SERINE ENZYMES RELEASED BY CULTURED NEOPLASTIC CELLS*

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Transformation of embryonic cells from various species by oncogenic viruses is associated with large increases in production of plasminogen activators, and similar enzymes are released by cultures and/or cell lines derived from human and animal neoplasms. These enzymes activate plasminogen by limited proteolysis, and several of them have been identified as serine proteases because they are irreversibly inactivated by low concentrations of the active site reagent diisopropylfluorophosphate (DFP, 1–6). The stability of the covalent bond formed by the incorporation of radioactive DFP into serine residues at active sites (7, 8) can be used to label both plasminogen activators and other serine enzymes; subsequent analysis of the reaction products by gel electrophoresis and autoradiography should yield an identifiable spectrum of labeled species that might provide insights about the secretory activities of cultured cells.

In this paper we present the initial results of such an approach to the comparison of serine enzymes released by transformed embryonic cells and their normal counterparts. To differentiate between serine proteases and esterases, and to obtain information about substrate specificity, we have taken advantage of the fact that incorporation of DFP can be blocked by prior reaction of enzymes with other active site reagents. The combination of these reactions can be used as a general procedure for identification of individual proteases in impure mixtures and particularly so for enzymes that are present at concentrations too low to permit detection by more traditional methods. We have also applied these methods to conditioned medium from several cultured human cell strains of neoplastic origin with a view of comparing the serine enzymes secreted by a variety of independently-derived cell types.

Materials and Methods

Cell Culture and Virus Infection. Cell cultures were prepared and maintained essentially as described by Unkeless et al. (1) using disposable plastic Petri dishes (100 mm diameter, Falcon Plastics, Division of BioQuest, Oxnard, Calif.) at 37°C in atmospheric air supplemented with 5%...
of CO₂. The medium was Dulbecco's modified (9) Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum. Mouse embryo cells prepared from C57 black embryos were seeded at a density of 2 × 10⁶ cells per plate. The cells were allowed to attach and grow for 16 h, were treated with 1 ml diethylaminoethyldextran (25 μg/ml, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) for 30 min, and infected with mouse sarcoma virus (MSV, obtained from Dr. H. M. Temin at the University of Wisconsin at Madison). Mock infected control cultures were treated in the same way as the infected cells except that no virus was added. Human neoplastic cell strains derived from a melanoma and a rhabdomyosarcoma (obtained from Dr. D. B. Rifkin, [5]) were seeded at a density of 2 × 10⁶ cells per plate.

**Serum-Free Culture Fluid.** Cultures that had grown to confluency were washed three times with a buffer containing 0.01 M NaPO₄, pH 7.4, 0.15 M NaCl, 1 mM CaCl₂, and 0.5 mM MgCl₂, and then incubated in 10 ml of serum-free medium for 16 h to yield conditioned culture fluid. The cell density at this stage was kept in the range 10⁻¹⁰ × 10⁶ per 100 mm plate. The cells were then recycled for 8 h in medium containing 10% serum before further incubation in serum-free medium and the procedure was repeated up to five times. The conditioned medium was centrifuged (5000 g, 10 min) at 4°C, the resulting supernate placed into dialysis tubes and concentrated 10-fold by packing in dry Sephadex G-150 at 4°C, and finally dialyzed against 0.1 M Tris-sulphate, pH 7.4, for 48 h at 4°C. Protein content in the concentrated and dialyzed medium was measured by the method of Lowry et al. (10).

**Assay for Plasminogen Activator.** Assays for fibrinolysis initiated by plasminogen activation were performed in plastic Petri dishes coated with bovine [³⁵S]fibrin as described previously (3). The assays were performed in 0.1 M Tris HC1, pH 8.1 containing 2 μg of human plasminogen in a final vol of 1 ml; incubation was at 37°C for the periods indicated in each experiment. 1 U of plasminogen activator was defined as the amount which released 5% of the radioactivity in 1 h from freshly activated plates.

[³⁵S]bovine fibrinogen was prepared as described by Unkeless et al. (1). Human plasminogen was prepared from plasma according to the method of Deutsch and Mertz (11) by two cycles of affinity chromatography on columns of Sepharose 4 B substituted with L-lysine.

**Labeling with [³H]DFP.** Concentrated and dialyzed conditioned culture fluid (1.5-2.5 ml) was incubated with [³H]DFP (50 μM, 3.9-10 Ci/mmol) for 16 h at 20°C, dialyzed for 48 h at 4°C against distilled water containing sodium dodecyl sulfate (SDS), lyophilized, and dissolved in 50 μl of a buffer containing 10% glycerol, 5% ethylene glycol, 0.062 M Tris HC1, pH 6.8. When labeling was performed in the presence of other competing protease inhibitors the latter were preincubated with concentrated conditioned medium for 1 h before addition of [³H]DFP. The concentration of SDS was equalized on both sides of the dialysis membrane and it was adjusted to yield a final concentration of 5% after lyophilizing and redissolving the sample in 50 μl buffer.

**Gel Electrophoresis.** SDS-polyacrylamide gel electrophoresis was performed in a stacking system (12), with slab gels (1.2 × 130 × 150 mm) with acrylamide concentrations as indicated in each case, and with a stacking layer consisting of 4% acrylamide. Buffers were as described by Laemmli (12). The gels were stained with 0.1% Coomassie Blue in 50% (wt/vol) trichloroacetic acid, and destained in 10% acetic acid. A mixture of protein markers was electrophoresed in each gel. These included basic pancreatic trypsin inhibitor (mol wt 6,500), lysozyme (mol wt 14,000), chymotrypsinogen (mol wt 25,000), ovalbumin (mol wt 45,000), bovine serum albumin (mol wt 68,000), β-galactosidase (mol wt 130,000), and bovine fibrinogen (mol wt 320,000). After electrophoresis some gels were sectioned longitudinally, each channel was frozen and cut into 1.1-mm slices with a fakir bed of razor blades, and the slices assayed for plasminogen activator as described above. To assay for [³H]DFP the slices were incubated for 72 h at 37°C in a mixture of Liquifluor/protosol/4 M ammonium hydroxide (50/5/1, vol/vol/vol) and counted in scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill., counting efficiency 30%); alternatively, [³H]DFP was detected in the gels by scintillation autoradiography as described by Bonner and Laskey (13).

**Chemicals.** The chemicals used in this work were obtained as follows: [³H]DFP from Amersham Corp., Arlington Heights, Ill.; urokinase Leo from Leo Pharmaceutical Industries, Ballerup, Denmark; p-nitrophenyl-p'-guanidinobenzoate (NPGB) and tert-butoxycarbonyl-ala-alala-pro-ala-p-nitroanilide and basic pancreatic trypsin inhibitor were gifts from Doctors E. Shaw, M. Zimmerman, and E. Truscheit, respectively; acrylamide, N,N',N' N'-tetramethylethylenediamine, N,N'-methylene bisacrylamide, ammonium persulfate, X-ray film RP/BS4, and dimethyl...
sulphoxide were from Eastman Kodak Co., Rochester, N. Y.; Sephadex G-150 and Sepharose-4B from Pharmacia Fine Chemicals; SDS (specially pure) from BDH Chemicals, Poole, England; \(^{[125]}\)iodide (carrier free) from Schwartz-Mann Div., Becton, Dickinson & Co. Biochemicals, Orangeburg, N. Y.; protosol and Liquifluor from New England Nuclear Corp., Boston, Mass.; Eagle's minimal essential medium, Dulbecco modified Eagle's medium, and fetal bovine serum from Grand Island Biological Co., Grand Island, N. Y. All other reagents were of the best commercially available grade.

Results

Analysis of Conditioned Media from Cultures of Normal and Transformed Mouse Embryo Cells. Serum-free conditioned media were collected, concentrated, reacted with \(^{[3]}\)HDFP, and analyzed by SDS-polyacrylamide gel electrophoresis. As seen in Fig. 1 a, the radioactivity profile of the sample from transformed cultures contained four distinct peaks, whereas none were observed in the normal control. Parallel differences in enzyme activity between normal and transformed cultures were found in gels of samples that were analyzed without having been exposed to DFP (Fig. 1 b). The coincidence of plasminogen activator activity and \(^{[3]}\)HDFP labeling in two of the peaks (Fig. 1 b and legend to Fig. 1) makes it seem likely that at least some of the incorporated DFP was due to reaction with these trypsin-like enzymes, but the fractional labeling represented by such enzymes could not be estimated from these observations alone, as DFP can bind covalently to proteins other than those containing active serine groups (14–17). Accordingly, the nature of the \(^{[3]}\)HDFP reactive proteins was characterized further by performing the labeling reaction in the presence of a variety of known, competing, protease inhibitors.

The results illustrated in Fig. 2 were obtained when conditioned media were incubated with \(^{[3]}\)HDFP in presence of NPGB, an inhibitor that acylates the active site of most known trypsin-like enzymes. In comparison with the results in Fig. 1, NPGB blocked the incorporation of \(^{[3]}\)HDFP into peaks I and II, but not into peaks III (Fig. 1) and IV. Since plasminogen activator is known both to share the substrate specificity of trypsin for arginine and lysine residues and to be inactivated by NPGB (3, 6, 18), it appears likely that peaks I and II were due to enzymes of this class. This conclusion is reinforced by the fact that all of the proteolytic activity associated with these two peaks was plasminogen dependent.

Because the concentrations of NPGB that are known to inhibit plasminogen activator are rather low (18), the complete block of \(^{[3]}\)HDFP labeling in peaks I and II (Fig. 2) suggests both that \(^{[3]}\)HDFP was bound only at the active site of the respective enzymes and that its incorporation could provide a measure of the number of enzyme molecules present. On this basis the specific catalytic activity of peak I was at least 10-fold greater than that of peak II when the extent of \(^{[3]}\)HDFP incorporation was related to enzyme assays as in Fig. 1. We have not characterized the kinetic parameters of peaks I and II in detail and therefore do not know whether the catalytic differences are due to changes in

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1 K. Dans and E. Reich. 1977. Plasminogen activator from cells transformed by oncogenic virus: inhibitors of the activation reaction. Manuscript submitted for publication.

2 Control experiments were performed to ensure that both maximal labeling and complete enzyme inactivation by DFP were achieved under the conditions used in Fig. 1 a.
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Fig. 1. (a) [$^3$H]DFP labeling and (b) plasminogen activation profile after gel electrophoresis of conditioned medium from cultures of normal and MSV-transformed mouse fibroblasts. 15 ml of medium were concentrated 10-fold, incubated with [$^3$H]DFP (50 μM, 10 Ci/mmol) for 16 h at 20°C, dialyzed against 0.17% SDS, lyophilized, and dissolved in 50 μl of buffer (10% glycerol, 5% ethylene glycol, 0.062 M Tris HCl, pH 6.8). Culture fluids for the plasminogen activator assays were treated identically except that the incubation with [$^3$H]DFP was omitted. The samples were electrophoresed in parallel on a slab gel with a linear gradient of 8–13.5% polyacrylamide for 18 h at 6 mA and 25–50 V. The gel was sectioned longitudinally and each strip was frozen and cut into 1.1-mm slices which were respectively (a) assayed for radioactivity (see experimental procedures) and (b) assayed for plasminogen activator in the [125I]fibrin plate assay for 2 h. (●—●) conditioned medium from MSV-transformed mouse fibroblasts containing 3,100 U of plasminogen activator. (○—○) conditioned medium prepared under identical conditions from an equal number of normal mouse fibroblasts containing no detectable plasminogen activator activity (less than 10 U). The localization of marker proteins in a stained strip of the gel and the estimated molecular weights of the peaks based on their electrophoretic mobilities are indicated. Prolonged assays (results not shown) revealed some plasminogen activator
Fig. 2. Effect of NPGB on [3H]DFP labeling of conditioned medium from MSV transformed mouse cells. (○—○) conditioned medium was labeled with [3H]DFP as described in Fig. 1; or (○—○) treated identically except that NPGB (100 μM) was added to the concentrated medium 1 h before [3H]DFP. Electrophoresis and analysis for radioactivity as described in legend to Fig. 1 except that the gel was constructed with a gradient of 6-16% polyacrylamide.

The radioactivity profiles of [3H]DFP-labeled culture fluids from normal and transformed mouse cells (Fig. 1a) consistently showed barely detectable small peaks and shoulders, suggesting the presence of additional [3H]DFP-labeled proteins whose existence might be more convincingly revealed using detection methods having greater sensitivity and resolving power. For this purpose, we applied the method for scintillation autoradiography recently reported by Bonnet and Laskey (13) which was designed for use with polyacrylamide gels containing tritium-labeled compounds. The results of an experiment comparing conditioned media from normal and transformed cultures are presented in Fig. 3. The staining patterns obtained from Coomassie Blue (a–d) show distinct differences between the normal and transformed cultures, but these are not detectably influenced by NPGB. Parallel specimens were incubated before electrophoresis with [3H]DFP, in presence or absence of NPGB, and analyzed by autoradiography for 5 days (e–h) or 17 days (i–l). As expected, the number of [3H]DFP-labeled bands detected was increased by longer periods of autoradiographic exposure; after 17 days of exposure there were 12 bands in transformed cell conditioned medium (Fig. 3k) and nine in the normal (Fig. 3i) and multiple differences between them. Five of the [3H]DFP-labeled proteins (E, F, G, H, and I) were present either exclusively or predominantly in transformed cell conditioned medium, while one (K) was detected only in media from normal cultures. Competition experiments with NPGB (Fig. 3f, h, j, and l) showed that bands E, F, H, I, and K were trypsin-like proteases, since [3H]DFP activity in the culture fluid from MSV-transformed cells corresponding to peak II in the radioactivity profile. The specific catalytic activity of peak II (calculated as radioactivity released in the fibrinolysis assay/[3H]DFP labeling) was more than 10-fold smaller than that of peak I.
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Fig. 3. Scintillation autoradiograms after gel electrophoresis of conditioned medium from normal and MSV transformed mouse fibroblasts after incubation with [3H]DFP, in the presence or absence of NPGB. The samples were prepared and electrophoresis performed as described in Fig. 1a, except that the specific activity of the [3H]DFP was 3.9 Ci/mmol. The gel was stained and scintillation autoradiograms prepared as described in Materials and Methods with exposure times as indicated. (a, e, and i): conditioned medium from normal cells labeled with [3H]DFP. (b, f, and j): conditioned medium from normal cells labeled with [3H]DFP after preincubation with 100 μM NPGB. (c, g, and k): conditioned medium from transformed cells labeled with [3H]DFP. (d, h, and l): conditioned medium from transformed cells labeled with [3H]DFP after preincubation with 100 μM NPGB. The localization of marker proteins in a stained lane of the gel, and the estimated molecular weights of the bands based on their electrophoretic mobilities, are indicated.

Each sample of culture fluid from transformed cells contained 1,420 plasminogen activator U while plasminogen activator was not detected in the culture fluid from the normal cells (below 3 U).

incorporation was largely inhibited under these conditions. By comparing the results in Fig. 3 with those in Figs. 1 and 2, we can identify band E (Fig. 3) as the predominant plasminogen activator released by transformed cells. It is significant that the region corresponding to plasminogen activator accounts for most of the serine enzymes found in transformed culture conditioned medium; it is also responsible for the major serine enzyme differences between transformed and normal cultures.

To obtain further data concerning bands in which [3H]DFP labeling was not affected by NPGB, experiments identical to that of Fig. 3 were performed by using known inhibitors with other specificities. As seen in Table I, three NPGB-resistant enzymes (D, G, and N) interacted with other inhibitors; these therefore probably represent serine esterases or proteases, although this level of characterization is not sufficient for their definitive identification. Two of these (D and N) were in culture medium from both normal and transformed cells, while one (G) was found predominantly in medium from transformed cells. The finding that the labeling of band E was blocked by benzamidine and l-arginine methyl ester, in addition to NPGB, is consistent with the known inhibitor spectrum of plasminogen activators from a variety of species (3, 18).
TABLE I

Inhibition of [3H]DFP Labeling of Proteins Found in Culture Fluid from MSV-Transformed Cells

| Inhibitors                     | Concentration | Proteins* |
|--------------------------------|---------------|-----------|
|                                | µM            | A | B | C | D | E | F | G | H | I | J | L | M | N |
| NPGB                           | 100           |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| PMSF†                          | 50            |   |   |   | + | + | + | + |   | - | - | - | - | - | - |
| Benzamidin                     | 100,000       |   |   |   |   |   |   |   | - | - | - | - | - | + | - |
| 1-Arginine methyl ester        | 100,000       |   |   |   |   |   |   |   | - | - | - | - | - | + | + |
| TPCK                           | 800           |   |   |   |   |   |   |   |   |   |   |   | - | - | - |
| tert-butoxycarbonyl-ala-ala-pro-ala-p-nitroanilide | 10,000 |   |   |   |   |   |   |   |   |   |   |   | + | - | - |
| Lima bean trypsin inhibitor    | 5             |   |   |   |   |   |   |   |   |   |   |   | - | - | - |
| Soybean trypsin inhibitor      | 5             |   |   |   |   |   |   |   |   |   |   |   | - | - | - |
| Basic pancreatic trypsin inhib.| 35            |   |   |   |   |   |   |   |   |   |   |   | - | - | - |

Experimental conditions as described in Fig. 3 except that preincubation with NPGB was substituted by preincubation with the listed inhibitors in concentrations as indicated. The letters refer to the bands in Fig. 3k.

* + Indicate that [3H]DFP incorporation was decreased by preincubation with the inhibitor; - that it was not.
† PMSF, phenyl methyl sulfonyl fluoride; TPCK, L-1-tosyl-2-phenylethylchloromethyl ketone.

Stained gels (Fig. 3a, b, c, and d) showed that the culture fluid contained considerable amounts of serum albumin. Addition of radioactive amino acids to the cultures did not lead to biosynthetic labeling of the albumin, which must therefore have been a persisting serum contaminant (unpublished results). This raised the possibility that some of the [3H]DFP-labeled proteins might likewise have been derived from the serum, and a sample of fetal bovine serum was therefore incubated with [3H]DFP and analyzed as described in Fig. 3. Only a single band of radioactivity was detected; this band migrated to the same place as did band B in the culture fluids. Hence the other bands detected in the culture fluids represent proteins that are either produced by the cells, or they are serum proteins that are rendered susceptible to [3H]DFP incorporation after exposure to the cultures.

Considerable differences between the protein contents of fluids from normal and transformed mouse cells were observed in the stained gels (Fig. 3a and c). For example, a consistent finding was the presence of two bands of high molecular weight (Fig. 3a, I, II) in fluids from normal cultures, which were either reduced in amount or absent in fluids from transformed cells. Biosynthetic labeling experiments showed that these two proteins were produced by the normal cells and they are probably related to the external glycoproteins studied in many other laboratories (19).

Neoplastic Cell Strains of Human Origin. To obtain information concerning enzymes released in cultures of human cells, several cell strains derived from human tumors were surveyed by using the approach described in Fig. 3 for murine material. The data from two such strains—a rhabdomyosarcoma and a melanoma—are presented in Fig. 4, where the enzymes in conditioned media can be compared with a preparation of human urinary urokinase that served as a reference standard. The conditioned medium from rhabdomyosarcoma cells contained 10 bands that were labeled with [3H]DFP, that from melanoma cells contained nine labeled species, while three proteins in urokinase were labeled by [3H]DFP. Competition experiments with NPGB (Fig. 4h, j, l, n, p, r, t, v,
FIG. 4. Scintillation autoradiogram obtained after gel electrophoresis of conditioned medium from two human tumor cell strains, and of urinary urokinase: \(^{3}H\)DFP labeling in presence or absence of NPGB. Experimental procedures as described in Fig. 3. The urokinase was dissolved in 1.5 ml 0.1 M Tris SO\(_4\), pH 7.2 before incubation with \(^{3}H\)DFP. (a, g, m, and s): conditioned medium from rhabdomyosarcoma cells labeled with \(^{3}H\)DFP. (b, h, n, and t): conditioned medium from rhabdomyosarcoma cells labeled with \(^{3}H\)DFP after preincubation with NPGB. (c, i, o, and u): conditioned medium from melanoma cells labeled with \(^{3}H\)DFP. (d, j, p, and v): conditioned medium from melanoma cells labeled with \(^{3}H\)DFP after preincubation with NPGB. (e, k, q, and w): urokinase labeled with \(^{3}H\)DFP. (f, l, r, and x): urokinase labeled with \(^{3}H\)DFP after preincubation with NPGB.

Preincubation with NPGB was for 1 h at 20 \(\mu\)M. Note: this concentration was fivefold lower than that used in the experiments described in Figs. 2 and 3. The content of plasminogen activator was, respectively: rhabdomyosarcoma cells—3,900 U; melanoma cells—5,300 U, and urokinase—8,000 U.

and x) showed that at least eight of the labeled bands in rhabdomyosarcoma conditioned medium were serine proteases (B, D, E, F, H, I, J, and M); the comparable numbers for melanoma conditioned medium and urokinase were seven (B, D, E, G, H, J, and K) and two (F and I), respectively.

The plasminogen activator profile of other samples from the same preparations was determined in a separate gel which was sliced and assayed for enzymatic activity (Fig. 5). The culture fluid from rhabdomyosarcoma cells contained four electrophoretically separable plasminogen activators, that from melanoma cells two, and urokinase two. Although the resolution of the gels sliced for determination of catalytic activity was low compared with the autoradiograms of \(^{3}H\)DFP labeling, comparison of the apparent molecular weights of the plasminogen activators and the serine proteases (Figs. 4 and 5) led to the following tentative identification: the plasminogen activators from rhabdomyosarcoma cells as the proteases D, E, F, and I (Figs. 4 g, m, s, and 5 a), the activators from melanoma cells as the proteases D and E (Fig. 4 i and 5 b), and the activators in urokinase as the proteases F and I (Figs. 4 k and 5 c). Hence, taken together, Figs. 4 and 5 show that two serine proteases and plasminogen activators D and E with apparent mol wt approximately 69,000 and 63,000 were common both to rhabdomyosarcoma and melanoma cultures but were not present in urokinase. Two serine proteases (F and I) with mol wt
FIG. 5. Plasminogen activator profile after gel electrophoresis of conditioned medium from human tumor cell strains and urinary urokinase. Experimental conditions were as described in Fig. 1b except that a 8-16% polyacrylamide gel was used for electrophoresis. (a) Conditioned medium from rhabdomyosarcoma cells containing 900 U plasminogen activator. Gel slices were assayed for 5 h. (b) Conditioned medium from melanoma cells containing 300 U plasminogen activator. Gel slices were assayed for 8 h. (c) Urokinase containing 1,000 U plasminogen activator. Gel slices were assayed for 8 h.
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approximately 58,000 and 36,000, which also were plasminogen activators, were common to urokinase and rhabdomyosarcoma conditioned medium, but neither of these was detected in culture medium from melanoma cells. Protease J, with a mol wt approximately 29,000, which had no detectable plasminogen activator activity was released from both rhabdomyosarcoma and melanoma cells. The electrophoretic mobility of this protease was indistinguishable from that of protease H in Fig. 3 g and h, which was previously observed in cultures of transformed mouse cells.

Discussion

The results in this paper may be considered in the following contexts: (a) the utility and applicability of the [3H]DFP labeling method, particularly when it is used in conjunction with enzyme assays and with other, competing, active site reagents; (b) the differences in serine enzyme profiles found in conditioned media from normal and transformed cultures; and (c) the properties of plasminogen activators released by different cell types.

(a) The reaction of DFP with protein is not limited to the active site of serine enzymes; DFP can form stable derivatives with the hydroxyl group of tyrosine residues, and with serine and/or threonine hydroxyls that are not located in enzymatically active centers (14-17). In spite of these shortcomings there is sound evidence to suggest that the incorporation of [3H]DFP can serve as a versatile and sensitive means for detecting and identifying serine enzymes, particularly serine proteases: thus, (1) both the rate and extent of nonspecific reactions are markedly reduced below pH 7.5 and at DFP concentrations below millimolar (14, 20); hence the risk of nonspecific labeling with [3H]DFP can be minimized by selecting the reaction conditions appropriately and with the reactivity of particular enzymes in mind. (2) It appears likely that the value of [3H]DFP for labeling serine enzymes will be greatest when the reaction is used in conjunction with enzyme assays and other enzymatic probes, such as known inhibitors. The large repertoire of well studied trypsin inhibitors, which includes a variety of macromolecules in addition to both reversible and irreversible active site blocking agents, provides a set of highly specific competing reagents for evaluating the tryptic character of a given [3H]DFP-labeled band. Similar, though less extensive groups of inhibitors are available for characterizing serine enzymes with chymotryptic, elastolytic, and other specificities.

With the exception of enzymes that function in unusual environments such as the vertebrate stomach, most extracellular proteases are catalytically active at pH close to neutrality, they have correspondingly neutral pH optima, and the vast majority are serine enzymes. Recent observations, and the result of a newly developed method suggest that most, and perhaps all serine proteases retain their catalytic activity after SDS-polyacrylamide gel electrophoresis under nonreducing conditions and can be visualized at very low concentrations as lytic bands. Thus, the combination of electrophoretic mobility, appropriate assays, and DFP labeling in the presence and absence of inhibitors should allow many of the extracellular proteases in crude tissue extracts, or complex

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body fluids, to be detected and identified; metalloproteinases, thiol proteinases, and the minority of serine enzymes that react poorly with DFP will obviously escape detection by this approach.

(b) The data in this paper, taken together with previously published work in this and other systems (1-3), demonstrate beyond doubt that transformation by sarcoma viruses is accompanied by a large increase in synthesis and secretion of plasminogen activators. In the present work, the increased release of enzyme after transformation by mSV was at least 100-fold; this is probably a minimal estimate in view of the fact that plasminogen activator is inactivated in conditioned medium. Because the phenotypic differences between normal and transformed cells are profound, a broad range of differences in the pattern of secreted enzymes might have been expected; hence, it was somewhat surprising to find that, in terms of the numbers of enzyme molecules released, the bands corresponding to plasminogen activator accounted for most of the difference in [3H]DFP labeling. This is clearly visible in the 5-day exposed autoradiograms in Fig. 3, which emphasize the contrast both between the normal and transformed cultures and between plasminogen activator and all other serine enzymes. The magnitude of the change in plasminogen activator production appears striking, and the fact that [3H]DFP labeling and catalytic activity are both completely abolished by low concentrations of NPGB (18) supports the assumption that this enzyme accounts for all of the labeling in band E (Fig. 3). Apart from plasminogen activator, the data also revealed the existence of multiple differences in serine enzyme patterns: there were at least six such differences of which four appeared to involve proteases with trypsin-like specificity. Owing to the limited amount of available information concerning these species we cannot as yet assess their biological significance. Moreover, it remains uncertain whether the other bands are secreted, or are the products of contaminating serum zymogens that might have become activated by exposure to cultured cells; this is in contrast with plasminogen activator, whose status as a cellular secretion product is well documented by several lines of evidence (1-6).

(c) The combined results of [3H]DFP labeling and enzyme assay showed the existence of several forms of plasminogen activator. In general, differences in the numbers of active bands and in their electrophoretic mobility were greater in samples from different species, than they were in a variety of cell types from a single species. It appears likely that the multiplicity of electrophoretic forms arises by progressive limited proteolysis of a high molecular weight precursor, but the full documentation of this process is beyond the scope of the present work.

Summary

Serine proteases or esterases released from cell cultures into the growth medium were converted to radioactive derivatives by active site labeling with tritiated DFP, both in the presence and absence of other competing active site reagents. The individual labeled enzymes were then identified by SDS-polyacrylamide gel electrophoresis and scintillation autoradiography.

Conditioned medium from embryonal mouse fibroblasts transformed by mouse sarcoma virus contained five serine enzymes that were not present in
medium from normal cells; two serine enzymes were released by both cell types, and one serine enzyme was found only in medium from normal cells. Two of the enzymes released by transformed cells were identified as plasminogen activators; these accounted for most of the serine enzyme labeling in transformed culture media and for most of the serine enzyme difference between normal and transformed cultures. The culture fluids from two cell strains of human neoplastic origin were examined by the same method. A rhabdomyosarcoma strain released eight serine enzymes (mol wt ranging from 22,500 to 102,000), four of which were plasminogen activators; seven serine enzymes (mol wt 26,000–102,000), including two plasminogen activators, were detected in medium from human melanoma cultures. In terms of electrophoretic mobility two of the plasminogen activators from rhabdomyosarcoma were identical with those from melanoma cultures, while the remaining two rhabdomyosarcoma activators coincided with activators found in commercial urokinase.

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