Specific identification of tissue kallikrein in exocrine tissues and in cell-free translation products with monoclonal antibodies

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A panel of six mouse monoclonal antibodies (IgG₁) has been prepared against purified rat urinary kallikrein (EC 3.4.21.35) and characterized. In radioimmunoassay, the antibody titres of ascitic fluid giving 50% binding to ¹²⁵I-kallikrein range from 1:2 × 10³ to 1:1 × 10⁶. Antibodies from four of the clones show no cross-reactivity with human urinary kallikrein, rat urinary esterase A or tonin. However, antibodies from a fifth clone cross-react with tonin and, from a sixth, with both urinary esterase A and tonin. Three of the kallikrein affinity-purified monoclonal antibodies inhibited, whereas one of the antibodies stimulated, kallikrein activity. Tissue kallikrein from rat submandibular-gland and pancreatic extracts and urine were labelled with [¹⁴C]di-isopropyl phosphofluoridate, immunoprecipitated with each of the six monoclonal antibodies and identified to be 38 kDa proteins, similar in size to purified rat urinary kallikrein. Western-blot analysis shows that ¹²⁵I-labelled kallikrein monoclonal antibodies (V₄D₁₁) bind directly to a 38 kDa protein in submandibular-gland and pancreatic extracts and urine. Cell-free translation products of submandibular-gland polyadenylated[poly(A)⁺]mRNA were immunoprecipitated with affinity-purified sheep anti-kallikrein antibodies and three monoclonal antibodies (V₄D₁₁, V₄G₆ and V₁C₃). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of these immunoprecipitates revealed that two kallikrein precursors with *M_r* values of 37000 and 35000 are encoded by submandibular-gland mRNA. The third monoclonal antibody, V₁C₃, which binds to active kallikrein, did not recognize either precursor form. Collectively, the data show that these monoclonal antibodies comprise a set of powerful and specific reagents for studies of tissue kallikreins.

INTRODUCTION

Kallikreins (EC 3.4.21.35) are a group of serine proteinases with specific and limited proteolytic actions (Schachter, 1980). Tissue kallikreins continue to be identified and isolated at new sites such as brain (Chao *et al.*, 1983), thyroid (Uchida *et al.*, 1982) and spleen (Chao *et al.*, 1984a) in addition to the long known localizations in the kidney, salivary glands, pancreas and intestinal mucosa (Fiedler, 1979). Multiple forms of tissue kallikrein have been reported in rat small intestine (Moriwaki *et al.*, 1980), pancreas (Matsas *et al.*, 1981), submandibular gland (Brantzaeg *et al.*, 1976), kidney (Nishimura *et al.*, 1983), and urine (Chao & Margolius, 1979). Heterogeneity has been attributed to variability in glycosylation as well as protein modifications (Pisano, 1975), but also probably reflects the existence of different gene products (Gerald *et al.*, 1984; Mason *et al.*, 1983).

Antisera specific for rat urinary kallikrein have been used for measurement of kallikrein by direct radioimmunoassay (Carretero *et al.*, 1978; Shimamoto *et al.*, 1979a; Lawton *et al.*, 1981), in immunohistochemical localization studies (Simson *et al.*, 1979; Ørstavik *et al.*, 1981), in purification and Western-blot analysis (Chao *et al.*, 1983), and in enzymic-activity studies (Chao & Margolius, 1979). Although all of these studies provided important information about kallikrein localization and levels, antibodies produced by conventional immuniz-

ation are limited in quantity, reproducibility and homogeneity, and thus possess only relative degrees of specificity. In addition, tissue kallikrein is now recognized to be a member of a large family of arginine esteropeptidases including the γ -subunit of nerve growth factor, an epidermal-growth-factor-binding protein, β -nerve-growth-factor endopeptidase, tonin and rat urinary esterase A (Bothwell *et al.*, 1979; Swift *et al.*, 1982; Chao, 1983; Mason *et al.*, 1983; McPartland *et al.*, 1983; Gerald *et al.*, 1984). Each member of this family shows chemical, physical and enzymic similarities as well as immunological cross-reactivity, so polyclonal antisera with the above-noted limitations are not the optimal antibody tools for future studies of specific tissue kallikreins. Thus a greater resolution among these proteins may be achieved through the use of monoclonal antibodies reacting with single antigenic determinants.

In the present paper we describe a panel of six monoclonal antibodies that bind to kallikrein and can affect rat tissue kallikrein activity and we show that these antibodies can be used in active-site labelling with immunoprecipitation and Western-blotting techniques. The incorporation of these antibodies into these biochemical studies has allowed the first specific tissue kallikrein identifications in submandibular gland, pancreas and urine; in addition, multiple precursor forms of submandibular-gland tissue kallikrein were also identified in cell-free translation products.

Abbreviations used: iPr₂P-F, di-isopropyl phosphofluoridate; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulphonic acid; NP40, Nonidet P40; SDS, sodium dodecyl sulphate; poly(A)⁺ RNA, polyadenylated RNA; DTT, dithiothreitol.

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EXPERIMENTAL

Enzyme purification and antibody isolation

Rat urinary kallikrein (Chao & Margolius, 1979), rat urinary esterase A (Chao, 1983), rat tonin (Demassieux *et al.*, 1976) and human urinary kallikrein (Shimamoto *et al.*, 1980) were purified to apparent homogeneity. Sheep anti-kallikrein antiserum was developed as described previously (Chao & Margolius, 1979). Fusion and cloning were carried out essentially as described by Oi & Herzenberg (1980) and Chao *et al.* (1983). The hybrid cells were selected and screened for kallikrein antibody production by either radioimmunoassay (Shimamoto *et al.*, 1980) or enzyme-linked immunosorbent assay (Voller *et al.*, 1976; Engvall & Perlman, 1972).

Antibody subclass was determined by an enzyme-linked immunosorbent assay using rabbit antisera against specific mouse immunoglobulin subclasses (IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgM) (Cappell Laboratories, West Chester, PA, U.S.A.). Monoclonal antibodies from ascites and (NH₄)₂SO₄-fractionated culture media were affinity-purified by kallikrein-agarose chromatography as described by Chao *et al.* (1983). The IgG concentration was determined by spectrophotometry at 280 nm.

Kallikrein assays

Kallikrein-like tosylarginine methylesterase activity of purified kallikrein was measured as described previously (Chao, 1978; Chao *et al.*, 1983). The kininogenase assay of Shimamoto *et al.* (1979b) was used to determine kinin-generating activity of rat urinary kallikrein and kinin generation was measured by a kinin radioimmunoassay (Shimamoto *et al.*, 1978). Purified bovine low-*M_r* kininogen was used as the substrate. The direct radioimmunoassay of kallikrein was carried out as previously described (Shimamoto *et al.*, 1979a).

Preparation of tissue extracts and urine

Male Sprague-Dawley rats (300–350 g) were killed and submandibular glands and pancreas were removed and homogenized as described by Chao *et al.* (1984a) and Woodley *et al.* (1985).

Overnight urine was collected into 50 ml tubes containing 100 µl of 1.0% soya-bean trypsin inhibitor, centrifuged at 10000 *g* for 15 min to remove debris, and then dialysed overnight against water at 4 °C. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Tissue-extract labelling and immunoprecipitation

Routinely, 100–200 µg of submandibular-gland extract, 1–5 mg of pancreatic extract and 0.5–1 ml of urine were used for an immunoprecipitation assay. The proteins in each extract were labelled by adding 0.4 µCi of [¹⁴C]Pr₂P-F (New England Nuclear, Boston, MA, U.S.A.; sp. radioactivity 113 mCi/mmol) and incubating for 1 h at 37 °C, followed by an overnight incubation at 4 °C. Labelled proteins were precipitated with the addition of 0.1 vol. of 10 mM-Chaps (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) and 25–50 µl of sheep anti-(rat kallikrein) antiserum, followed by an overnight incubation at 4 °C. Immune complexes were collected by centrifugation in a Microfuge for 10 min. The pellets were washed twice in phosphate-buffered saline (1.4 mM-KH₂PO₄/8 mM-Na₂HPO₄/136 mM-NaCl/2.6 mM-KCl),

containing 1 mM-Chaps, and prepared for electrophoresis.

Monoclonal-antibody immune complexes were precipitated from tissue extracts with a double-antibody precipitation procedure using a rabbit antiserum against mouse IgG heavy and light chains. (NH₄)₂SO₄-fractionated monoclonal media (75 µl) and rabbit anti-(mouse IgG heavy and light chain) antiserum (50 µl, Cappell Laboratories) were added to the extracts and incubated overnight at 4 °C. The immune precipitates were collected by centrifugation in a Microfuge for 15 min. The pellets were washed three or four times in the wash buffer {phosphate-buffered saline [10 mM-NaH₂PO₄/Na₂HPO₄(pH 7.4)/150 mM-NaCl], containing 0.1% bovine serum albumin, 0.02% NaN₃, 0.5% NP40 and 0.1% SDS} and prepared for electrophoresis. The immunoprecipitates were analysed on a 7.5–17.5%-(w/v)-polyacrylamide gel as described by Laemmli (1970) and detected by fluorography.

Western-blot analyses

A 60 µl portion of urine or tissue extract from pancreas (600 µg) or submandibular gland (200 µg) was reduced, denatured, and electrophoresed along with purified rat urinary kallikrein on a gradient (7.5–17.5%) polyacrylamide slab gel containing 0.1% SDS as described above. Proteins were then electrophoretically transferred on to nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.; 0.45 µm) by using a Trans-Blot Cell (Bio-Rad, Richmond, CA, U.S.A.) as described by Burnette (1981). Affinity-purified monoclonal antibodies or polyclonal antibodies were iodinated and separated from free iodine as described previously (Shimamoto *et al.*, 1980). Kallikreins were bound directly with ¹²⁵I-labelled monoclonal antibodies or ¹²⁵I-labelled sheep anti-kallikrein antibodies by the method of Schenk & Leffert (1983) and autoradiographed.

Preparation of poly(A)⁺ RNA

Tissues were removed from anaesthetized Sprague-Dawley rats, rapidly frozen in liquid N₂ and then pulverized with a mortar and pestle. Total RNA was prepared from the homogenate by the guanidinium thiocyanate extraction procedure of Chirgwin *et al.* (1979) and poly(A)⁺ RNA was isolated by two cycles of chromatography on an oligo(dT)-cellulose column (Collaborative Research, Lexington, MA, U.S.A.).

Translation of exogenous mRNA in rabbit reticulocyte system

The reaction mixture was prepared as described by Paterson *et al.* (1977) and contained, in a final volume of 25 µl: 10 µl of lysate, 10 µl of master mix (2.5 mM-ATP, 1 mM-GTP, 1.5 mM-spermidine, 20 mM-creatine phosphate, 64 mM-Hepes, 5 mM-DTT, 64 µM-amino acids except methionine, 300 mM-potassium acetate, 4.5 mM-magnesium acetate), 10–15 µCi of [³⁵S]methionine (400–800 Ci/nmol; New England Nuclear), 0.1–2 µg of mRNA in water, and 0.3 µg of creatine phosphokinase. The reaction mixtures were incubated at 30 °C for 1 h and trichloroacetic acid-insoluble radioactivity was determined.

Immunoprecipitation of translation products

Insoluble material was removed from translation products by centrifuging for 15 min in a Microfuge. The

Table 1. Characterization of anti-(rat tissue kallikrein) monoclonal and polyclonal antibodies

Antibodies	Ig subclass*	10 ⁻³ × M _r †		Binding titre‡	Enzyme inhibition§	
		Light chain	Heavy chain		Tosylarginine methylesterase	Kininogenase (M)
Monoclonal						
V ₄ G ₆	IgG ₁	27.5	50.5	2.0 × 10 ⁵	—	—
V ₁ C ₃	IgG ₁	26.5	50.5	1.0 × 10 ⁶	—	9.3 × 10 ⁻⁸
V ₄ D ₁₁	IgG ₁	25.5	50.5	1.4 × 10 ⁴	+	4.4 × 10 ⁻⁹
E ₉ C ₆	IgG ₁	25.5	50.5	6.0 × 10 ⁵	—	6.0 × 10 ⁻⁹
H ₁ F ₃	IgG ₁	26.5	50.5	8.0 × 10 ⁴	—	—
A ₁₁ C ₄	IgG ₁	25.5	50.5	2.0 × 10 ³	+	3.3 × 10 ⁻⁸
Polyclonal						
Sheep	—	28.5	52.5	8.0 × 10 ⁵	+	1.7 × 10 ⁻⁹

* Determined by enzyme-linked immunosorbent assay and Ouchterlony double-diffusion analysis with monospecific antiserum against each subtype.

† M_r values for the purified antibodies were determined from SDS/polyacrylamide-gel electrophoresis.

‡ Binding titre is expressed as the reciprocal of final dilution of monoclonal ascitic fluid or sheep antiserum required to bind 50% of total 125 I-labelled rat urinary kallikrein in the standard radioimmunoassay.

§ Enzyme inhibition is expressed as the molarity of affinity-purified antibody required to inhibit 50% of the kininogenase activity of 1.5 nM (1.5×10^{-9} M)-rat urinary kallikrein. A negative entry indicates no inhibitory effect.

supernatants were then diluted in 3 vol. of wash buffer (Ivell & Richter, 1982) containing 10 mM-NaH₂PO₄, pH 7.6, 1 mM-EDTA, 1% NP40, 1% sodium deoxycholate, 0.3% SDS, and 2 mM-DTT and 20–80 μ l of affinity-purified polyclonal, or affinity-purified monoclonal, antibody and second antibody (rabbit anti-mouse IgG, Cappel) were added for each precipitation. The mixture was incubated overnight at 4 °C and precipitates were collected by centrifugation for 15 min in a Microfuge. The pellets were washed in wash buffer three or four times and were analysed on polyacrylamide gels containing 0.1% SDS, the radioactive protein bands being detected by fluorography.

RESULTS

Properties of anti-kallikrein monoclonal antibodies

Six hybridomas secreting anti-kallikrein monoclonal antibodies were isolated and designated V₁C₃, V₄G₆, V₄D₁₁, E₉C₆, A₁₁C₄ and H₁F₃. A comparison of the molecular properties of these monoclonal antibodies with the sheep polyclonal antibodies is shown in Table 1. Antibodies from four of the clones (V₁C₃, V₄D₁₁, V₄G₆, E₉C₆) show no cross-reactivity with human urinary kallikrein, rat urinary esterase A or tonin. However, antibodies from A₁₁C₄ react with tonin, and those from H₁F₃ react with both esterase A and tonin. These antibodies diluted in a range of $1:4 \times 10^2$ to $1:4 \times 10^6$ show a concentration-dependent antigen-binding activity when analysed by radioimmunoassay (Fig. 1). The antibody titres of ascitic fluid giving 50% binding to 125 I-kallikrein range from $1:2 \times 10^3$ to $1:1 \times 10^6$ (Table 1).

Effects of antibodies on kallikrein activity

Antibody effects on kallikrein activity were measured by using both the tosylarginine methylesterase assay and a kininogenase assay. Monoclonal antibodies from

Fig. 1. Kallikrein antibody titration curves

Ascitic fluid or antiserum was diluted in phosphate-buffered saline (0.14 M-NaCl/0.01 M-Na₂HPO₄/NaH₂PO₄, pH 7.0) containing 1% bovine serum albumin to the indicated final concentrations. A portion (0.1 ml) was incubated with 0.1 ml of 125 I-labelled rat urinary kallikrein (approx. 9000 c.p.m.) in a final volume of 0.4 ml at 4 °C for 18–24 h. Antibody-bound enzyme was separated from the free form with a poly(ethylene glycol) separation technique. Each point represents the mean of four experiments in duplicate. Symbols: ●, E₉C₆; ○, V₄G₆; ▲, V₁C₃; △, V₄D₁₁; ■, H₁F₃; □, A₁₁C₄; ×, sheep anti-(rat tissue kallikrein).

Fig. 2. Effects of antibody kallikrein kininogenase activity

A portion (50 μ l) of rat urinary kallikrein (25 ng) was preincubated with 50 μ l of affinity-purified monoclonal or sheep anti-kallikrein antibodies (final concentrations as indicated) for 30 min at 37 °C. Immediately after preincubation, 400 μ l of 0.1 M-sodium phosphate buffer, pH 8.5, containing 3 mM-phenanthroline, 30 mM-EDTA and 1.5 μ g of bovine low- M_r kininogen, was added to the assay mixture. This was incubated for 30 min at 37 °C. The reaction was stopped by boiling for 15 min. The kinin generated was measured by radioimmunoassay with bradykinin as a standard. Activity is expressed as the percentage of control kinin generation after 30 min of preincubation in water alone. Each point represents the results of two to four experiments carried out in duplicate. Symbols: ●, E₉C₆; △, V₄D₁₁; □, A₁₁C₄; ■, H₁F₃; ▲, sheep anti-kallikrein antibody.

ascites or (NH₄)₂SO₄-fractionated media as well as the polyclonal sheep antiserum were purified by kallikrein-affinity chromatography (Chao *et al.*, 1983) to apparent homogeneity (results not shown). Fig. 2 shows the effect of four of the monoclonal antibodies on kallikrein kininogenase activity; the other two show no significant effect. Antibodies from clone H₁F₃ are shown to stimulate kininogenase activity over the concentration range tested. Antibodies from A₁₁C₄, V₄D₁₁ and E₉C₆ markedly inhibit kininogenase activity to the extent that they parallel the inhibition of the polyclonal antibodies. The molar concentrations for 50% inhibition are shown in Table 1. Similar results with each antibody were also found when the esterase assay (results not shown) was used, with the exception that antibodies from clone E₉C₆ stimulated kallikrein esterase activity. This effect is opposite to that shown with the same antibody in the kininogenase assay.

Identification of kallikrein in total tissue extracts

Active-site labelling. Submandibular-gland extract was prelabelled with [¹⁴C]iPr₂P-F and immunoprecipitated with polyclonal antiserum or each of the six monoclonal antibodies. Separation of the immunoprecipitates on a polyacrylamide slab gel (Fig. 3) shows that a single band (M_r approx. 38000) is precipitated from [¹⁴C]iPr₂P-F-labelled submandibular-gland extract by five of the monoclonal antibodies (lanes d-h) and two bands recognized by the sixth monoclonal antibody (lane i). The mobility of this band is similar to that of purified urinary kallikreins, which migrate as two forms (M_r approx. 38000 and 36000) (lanes b and k). The polyclonal antiserum, on the other hand, recognizes multiple bands, which include a broad band with M_r 38000-36000 and three additional bands of M_r 28000, 17000, and 13000 (lane c).

Fig. 4 shows the [¹⁴C]iPr₂P-F labelled immunoprecipitated proteins from submandibular-gland (lanes b and c) and pancreatic (lanes d and e) extracts and urine (lanes f and g). As in Fig. 3, one band (M_r 38000) was immunoprecipitated from submandibular-gland extract by both the monoclonal antibodies (lane b) and polyclonal antibodies (lane c). Polyclonal antibodies, however, precipitated four additional bands (M_r approx. 28000, 21000, 17000 and 13000). A predominant protein species with M_r 38000 was recognized in the pancreatic extract by both monoclonal (lane d) and polyclonal antibodies (lane e), with a minor species of M_r 25000. Urine contains an immunoprecipitable protein that is recognized by both monoclonal and polyclonal antibodies at a position corresponding to M_r 38000 (lanes f and g), a value that is similar to that for purified urinary kallikrein.

Western-blot analyses. Tissue extracts from submandibular gland and pancreas and from urine were electrophoresed and analysed by Western blotting. Fig. 5(a) shows total tissue and urinary protein stained with Coomassie Blue, whereas Fig. 5(b) shows Western-blot analyses with the monoclonal antibody V₄D₁₁. A single protein of M_r ~ 38000 was identified in submandibular-gland (lane ii) and pancreatic (lane iii) extracts and it had an apparent migration similar to that of purified urinary kallikrein (lane i). The monoclonal antibody recognized two protein species in urine (lane iv) one with M_r ~ 92000 and the other with a migration similar to that of submandibular-gland, pancreatic and purified urinary kallikrein.

Analysis of cell-free translation products

Cell-free translation products were immunoprecipitated with both affinity-purified sheep anti-kallikrein and three monoclonal antibodies and analysed on SDS/polyacrylamide gels (Fig. 6). Antibodies from two (V₄D₁₁ and V₄G₆) of the hybridomas specifically recognized two major proteins (lanes f and g), with M_r 37000 and 35000, which migrate slightly faster than the purified enzyme (lane e). Antibody (V₁C₃) from a third hybridoma failed to recognize these primary translation products (lane h), although it is capable of binding to active kallikrein (Fig. 1). Affinity-purified sheep anti-kallikrein antibodies also recognized the same proteins, but, in addition, also recognized a third protein with M_r 20000 (lane d).

Fig. 3. SDS/polyacrylamide-gel electrophoresis of active-site-labelled rat submandibular-gland serine proteinases

Submandibular-gland homogenates (approx. 200 μ g of protein) were incubated with [14 C]jPr₂P-F for 1 h at 37 °C and then overnight at 4 °C and sheep anti-kallikrein antiserum or ascitic fluid (50 μ l) from each of the six hybridomas was added. The total labelled proteins and immunoprecipitates were electrophoresed on a linear-gradient (7.5–17.5%, w/v) polyacrylamide gel containing 0.1% SDS. The protein bands were revealed by fluorography. Lane (a), total labelled proteins; lanes (b) and (k), purified kallikrein; lane (c), labelled proteins precipitated by sheep anti-kallikrein antiserum; lanes (d)–(j), labelled proteins precipitated by monoclonal ascitic fluid from clones V₁C₃ (d), V₄G₆ (e), V₄D₁₁ (f), E₉C₆ (g), A₁₁C₄ (h), H₁F₃ (i) and affinity-purified V₁C₃ (j); M_r markers were: phosphorylase b, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; soya-bean trypsin inhibitor, 20100; and α -lactalbumin, 14400.

DISCUSSION

A panel of six hybridomas secreting monoclonal antibodies to rat tissue kallikrein have been identified and characterized. These monoclonal antibodies have distinctive properties based upon their subunit M_r values, abilities to competitively bind to 125 I-kallikrein, cross-reactivities with other kallikrein-like enzymes and their effects on kallikrein enzymic activities. Together they serve as unique tools for the study of tissue kallikrein structure and function.

The fact that tissue kallikrein belongs to a group of structurally and immunologically related arginine ester-peptidases is illustrated by the isolation of two hybridomas that produce antibodies cross-reactive with two kallikrein-related enzymes: urinary esterase A and tonin. Clone H₁F₃ produces antibodies that recognize a

Fig. 4. Electrophoretic analysis of active-site-labelled rat tissue serine proteinases

Tissue homogenates and purified urinary kallikrein were labelled with [14 C]jPr₂P-F as described in Fig. 3. Labelled proteins were precipitated with monoclonal antibody from clone V₁C₃ (lanes b, d and f) and sheep anti-kallikrein antiserum (lanes c, e and g). The immunoprecipitates of the labelled proteins were electrophoresed on a linear-gradient (7.5–17.5%) polyacrylamide gel containing 0.1% SDS. The protein bands were revealed by fluorography. Lane (a), purified kallikrein; lanes (b) and (c), proteins precipitated from submandibular-gland homogenate; lanes (d) and (e), proteins precipitated from pancreatic homogenate; and lanes (f) and (g), proteins precipitated from urine.

Fig. 5. Western-blot analysis of rat tissue homogenates by ^{125}I -labelled monoclonal anti-kallikrein antibodies

Proteins were separated in a linear gradient (7.5–17.5%) polyacrylamide gel containing 0.1% SDS and then either stained with Coomassie Blue (a) or Western-blotted with ^{125}I -labelled monoclonal anti-kallikrein antibodies

Fig. 6. Electrophoretic analysis of kallikrein-immunoreactive polypeptides from rat submandibular-gland mRNA-directed translation products

Submandibular-gland mRNA was translated in a rabbit reticulocyte system with [^{35}S]methionine as the radiolabel. Labelled proteins and immunoprecipitates were separated on a linear-gradient (7.5–17.5%) polyacrylamide gel and revealed by fluorography. Lane (a), total translation products and lane (b), immunoprecipitation products with affinity-purified sheep anti-kallikrein antibodies, with no exogenously added mRNA respectively; lane (c), total submandibular-gland poly(A)⁺ mRNA translation products; lane (d), immunoprecipitate of translation products with affinity-purified sheep anti-kallikrein antibodies; lane (e), purified ^{125}I -kallikrein; lanes (f), (g) and (h), immunoprecipitates with monoclonal antibodies V₄G₆, V₄D₁₁ and V₁C₃ respectively. A double-antibody method was used in which rabbit anti-mouse IgG was the second antibody. Lane (i) shows non-specific precipitates from a translation mixture with the second antibody alone. M_r markers were the same as in Fig. 3.

determinant common to kallikrein, esterase A and tonin, whereas clone A₁₁C₄ shows cross-reactivity only with kallikrein and tonin. These data imply that kallikrein has at least two different domains in common with tonin. The clone V₁C₃, only recognizing active kallikrein, has been used for prokallikrein-activation studies (Woodley *et al.*, 1985) and for the development of a radioimmunoassay measuring active and pro-kallikrein.

The enzymic-activity studies provide information about possible antibody-binding sites and suggest the potential usefulness of the antibodies as reagents with which to specifically manipulate enzymic activity. Three clones (A₁₁C₄, V₄D₁₁, and E₆C₆) produce antibodies that inhibited kininogenase activity. This inhibition parallels the inhibition seen with polyclonal anti-kallikrein antibody. It is reasonable to suggest that antibodies from clones V₄D₁₁ and A₁₁C₄ are possibly active-site-directed, since inhibition was seen in both the kininogenase and tosylarginine methyl-esterase assays. Thus antibodies

from clone V₄D₁₁ (b). The antibody binding was revealed by autoradiography. The gels contain purified urinary kallikrein (lane i), submandibular-gland homogenate (lane ii), pancreatic homogenate (lane iii), urine (lane iv), and M_r markers (a, lane v).

from clone V₄D₁₁ may be particularly useful as highly specific and potent tissue-kallikrein inhibitors (Table 1). In contrast, antibodies from clone H₁F₃ stimulated kininogenase activity. Although the cause of this stimulation is unknown, it is reasonable to suggest that the antibody binding causes a conformational change in the enzyme.

These monoclonal antibodies have specifically identified tissue kallikrein as a 38 kDa protein in total tissue homogenates from rat submandibular gland, pancreas and urine when the two different approaches of active-site labelling with immunoprecipitation and Western blotting were used. There are at least eight to ten serine proteinases that are labelled efficiently with [¹⁴C]iPr₂P-F in submandibular-gland tissue homogenates (Fig. 3). Four of these bands can be recognized by sheep anti-(rat kallikrein) antiserum, indicating that there are structural similarities between these proteins (Figs. 3 and 4). However, antibodies from four of the monoclonal cell lines recognize a single 38 kDa protein species in submandibular gland (Fig. 3). It is interesting to note that Western-blot analysis with monoclonal antibodies revealed a 92 kDa species in urine in addition to the 38 kDa species. The fact that this protein was not active-site-labelled implies that either this is a precursor to kallikrein whose active site is inaccessible to [¹⁴C]iPr₂P-F, or perhaps that this protein represents an interesting protein-inhibitor complex. The latter possibility is more likely in view of the finding of a 92 kDa SDS-stable kallikrein-inhibitor complex in both human and rat serum (Chao *et al.*, 1984b).

Molecular analysis of the kallikrein gene(s) and gene products was initiated by examining the primary biosynthetic products directed by poly(A)⁺ mRNA from rat submandibular gland. Analysis of the cell-free translation products immunoprecipitated with purified monoclonal and polyclonal anti-kallikrein antibodies by SDS/polyacrylamide-gel electrophoresis yielded two polypeptides with *M_r* 37000 and 35000. The fact that the migration of these two peptides is similar to, but not identical with, that of purified kallikrein, which migrates as two isoenzymes (*M_r* 38000 and 36000) (Fig. 6, lane *e*) is not surprising, since products synthesized *in vitro* are not processed or glycosylated in the reticulocyte system. The fact that monoclonal antibodies recognize these two polypeptides implies that they are kallikrein precursor peptides. The identification of two cDNA molecules from mouse submandibular gland encoding kallikrein-like proteins and the fact that the mouse genome has at least twelve homologous genes in a kallikrein family (Nordeen *et al.*, 1982), support the idea of multiple kallikrein-like mRNA species and multiple kallikrein precursors.

In summary, the present studies indicate that this panel of monoclonal anti-kallikrein antibodies are specific and useful reagents for the identification, purification, quantification and localization of kallikrein, and potentially for cellular physiological studies. Furthermore, they provide tools for studies of kallikrein-precursor synthesis and for further studies of kallikrein synthesis *de novo* occurring in tissue under the influence of various hormones or drugs or in disease states (Chao & Margolius, 1983; Miller *et al.*, 1984).

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