation, and resolved on SDS/PAGE under reducing conditions. The cell extract from HB101 cells harbouring the pET3b vector without the RKBP cDNA insert was applied onto SDS/PAGE as a control. As shown by Coomassie Blue staining in Figure 2, an additional major protein appears in the cells harbouring the RKBP-expressing construct (lane 2), compared with the pET3b vector control (lane 1). This protein has an apparent molecular mass of 43 kDa and was recognized by an anti-RKBP antibody

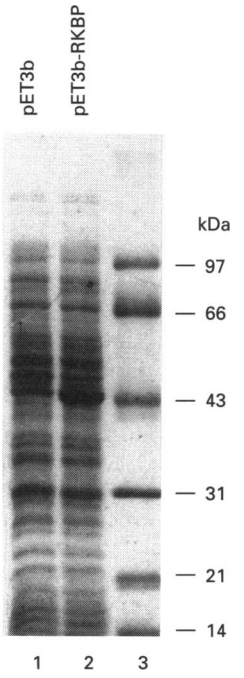

Figure 2 SDS/PAGE of the crude *E. coli* cell extract

A sample of 40 μg of the cell extracts from *E. coli* harbouring the pET3b vector alone (lane 1) and the RKBP-pET3b expression construct (lane 2) were resolved in SDS/PAGE and were stained with Coomassie Blue.

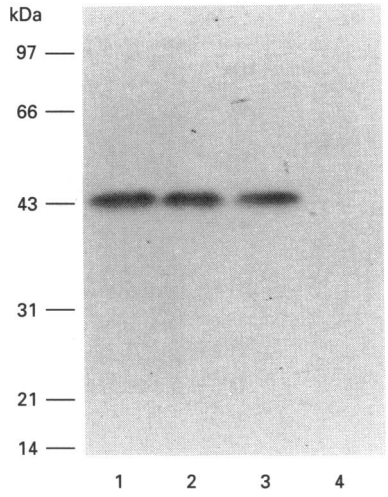

Figure 3 Western-blot analysis of the recombinant RKBP

The Western-blot analysis used a polyclonal antibody against the native RKBP. Lane 1; crude *E. coli* extract producing recombinant RKBP; lane 2, fraction from the DEAE-Sepharose CL-6B column; lane 3, fraction from the Mono P 5/5 column; lane 4, extract of *E. coli* harbouring the pET3b vector alone.

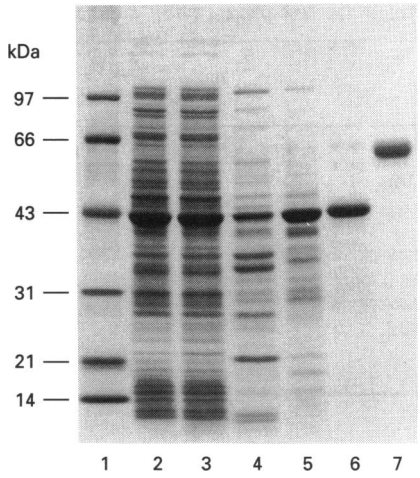

Figure 5 SDS/PAGE of the recombinant RKBP

Crude *E. coli* extracts and fractions from each purification step were resolved on SDS/PAGE and were stained with Coomassie Blue. Lane 1, molecular mass marker; lane 2, crude *E. coli* extract; lane 3, fraction after polyethylenimine precipitation; lane 4, fraction from the DEAE-Sepharose CL-6B column; lane 5, fraction from the hydroxyapatite column; lane 6, fraction from the Mono P 5/5 column; lane 7, purified native RKBP.

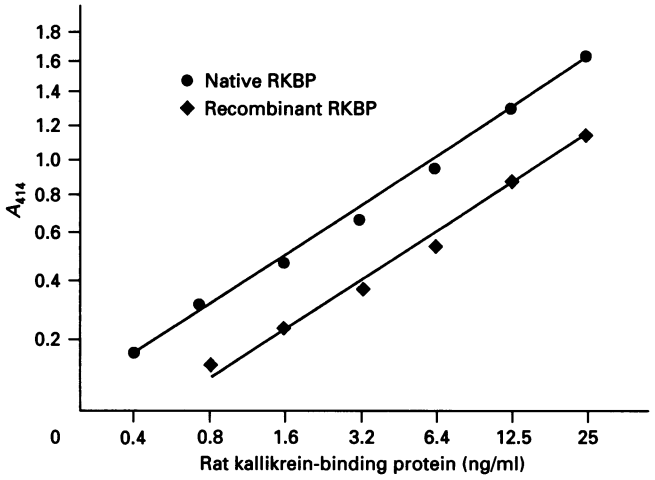

Figure 4 E.l.i.s.a. of the native and the recombinant RKBP

E.l.i.s.a. with an anti-RKBP polyclonal antibody showed a linear dose-dependent curve for the recombinant RKBP that was parallel with the standard curve for the native RKBP.

in Western-blot analysis as a single band (Figure 3, lane 1). To determine the yield of the recombinant protein, the concentration of RKBP in the crude *E. coli* extract was measured by a specific e.l.i.s.a. using a polyclonal antibody against the native RKBP (Figure 4). The purified RKBP was used as standard in the e.l.i.s.a. The average yield was 24.2 mg of recombinant RKBP per litre of *E. coli* culture, which accounts for 4.6% of the total protein.

To identify the activities of the recombinant protein, the crude cell extract was subjected to a kallikrein-binding assay. The crude extract from RKBP-expressing cells and from pET3b vector control cells was incubated with ^{125}I-labelled kallikrein and the formation of high-molecular-mass complexes was examined. Incubation of an aliquot of the cell extract with ^{125}I-labelled rat tissue kallikrein resulted in the formation of an SDS-stable high-molecular-mass complex in the cells expressing RKBP, but not in the pET3b control. This result suggests that the kallikrein-binding activity derives from the recombinant RKBP, rather than components of the *E. coli* extracts.

Recombinant RKBP was purified by DEAE-Sepharose CL-6B hydroxyapatite Bio-Gel HPHT and Mono P 5/5 column chromatography as described in the Experimental section. The fractions after each purification step were resolved on SDS/PAGE under reducing conditions and were stained with Coomassie Blue as shown in Figure 5. After the Mono P 5/5 column treatment, RKBP appeared to be homogenous in SDS/PAGE (Figure 5, lane 6). The purified recombinant RKBP had a molecular mass of 43 kDa, while the native RKBP was 60 kDa (Figure 5, lane 7), a difference that may be attributed to glycosylation.

N-terminal sequence analysis of the recombinant RKBP

To verify the fidelity of the expression, purified recombinant RKBP was subjected to N-terminal amino-acid sequencing. The sequence of the N-terminal ten amino-acid residues was obtained. As shown in Table 1, the amino-acid sequence of recombinant RKBP matches the N-terminal sequence of the native RKBP, except for one additional Met residue at the N-terminus of the recombinant protein. The additional Met is encoded by the imported ATG translational initiation codon in RKBP cDNA. This result confirmed the authenticity of the recombinant RKBP and the accuracy of the expression.

Immunological characteristics

To confirm the immunological identity of the recombinant protein produced by *E. coli* with native RKBP, both crude cell extracts and purified recombinant RKBP were subjected to Western-blot analysis using an anti-RKBP polyclonal antibody.

Table 1 Comparison of the recombinant RKBP with native RKBP

Parameter	Native RKBP	Recombinant RKBP
Source	Rat serum	*E coli* expression
Molecular mass (kDa)	60	43
N-terminal sequence	123456789 DGILGRDTL	123456789 MDGILGRDTL
Molecular mass of complex with rat tissue kallikrein (kDa)	92	75
Molecular mass of complex with T-kininogenase (kDa)	89	72
Isoelectric point	4.2–4.6	4.8–5.2

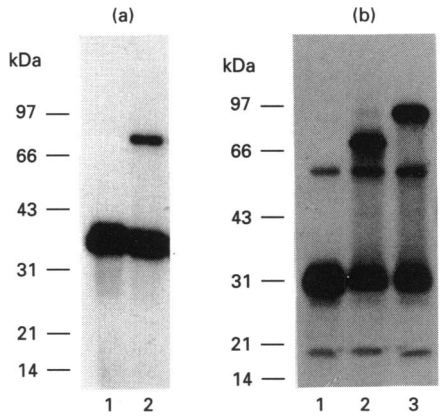

Figure 6 Complex formation of RKBP with kallikreins

^{125}I-labelled rat tissue kallikrein (**a**) or T-kininogenase (**b**) were incubated with purified recombinant and native RKBP followed by SDS/PAGE under non-reducing conditions. The high-molecular-mass complexes of recombinant RKBP with rat tissue kallikrein or with T-kininogenase were visualized by autoradiography. Lane 1, labelled tissue kallikrein or T-kininogenase alone; lane 2, labelled tissue kallikrein or T-kininogenase incubated with recombinant RKBP; lane 3, labelled T-kininogenase incubated with native RKBP.

Figure 3 shows that the recombinant RKBP was recognized by anti-RKBP antibody as single bands in the crude *E. coli* extract (lane 1), DEAE column fractions (lane 2) and Mono P column purified recombinant RKBP (lane 3). The immunoreactive RKBP had a molecular mass of 43 kDa (Figure 3, lanes 1–3), which is consistent with the molecular mass of the purified recombinant protein stained with Coomassie Blue, as seen in SDS/PAGE (Figure 5, lane 6). The *E. coli* extract containing pET3b vector alone was used as a control in the Western-blot analysis and no *E. coli* component was recognized by the anti-RKBP antibody (Figure 3, lane 4).

The immunological identity of the native and recombinant RKBPs was further examined by an e.l.i.s.a. using a polyclonal antibody against native RKBP. Both native and recombinant RKBPs were diluted serially and were measured by e.l.i.s.a. as described above. The A_{414} of each dilution was plotted as a function of protein concentration to give a dilution curve. As shown in Figure 4, the linear dose-dependent curve for the recombinant RKBP is parallel with the native RKBP standard curve, indicating their immunological identity (Figure 4). There was no cross-reactivity between anti-RKBP antibody and other serine proteinase inhibitors in the e.l.i.s.a.

Biochemical characterization of recombinant RKBP

The biochemical properties of the recombinant RKBP were compared with those of the native protein, as shown in Table 1. They differ both in molecular mass and in charge. The native protein has a molecular mass of 60 kDa while the recombinant RKBP has a molecular mass of 43 kDa, which is very close to the calculated value for RKBP (44.6 kDa), based on the translated amino-acid sequence, and to the molecular mass of the de-glycosylated RKBP (45 kDa), as reported by Pages et al. (1990). The isoelectric point of the recombinant RKBP is pH 4.8–5.2, as determined by isoelectric focusing. The pI of the recombinant RKBP was also calculated at 5.2, using the GCG program. Native RKBP has a lower pI value, pH 4.2–4.6 (Chao et al., 1990). These differences may be attributed to the lack of glycosylation in *E. coli*-expressed proteins.

In order to examine its enzyme-binding specificity, purified recombinant RKBP was subjected to a kallikrein-binding assay as described in the Experimental section. The recombinant and native RKBPs were incubated with ^{125}I-labelled rat tissue kallikrein (rK1), tonin (rK2), S3 protein (rK9) and T-kininogenase (rK10), respectively, and the complex formation was examined. As shown in Figure 6(a), after incubation with ^{125}I-labelled rat tissue kallikrein (rK1), recombinant RKBP formed a 75 kDa complex (lane 2), while native RKBP formed a 92 kDa complex with rat tissue kallikrein (Chao et al., 1990). Figure 6(b) shows that ^{125}I-labelled T-kininogenase (rK10) formed a 72 kDa complex with the recombinant RKBP (lane 2), while it formed an 89 kDa complex with the native RKBP (lane 3) under non-reducing conditions (Figure 6b). The recombinant RKBP bound to mouse tissue kallikrein (mK1) and human tissue kallikrein (hK1) as well (for nomenclature see Berg et al., 1992), forming a 76 kDa and a 70 kDa complex, respectively (data not shown). These complexes were resistant to boiling and to SDS treatment, suggesting a covalent linkage between RKBP and the tissue kallikreins or T-kininogenase. In contrast the recombinant RKBP did not bind to tonin (rK2) or to S3 protein (rK9) under the same conditions (data not shown), which indicates binding specificity between the kallikrein-binding protein and its target enzymes. The results showed that the binding specificity of the recombinant RKBP is consistent with that of the native RKBP.

DISCUSSION

In the present study we have expressed rat kallikrein-binding protein at a high yield using the T7 polymerase/promoter expression system. The recombinant protein was recognized by an anti-RKBP antibody and its N-terminal amino-acid sequence matched that of native RKBP, demonstrating the fidelity of the expression. The recombinant RKBP was soluble and functionally active and can be used therefore to study its structural and functional relationships by site-directed mutagenesis.

In constructing the expression vector we have manipulated the RKBP cDNA using the PCR technique, to confer a suitable restriction site at the desired position, while keeping the correct reading frame. *E. coli* lacks the eukaryotic machinery for protein modification and cannot remove the signal peptide from a mature protein. The signal peptide may potentially affect the folding and the activity of the recombinant protein, therefore the sequence coding for the signal peptide was deleted from the cDNA in the PCR. The 5′ PCR primer contains an unannealed *Nde*I site immediately 5′ to the cDNA sequence coding for the

mature peptide. The *Nde*I site provides a translational initiation codon as well, which accounts for the additional Met residue at the N-terminal of the recombinant protein.

The recombinant and native RKBPs have identical N-terminal amino-acid sequences and immunological properties. However they showed differences in their size and charge. These differences may be ascribed to a lack of protein-glycosylation machinery in *E. coli*. Native RKBP contains five potential glycosylation sites, according to the consensus sequence (Chao et al., 1990). Since glycosylation alters the pI value of the protein by introducing carbohydrate moieties to some amino-acid residues, native RKBP has a lower pI value than the recombinant RKBP. As the result of the glycosylation the molecular mass of the native protein is higher than that of the recombinant RKBP, which results in the different molecular masses of their complexes with tissue kallikrein (rK1) and T-kininogenase (rK10).

Like native RKBP, recombinant RKBP can form SDS- and heat-stable complexes with rat tissue kallikrein (rK1) and with T-kininogenase (rK10) *in vitro*. Both tissue kallikrein and T-kininogenase are serine proteases that function via limited proteolysis of their specific kininogen substrates (Chagas et al., 1992). In rat, tissue kallikrein cleaves both low- and high-molecular-mass kininogens to release vasoactive bradykinin (Kato et al., 1985). T-kininogenase processes T-kininogen and releases T-kinin (Barlas et al., 1987). Previous studies showed that rat tissue kallikrein is expressed in the kidney and the submandibular gland (Ashley and MacDonald, 1985). Our recent results indicate that T-kininogenase is also expressed both in the kidney and in the submandibular gland (Ma et al., 1992). Tissue kallikrein and T-kininogenase may be involved in the regulation of water and salt excretion in the kidney, as well as in the regulation of blood pressure. Kallikrein-binding protein may interact with kallikrein firstly by forming a reversible complex and then by forming an irreversible complex (Chao et al., 1990). By binding with tissue kallikrein and T-kininogenase irreversibly, RKBP may inhibit their proteolytic activities and may induce a series of physiological changes as a consequence. Kallikrein-binding protein may also modulate the activity of the kallikreins by influencing their delivery, internalization or degradation (Chao and Chao, 1988). Xiong et al. (1992) reported recently that human kallikrein-binding protein prolongs the half-life of human tissue kallikrein in the circulation of rats and may regulate the rate of kallikrein catabolism *in vivo*. It was postulated that kallikrein-binding protein changes its structure after binding with kallikrein so that it is recognized by a receptor that mediates the clearance of the complex (Xiong et al., 1992).

The binding of kallikrein-binding protein with its target serine proteases is enzyme-specific. Both recombinant and native RKBP showed strong binding with two kallikrein gene family members, tissue kallikrein (rK1) and T-kininogenase (rK10), but not with other members, such as tonin (rK2) and S3 protein (rK9). Rat tissue kallikrein, T-kininogenase, tonin and S3 protein belong to the rat tissue kallikrein gene family. They share more than 80% amino-acid sequence similarity and have a similar tertiary protein structure (Bode et al., 1983; Wines et al., 1991). Despite the similarity of structure, they have divergent substrate specificities because of divergent key amino-acid residues (Elmoujahed et al., 1990; Gutman et al., 1991; Chagas et al., 1992). T-kininogenase retains all the key amino-acid residues contributing to the substrate-binding preference of tissue kallikrein (Ma et al., 1992), while tonin and S3 protein have distinct substrate-binding pockets from that of tissue kallikrein (Ashley and MacDonald, 1985). As a result tonin and S3 protein have very different substrate-binding specificities from tissue kallikrein (Wang et al., 1992; Moreau et al., 1992). Whether the differences in these key residues accounts for the fact that RKBP binds to tissue kallikrein and to T-kininogenase, but not to tonin and S3 protein remains to be investigated.

Kallikrein-binding protein shares a high structural similarity with human α_1-antitrypsin and α_1-antichymotrypsin (Chai et al., 1991; Zhou et al., 1992). However, they have shown distinct binding specificities toward their target proteases. The different enzyme-binding specificities may be determined by the divergent amino-acid residues in their reactive centres (Chao et al., 1990; Chai et al., 1991). It will be interesting to evaluate the altered proteinase-binding specificity of RKBP after the substitution of amino-acid residues at its reactive site. This RKBP expression system provides a basis for studying the interactions of kallikrein with its specific inhibitors. It establishes also a system for studying the structure–function relationships of RKBP through site-directed mutagenesis.

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