Identification of a new tissue-kallikrein-binding protein

Julie CHAO,*§ David M. TILLMAN,† Maoyin WANG,*† Harry S. MARGOLIUS*‡ and Lee CHAO†

*Department of Pharmacology, †Department of Biochemistry and ‡Department of Medicine, Medical University of South Carolina, Charleston, SC 29425, U.S.A.

We have identified a tissue-kallikrein-binding protein in human serum and in the serum-free culture media from human lung fibroblasts (WI-38) and rodent neuroblastoma × glioma hybrid cells (NG108-15). Purified and ^{125}I -labelled tissue kallikrein and human serum form a $\sim 92000\text{-}M_r$ SDS-stable complex. The relative quantity of this complex-formation is measured by densitometric scanning of autoradiograms. Complex-formation between tissue kallikrein and the serum binding protein was time-dependent and detectable after 5 min incubation at 37 °C, with half-maximal binding at 28 min. Binding of ^{125}I -kallikrein to kallikrein-binding protein is temperature-dependent and can be inhibited by heparin or excess unlabelled tissue kallikrein but not by plasma kallikrein, collagenase, thrombin, urokinase, α_1 -antitrypsin or kininogens. The kallikrein-binding protein is acid- and heat-labile, as pretreatment of sera at pH 3.0 or at 60 °C for 30 min diminishes complex-formation. However, the formed complexes are stable to acid or 1 M-hydroxylamine treatment and can only be partially dissociated with 10 mM-NaOH. When kallikrein was inhibited by the active-site-labelling reagents phenylmethanesulphonyl fluoride or D-Phe-D-Phe-L-Arg-CH₂Cl no complex-formation was observed. An endogenous $\sim 92000\text{-}M_r$ kallikrein–kallikrein-binding protein complex was isolated from normal human serum by using a human tissue kallikrein–agarose affinity column. These complexes were recognized by anti-(human tissue kallikrein) antibodies, but not by anti- α_1 -antitrypsin serum, in Western-blot analyses. The results show that the kallikrein-binding protein is distinct from α_1 -antitrypsin and is not identifiable with any of the well-characterized plasma proteinase inhibitors such as α_2 -macroglobulin, inter- α -trypsin inhibitor, C₁-inactivator or antithrombin III. The functional role of this kallikrein-binding protein and its impact on kallikrein activity or metabolism *in vivo* remain to be investigated.

INTRODUCTION

Tissue kallikreins (EC 3.4.21.35) are now being located in new tissue sites, including the brain, pituitary gland, pineal body and the vascular wall (Chao *et al.*, 1983, 1986a; Simson *et al.*, 1985; Nolly *et al.*, 1985). We have shown that kallikrein-synthetic rate, mRNA concentrations and the quantities of active or prokallikrein can be altered by dietary manoeuvres or hormones (Chao & Margolius, 1983; Miller *et al.*, 1984; Ando *et al.*, 1985; Gerald *et al.*, 1986; Chao *et al.*, 1986a). Specific tissue-kallikrein inhibitors, or modulators of enzyme activity or metabolism, have not been found. Among the known plasma proteinase inhibitors, none is an effective inhibitor of tissue kallikreins. α_2 -Macroglobulin and C₁-inactivator are potent inhibitors of plasma kallikrein, but do not bind or inhibit tissue kallikreins (Vahtera & Hamberg, 1976; Sakamoto & Nishikaze, 1980). We and others have found that purified and ^{125}I -labelled tissue kallikreins or unlabelled enzyme appear to be complexed to form some higher- M_r entities when they were added to human or rodent serum (Rabito *et al.*, 1980; Lawton *et al.*, 1981; Shimamoto *et al.*, 1984).

A new group of proteinase inhibitors named protease nexins have been found in the serum-free conditioned media of a variety of transformed or malignant cell lines (Baker *et al.*, 1980; Eaton & Baker, 1983; Scott *et al.*, 1985). Protease nexins formed SDS-stable complexes with thrombin and several other serine proteinases (Baker *et al.*, 1980; Scott & Baker, 1983; Scott *et al.*,

1985). These findings prompted us to search for a 'nexin-like' tissue-kallikrein-binding protein. In the present paper we describe a new specific tissue-kallikrein-binding protein in human serum and in conditioned culture media from human lung fibroblasts (WI-38) and rodent neuroblastoma × glioma hybrids (NG108). An endogenous tissue-kallikrein-binding protein complex was also identified.

EXPERIMENTAL

Purification and agarose-immobilization of kallikreins

Human and rat urinary kallikreins were purified to homogeneity, and antisera were generated in rabbits or sheep (Chao & Margolius, 1979; Shimamoto *et al.*, 1980). Human urinary kallikrein (2 mg/ml in 0.1 M-Mops/NaOH buffer, pH 7.3) was mixed with 25 ml of Affi-Gel 15 that had been prewashed with cold distilled water on a sintered-glass filter. Incubation was continued with gentle shaking for 4 h at room temperature and then for 16 h at 4 °C. Then 0.1 vol. of 1 M-ethanolamine/HCl buffer, pH 8.0, was added to block any remaining sites that had not reacted. After incubation for 1 h at 23 °C, the conjugate was collected on Whatman no. 40 filter paper and washed several times with water. The filtrate was collected for measurement of remaining protein. More than 80% of kallikrein was immobilized. Rat tissue kallikrein was coupled to Affi-Gel 15 as described previously (Chao *et al.*, 1983). The kallikrein–agarose

§ To whom correspondence and reprint requests should be addressed.

columns were then equilibrated with 0.1 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.0. Purified kallikreins were labelled with ^{125}I by using the lactoperoxidase method as described previously (Shimamoto *et al.*, 1980). Rat urinary esterase A and rat tonin were purified as described previously (Chao, 1983; Demassieux *et al.*, 1976).

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed under denaturing conditions in a 7.5–15% linear-gradient gel (Chao *et al.*, 1983). The gels were stained with 0.25% Coomassie Brilliant Blue in 9% (v/v) acetic acid/45% (v/v) methanol and destained in 9% acetic acid/45% methanol. Slab gels were dried and prepared for autoradiography at -70°C for 24 h.

Western-blot analyses

Kallikrein and kallikrein–kallikrein-binding protein complexes were detected by autoradiography with ^{125}I -Protein A binding as described previously (Chao *et al.*, 1983). Electrophoresis was performed in a 7.5–17.5% linear-gradient polyacrylamide gel containing 0.1% SDS. The proteins were then electrophoretically transferred to nitrocellulose as described by Burnette (1981). Electrophoretic transfer was performed at 30V for 18 h at 4°C . The unstained blots were soaked for 1 h at 40°C in 0.02 M-Tris/HCl buffer, pH 7.4, containing 0.10 M-NaCl and 5% (w/v) bovine serum albumin to saturate unoccupied protein-binding sites. The blot was incubated for 2 h at room temperature with affinity-purified rabbit anti-(human urinary kallikrein) antibody (5 $\mu\text{g}/\text{ml}$) or rabbit anti-(human α_1 -antitrypsin) serum (1:200 dilution) in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 3% bovine serum albumin and 0.05% Nonidet P-40. The blots were washed with 0.15 M-NaCl/0.01 M-Tris/HCl buffer, pH 7.4, containing 0.05% Nonidet P-40 and incubated for 1 h at room temperature with ^{125}I -Protein A (5×10^5 c.p.m./ml) for 1 h at room temperature. The solution was aspirated and then washed with shaking in 0.05 M-Tris/HCl buffer, pH 7.4, containing 5 mM-EDTA, 0.15 M-NaCl, 0.25% gelatin, 0.5% Triton X-100 and 0.1% SDS at room temperature for 2 h. The sheet was affixed to a sheet of filter paper and exposed to Kodak X-Omat AR film at -70°C with Cronex Lightning Plus intensifying screen for 6–24 h.

Cell cultures

Human diploid lung fibroblast cells (WI-38) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). A neuroblastoma $\times$ glioma hybrid cell line (NG108-15) was a gift from Dr. M. Nirenberg (National Institutes of Health, Bethesda, MD, U.S.A.). The lung fibroblasts were grown in Eagle's Minimal Essential Medium (Grand Island Biological Co.) containing 10% (v/v) foetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. The neuroblastoma $\times$ glioma hybrids were cultured at 37°C in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) containing 10% foetal bovine serum, 0.1 mM-hypoxanthine, 1 μM -aminopterin, 16 μM -thymidine, 4 g of glucose/l and 10 mM-Hepes/NaOH buffer, pH 7.4 in a humidified atmosphere of 5% CO_2 in air. The cell-culture media were supplemented with 100 units of penicillin/ml and 100 μg of streptomycin/ml.

To evaluate the presence of kallikrein-binding protein

in serum-free conditioned media, either WI-38 or NG108-15 cells were grown to confluence in medium containing 10% foetal calf serum and then trypsinized and centrifuged. The cell pellets were washed three times in 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.3. WI-38 cells were resuspended at 1×10^5 – $2 \times 10^5/\text{ml}$ in serum-free Eagle's Minimal Essential Medium containing 10 ng of epidermal growth factor/ml and incubated for 4–5 days. NG108-15 cells were resuspended at 2×10^5 – $4 \times 10^5/\text{ml}$ in serum-free Dulbecco's modified Eagle's medium containing 4 g of glucose/l and 10 ng of epidermal growth factor/ml and incubated for 3 days. Opti-Minimal Essential Medium (a modification of Eagle's Essential Mineral Medium; Grand Island Biological Co.) was also used to produce conditioned media. Conditioned serum-free medium was concentrated 50–100-fold under vacuum on Immersible CX (Millipore).

Immunoprecipitation of human urinary kallikrein–kallikrein-binding protein complexes

Human serum samples (0.4 ml) were preincubated with 0.7 ml of water and 5 μg of human urinary kallikrein for 2 h at 37°C , and then for 16 h at 4°C . Undiluted goat anti-kallikrein serum (0.1 ml) was added and the mixture was incubated at 4°C for 16 h. Immunoprecipitates were pelleted by centrifugation for 30 min at 4°C in a Microfuge, washed once in 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.3, and re-centrifuged. Extraction buffer [0.06 M-Tris/HCl buffer, pH 6.8, containing 15% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 2.5% (w/v) SDS] was then added and the samples were boiled for 5 min. Portions (0.05–0.1 ml) were run on a 7.5–15% gradient acrylamide gel containing 0.1% SDS as described previously (Chao, 1983).

Kallikrein–agarose affinity chromatography

Human or rat sera were diluted 1:3 in 0.2 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.0 and applied to human or rat kallikrein–Affi-gel 15 columns (1.5 cm $\times$ 15 cm) equilibrated with the same buffer. Kallikrein–kallikrein-binding protein complexes were eluted with 0.1 M-acetic acid. The eluted fractions were adjusted to pH 7.0 with 1 M-NaOH. The protein concentration of each fraction was measured by the absorbance at 280 nm.

Materials

Purified bovine low- M_r kininogen was a gift from Dr. S. Iwanaga and Dr. H. Kato (Protein Research Institute, University of Osaka, Osaka, Japan). Highly purified human low- M_r kininogen and human urokinase were provided by Dr. Kazuaki Shimamoto (Sapporo Medical College, Sapporo, Japan). Purified human plasma kallikrein was a gift from Dr. Allen P. Kaplan (State University of New York at Stony Brook, NY, U.S.A.) and D-Phe-D-Phe-L-Arg- CH_2Cl was a gift from Dr. C. Kettner and Dr. E. Shaw (Brookhaven National Laboratory, Brookhaven, NY, U.S.A.). Purified human thrombin was a gift from Dr. Alan Cardin (University of Cincinnati, College of Medicine, Cincinnati, OH, U.S.A.), and was originally provided by J. W. Fenton (Division of Laboratories and Research, New York State Department of Health, Albany, NY, U.S.A.). Purified human α_1 -antitrypsin and collagenase were purchased

Fig. 1. Time course of complex-formation between ^{125}I -labelled human urinary kallikrein and human serum

^{125}I -labelled human urinary kallikrein (10000 c.p.m.; specific radioactivity $73.2 \mu\text{Ci}/\mu\text{g}$) was incubated with human serum ($2 \mu\text{l}$) at 37°C in $0.15 \text{ M-NaCl}/0.01 \text{ M-sodium phosphate buffer}$, pH 7.0, in a total volume of $30 \mu\text{l}$. At different times, the binding was stopped by adding $30 \mu\text{l}$ of the extraction buffer ($0.13 \text{ M-Tris/HCl buffer}$, pH 6.8, containing 30% glycerol, 10% 2-mercaptoethanol and 5% SDS) and heated at 100°C for 5 min. Electrophoresis was performed in a 7.5–15% linear-gradient polyacrylamide gel. An autoradiogram was prepared. Lanes 1–9, ^{125}I -labelled human urinary kallikrein incubated with human serum for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 30 min (lane 4), 60 min (lane 5), 90 min (lane 6), 120 min (lane 7), 150 min (lane 8) or 180 min (lane 9); lane 10, ^{125}I -labelled human urinary kallikrein alone.

from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Antisera to human kininogens were obtained from Nordic Immunological Laboratories (El Toro, CA, U.S.A.). Antisera to human α_1 -antitrypsin, α_2 -macroglobulin, inter- α -trypsin inhibitor, C_1 -inactivator and antithrombin III were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.).

RESULTS

Identification of circulating tissue-kallikrein-binding protein

When ^{125}I -labelled human tissue kallikrein was incubated with human serum, an $\sim 92000\text{-}M_r$ SDS-stable complex is formed (Fig. 1). Complex-formation between tissue kallikrein and the serum-binding protein was time-dependent and detectable after only 5 min incubation at 37°C , with half-maximal binding at 28 min (Fig. 1). Maximal complex-formation occurred when serum was pretreated at $37\text{--}45^\circ\text{C}$, but no complex was detected when serum was pretreated at 60°C for 30 min. However, when ^{125}I -kallikrein and serum were incubated together for 30 min at 60°C , complex-formation was still apparent. These results indicate that the binding protein itself is heat-labile. This protein is also acid-labile, as pretreatment of serum at pH 3.0 for 120 min at 4°C diminished complex-formation to 3–5% of the control as measured by densitometric scanning of the autoradiograms. However, the formed complex itself is resistant to the same acid treatment at different temperatures (results not shown).

Fig. 2. Effects of hydroxylamine or alkali treatment on kallikrein-binding protein complexes

^{125}I -labelled rat urinary kallikrein (10000 c.p.m.) was incubated with $2 \mu\text{l}$ of rat serum (lanes 1–3) or water (lane 4) or human serum (lanes 5–7). ^{125}I -labelled human urinary kallikrein (10000 c.p.m.) was incubated with $2 \mu\text{l}$ of human serum (lanes 8–10) or water (lane 11). Incubation was carried out for 90 min at 37°C . Each mixture was treated with 0.2% SDS. To each mixture, an equal volume of $0.15 \text{ M-NaCl}/0.01 \text{ M-sodium phosphate buffer}$, pH 7.0, was added to the control (lanes 1, 5 and 8), 2 M-hydroxylamine, pH 7.4 (lanes 2, 6 and 9), or 20 mM-NaOH (lanes 3, 7 and 10) and incubated for 60 min at 37°C . The samples were then desalted by centrifugation on Sephadex G-25 columns, boiled in extraction buffer and electrophoresed on a 7.5–15% linear-gradient polyacrylamide gel as described in the legend to Fig. 1.

Tissue kallikrein–kallikrein-binding protein complexes were not dissociated by 1 M-hydroxylamine and only slightly dissociated by 10 mM-NaOH (Fig. 2). This Figure shows that a purified rat tissue kallikrein also forms complexes of similar size in human serum that are relatively resistant to both hydroxylamine and alkali treatment. In addition, two higher- M_r rat kallikrein-containing bands are seen in rat serum (Fig. 2, lanes 1–3). Human tissue kallikrein also forms a $92000\text{-}M_r$ and two higher- M_r SDS-stable complexes with binding protein(s) in rat serum (results not shown). These may represent polymeric forms of the rat binding proteins or multiple binding proteins encoded by different genes. Fig. 3 illustrates competition for human serum kallikrein-binding protein between ^{125}I -labelled human tissue kallikrein and unlabelled kallikreins and other human proteinases and proteins. As expected, unlabelled human tissue kallikrein competes with the labelled enzyme whereas human plasma kallikrein, urokinase, thrombin and collagenase have no effect on complex-formation. Neither low- M_r kininogen substrates of bovine or human origin nor α_1 -antitrypsin affect complex-formation. Interestingly, rat urinary kallikrein and a kallikrein-related kininogenase, rat urinary esterase A, but not rat tonin, also compete with the labelled human tissue kallikrein. Finally, heparin competes in the binding assays (Fig. 3, lane 9). Heparin in a range of concentrations (heparin/kallikrein ratio 10, 100 and 1000, w/w) has no inhibitory effect on the esterolytic or kininogenase activity of the enzyme (results not shown). Therefore heparin appears to prevent complex-formation by binding to the kallikrein-binding protein in human

Fig. 3. Competition between ^{125}I -kallikrein binding to kallikrein-binding protein by non-labelled kallikrein and other proteins

(a) ^{125}I -labelled human urinary kallikrein (10000 c.p.m.) and human serum (2 μl) were incubated for 90 min at 37 °C in the absence (lane 1) or in the presence of human plasma kallikrein (5 μg) (lane 2), human urinary kallikrein (1 μg) (lane 3), human low- M_r kininogen (5 μg) (lane 4), bovine low- M_r kininogen (5 μg) (lane 5), rat urinary kallikrein (5 μg) (lane 6), rat urinary esterase A (5 μg) (lane 7), rat tonin (5 μg) (lane 8), heparin (1 unit) (lane 9), human thrombin (5 μg) (lane 10), human urokinase (5 μg) (lane 11) or collagenase (5 μg) (lane 12). (b) ^{125}I -labelled human urinary kallikrein (10000 c.p.m.) and human serum (2 μl) were incubated for 90 min at 37 °C (lane 1) in the presence of unlabelled human urinary kallikrein (1 μg) (lane 2), human α_1 -antitrypsin (5 μg) (lane 3), α_1 -antitrypsin (10 μg) (lane 4) or ^{125}I -labelled human urinary kallikrein alone (lane 5). Experimental procedures were then as described in the legend to Fig. 1.

serum. When purified tissue kallikreins were active-site-labelled and enzymically inhibited with phenylmethanesulphonyl fluoride or D-Phe-D-Phe-L-Arg-CH₂Cl and subsequently ^{125}I -labelled, and then incubated with human serum, no complex-formation was observed (Fig. 4).

Identification of a secreted tissue-kallikrein-binding protein

An ~ 92000 - M_r SDS-stable complex was formed after incubation of ^{125}I -labelled rat or human tissue kallikrein with serum-free conditioned medium from the rodent neuroblastoma $\times$ glioma hybrid cells (NG108-15) or human lung fibroblasts (WI-38) (Figs. 5a and 5b). These complexes were not formed between kallikrein and the concentrated control serum-free medium (results not shown). No complex-formation between kallikrein and homogenized cell extracts containing amounts of protein comparable with those in the concentrated medium samples could be detected (Figs. 5a and 5b). The results indicate that the tissue-kallikrein-binding protein is secreted by these cell lines.

Detection of endogenous kallikrein-kallikrein-binding protein complexes

An endogenous kallikrein-kallikrein-binding protein complex was isolated from human serum by a human tissue kallikrein-agarose affinity column. The endogenous ~ 92000 M_r complex was recognized specifically by an affinity-purified anti-(human kallikrein) antibody (Fig. 6a) and not recognized by an anti- α_1 -antitrypsin serum in Western-blot analyses (Fig. 6b).

Differentiation of kallikrein-binding protein from α_1 -antitrypsin and other proteinase inhibitors

Although human α_1 -antitrypsin did not show competition with labelled kallikrein for the binding protein (Fig. 3b) and the endogenous kallikrein-kallikrein-binding protein complex was not recognized by an anti- α_1 -

antitrypsin serum (Fig. 6b), further studies were carried out to distinguish between α_1 -antitrypsin and the kallikrein-binding protein. After human urinary kallikrein was incubated with human serum and immunoprecipitated by an anti-(tissue kallikrein) serum (Shimamoto *et al.*, 1980), immunoprecipitates were separated and analysed by Western blotting with affinity-purified anti-(human urinary kallikrein) antibody (Fig. 7a) or anti- α_1 -antitrypsin serum (Fig. 7b). The

Fig. 4. Active-site-blocked kallikrein and kallikrein-binding protein complex-formation

(a) ^{125}I -labelled human kallikrein. (b) ^{125}I -labelled D-Phe-D-Phe-L-Arg-CH₂Cl-blocked human kallikrein. (c) ^{125}I -labelled phenylmethanesulphonyl fluoride-blocked human kallikrein. ^{125}I -kallikrein was in each case incubated with water (lanes 1) or 2 μl of human serum (lanes 2) or 2 μl of rat serum (lanes 3). No complex-formation was noted with the active-site-blocked kallikreins (b and c, lanes 2). The experimental procedures are as described in the legend to Fig. 1.

Fig. 5. Complex-formation of ^{125}I -kallikrein and kallikrein-binding protein in serum-free cell-culture media.

^{125}I -labelled human urinary kallikrein (10000 c.p.m.) (a) or ^{125}I -labelled rat urinary kallikrein (10000 c.p.m.; specific radioactivity 71.8 $\mu\text{Ci}/\mu\text{g}$) (b) was incubated with water (lanes 1), homogenized cell extracts (approx. 2 mg/ml) (30 μl) (lanes 2 and 4) or serum-free media (50-fold concentrated) (30 μl) (lanes 3 and 5) of neuroblastoma $\times$ glioma hybrids (lanes 2 and 3) or human lung fibroblasts (lanes 4 and 5), for 90 min at 37 $^{\circ}\text{C}$. The mixtures were then boiled in extraction buffer and electrophoresed as described in the legend to Fig. 1.

Fig. 6. Western-blot analyses of endogenous kallikrein-kallikrein-binding protein complexes

(a) Immuno-blotting with anti-(human urinary kallikrein) antibody. (b) Immuno-blotting with anti- α_1 -antitrypsin serum. Purified human urinary kallikrein (5 μg) (lanes 1), endogenous kallikrein-kallikrein-binding protein complexes purified from human serum by a kallikrein-agarose affinity column (lanes 2) and human α_1 -antitrypsin (5 μg) (lanes 3) were studied. Electrophoresis was performed in a 7.5–15% linear-gradient polyacrylamide gel containing 0.1% SDS, and proteins were electrophoretically transferred to nitrocellulose. The blots were incubated either with rabbit anti-(human urinary kallikrein) antibody or anti- α_1 -antitrypsin serum, followed by ^{125}I -Protein A binding. Antibody binding was detected by autoradiography.

Fig. 7. Western-blot analyses of unlabelled purified human tissue kallikrein and kallikrein-binding protein complexes

(a) Immuno-blotting with anti-(human urinary kallikrein) antibody. (b) Immuno-blotting with anti- α_1 -antitrypsin serum. Human urinary kallikrein (5 μg) was added to 0.4 ml of human serum and precipitated by anti-kallikrein serum as described in the Experimental section. Purified human urinary kallikrein (5 μg) (lanes 1), purified human α_1 -antitrypsin (5 μg) (lanes 2), immunoprecipitated complexes of human urinary kallikrein and kallikrein-binding protein from normal human serum (lanes 3) and α_1 -antitrypsin-deficiency (ZZ type) human serum (lanes 4) were studied.

Figure shows that an $\sim 92000\text{-}M_r$ complex was recognized by the anti-kallikrein antibody but not recognized by anti- α_1 -antitrypsin serum. However, in the same immunoprecipitates that contain the SDS-stable $\sim 92000\text{-}M_r$ kallikrein-kallikrein-binding protein complex, a protein of M_r 54000, identical with purified α_1 -antitrypsin, was recognized by the anti- α_1 -antitrypsin serum (Fig. 7b). The results show that tissue kallikrein can form an immunoprecipitable complex with serum α_1 -antitrypsin, but that the complex is dissociable by the SDS treatment at 100 $^{\circ}\text{C}$, hence the single 54000- M_r band in Fig. 7(b). In contrast, the immunoprecipitated kallikrein-kallikrein-binding protein complex is not affected by this treatment (Fig. 7a).

The binding of human tissue kallikrein to several well-characterized human plasma proteinase inhibitors is shown in Table 1. ^{125}I -kallikrein was incubated with human serum and any complexes formed were subject to immunoprecipitation by antisera to kallikrein and the other proteins. Some kallikrein immunoprecipitation was detected with antiserum to α_1 -antitrypsin, but minimal kallikrein was detected in the initial or washed immunoprecipitates produced by the antisera to α_2 -macroglobulin, inter- α -trypsin inhibitor, kininogens, C_1 -inactivator and antithrombin III. Finally, no significant difference in tissue kallikrein-kallikrein-binding protein complex-formation, measured by quantitative densitometry, could be found between normal subjects or patients with α_1 -antitrypsin deficiency.

Table 1. Identification of human tissue-kallikrein-binding protein complexes

¹²⁵I-labelled human urinary kallikrein (280000 c.p.m.) was incubated with human serum (140 μ l) in a total volume of 420 μ l at 37 °C for 120 min. Antiserum (25 μ l) to human urinary kallikrein and 0.5 μ g of purified human urinary kallikrein carrier was added to a portion (30 μ l) of the ¹²⁵I-kallikrein/human serum mixture. Antisera (50 μ l) to α_1 -antitrypsin, α_2 -macroglobulin, inter- α -trypsin inhibitor, low- M_r kininogen, high- M_r kininogen, C₁-inactivator and antithrombin III were added to portions (30 μ l) of the ¹²⁵I-kallikrein/human serum mixture. Incubations were carried out at 4 °C for 48 h. Immunoprecipitates were collected by centrifugation, unwashed and washed twice with 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.0.

Antiserum added	Unwashed immunoprecipitates (c.p.m.)			Washed immunoprecipitates (c.p.m.)		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Urinary kallikrein	7764	7185	5041	7702	7137	3185
α_1 -Antitrypsin	2324	1708	1023	1701	1278	712
α_2 -Macroglobulin	455	569	540	194	213	195
Inter- α -trypsin inhibitor	408	806	506	128	187	195
Low- M_r kininogen	415	372	250	173	184	272
High- M_r kininogen	—	403	562	—	153	151
C ₁ -inactivator	496	836	319	167	215	130
Antithrombin III	422	527	661	202	348	306

DISCUSSION

We have identified a new tissue-kallikrein-binding protein in human serum and in serum-free culture media from human or rat cultured cell lines. This kallikrein-binding protein is distinct from α_1 -antitrypsin, the only other serum protein known to inhibit tissue kallikrein (Geiger *et al.*, 1981).

Tissue kallikrein and the binding protein form a SDS-stable complex with an apparent M_r of 92000 on SDS/polyacrylamide-gel electrophoresis. From this estimated M_r and that of tissue kallikreins (M_r approx. 38000–40000), an M_r of about 52000–54000 would be expected for the binding protein. However, it is also possible that a peptide fragment is released from the kallikrein-binding protein during complex-formation, as has been observed for thrombin–protease nexin interaction (Scott & Baker, 1983). Thus more accurate estimation of the M_r of kallikrein-binding protein must await its purification.

Binding is specific, as it is competitively inhibited only by an excess of tissue kallikreins, but not other serine proteinases such as plasma kallikrein, thrombin, urokinase or collagenase. In addition, complex-formation is not inhibited by α_1 -antitrypsin or purified low- M_r kininogens, eliminating the possibilities that α_1 -antitrypsin or kininogens are the identified binding protein. The protein is neither antithrombin III nor a protease nexin because of the following findings. First, heparin clearly inhibits the complex-formation between kallikrein and the binding protein (Fig. 3b), but enhances binding between thrombin and protease nexin or between thrombin and antithrombin III (Baker *et al.*, 1980; Scott & Baker, 1983). Secondly, hydroxylamine or alkali treatment had no significant effects on kallikrein–kallikrein-binding protein complex dissociation, but the thrombin–protease nexin or thrombin–antithrombin III complexes are readily dissociable by hydroxylamine or alkali (Baker *et al.*, 1980; Scott & Baker, 1983). Finally, thrombin did not compete with tissue kallikrein in the binding studies.

Although we could demonstrate tissue kallikrein– α_1 -

antitrypsin complex-formation, the binding protein is distinct from α_1 -antitrypsin. That is, when ¹²⁵I-labelled kallikrein was incubated with serum, binding to α_1 -antitrypsin could be detected by immunoprecipitation with anti- α_1 -antitrypsin serum (Table 1). The complex between added unlabelled kallikrein and serum α_1 -antitrypsin could be immunoprecipitated by an anti-kallikrein serum, and the α_1 -antitrypsin could be dissociated from the immunoprecipitates in SDS-containing buffer, and subsequently identified in Western blotting with anti- α_1 -antitrypsin serum (Fig. 7b). In contrast, the ~92000- M_r kallikrein–kallikrein-binding protein complex from the same immunoprecipitates cannot be recognized by α_1 -antitrypsin antiserum (Fig. 7b). Although the M_r of α_1 -antitrypsin is similar to that estimated for kallikrein-binding protein (approx. 52000–54000), these results showed that the kallikrein– α_1 -antitrypsin complex is SDS-unstable whereas the kallikrein–kallikrein-binding protein complex is SDS-stable. It is also notable that the binding of α_1 -antitrypsin to tissue kallikrein could only be detected after several hours of incubation. Finally, the results of the competition studies (Fig. 3b) showed that excess α_1 -antitrypsin did not diminish the formation of the kallikrein-binding protein complexes. Collectively, the data indicate that kallikrein-binding protein is a new tissue-kallikrein-binding protein, distinct from any described previously.

When rat or human tissue kallikreins were first inhibited by incorporating the active-site-labelling reagents phenylmethanesulphonyl fluoride and D-Phe-D-Phe-L-Arg-CH₂Cl, the active-site-blocked enzymes did not form complexes with the kallikrein-binding protein. These findings suggest the possibility that the active-site centre of tissue kallikrein may be involved in the complex-formation. Confusing this suggestion are the data in Fig. 2, which show that low- M_r kininogen does not interfere with binding protein–enzyme interaction. This result might mean that active-site blockade affects the ability of enzyme to interact with binding protein at some auxiliary binding site(s). The nature of the kallikrein–kallikrein-binding protein interaction awaits

studies dependent upon the prior purification of the protein.

There is a lack of species specificity for the kallikrein-binding proteins. ^{125}I -labelled human tissue kallikrein forms SDS-stable complexes with binding protein(s) present in human and rat serum. Similarly, ^{125}I -labelled rat tissue kallikrein not only formed an $\sim 92000\text{-}M_r$ complex with rat serum, but also formed a similar complex with human serum (Fig. 2, lanes 1–7). Several SDS-stable higher- M_r protein bands were also formed between rat tissue kallikreins and rat serum in addition to the $\sim 92000\text{-}M_r$ complex (Fig. 2, lanes 1–3). These bands may represent polymeric forms or other binding proteins. Furthermore, rat tissue kallikrein (Chao & Margolius, 1979) and rat arginine esterase A (Chao, 1983), a tissue-kallikrein-related kininogenase, were able to compete with human tissue kallikrein in binding to human serum kallikrein-binding protein (Fig. 3a).

The present study also demonstrates an endogenous $\sim 92000\text{-}M_r$ kallikrein–kallikrein-binding protein complex in human serum (Fig. 6). Such a complex is also present in rat serum (results not shown). The finding that kallikrein–kallikrein-binding protein complex could be isolated by chromatography on kallikrein–agarose affinity column(s) suggests that the covalent complex might have additional non-covalent binding sites. Circulating autoantibodies to kallikrein have also been isolated with the same affinity column (Chao *et al.*, 1984), and perhaps they are responsible for the non-covalent binding of the complex with the kallikrein–agarose affinity column.

In other studies, we have found an $\sim 92000\text{-}M_r$ protein in rat urine and kidney that could be recognized by a specific monoclonal antibody to rat tissue kallikrein (Woodley *et al.*, 1985; Chao *et al.*, 1986b). Cell-free translation studies directed by brain, salivary, pancreatic and kidney mRNAs have shown that the M_r values of rat tissue preprokallikreins vary from 30000 to 43000 (Chao *et al.*, 1986b). Therefore the $\sim 92000\text{-}M_r$ renal immunoreactive material (Woodley *et al.*, 1985; Chao *et al.*, 1986b) cannot be a kallikrein precursor and most probably represents another kallikrein-binding protein complex. The functional significance of this binding protein is unknown, but the unique ability of the protein to bind tissue kallikreins suggests possible regulation of kallikrein activity, delivery, internalization or metabolism.

This work was supported by U.S. Public Health Service Grants HL29397, HL33552, HL17705 and HL29566. J. C. is a Research Career Development Awardee of the N.H.L.B.L.

REFERENCES

- Ando, T., Chao, J., Miller, D. H. & Margolius, H. S. (1985) *Clin. Res.* **33**, 582A
- Baker, J. B., Low, D. A., Simmer, R. L. & Cunningham, D. D. (1980) *Cell* (Cambridge, Mass.) **21**, 37–45
- Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
- Chao, J. (1983) *J. Biol. Chem.* **258**, 4434–4439
- Chao, J. & Margolius, H. S. (1979) *Biochem. Pharmacol.* **28**, 2071–2079
- Chao, J. & Margolius, H. S. (1983) *Endocrinology* (Baltimore) **113**, 2221–2225
- Chao, J., Woodley, C., Chao, L. & Margolius, H. S. (1983) *J. Biol. Chem.* **258**, 15173–15178
- Chao, J., Chao, L., Tillman, D. M. & Margolius, H. S. (1984) *Proc. Int. Congr. Kinin* 29A
- Chao, J., Chao, L., Swain, C. C., Tsai, J. & Margolius, H. S. (1986a) *Endocrinology* (Baltimore), in the press
- Chao, J., Chao, L., Woodley, C. M., Gerald, W. & Margolius, H. S. (1986b) *Adv. Exp. Med. Biol.*, in the press
- Demassieux, S., Boucher, R., Griso, C. & Genest, J. (1976) *Can. J. Biochem.* **54**, 788–794
- Eaton, D. L. & Baker, J. B. (1983) *J. Cell. Physiol.* **117**, 175–182
- Geiger, R., Stuckstedte, U., Clausnitzer, B. & Fritz, H. (1981) *Hoppe-Seyler's Physiol. Chem.* **362**, S317–S325
- Gerald, W. L., Chao, J. & Chao, L. (1986) *Biochim. Biophys. Acta* **867**, 16–23
- Lawton, W. J., Proud, D., Frech, M. E., Pierce, J. V., Keiser, H. S. & Pisano, J. J. (1981) *Biochem. Pharmacol.* **30**, 1731–1737
- Miller, D. H., Chao, J. & Margolius, H. S. (1984) *Biochem. J.* **218**, 37–43
- Nolly, H., Scicli, A., Scicli, G. & Carretero, O. A. (1985) *Circ. Res.* **56**, 816–821
- Rabito, S. F., Scicli, A. G. & Carretero, O. A. (1980) in *Enzymatic Release of Vasoactive Peptides* (Gross, F. & Vogel, G., eds.), pp. 247–258, Raven Press, New York
- Sakamoto, W. & Nishikaze, O. (1980) *Biochim. Biophys. Acta* **633**, 305–309
- Scott, R. W. & Baker, J. B. (1983) *J. Biol. Chem.* **258**, 10439–10444
- Scott, R. W., Bergman, B. L., Bonpai, A., Hersh, R. T., Rodriguez, H., Jones, B. N., Barreda, C., Watts, S. & Baker, J. B. (1985) *J. Biol. Chem.* **260**, 7029–7034
- Shimamoto, K., Chao, J. & Margolius, H. S. (1980) *J. Clin. Endocrinol. Metab.* **51**, 840–848
- Shimamoto, K., Mayfield, R. K., Margolius, H. S., Chao, J., Stroud, W., & Kaplan, A. P. (1984) *J. Lab. Clin. Med.* **103**, 731–738
- Simson, J. A. V., Dom, R., Chao, J., Woodley, C., Chao, L. & Margolius, H. S. (1985) *J. Histochem. Cytochem.* **33**, 951–953
- Vahtera, E. & Hamberg, U. (1976) *Biochem. J.* **157**, 521–524
- Woodley, C., Chao, J., Margolius, H. S. & Chao, L. (1985) *Biochem. J.* **231**, 721–728

Received 16 January 1986/28 April 1986; accepted 24 June 1986