The existence of proteins in BALB/c 3T3 cells which interact strongly with trypsin was investigated in order to learn more about how the activities of proteases with trypsin-like specificity might be regulated in mammalian cells. Cell extracts were found to contain 7 to 12 pmol/million cells of a protein (TCP) which inhibits trypsin. The trypsin-TCP complex which forms upon incubation of cell extracts with trypsin was demonstrated to have a Stokes' radius of 3.55 nm, a sedimentation coefficient of 4.5 S, and a molecular weight of 60 to 63 × 10^6 by gel filtration and sucrose density gradient centrifugation. Gel filtration of the cell extracts prior to reaction with trypsin revealed that 90% of the trypsin-inhibitory activity of the extracts had an elution volume corresponding to that of an MD = 42,500 globular protein standard. This MD = 42,500 trypsin-inhibitory fraction also inhibited thrombin, but it did not inhibit chymotrypsin. After incubation of trypsin with the MD = 42,500 trypsin-inhibitory fraction, the radiochemical label of the trypsin co-eluted with trypsin-TCP upon gel filtration. This result supports the view that a single protein or group of proteins with a molecular weight of about 42,500 combines with trypsin (MD = 23,300) to form a M = 60 to 63 × 10^6 catalytically inactive trypsin-TCP complex. The trypsin-TCP complex could be dissociated by incubation with excess lima bean trypsin inhibitor (LBTI). Experiments using trypsin-TCP in which either the trypsin or the TCP was radiochemically labeled indicated that the incubation with LBTI resulted in formation of a trypsin-LBTI complex with concomitant release of free TCP.

Proteases with trypsin-like specificity appear to play important roles in cellular physiology. Most of the known activation reactions of prohormones and proenzymes are mediated by such proteases (1). Furthermore, rapidly proliferating cells and certain transformed cell lines elaborate more plasminogen activator than their normal counterparts (e.g. Refs. 2 and 3), suggesting that this protease (which has a trypsin-like specificity) may play an important role in the regulation of cell growth and migration. Interestingly, addition of low levels of proteases with trypsin-like specificity has been found to alter cellular morphology and adhesion (4), mobility of cell-surface components (5), cyclic nucleotide levels (6), sugar transport (7), complement of cell surface proteins (7, 8), growth control (9, 10), and cytoskeletal structure (11) of normal animal cells and to cause these cells to assume phenotypes similar to those of transformed cells. These effects and the effects of protease inhibitors on growing cells (12, 13) provide additional circumstantial evidence that trypsin-like proteases may play an important role in maintaining certain phenotypes which are characteristic of the transformed state. In an effort to learn more about how the activities of both endogenous and exogenous trypsin-like proteases are controlled in mammalian cells, we initiated studies of mammalian cell proteins which interact strongly with trypsin. The work reported here demonstrated that mouse fibroblasts contain at least one such protein which binds and inhibits trypsin and thrombin.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagles medium and fetal calf serum were obtained from GIBCO. Trypsin and chymotrypsin (bovine, lyophilized) were obtained from Worthington. Bovine serum albumin (B grade) and human thrombin (lyophilized A grade) were obtained from Calbiochem. Egg yolk-white ovomucoid trypsin inhibitor (type II-O) was obtained from Sigma. Carrier-free Na"I and [1-"C]acetate were obtained from Amersham. "C-labeled L-isoleucine, L-leucine, and L-valine were obtained from Schwarz/Mann.

Buffers used in this work include: PBS at pH 7.4, which consisted of 137 mM NaCl, 2.5 mM KCl, 9.5 mM phosphate, 0.9 mM MgCl2; PBS-BSA and PBS-0.1 BSA, which consisted of PBS containing BSA at 1 and 0.1 mg/ml, respectively; and PBS-BSA/Triton, which consisted of 0.25% Triton X-100 in PBS-BSA.

Iodinated trypsin was prepared by a method similar to that described by Cuatrecasas and Hollenberg (14) for iodinating proteins. Typically, carrier-free Na"I ([5 mCi in approximately 50 μl at pH 5 to 13] was mixed with 2 mg of trypsin (in 2.0 ml 0.25 mM sodium phosphate buffer, pH 7.4). Iodination was initiated by the addition of a freshly prepared solution of chloramine-T (1.6 mg in 100 μl of water), whereupon the reaction mixture was agitated for 20 to 30 s and sodium metabisulfite (3.2 mg in 100 μl of water) was added. After 20 to 40 s of gentle agitation, 1 ml of 0.1 M Tris (pH 7.5) containing 2 mg of BSA was added to the reaction mixture. "C-Trypsin was purified from the reaction mixture by affinity chromatography on a column of CHOM-Sepharose using a method similar to that of Robinson et al. (15) and stored in the pH 2.5 eluting buffer in the dark at 4°C. Calcium ion was omitted from eluting buffers during affinity chro-

The abbreviations used are: PBS, phosphate-buffered saline; BSA, bovine serum albumin; CHOM, chicken ovomucoid trypsin inhibitor; CHOM-Sepharose, CHOM covalently linked to Sepharose; DME medium, Dulbecco's modified Eagle's medium; LBTI, lima bean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; PMS-trypsin, the inactive phenylmethanesulfonyl derivative of the active site serp residue in trypsin; TCP, trypsin-combining protein; SV40, simian virus 40.

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Trypsin-inhibitory Protein of Mouse Fibroblasts

matography in order to avoid precipitation of calcium phosphate. The 
125I-trypsin was rechromatographed on CHOM-Sepharose before use in 
the experiments. The initial specific radioactivity of the purified 125I-
trypsin was about 15 nCi/pmol and its specific enzymatic activity was 
the same as affinity-chromatographed trypsin which had not been 
iodinated. The time-dependent decrease in the ratio of radioactivity/ 
enzymatic activity of the 125I-trypsin was that expected from the decay 
technique used to 125I.

PMS-125I-trypsin was prepared by a method similar to that of Gold 
(16). A solution of 0.1 M PMSF (in 95% ethanol) was added to a solution 
of 125I-trypsin in PBS to give a final concentration of 1 mM PMSF. After 1 h in the dark at 25°C, PMS-125I-trypsin was purified by chromatography by chromatography with Sepharose 4B (to remove the active trypsin), followed by gel filtration on Sephadex 
G-25 (eluted with 0.05 M NaCl). After gel filtration, PMS-125I-trypsin 
had a specific enzymatic activity which was less than 0.01% of that of 
fully active 125I-trypsin. PMS-125I-trypsin was stored at 4°C, neutral 
ph, in the dark.

125I-ovalbumin was prepared by iodinating ovalbumin by the 
method used in the preparation of 125I-trypsin, except that no BSA 
was added and the product was separated from low molecular weight 
materials in the reaction mixture by dialysis.

Acetylated protein standards containing radiochemical label were 
prepared as described by Cuesta (17), using [1-14C]acetic anhy-
dride (28.8 pCi/mer)

Cell Culture—Cells were grown in DME medium at 37°C in a 
humidified atmosphere containing 5% CO2. Powdered DME medium 
was dissolved in glass distilled water, adjusted to pH 4.0 to pH 6.0 
with 0.125 NIH unit of thrombin in 

Preparation of Cell Extracts—Monolayers of cells on tissue culture 
dishes were washed six times with PBS to remove serum 
proteins. The PBS-washed cells then were disrupted with PBS-BSA-
Triton (0.5 mL/20 cm² plate), transferred with the aid of silicone 
rubber scraper to a polypropylene tube, and centrifuged at 12,000 × g for 2 min in order to remove cellular debris.

Extracts from metabolically labeled cells were purified by absor-
ation on an agarose support to which trypsin was covalently bound via a 
cleavable phenyl ester linkage (18). Cell extracts (0.5 mL) were 
incubated for 45 min with 0.4 mL of a resin consisting of trypsin-linked 
agarose beads which had been diluted with Sepharose CL-6B to a trypsin 
level of 2.2 mmol/mL of resin. The resin was washed eight times with PBS and twice with 1 M NaCl in PBS. Covalently bound material 
was released from the resin by a 10-min incubation with 1 M hydrox-
yamine at pH 7.0 containing 1 mg/mL of BSA.

GBF technique was used to determine Stokes' radii (19), to estimate molecular weight (20), and to separate trypsin from trypsin-containing complexes. Samples were chromatographed (at 
4°C) on a column of Sephadex G-100 (superfine) or Sepharose CL-6B as indicated in the text. The column was eluted at a flow rate of 1.5 
ml/hr with a PBS buffer containing 0.06 M potassium phosphate buffer (pH 6.8), and fibrinogen (72 g in 40 mL) was added. The thrombin-
catalyzed conversion of fibrinogen to fibrin was monitored by meas-
uring the time dependence of the increase in absorbance of the 
solution at 350 nm due to the increase in turbidity of the solution 
which accompanies formation of fibrin. The absorbance versus time 
plots were sigmoidal, and the extent of inhibition of thrombin was assessed from comparisons of the plots obtained in the presence of cell 
extacts with those obtained in the absence of extracts at several 
levels of thrombin.

Electrophoresis—Polyacrylamide slab gel electrophoresis was performed 
at pH 4.3 as described by Brewer and Ashworth (30). Radio-
active bands on slab gels were detected by autoradiography with Kodak RP-Royal X-Omat film.

RESULTS AND DISCUSSION

Gel filtration of 125I-trypsin which had been incubated with extracts from BALB/c 3T3 cells revealed the presence of material which formed a stable complex with the trypsin. In a typical experimental 
procedure, washed cells on tissue culture dishes were disrupted by treatment with Triton X-100 in a PBS-BSA buffer. The resulting cell extracts then were incubated with 
125I-trypsin and subjected to gel filtration on a column of 
Sephadex G-100. The elution profiles in Fig. 1 show that, after reaction with the cell extracts, most of the radiochemical label 
eluted as a single symmetrical peak with an elution volume smaller than that observed for unreacted 125I-trypsin. These 
observations are consistent with the view that a protein (or group of proteins of similar Mr) in the cell extracts formed a complex with trypsin. The small peak in Fig. 1B at the elution position of trypsin is ascribed to unreacted excess trypsin. This peak was not seen when the amount of added trypsin was less than 8 pmol/10⁶ cells extracted.

Control experiments were carried out in order to verify that the decrease in elution volume of 125I-trypsin upon its reaction with cell extract indeed reflected formation of a complex between trypsin and a trypsin-combining protein (TCP) in 
the cell extracts. Formation of the complex was found to be 
dependent upon the presence of cellular material. The trypsin-
TCP complex was not formed when 125I-trypsin was incubated with PBS-BSA-Triton extracts of cell-free tissue culture plates that had been incubated with growth medium and 
washed in a manner similar to that used for plates containing 
cells. Since the fetal calf serum used in the growth medium is a
Trypsin-inhibitory Protein of Mouse Fibroblasts

In order to demonstrate that the radioactivity in the first peak represents the trypsin-TCP complex, the peak fraction was incubated for 200 min at room temperature in the presence of 1 mg/ml of lima bean trypsin inhibitor (LBTI) and then rechromatographed on a column of Sephadex G-100. Two peaks of radioactivity were observed in the elution profile (Fig. 3B). The first peak had an elution volume corresponding to the trypsin-TCP complex, and the second peak had a larger elution volume, suggesting that it might be free TCP. This view was tested in a duplicate experiment. As in the first experiment, the original trypsin-TCP complex was incubated with LBTI and rechromatographed on the Sephadex G-100 column. In this experiment, however, the eluent was analyzed for both radioactivity and trypsin-inhibitory activity. Fig. 4 shows that the peak postulated to be free TCP coincides with a peak of trypsin-inhibitory activity distinct from the peak for free LBTI. These results suggest that incubation with LBTI converted a substantial fraction of the trypsin-TCP complex to a trypsin-LBTI complex with concomitant release of free radiochemically labeled (and functionally competent) TCP. Several additional experiments were carried out to substantiate this conclusion.

The size of TCP in cell extracts prior to reaction with trypsin was determined by subjecting cell extracts to chromatography on a Sephadex G-100 column and analyzing the eluent for trypsin-inhibitory activity. As shown in Fig. 5A, the position of the peak of trypsin-inhibitory activity corresponds to that attributed to TCP which was liberated from the trypsin-TCP complex by LBTI (Figs. 3B and 4). This result indicates that formation of the trypsin-TCP complex occurs without the loss of a substantial portion of the TCP molecule. The observation that this peak represents 90% of the trypsin inhibitory activity in the cell extracts suggests that TCP is the major cellular protein which binds trypsin. As would be predicted, incubation of the material with trypsin-inhibitory activity with 125I-trypsin followed by gel filtration resulted in the appearance of a peak of radiochemical label at the position of trypsin-TCP (Fig. 5B).

The 125I-trypsin used throughout this work was purified by affinity chromatography in order to ensure that all of the radiochemical label in solutions of 125I-trypsin was associated with a catalytically active form of trypsin. Furthermore, the functional equivalence of radiochemically labeled trypsin and unlabeled trypsin was demonstrated by dilution with unlabeled trypsin. Dilution of 30 pmol of 125I-trypsin with 17 pmol of pure unlabeled trypsin prior to incubation of the 125I-trypsin with the cell extracts from 106 cells resulted in a 32% decrease in the amount of radiochemical label which eluted at the position of trypsin-TCP. The observed 32% decrease in the specific radioactivity of trypsin-TCP is in reasonable agreement with the 37% decrease in radioactivity that would be expected if labeled and unlabeled trypsin were equivalent in their reactivities toward TCP.

The trypsin-TCP obtained when cell extracts were incubated with 125I-trypsin and subjected to gel filtration was rechromatographed in order to study the stability of the trypsin-TCP complex. Fig. 6A shows that the elution position of trypsin-TCP was not altered by a second gel filtration and that very little radiochemical label was eluted in the position corresponding to free trypsin. Although this observation indicates that TCP binds trypsin tightly, dissociation of the complex was demonstrated by incubation of trypsin-TCP with LBTI. Thus, when a 4.1 nM sample of trypsin-TCP obtained on gel filtration was incubated in the presence of 1 mg/ml of LBTI at 25°C for 150 min and then rechromatographed, the size of the trypsin-TCP peak decreased. This decrease in the amount of radiochemical label at the elution
Trypsin-inhibitory Protein of Mouse Fibroblasts

Fig. 2 (left). Gel filtration profiles of trypsin-TCP and the trypsin complexes of proteins in fetal calf serum. Samples were chromatographed on a column (1.3 x 46 cm) of Sepharose CL-6B. The arrows indicate the elution positions of Blue Dextran, [14C]acetyl-thyroglobulin (MW = 670,000), [14C]acetyl-apoferritin (MW = 480,000), [14C]acetyl-phosphorylase α (MW = 360,000), [14C]acetyl-γ-globulins (MW = 150,000), alkaline phosphatase (MW = 80,000), and 125I-trypsin (MW = 23,300). A, elution profile of a sample of the cell extract that had been incubated with 2.5 pmol of 125I-trypsin/10⁶ cells extracted. B, elution profile of a sample of fetal calf serum that had been incubated with 0.75 pmol of 125I-trypsin/μl of serum. The two major peaks are probably trypsin complexes of α₂-macroglobulin and α₂-anti-trypsin.

Fig. 3 (center). The elution behavior (upon gel filtration on a column (1 x 40 cm) of Sephadex G-100 (superfine)) of trypsin-binding material in extracts of metabolically labeled cells. The arrows indicate the elution positions of alkaline phosphatase (MW = 80,000) and d-serine dehydratase (MW = 46,000). A, gel filtration profile of 0.5 ml of the material released from the trypsin-linked agarose resin upon treatment with 1 M hydroxylamine. Samples from each fraction were assayed by liquid scintillation counting. Results are expressed as total counts per min above background/0.5-ml fraction. B, elution profile of the trypsin-TCP complex peak (Fraction 31) from A after it had been incubated for 200 min at 25°C with 1 mg/ml of LBTI. A 0.4-ml portion of each fraction (0.5 ml) was counted. Results are expressed as total counts per min above background/0.5-ml fraction.

Fig. 4 (right). Elution profile (upon gel filtration on a column (1 x 40 cm) of Sephadex G-100 (superfine)) of the trypsin-TCP complex after it had been incubated for 200 min with 1 mg/ml of LBTI as described in the legend to Fig. 3B. A 0.4-ml portion of each 0.5-ml fraction was assayed for radioactivity, and the remaining 0.1 ml was assayed for trypsin inhibitory activity. Results are plotted as counts above background/50 min/0.5-ml fraction. — and — —, total radioactivity and trypsin inhibitory activity of each fraction, respectively. The second peak of trypsin inhibitory activity co-elutes with LBTI.

Fig. 5. Gel filtration profile of the trypsin-inhibitory activity in extracts from BALB/c 3T3 cells before and after incubation with trypsin. A, gel filtration profile of TCP before reaction with trypsin. The cell extracts from 6 x 10⁶ cells were subjected to gel filtration on a column (1 x 45 cm) of Sephadex G-100 (superfine) and each fraction was assayed for its ability to inhibit trypsin. The left ordinate indicates the relative molecular weights of the protein standards (C). The arrow indicates the elution position of glycine. B, gel filtration profile of trypsin-TCP formed upon reaction of a portion of the peak fraction from Fig. 5A with an 8% excess of 125I-trypsin. The arrows indicate the elution positions of the internal standards, [14C]acetyl-thyroglobulin (MW = 670,000), alkaline phosphatase (MW = 80,000), and d-serine dehydratase (MW = 46,000).

Fig. 6. Gel filtration profile of the trypsin-TCP fraction from Fig. 1B using a column (1 x 45-cm) of Sephadex G-100 (superfine). The arrows indicate the elution positions of the internal standards: [14C]acetyl-thyroglobulin (MW = 670,000), alkaline phosphatase (MW = 80,000), and d-serine dehydratase (MW = 46,000). A, elution profile of an aliquot of the trypsin-TCP fraction which had been incubated for 150 min at 0°C and chromatographed. B, elution profile of an aliquot of the trypsin-TCP fraction which had been incubated for 150 min at 25°C with 1 mg/ml of LBTI and chromatographed. C, elution profile of the 125I-trypsin-LBTI complex that formed upon mixing 125I-trypsin with excess LBTI.
Trypsin-inhibitory Protein of Mouse Fibroblasts

Fig. 7 (left). Sedimentation profile of trypsin-TCP in a sucrose gradient. \(^{125}\text{I}\)-trypsin-TCP was formed by mixing cell extract with an amount of 15 nM \(^{125}\text{I}\) -trypsin that was less than the estimated amount of TCP. An aliquot of this mixture was centrifuged in a sucrose gradient (5 to 20%) as described under "Experimental Procedures." Sedimentation positions of the following protein standards: bovine ribonuclease (1.78 S), bovine \(^{125}\text{I}\)-trypsin (2.48 S), \(^{125}\text{I}\) -acetetyl-bovine serum albumin (4.41 S), E. coli alkaline phosphatase (6.0 S), and rabbit muscle aldolase (7.35 S).

Fig. 8 (right). Inhibition of trypsin-catalyzed hydrolysis of \(N^\circ\)-benzoyl-L-arginine \(p\)-nitroanilide by cell extracts. Cell extract position of trypsin-TCP was accompanied by the appearance of a second peak of radioactivity at the elution position of the trypsin-LBTI complex (Fig. 6, B and C). These results are consistent with those obtained earlier for dissociation of metabolically labeled TCP from the trypsin-TCP complex. From the relative amounts of the trypsin-TCP and trypsin-LBTI complexes, it appeared that 44% of the trypsin-TCP complex had dissociated during the 150-min incubation with LBTI. The failure to obtain complete dissociation of the trypsin-TCP complex in the 150-min incubation period is probably due to slow dissociation of trypsin-TCP. The observation that longer incubation times of trypsin-TCP with LBTI result in increased yields of trypsin-LBTI supports this view; however, the reaction of trypsin-TCP with LBTI has not been studied in sufficient detail to determine whether the trypsin-TCP fraction contains material which does not dissociate.

Molecular weight determination of the trypsin-TCP complex and free TCP was carried out in order to determine the binding stoichiometry of the complex. Comparison of the elution volume of \(^{125}\text{I}\)-trypsin, with those of protein standards (Fig. 1), indicates that the Stokes' radius of trypsin increases from 2.26 to 3.25 nm upon formation of the trypsin-TCP complex. Additional information concerning the size of trypsin-TCP is presented in Fig. 7. This figure illustrates the sedimentation profile of radiochemical label in a mixture containing the cell extracts and \(^{125}\text{I}\)-trypsin. In this experiment the amount of trypsin was less than the amount of TCP, so that a peak at the position of uncomplexed trypsin is not seen. A value of 4.5 S for the sedimentation coefficient of the trypsin-TCP complex was obtained from a comparison of its sedimentation position with that of the protein standards (Fig. 7). A molecular weight of 60,000 for the trypsin-TCP complex was calculated from the value of the sedimentation coefficient \((s_{20,w})\) and the Stokes' radius \((a)\) of the complex, using the equation:

\[
M_r = \frac{6 \pi \eta a s_{20,w}}{1 - \frac{a}{\theta}}
\]

where \(\eta\) is the viscosity and \(\rho\) is the density of water at 20°C, \(N\) is Avogadro's number, and \(a\) is the partial specific volume of the complex, which was assumed to be 0.725 g/cm³, the value of \(\theta\) for many proteins. A similar molecular weight (63,000) was obtained for trypsin-TCP from a comparison of its elution volume on gel filtration to the elution volumes of globular proteins of known molecular weight. The agreement between these molecular weights (60,000 and 63,000) is consistent with the view that trypsin-TCP has a shape similar to that of the globular protein standards. A comparison of the elution volume of the free TCP peak with the elution positions of the protein standards (Fig. 5A) indicates that the protein in this peak has a Stokes radius of 2.9 nm. Assuming that the protein in this peak is similar in shape and partial specific volume to the globular protein standards, the elution volume of this protein indicates that its molecular weight is 42,500. The correspondence between the sum of the molecular weights of trypsin (23,300) and TCP (42,500) with that of the trypsin-TCP complex (60 to 63 \(\times 10^3\)) is consistent with a 1:1 reaction between trypsin and TCP which occurs without loss of a substantial portion of the TCP molecule.

An estimate of TCP levels in BALB c/3T3 cells was obtained by adding trypsin to cell extracts in three equal portions, and assaying the extracts for trypsin activity toward \(N^\circ\)-toluenesulfonyl-L-arginine methyl ester after each addition. The trypsin in the first two aliquots was completely inhibited by the cell extracts, whereas the trypsin in the third aliquot was only partially inhibited. The correspondence between the extent of trypsin inhibition (85% of the added trypsin; 8 pmol/10⁶ cells), the recovery of radiochemical label after gel filtration (Fig. 1B) in the \(^{125}\text{I}\)-trypsin-TCP peak (71% of that applied to the column), and the recovery of radiochemical label in the \(^{125}\text{I}\)-trypsin peak (8% of that applied to the column) indicates that most of the added trypsin reacted with TCP to form an inactive trypsin-TCP complex. Depending upon the cell extract, 7 to 12 pmol of trypsin was inhibited by an extract from 10⁶ cells. As shown in Fig. 8, TCP in cell extracts also inhibited the catalytic activity of trypsin toward \(N^\circ\)-benzoyl-L-arginine \(p\)-nitroanilide. Complete inhibition of the 3.8 pmol

\(^3\) If the complex had a less symmetrical shape than that of the globular protein standards, the complex would be expected to co-elute with a globular protein standard whose molecular weight was higher than that determined for the complex using Equation 1.
of trypsin present in this determination was achieved by an extract of 3.3 \times 10^6 cells. Assuming TCP binds trypsin in a 1:1 ratio, this result indicates the presence of 11 pmol of TCP in the extract from 10^6 cells. This value is in agreement with the values obtained using N\textsuperscript{\textomega}-toluenesulfonyl-L-arginine methyl ester to assay trypsin.

Titration of TCP with trypsin was also accomplished using polyacrylamide gel electrophoresis to monitor formation of the complex. Cell extracts were treated with varying amounts of \(^{125}\text{I}\)-trypsin and then electrophoresed. The autoradiogram depicted in Fig. 9 shows that one major band of radioactivity was observed when cell extracts were incubated with less than about 9 pmol of \(^{125}\text{I}\)-trypsin/10^6 cells, whereas incubations of cell extracts with greater than about 9 pmol of \(^{125}\text{I}\)-trypsin/10^6 cells resulted in a pronounced increase in the intensity of a second band of radioactivity which co-migrated with \(^{125}\text{I}\)-trypsin. These results, which indicate that there are approximately 9 pmol of TCP present in this extract from 10^6 cells, are consistent with those of previous determinations of TCP levels in cell extracts.

The reaction of the catalytically inactive phenylmethanesulfonyl derivative of \(^{125}\text{I}\)-trypsin with cell extracts was studied in order to further characterize the mode of interaction of trypsin with TCP. When this derivative of \(^{125}\text{I}\)-trypsin was added to the cell extracts instead of \(^{125}\text{I}\)-trypsin, essentially all of the radiochemical label had an elution volume equal to that of the unreacted derivative. The inability of the cell extracts to alter the elution volume of PMS, \(^{125}\text{I}\)-trypsin indicates that trypsin must contain an unblocked active site in order to bind to TCP.

The specificity of TCP was further characterized by studying the inhibitory activity of TCP toward trypsin, thrombin, and chymotrypsin. In the nanomolar concentration range, TCP (from the \(M_c = 42,500\) fraction of Fig. 5A) completely inhibited the catalytic activity of trypsin but had no effect on the catalytic activity of chymotrypsin toward the substrate N-benzoyl-L-tyrosine ethyl ester. The \(M_c = 42,500\) fraction was also observed to inhibit thrombin-catalyzed hydrolysis of N\textsuperscript{\textomega}-toluenesulfonyl-L-arginine methyl ester; however, the insta-

![FIG. 9. Slab gel electrophoresis of cell extracts which had been reacted with varying amounts of \(^{125}\text{I}\)-trypsin. Extracts from cells were incubated for 30 min with varying amounts of \(^{125}\text{I}\)-trypsin, treated with PMSF (1 mM), and subjected to electrophoresis. The amounts of trypsin used/10^6 cells extracted were 2.7 pmol (Well 1), 4.4 pmol (Well 2), 6.2 pmol (Well 3), 8.9 pmol (Well 4), 13.3 pmol (Well 5), and 17.8 pmol (Well 6). The arrow indicates the migration position of \(^{125}\text{I}\)-trypsin.](image)

\(^{22}\text{Br}\)I

of dilute solutions of thrombin precluded determination of the molar amount of thrombin which was inhibited. Separate studies wherein the catalytic activity of thrombin toward fibrinogen was examined indicated that thrombin, like trypsin, was inhibited by cell extracts.

Studies of the binding of \(^{125}\text{I}\)-trypsin to intact cells on culture dishes were initiated in order to determine whether TCP was a cell-surface protein. Unfortunately, these studies were confounded by the low amount of trypsin (<1 pmol/10^6 cells) which bound to the cells and by the high nonspecific binding of \(^{125}\text{I}\)-trypsin to culture dishes which had been incubated with serum.

The properties of TCP suggest that it is distinct from other mammalian protease inhibitors which have been characterized. TCP differs in size from known serum inhibitors including mouse \(\alpha\)-anti-trypsin which has a molecular weight of 58,200 (31). Size also distinguishes TCP from inhibitors recently found in cartilage (32) and bovine cardiac muscle (33). Moreover, TCP differs from the less specific inhibitors which, unlike TCP, inhibit both chymotrypsin and trypsin (34–37). The presence of TCP in other rodent cell lines was investigated using \(^{125}\text{I}\)-trypsin in experiments similar to those described for BALB/c 3T3 cells. A complex similar to trypsin-TCP was formed with extracts of the cell lines tested which include SV40-transformed BALB/c 3T3, Swiss 3T3, SV40-transformed Swiss 3T3, BHK-21/c13 and normal rat kidney cells. Further work is necessary, however, in order to determine the pattern of occurrence of TCP in mammalian cell lines.

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Trypsin-inhibitory Protein of Mouse Fibroblasts