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Purification of Plasminogen Activator from Rous Sarcoma Virus Transformed Chick Embryo Fibroblasts Treated with the Tumor Promoter Phorbol 12-Myristate 13-Acetate†

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ABSTRACT: Rous sarcoma virus (RSV) transformed chick embryo fibroblasts produce a specific serine protease, plasminogen activator at levels 25–100-fold greater than nontransformed cultures. Treatment of transformed cultures with the tumor promoter phorbol 12-myristate 13-acetate (PMA) further enhances the production of plasminogen activator in the transformed cultures thereby permitting sufficient quantities of crude enzyme to be harvested for purification studies [Goldfarb, R. H., & Quigley, J. P. (1978) *Cancer Res. 38*, 4601]. The plasminogen activator harvested from the culture supernatants of PMA-treated, transformed cultures has now been purified to apparent homogeneity by multiple affinity chromatography on *p*-aminobenzamidine–Sepharose 4B, Sephadex G-100 gel filtration, and sulfopropyl cation-exchange chromatography. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicates that plasminogen activator is isolated as two enzymatically active forms. The major form is a 46 000–48 000-dalton species, sometimes detectable as a doublet on gel electrophoresis. A minor form of plasminogen activator comprising 8–12% of the total activity is present in a 26 000-dalton species. The fibrinolytic activity of both forms of the enzyme is dependent upon the presence of plasminogen in the assay system. A metabolic label ([^{3}H]leucine) is incorporated into both forms of plasminogen activator, demonstrating that the enzyme is a biosynthetic product of the transformed cells and not derived from serum. The specific inhibitor, diisopropyl fluorophosphate (DFP), inhibits the isolated enzyme, and, in addition, tritium from radiolabeled [^{3}H]DFP is incorporated into the enzyme, confirming that purified plasminogen activator is a serine protease. The employment of purified plasminogen activator to examine the catalytic role of the enzyme in malignant transformation and cellular regulation is discussed.

Proteolytic enzymes, which have the ability to stimulate cell growth, enhance cellular migration, and modify cellular and extracellular protein components, are candidates as catalytic regulatory molecules that could be responsible, directly or indirectly, for several of the phenotypic features of the malignant cell. Increased levels of proteolytic activity are often associated with malignant tumors in vivo and malignant transformation in culture [see review by Quigley (1979)]. In addition, numerous studies have shown that nontransformed cells, when treated with exogenously added proteolytic enzymes, transiently display several phenotypic properties that are characteristic of transformed cells [see review by Noonan (1978)]. Conversely, several specific protease inhibitors, upon addition to transformed cells in culture, partially restore "normal" cellular characteristics (Weber, 1975).

A role for specific proteolysis in malignant transformation has been strengthened by the demonstration of increased fibrinolytic activity associated with various transformed cell cultures (Unkeless et al., 1973; Ossowski et al., 1973a). The fibrinolytic activity was shown to be an early event in malignant transformation and dependent upon the interaction of a cellular serine protease, plasminogen activator (PA), and a normal serum component, plasminogen (Unkeless et al., 1974a; Quigley et al., 1974; Christman et al., 1977). The rapid formation of plasmin, through proteolytic activation of serum plasminogen by plasminogen activator, thus leads to further protease activity generated within the milieu of the malignant cell. Plasmin in turn has regulatory potential since this protease can alter cell surface proteins, modify cytoskeleton components, and induce cell division (Hynes, 1973; Pollack & Rifkin, 1975; Blumberg & Robbins, 1975). Plasminogen activator production and the generation of plasmin have been correlated with growth in agar (Ossowski et al., 1973b; Pollack et al., 1974), tumorigenicity of viral transformants in nude mice (Pollack et al., 1975), tumorigenicity of malignant melanoma cells (Christman et al., 1975), and a temperature-sensitive expression of the sarcoma gene product (Unkeless

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et al., 1973; Rifkin et al., 1975). In addition, transformed cell morphology (Ossowski et al., 1974), migration (Ossowski et al., 1975), and adhesion (Weber, 1975) have been reported to be dependent, in part, upon plasminogen or the conversion of plasminogen to plasmin.

Enhanced levels of plasminogen activator have also been observed in a number of normal cell culture systems including kidney cells, lung cells, activated macrophages, and trophoblast cells. In addition, studies on some tumorigenic cell lines and clonal isolates have failed to show a direct correlation between plasminogen activator levels and malignant transformation in culture [see review by Quigley (1979)]. Thus, the exact function and mode of action of the enzyme remains unclear. In order to correlate the biochemical properties of plasminogen activator with biological function, it will be necessary to examine and employ a highly purified plasminogen activator molecule and an antibody raised against it. The RSV[1]-transformed chick embryo fibroblast culture system, in which the transformed phenotype has been well characterized, in which tumor virus–tumor promoter interactions have been well studied, and for which mutants temperature-sensitive for malignant transformation exist, appears to be an ideal system for the critical analysis of the role of plasminogen activator in malignant transformation. However, the purification to homogeneity of plasminogen activator from this culture system has been prevented by several obstacles including the difficulties in solubilizing membrane-associated forms of plasminogen activator, the autocatalytic nature of the enzyme, and the extremely low amounts (~ 1 μg/L) of enzyme present in extracellular serum-free media (Unkeless et al., 1974a; Quigley, 1976). The recent finding in our laboratory that the tumor promoter, phorbol 12-myristate 13-acetate (PMA), synergistically increases plasminogen activator activity 10–12-fold above already transformed cultures and 500–1000-fold over normal, untreated cultures (Goldfarb & Quigley, 1978) has led to the accumulation of large amounts of enzyme-enriched culture fluid. This enrichment of crude enzyme in combination with modified culture conditions has surmounted the obstacles that have precluded plasminogen activator purification. This report describes the purification and partial characterization of plasminogen activator from cultures treated with PMA. The pure enzyme can now be used directly in culture and for the preparation of specific antisera in order to analyze its role in cellular regulation and malignant transformation.

Materials and Methods

Materials. Fibrinogen (65% clottable) was purchased from Miles Laboratory (Elkhart, IN). Media and sera were obtained from Flow Laboratories (Rockville, MD) or Grand Island Biological Co. (Grand Island, NY). Tissue culture ware was purchased from Falcon Plastics (Oxnard, CA), Linbro Chemical Co. (New Haven, CT), or Corning (Corning, NY). PMA was a gift of Dr. Walter Troll, New York University Medical Center, or was purchased from Dr. Peter Borchert, University of Minnesota Medical School. PMA was stored and diluted in acetone. [^{3}H]Diisopropyl fluorophosphate (DFP, 5 mCi/mL, 3.4 Ci/mmol), carrier-free [^{125}I]NaI, and [^{3}H]leucine (0.5 mCi/mL, 40 Ci/mmol) were purchased from New England Nuclear Inc. (Boston, MA) or Amersham (Arlington Heights, IL). Benzamidine, crystalline bovine serum albumin (BSA), DFP, *p*-aminobenzamidine, and carbodiimide were purchased from Sigma Chemical Co. (St. Louis, MO). *N,N,N′,N′*-Tetramethylethylenediamine (Temed), acrylamide, *N,N′*-methylenebis(acrylamide), and dimethyl sulfoxide were purchased from Eastman (Rochester, NY). Urokinase (2240 Ploug units/vial) was purchased from Leo Pharmaceutical Products (Denmark). Sepharose 4B, CH Sepharose 4B, G100 Sephadex, SP C25 Sephadex, cytochrome *c*, ovalbumin, and chymotrypsinogen were purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

A Bellco roller bottle apparatus was used with disposable, roller bottles for batch affinity chromatography. Protein determinations were performed on a Gilford spectrophotometer (Oberlin, OH) or on a Perkin-Elmer fluorescent spectrophotometer (Elmwood Park, NJ). Radioactive samples were counted in Beckman liquid scintillation counters or Packard (Downers Grove, IL) γ counters. A Vertis (Gardiner, NY) lyophilizer and Vertis lyophilization vessels were used.

Cell Cultures and Viral Infections. Primary cultures of chick embryo fibroblasts were prepared from 11–12-day-old embryos (Temin, 1960). The cultures were grown in minimal essential medium supplemented with 10% fetal bovine serum and incubated at 37 °C in an incubator containing 10% CO_2. Chick fibroblast secondary cultures were infected with the Schmidt–Ruppin strain of RSV, antigenic subgroup A or D (obtained from Dr. H. Hanafusa, The Rockefeller University), at a multiplicity of 1–5 focus-forming units/cell.

Preparation of Extracellular Plasminogen Activator. Cultures of RSV-transformed fibroblasts (second, third, and fourth passage), following initial plating, were grown in culture medium supplemented with 5% plasminogen-depleted fetal bovine serum until cells were just confluent. The cultures were changed to medium supplemented with 5% plasminogen-depleted fetal calf-serum containing PMA at 100 ng/mL (Goldfarb & Quigley, 1978). Twelve to twenty-four hours later, cultures were washed 2 times with phosphate-buffered saline ($NaCl/P_i$) and then 1 time with medium. The cultures were then incubated in serum-free medium containing PMA at a concentration of 100 ng/mL, and the harvesting schedule was initiated. During enzyme harvesting conditions, medium was removed every 8–14 h and replaced with fresh medium containing PMA (100 ng/mL). The serum-free conditioned medium is referred to as harvest fluid (HF) and is the source of extracellular plasminogen activator. HF was immediately centrifuged at 2000 rpm for 10 min at 4 °C to remove whole cells and cellular debris. The supernatants were acidified to pH 3.5 by the careful addition of 1 N HCl and then stored at −20 °C.

Enzyme Assay. The plasminogen activator assay was based on the hydrolysis of [^{125}I]fibrin, coated onto tissue culture wells. Fibrinogen, free of plasminogen, was purified according to published methods (Mosseson, 1962) and radiolabeled by lactoperoxidase-catalyzed iodination (Martin et al., 1976) or by the iodination method previously described (Goldfarb & Quigley, 1978). Plasminogen was purified from fetal bovine serum by lysine–Sepharose 4B affinity chromatography as described (Quigley et al., 1974). The [^{125}I]fibrinogen was coated on tissue culture wells (10 μg/cm^2, 500–700 cpm/μg) and dried at 45 °C for 72 h. Following drying the [^{125}I]-fibrinogen-coated wells were incubated at 37 °C for 2.5 h in medium supplemented with fetal bovine serum. Thrombin and other factors contained in the serum converted [^{125}I]fibrinogen to insoluble [^{125}I]fibrin. Each well was washed 2 times with $NaCl/P_i$ and washed an additional time just prior to the assay

[1] RSV, Rous sarcoma virus; PMA, phorbol 12-myristate 13-acetate; $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl fluorophophate; $NaCl/P_i$, phosphate-buffered saline; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; PA, plasminogen activator.

with 0.1 M Tris, pH 8.1. The assay incubation mixture contained 5–10 μg of purified plasminogen, 0.02% Triton X-100, 1 mL of 0.1 M Tris, pH 8.1, and an aliquot of the plasminogen activator containing sample. The radioactivity released from the wells was determined by direct γ counting. One unit of plasminogen activator activity is equivalent to the removal of 2000 cpm of ^{125}I in 1 h at 37 °C from wells containing ~40 000 cpm.

Determination of Protein. Protein was determined by published methods (Lowry et al., 1951) with bovine serum albumin as the standard. Fluorescamine was also used in a fluorometric determination of protein (Bohlen et al., 1973). Protein from culture fluids metabolically labeled for 2–3 days with [^{3}H]leucine (2–10 μCi/mL) was determined by precipitation of radioactivity with ice-cold 5% trichloracetic acid onto millipore filters. Dried filters were dissolved in a liquid scintillation cocktail (Omnifluor–toluene) and counted in a β counter. Protein concentration was shown to be directly proportional to the amount of acid insoluble ^3H when compared to values derved from spectrophotometric or fluorescent determinations.

Affinity Ligand. *p*-Aminobenzamidine was linked to CH Sepharose 4B by carbodiimide coupling according to the methods of Pharmacia Fine Chemicals. The ligand was reacted in a 20-fold mole excess over CH Sepharose 4B. Carbodiimide was added to a final concentration of 0. 1 M. The pH of the reaction mixture was maintained between 4.5 and 6.0 for 24 h by the addition of 1 N HCl or 1 N NaOH.

Harvest Fluid Adsorption with p-Aminobenzamidine–CH Sepharose 4B. Triton X-100 was added to HF to a final concentration of 0.5% and adjusted to pH 7.0. The HF was then adsorbed onto *p*-aminobenzamidine–CH Sepharose 4B previously equilibrated in 0.1 M sodium phosphate, 0.4 M sodium chloride, and 0.5% Triton, pH 7.0. In addition to standard column chromatography, modifications were introduced to allow for the batch adsorption of large volumes of HF. One to two liters of HF was added to disposable tissue culture roller bottles (Corning) containing 100 mL of *p*-aminobenzamidine–CH Sepharose 4B. Following 30 min of rotation (4 C) at maximum speed on a roller bottle apparatus, the beads were centrifuged at 1000 rpm for 10 min at 4 °C, and nonadsorbed material was decanted. Following decantation a new batch of culture fluid was added to the centrifuged beads and was processed as before. In this manner large volumes of HF (8–10 L) could be routinely adsorbed to 100 mL of affinity beads in 6–10 h.

Following batch adsorption, the beads were packed into a siliconized column. The column was washed with 3 column volumes of 0.1 M sodium phosphate, 0.4 M NaCl, and 0.5% Triton, pH 7.0, and then plasminogen activator was eluted in minimal volumes of 0.1 M ammonium acetate, 0.4 M NaCl, 0.5 M benzamidine, and 0.5% Triton, pH 4.0. Fractions (10 mL) were collected at a rate of 40–50 mL/h. Following dialysis of each fraction against 0.1 M sodium phosphate and 0.4 M NaCl, pH 7.0, to remove free benzamidine, enzyme activity and protein content were determined. Plasminogen activator containing fractions were concentrated by a second round of affinity chromatography. Active fractions from the first column were pooled and dialyzed further against 0.1 M sodium phosphate, 0.4 M NaCl, and 0.5% Triton, pH 7.0. The sample was then chromatographed on *p*-aminobenzamidine–CH Sepharose 4B under the previously described conditions.

Molecular Sieve Gel Chromatography. Active fractions, partially purified and concentrated by two rounds of affinity chromatography, were pooled and dialyzed against 0.05 M sodium phosphate and 0.8 M NaCl, pH 7.4. The sample was then carefully loaded onto a molecular sieving gel (G100 Sephadex) that had been packed into a siliconized column and equilibrated with the same buffer at 4 °C. The gel bed was stabilized with a G10 overlay. A small volume of sample was chromatographed on large bed volumes of G100 (e.g., 12 mL over 300 mL). Fractions of 5 mL were collected at 4 °C with flow rates of 15–25 mL/h. Plasminogen activator and protein content were determined for each fraction.

Cation-Exchange Chromatography. Plasminogen activator containing fractions from G100 Sephadex chromatography were dialyzed against 0.05 M ammonium acetate, pH 5.25, and loaded onto siliconized columns packed with the cation-exchange resin sulfopropyl-Sephadex C25 (equilibrated with 0.05 M ammonium acetate, pH 5.25, at 4 °C). After the column was washed with several column volumes of the same buffer, a stepwise elution was performed with increasing concentrations of ammonium sulfate (0.15, 0.30, and 0.50 M) buffered to pH 5.25 with 0.05 M sodium acetate. Fractions were collected at a flow rate of 25 mL/h. Enzyme activity and protein content were determined for each fraction.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide slab gels were prepared according to reported methods (Laemmli, 1970) with upper stacking gels of 4% acrylamide and lower resolving gels of 10% acrylamide. Samples were run for 3–4 h at 100 V. Gels were divided into lanes for protein staining and/or measuring plasminogen activator activity. Protein lanes were stained with 0.25% Coomassie blue in 50% methanol and 8% acetic acid for 1 h at 37 °C and destained overnight with several changes of 5% methanol and 12% acetic acid. Lanes to be assayed for enzyme activity were excised from the slab gel, soaked in three changes of 0.1 M Tris and 0.5% Triton, pH 8.1 (C. J. Scheiner, personal communication), for 1 h at room temperature, and cut into 1-mm slices. Each slice was placed in a tissue culture well containing [^{125}I]fibrin, with or without plasminogen, and assayed for plasminogen activator activity as described above.

Preparation of DFP. Commercially available DFP (5.4 M) was carefully diluted and stored in 2-propanol in tightly sealed tubes at −20 °C. [^{3}H]DFP was also diluted and stored in 2-propanol at −20 °C.

[^3H]DFP Labeling. Column-purified plasminogen activator (PA) samples were incubated with [^{3}H]DFP (100–500 μCi/mL) for 60 min at 37 °C. Control cultures were treated with propylene glycol and incubated at 4 °C. Following incubation, samples were dialyzed against 0.001% $NaDodSO_4$, lyophilized, and prepared for gel electrophoresis.

Fluorography. Fluorography of polyacrylamide gel lanes containing [^{3}H]DFP-treated purified fractions or purified fractions that contained incorporated [^{3}H]leucine were carried out exactly as described (Bonner & Lasky, 1974).

Results

Trial Affinity Chromatography. Serum-free culture fluid was harvested from PMA-treated cultures of RSV-transformed chick fibroblasts as described in a previous publication (Goldfarb & Quigley, 1978) and under Materials and Methods. Affinity chromatography was chosen as a first step in purification since it would allow the plasminogen activator present in large volumes of culture fluid to be partially purified and concentrated to small volumes, under stable, nondegradative conditions.

It was reported that *p*-aminobenzamidine competitively inhibits urokinase, the plasminogen activator produced in the kidney and excreted in the urine (Geratz & Cheng, 1975); in addition, it has been shown that a plasminogen activator se-

creted by ovarian carcinoma cells and immunologically identical with urokinase may be partially purified by *p*-aminobenzamidine affinity chromatography (Astedt & Holmberg, 1976). In order to determine whether *p*-aminobenzamidine linked to Sepharose 4B could function as an affinity ligand for the plasminogen activator from RSV-transformed fibroblasts, we conducted small-scale trial experiments.

Serum-free, harvest fluid was loaded onto a column of *p*-aminobenzamidine–CH Sepharose 4B. Ninety percent of the applied plasminogen activator activity became bound to the affinity ligand and was eluted with a buffer that contained a neutral detergent (Triton X-100) and the competitive inhibitor benzamidine buffered to pH 4.0. The presence of the detergent in all buffers was shown to be essential for optimal recovery. Only a small percentage (<10%) of the plasminogen activator activity that was adsorbed onto the column could be eluted in pH 4.0 buffer in the absence of benzamidine. This is in contrast to mammalian plasminogen activator adsorbed onto similar *p*-aminobenzamidine affinity columns (Astedt & Holmberg, 1976; Holmberg et al., 1976). The enzyme from transformed chick embryo cultures thus requires an additional elution step, incorporating both pH 4 conditions and the presence of benzamidine. In a control experiment an equal amount of harvest fluid was loaded onto a column of unlinked Sepharose 4B and eluted under identical conditions. Little or no PA was adsorbed to unlinked Sepharose 4B and was eluted in the void volume of the column.

These results indicated that *p*-aminobenzamidine serves as an efficient affinity ligand for chick plasminogen activator. However, proof of successful affinity chromatography of plasminogen activator required the demonstration that the enzyme could be separated from the bulk of contaminating proteins. Technical problems, however, were associated with the determination of protein. The dilute nature (in terms of the protein concentration) of serum-free culture supernatants, the extremely low amounts of plasminogen activator protein being purified, and the necessity of using solutions which interfere with standard protein detection methods (benzamidine and Triton), made protein quantitation difficult. These problems were overcome through the employment of relatively large amounts of starting material, extensive dialysis to remove interfering substances, and the monitoring of protein by either fluorescence spectroscopy following reaction with fluorescamine (Bohlen et al., 1973) or trichloroacetic acid precipitation of macromolecules endogenously labeled in culture with [^{3}H]leucine.

Affinity Chromatography of Plasminogen Activator from Large Volumes of Culture Fluids. It was demonstrated that *p*-aminobenzamidine–CH Sepharose 4B columns had the ability to bind large amounts of chick plasminogen activator. A column with a 20-mL bed volume had the capacity to bind >80–90% of the plasminogen activator contained in 10 L of harvest fluid (approximately 100–200 μg of enzyme). Additional experiments indicated that 50 μg of trypsin could be bound by a 1.0-mL bed volume of the same preparation of *p*-aminobenzamidine–CH Sepharose 4B. Furthermore, in contrast to another affinity ligand, L-arginine methyl ester, the CH Sepharose 4B ligated with *p*-aminobenzamidine could be reused for plasminogen activator adsorption after reequilibration of the column with the wash buffer. Thus a small affinity column has a relatively large plasminogen activator binding capacity and is also ideally suited as a concentration vehicle for large volumes of culture fluid.

Harvest fluid (9.1 L, 76.5 units of PA/mL) from PMA-treated cultures was adsorbed in batch fashion (see Materials and Methods) onto *p*-aminobenzamidine–CH Sepharose 4B. The beads containing the adsorbed material was then packed into a column, washed, and then eluted with a buffer containing benzamidine under the conditions used for the small, trial experiments. Following dialysis, fractions were pooled and assayed for enzyme activity and protein content. The results shown in Figure 1A demonstrate that the majority of the protein is not adsorbed to the *p*-aminobenzamidine–Sepharose 4B. Ninety percent of the plasminogen activator activity was recovered in the benzamidine-containing fractions which contain only 10–15% of the protein. The total recovery of plasminogen activator and protein from the column was 98% and 91%, respectively.

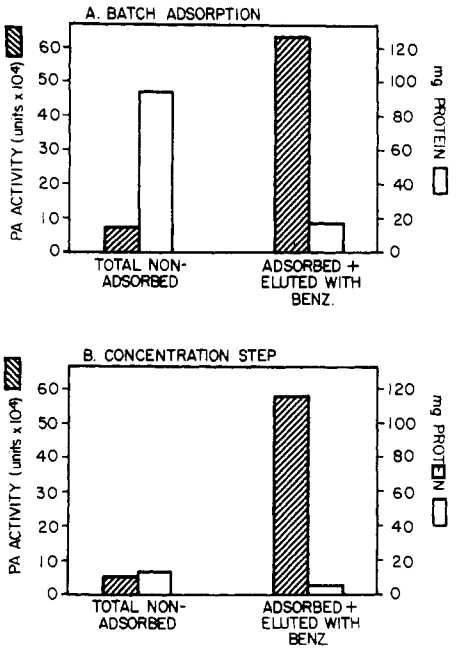

FIGURE 1: Affinity chromatography (steps 1 and 2). (A) The harvest fluid from PMA-treated RSV-transformed fibroblasts (9.1 L, 76.5 units of PA/mL) was brought to 0.5% Triton, reneutralized to pH 7.0, and batch adsorbed onto *p*-aminobenzamidine–CH Sepharose 4B as described under Materials and Methods. Following batch adsorption, the beads were packed into a siliconized 2.5 × 40 cm column and washed with 100 mL of 0.1 M sodium phosphate, 0.4 M NaCl, and 0.5% Triton, pH 7.0. The column was then eluted with 100 mL of 0.1 M ammonium acetate, 0.4 M NaCl, and 0.5% Triton, pH 4.0, containing 0.5 M benzamidine. Fractions (10 mL) were collected with a flow rate of 40 mL/h. The material which did not bind to the *p*-aminobenzamidine–Sepharose during batch adsorption and the material eluted with the pH 7.0 buffer were pooled (total nonadsorbed). The fractions eluted with the pH 4.0, benzamidine-containing buffer were also pooled. Both pooled fractions were dialyzed and assayed for plasminogen activator activity and protein content. (B) The dialyzed, plasminogen activator containing fraction from step 1 (130 mL, 4822 units of PA/mL) was concentrated by passing it a second time over *p*-aminobenzamidine–Sepharose 4B. The column was washed with the pH 7.0 buffer and then eluted with the pH 4.0, benzamidine-containing buffer as described above for step 1. Fractions were pooled as indicated and assayed for enzyme activity and protein content.

Concentration and Further Purification of Plasminogen Activator by a Second Round of Affinity Chromatography. Although the active fractions obtained by affinity chromatography (Figure 1A) represented partial purification and were concentrated from 9100 to 130 mL, this volume had to be further concentrated prior to gel filtration. Ultrafiltration on several types of filters either gave poor recoveries or resulted in enzyme aggregation. Similarly, lyophilization caused aggregation of plasminogen activator and also led to partial loss of enzyme activity by adsorption onto lyophilization vessels. In contrast, a second passage over a *p*-aminobenzamidine

Table I: Purification of Plasminogen Activator

	units of enzyme[c]	protein[c] (mg)	sp act. (units/mg)	x-fold purification
original harvest fluid	696 500 (100)	110.16 (100)	6 322	
first affinity step (benzamidine-eluted fractions)	626 860 (90)	16.36 (14.9)	38 316	6.1
second affinity step (benzamidine-eluted fractions)	578 100 (83)	5.07 (4.6)	114 023	18.03
G100[a] chromatography	318 530 (46)	0.31 (0.28)	1 027 516	162.0
ion-exchange[b] chromatography	296 233 (43)	0.07 (0.06)	4 231 900	669.0

[a] Yield of enzyme and protein from G100 Sephadex column is based on pooled fractions 35–47 (Figure 2). [b] Yield of enzyme and protein from SP Sephadex column is based on pooled 0.15 and 0.30 M elution fractions (Figure 3). [c] Percent yield in parentheses.

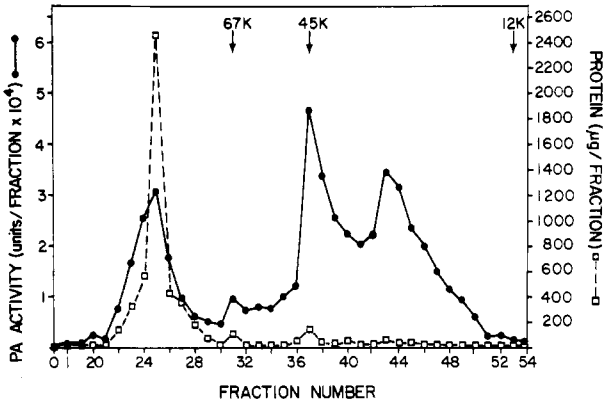

FIGURE 2: G100 Sephadex chromatography (step 3). The plasminogen activator containing fractions concentrated by affinity chromatography (Figure 1B, 12 mL, 48175 units of PA/mL) were dialyzed against 0.05 M sodium phosphate and 0.8 M NaCl, pH 7.4, and chromatographed on a siliconized 2.5 × 83 cm column containing G100 Sephadex equilbrated and standardized in the same buffer. Fractions (5 mL) were collected at a flow rate of 25 mL/h by using the same buffer. Each fraction was assayed for enzyme activity by the [^{125}I]fibrin plate method and for protein determination by fluorescence spectroscopy as described under Materials and Methods. The column had previously been standardized with BSA (67K), ovalbumin (45K), and cytochrome *c* (12K).

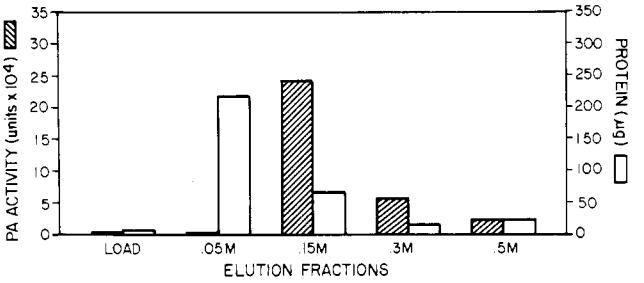

FIGURE 3: Ion-exchange chromatography (step 4). The plasminogen activator containing fractions from the G100 column (Figure 2, fractions 35–47, 26 mL, 12251 units/mL) were pooled, dialyzed against 0.05 M ammonium acetate, pH 5.25, and loaded onto a 16 × 1.5 cm column containing sulfopropyl Sephadex, equilibrated with the same buffer. The column was washed with 50 mL of the same buffer and then eluted in succession with 50 mL of 0.15, 0.30, and 0.50 M ammonium sulfate, pH 5.25. Fractions were pooled and assayed for enzyme activity and for protein by fluorescence spectroscopy.

affinity column, proved to be a rapid, gentle, specific, and efficient method for plasminogen activator concentration in preparation for gel filtration.

The active fractions from the first affinity column (130 mL, 4822 units of PA/mL) were concentrated by a second round of *p*-aminobenzamidine affinity isolation. The results shown in Figure 1B demonstrates that 92% of the recovered plasminogen activator (85% recovery) was eluted with benzamidine. The active fractions were concentrated from 130 to 12 mL. The results also demonstrate that the enzyme was further purified, albeit slightly, by this concentration step since only 31% of the recovered protein was associated with the active fractions. The yield of protein at this step was 4.6% based upon the starting culture fluid (Table I).

Gel Filtration. The fractions containing concentrated, partially purified plasminogen activator from the second affinity chromatography (12 mL, 48175 units of PA/mL) were dialyzed and chromatographed on a G100 Sephadex column that had been standardized with protein markers. A number of trial experiments employing different buffers and salt solutions indicated that the high-salt containing buffer was essential for optimal gel filtration and recovery of enzyme activity. Following G100 Sephadex chromatography, an aliquot of each fraction was assayed for plasminogen activator activity and for protein content. The results are shown in Figure 2. Fractions 35–47 contained 55% of the recovered activity and only 6.1% of the recovered protein. The protein distribution also indicates that 88% of the recovered protein is in the void volume (fractions 22–28). The void fractions also contained 23% of the recovered plasminogen activator activity. The peak fractions of enzyme activity (fraction 37 and fraction 43) had calculated molecular weights of 45 000 and 28 000. The total recovery of plasminogen activator from the column was 90% while the amount of activity present in the two peak regions represented a recovery from the starting harvest fluid of 46%. The recovery of protein from the column was 90%, and the amount of protein present in the pooled peak regions represented 0.28% of the starting protein present in the harvest fluid (Table I).

$NaDodSO_4$ gel electrophoresis demonstrated that each peak fraction contained both 45 000- and 26 000-dalton forms of plasminogen activator (results not shown). The peak fractions were therefore pooled for further purification. Gel electrophoresis also confirmed that a large amount of contaminating protein was found in the void fractions. The plasminogen activator present in the void fractions (23%) was either aggregated or nonspecifically stuck to contaminating proteins. Subsequent experiments have shown that when the enzyme activity contained in the void fractions is rechromatographed on G100 Sephadex columns, ~50% of the applied activity now becomes included in the column and chromatographs in the 45 000–50 000 and 25 000–30 000 molecular weight regions (results not shown).

Cation-Exchange Chromatography. Ion-exchange chromatography was employed as the next purification step. Cation-exchange chromatography had been reported as a partial purification step for plasminogen activator (Unkeless et al., 1974a; Christman & Acs, 1974). The active fractions (35–47) from the G100 Sephadex column (26 mL, 12251 units of PA/mL) were dialyzed and applied onto a sulfopropyl cation-exchange column. The column was then eluted with a stepwise gradient of increasing ammonium sulfate (Figure 3). The bulk of the contaminating protein (69%) and only a small amount of enzyme activity (1%) were eluted at low

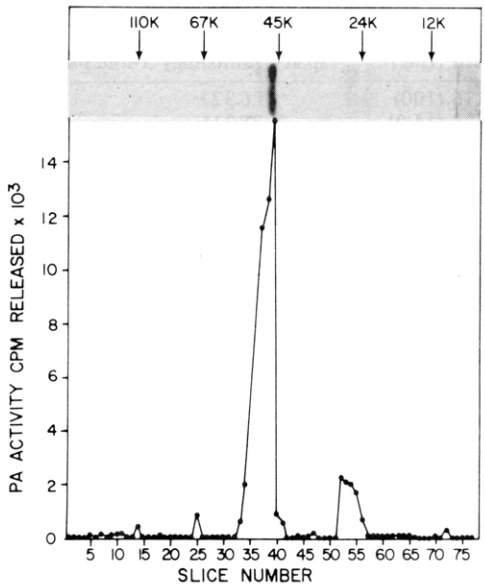

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of plasminogen activator following the complete purification scheme. The active fractions isolated in 0.15 and 0.30 M ammonium sulfate elution from the ion-exchange column (Figure 3) were pooled, dialyzed against 0.001% $NaDodSO_4$ at 4 °C, lyophilized to dryness, run on a slab gel, and stained for protein. An enzymatically active sample from the same pool was electrophoresed in an adjacent lane on the slab gel, sliced, and assayed for plasminogen activator activity as described under Materials and Methods.

ionic strength, whereas 76% and 17% of the recovered plasminogen activator activity and 21% and 5% of the recovered protein was eluted at 0.15 and 0.30 M ammonium sulfate, respectively. The final yield of enzyme at this step was 43% and that of protein was 0.06%, based on the starting culture fluid (Table I). Since $NaDodSO_4$ gel electrophoresis showed that the stained protein profile was similar for both the 0.15 and 0.30 M fractions and since the specific activity of plasminogen activator in each fraction was similar (results not shown), the fractions were combined for subsequent analysis. The plasminogen activator eluted under the above described conditions was stable for at least 6 months when stored at either −20 or −70 °C.

$NaDodSO_4$ Gel Electrophoresis Analysis of Plasminogen Activator. Active fractions isolated in 0.15 and 0.30 M ammonium sulfate from the ion-exchange column (Figure 3) were pooled, extensively dialyzed at 4 °C against 0.001% $NaDodSO_4$, lyophilized, and subjected to gel electrophoresis. The determination of plasminogen activator activity in the gel was performed as described under Materials and Methods. The results in Figure 4 show two Coomassie blue stainable regions, one at 46 000–48 000 daltons and another at 23 000–26 000 daltons; the enzyme activity profile demonstrated a major plasminogen activator activity of 46 000–48 000 daltons and a minor activity of 23 000–26 000 daltons. Stained protein bands consisting of distinct doublets were resolved in both the 46 000–48 000-dalton and the 23 000–26 000-dalton regions of the gel (Figure 4). One of the stained bands in the 46 000–48 000 dalton region doublet was completely coincident with the major enzymatic activity (46 000 daltons) whereas the less distinct doublet band (48 000 daltons) was congruous with a shoulder on the activity profile peak (Figure 4). The recovery of enzyme activity for the gel shown in Figure 4 was 82%; 76% of the recovered plasminogen activator was under the major peak whereas 12% was under the minor peak. The one-slice peaks of enzymatic activity observed at 110 000 and 70 000 daltons may not be significant and only represent minor fluctuations in the [^{125}I]fibrin assay. However, they may also represent trace amounts of other multiple forms of plasminogen activator (see Discussion). The exact coincidence of enzyme activity with the only stainable bands indicated that plasminogen activator had been purified to apparent homogeneity in two molecular weight forms.

Further Studies on Purified Forms of Plasminogen Activator. It was important to determine whether the isolated plasminogen activator is a biosynthetic product of the transformed cultures and not a contaminating plasminogen activator adsorbed by the cells from the fetal calf serum used in the growth of the cultures. Plasminogen activator was therefore isolated from cultures that had been grown in the presence of [^{3}H]leucine until cellular protein had reached constant specific activity. The enzyme purified from 7.5 L of culture fluid from cells endogenously labeled with [^{3}H]leucine is shown in Figure 5A. The results show two stainable bands of protein at 48 000 and 26 000 daltons, and the activity profile shows major plasminogen activator activities at 48 000 and 26 000 daltons and is similar to the results shown in Figure 4. In this preparation, a doublet at 48 000 daltons was not resolved, although a shoulder on the activity profile was clearly evident. The results in Figure 5A also show that the enzymatic activity of the purified protease is completely dependent upon the presence of plasminogen in the assay (open squares). The recovery of enzyme activity from the gel was 88%. Of the recovered activity, 81% was under the major peak and 8% was under the minor peak. Fluorographic analysis of this gel shows that [^{3}H]leucine, used to metabolically label the cultures was indeed incorporated into both the 48 000- and 26 000-dalton forms of the enzyme (Figure 5B). The results show that the metabolic label is exactly coincident with the stained bands of plasminogen activator.

The specificity of the purified plasminogen activator for different types of plasminogen was also used as a test for the origin of the enzyme. It has been reported that plasminogen activator from RSV-transformed chick fibroblasts has the capacity to activate both chicken and bovine plasminogen, whereas mammalian plasminogen activators cleave only the latter type of plasminogen (Quigley et al., 1974). The purified chick enzyme was therefore compared to crude bovine kidney cell plasminogen activator for specificity in activating chicken and bovine plasminogen. The results showed that the purified chick enzyme activates both chicken plasminogen and bovine plasminogen, whereas the plasminogen activator derived from the bovine cultures cleaves only the bovine zymogen (results not shown). Therefore, the purified plasminogen activator was not a bovine enzyme derived from the fetal bovine serum employed in the culture medium but is indeed an avian enzyme and a biosynthetic product of the RSV-transformed cultures.

That the isolated plasminogen activator is a serine protease was ascertained by employing DFP, the active-site titrant of serine proteases. The enzymatic activity of the purified enzyme is irreversibly inhibited by DFP to >98%. Furthermore, fluorographic analysis of purified plasminogen activator that had been incubated with [^{3}H]DFP prior to $NaDodSO_4$ gel electrophoresis showed that the two molecular weight forms of the enzyme incorporated [^{3}H]DFP (Figure 6). The 26 000-dalton form of plasminogen activator incorporated substantially less DFP than the 46 000–48 000-dalton forms of the enzyme; this is consistent with the finding that the lower molecular weight form of plasminogen activator has less enzymatic activity than the higher molecular weight form.

Comparison of Purified RSV-Transformed Chick Fibroblast Plasminogen Activator Activity with Urokinase. The

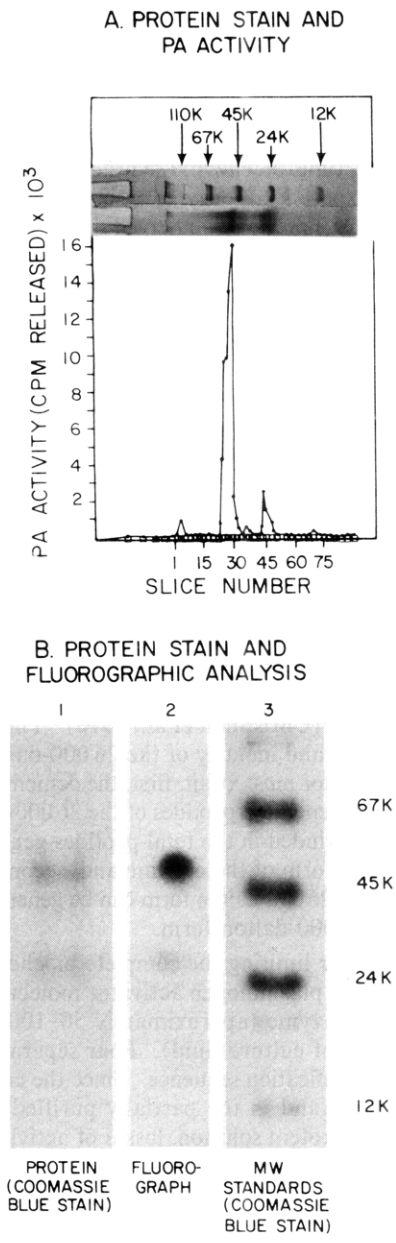

FIGURE 5: $NaDodSO_4$ gel electrophoresis of plasminogen activator isolated from [^{3}H]leucine-labeled cultures. (A) Harvest fluid from [^{3}H]leucine-labeled, PMA-treated, RSV-transformed cultures was processed for plasminogen activator activator purification as described in the text. The active fractions isolated in 0.15 and 0.30 M ammonium sulfate from an ion-exchange column were pooled, dialyzed against 0.001% $NaDodSO_4$ at 4 °C, lyophilized to dryness, run on a slab gel, and stained for protein. An enzymatically active sample of the same pool was electrophoresed in an adjacent lane on the same slab gel, sliced and assayed for plasminogen activator activity (closed circles) as described under Materials and Methods. The open squares indicate the activity present when the assay is performed in the absence of added plasminogen. (B) An aliquot of the exact same plasminogen activator preparation shown in (A) was prepared for gel electrophoresis as described above. The gel was then stained and processed for fluorographic analysis of ^{3}H-containing protein as described under Materials and Methods. The stained, dried, and processed gel lane containing the purified plasminogen activator is shown in lane 1. The corresponding fluorograph is shown in lane 2 and demonstrates that [^{3}H]leucine was incorporated into the two molecular weight forms of plasminogen activator. The stained, dried, gel lane containing the standard proteins is shown in lane 3 for molecular weight reference.

plasminogen activator assays always included a known amount of urokinase standard (0.22 Ploug units). The slight, day-to-day variations in the [^{125}I]fibrin assay system could be controlled in this way, and thus normalized values were obtained in calculating yield and recoveries from each of the sequential steps of the purification. Although the enzyme units reported (Figures 1–3 and Table I) are based on the amount of [^{125}I]fibrin released, the use of standard urokinase in the assay allows for the calculation that 1 Ploug unit equals 27.26 reported units. The specific activity of the purified plasminogen activator is therefore 155 237 Ploug units/mg of protein which corresponds to 222 050 CTA units/mg of protein or 231 288 International units/mg of protein.

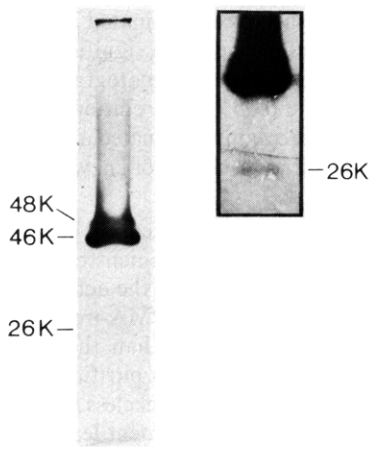

FIGURE 6: Fluorographic analysis of purified plasminogen activator that had been incubated with [^{3}H]DFP. Purified plasminogen activator was incubated in 1 mL of 0.1 M Tris, pH 8.1, containing 500 μCi of [^{3}H]DFP (0.15 mM DFP) for 1 h at 37 °C. Following incubation, nonradioactive DFP was added to bring the final concentration to 10 mM DFP. The sample was then dialyzed against 0.001% $NaDodSO_4$ at 4 °C, lyophilized to dryness, and separated by $NaDodSO_4$ gel electrophoresis. The resulting gel was processed for fluorographic analysis of ^{3}H-containing proteins as described under Materials and Methods. The gel lane shown represents a 30-day fluorographic exposure time with the 48 000- and 46 000-dalton forms of plasminogen activator clearly discernible while the 26 000-dalton form of the enzyme is barely detectable. The inset is a magnification of the 20 000–50 000-dalton region of the fluorographic analysis following a 90-day exposure of the gel and substantiates that the 26 000-dalton form of plasminogen activator incorporated [^{3}H]DFP.

Discussion

The purification of plasminogen activator from RSV-transformed fibroblasts represents the first step in a planned attempt to use the purified molecule to examine the biochemical role of the enzyme in the manifestation of the transformed phenotype in the chick fibroblast system. The purification of the enzyme from tumor promoter-treated, transformed cultures has been accomplished by multiple affinity chromatography, gel filtration, and ion-exchange chromatography. Analysis by $NaDodSO_4$ gel electrophoresis has demonstrated that the isolated plasminogen activator is composed of enzymatically active, multiple molecular weight forms.

Affinity chromatography was optimal as the first step in purification since large volumes of crude material could be concentrated to small volumes under stable conditions (low pH and in the presence of the inhibitor, benzamidine). Although the binding of plasminogen activator to the affinity ligand *p*-aminobenzamidine appears to be specific, the elution of enzyme from the ligand is far from being a "one-step" purification, since other contaminating proteins are eluted with benzamidine. The employment of a second affinity adsorption and elution procedure further reduces the contamination but leads only to an ~95% pure molecule (Figure 1B and Table I). Gel filtration functioned best as the second method of the purification sequence since comtaminants of both high and

low molecular weight were eliminated by this procedure (Figure 2) and therefore yielded a significant enrichment of PA (Table I). Ion-exchange chromatography completed the purification since the 0.05 M salt elution fraction from the sulfopropyl Sephadex column eliminated a substantial amount of protein and only trace amounts of enzyme activity (Figure 3).

The results of the overall purification of plasminogen activator as summarized in Table I demonstrate that the enzyme has been purified ~700-fold over the activity present in the crude harvest fluid obtained from PMA-treated cultures. This enrichment is substantially less than the 2600-fold purity previously reported for a partially purified plasminogen activator from untreated cultures (Unkeless et al., 1974a). This is most likely because PMA treatment leads to a 10–20-fold enhancement of plasminogen activator in the starting culture fluids (Goldfarb & Quigley, 1978), and, in addition, our use of complete serum-free harvesting conditions leads to a concomitant decrease in the total protein content of culture fluids. Therefore, since our starting culture fluid is substantially enriched in enzyme and also reduced in contaminating serum proteins, the final 700-fold purification is not an unreasonable figure. It should also be noted that when acid precipitation of macromolecules containing endogenously incorporated [^{3}H]leucine was used as a more sensitive assay for monitoring protein, the purification of plasminogen activator was 2000-fold (results not shown). We therefore conclude that the overall purification of plasminogen activator from PMA-treated, RSV-transformed fibroblast cultures is between 700- and 2000-fold.

The results of gel electrophoresis indicated that the isolated enzyme consists of several catalytically active molecular weight forms. The results shown in Figures 4 and 5, plus the results of five additional purification runs (data not shown), have led to the following conclusion: Pure plasminogen activator isolated from transformed chick cells exists as two enzymatically active molecular weight forms of approximately 46 000 and 26 000 daltons. In some experiments (Figure 4 and unpublished observations) two distinct protein bands with enzymatic activity may be resolved at 46 000 and 48 000 daltons. Similarly, the 26 000-dalton form is at times resolvable into distinct bands, each of which has some enzymatic activity. Occassionally, plasminogen activator activity, with concomitant stained protein, is also detected at 39 000 daltons, and enzyme activities with molecular weight forms >50 000 (i.e., 70 000 and 110 000 daltons) have also been detected. Although we have been unable to consistently detect the latter forms, they may represent high molecular weight precursor forms or unstable forms of plasminogen activator.

Although some plasminogen activators have only a single, predominant molecular weight form, such as the enzyme purified from SV40 transformed hamster cells (50 000 daltons) (Christman & Acs, 1974), it is clear that for many cell types, plasminogen activators can indeed exist in various molecular weight species. Multiple forms of plasminogen activator exist for urokinase (White et al., 1966; Soberano et al., 1976), the enzyme from ovarian carcinoma cells (Holmberg et al., 1976; Astedt & Holmberg 1976), pig heart (Cole & Bachmann, 1977), cultured human pancreatic carcinoma cells (Wu et al., 1977), mouse macrophages (Unkeless et al., 1974b), and human rhabdomyosarcoma cells (Dano & Reich 1978). It is suggested in many of the above studies that the multiplicity of electrophoretic forms of plasminogen activator might arise by progressive, limited proteolysis of a high molecular weight precursor.

The 26 000-dalton form of purified plasminogen activator described in this report may result from limited proteolysis of either the 46 000-dalton form of the enzyme or a large molecular weight precursor which has not yet been consistently detected. This proteolytic processing may occur either within the cells at the time of enzyme synthesis, during cellular processing and export of the enzyme, or during the purification procedure. We have previously observed that the 26 000-dalton form of the enzyme comprises only 1–4% of the total plasminogen activator activity of crude culture fluids (Goldfarb & Quigley 1978). The 26 000-dalton form of the purified plasminogen activator, however, represents as much as 8–12% of the total enzymatic activity (Figures 4 and 5), suggesting that the low molecular weight form is generated, at least in part, during the purification procedure.

It is also possible that the 26 000 dalton-species of plasminogen activator may be the product of a separate gene. Vetterlein et al. (1979) reported that several molecular weight forms of plasminogen activator can be produced by human cells in culture, and only some of them exhibit cross-reactivity to anti urokinase IgG. It has also been reported that two antigenically distinguishable hamster plasminogen activators can be produced in the same culture, and it was suggested that they represented separate gene products under different regulatory constraints (Christman et al., 1978). The final proof of both the nature and identity of the 26 000-dalton form of plasminogen activator must await, first, the demonstration that all proteolytically generated peptides of the 26 000-dalton form overlap and are included in the total peptides generated from the 46 000-dalton form of the enzyme and, second, the demonstration that the 26 000-dalton form can be generated in vitro from the pure 46 000-dalton form.

The major factor limiting the complete biochemical characterization of the plasminogen activator molecule has been the low yield of enzyme (approximately 50–100 μg of pure enzyme per 10 L of culture fluid). Four separate steps are required in the purification sequence. Since the enzyme, both in the crude form and in the partially purified form, is in extremely dilute protein solution, losses of activity are often encountered during the purification due to nonspecific adsorption, dilution denaturation, and autodigestion. A single-step purification employing an anti plasminogen activator IgG covalently linked to a solid support would be extremely beneficial in acquiring sufficient amounts of purified enzyme in a relatively short period of time. Therefore, the pure plasminogen activator isolated by the above described procedures will now be used for the production of an anti plasminogen activator antisera. An antisera against purified plasminogen activator could also be used for examining the subcellular processing of the enzyme, for detecting precursor zymogens that share molecular domains with the active enzyme, and for immunoprecipitating newly synthesized plasminogen activator, thereby allowing for cell-free protein synthesis studies. If the anti plasminogen activator IgG also inhibits the catalytic activity of the enzyme it could be used to determine the effect of plasminogen activator on the various manifestations of the malignant phenotype.

Although complete biochemical characterization of the enzyme in terms of its structural and catalytic properties is far from complete, the availability for the first time of pure plasminogen activator isolated from RSV-transformed chick fibroblasts should allow for direct studies on the function and role of the enzyme in the chick culture system. Since purified proteases exogenously added to normal cells have long served as models for putative malignant cell proteases [see review by

Noonan (1978)], it will now be possible to directly test the catalytic and functional potential of purified chick plasminogen activator as an endogenous, malignant cell protease. The ability of a purified, endogenous protease, which is elevated upon transformation, to induce pleiotropic effects in chick cultures will be a more critical test of the relationship between enhanced protease activity and the transformed phenotype.

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