ASSOCIATION OF A PROTEASE (PLASMINOGEN ACTIVATOR) WITH A SPECIFIC MEMBRANE FRACTION ISOLATED FROM TRANSFORMED CELLS

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ABSTRACT

The intracellular distribution of a specific protease, plasminogen activator (PA), has been examined in Rous sarcoma virus-transformed chick embryo fibroblasts (RSV-CEF). Cellular homogenates were fractionated by differential centrifugation followed by sucrose gradient centrifugation. The activities and the percent distribution of a series of marker enzymes, specific for different subcellular organelles, were compared to those of PA. Normal CEF have been similarly fractionated and the relatively low amount of PA activity present in these cells has been analyzed in terms of its subcellular distribution.

A membrane fraction was isolated from the RSV-CEF that contained the bulk of the PA activity and less than 8% of the total cellular protein. The specific activity of the PA in this fraction is 40-fold higher than that of a comparable fraction isolated from companion cultures of normal cells. This fraction contains little or no nuclear and cytoplasmic material and is contaminated only to a relatively small degree with mitochondria, lysosomes, and endoplasmic reticulum. Significant amounts of a putative Golgi membrane marker are present in this fraction. The relatively high specific activities of Na⁺,K⁺-ATPase, 5'-nucleotidase, and [³H]fucose indicate that the fraction is enriched in surface membrane. Further purification of the fraction by equilibrium centrifugation on shallow sucrose gradients reduces further the contaminating activities and results in a PA distribution that closely parallels the distribution of the membrane enzyme, 5'-nucleotidase. PA was not released from its membrane association by hypotonic and hypertonic extraction and ultrasonication, while granule-bound enzymes were released by these treatments. The PA activity from hamster SV40 cells fractionated the same way as that of RSV-CEF. These results suggest that a protease that is dramatically enhanced upon malignant transformation is associated with "plasma membrane-like" elements of the cell and may serve as an intrinsic modifier of cell surface proteins after malignant transformation.
Numerous in vitro studies have also indicated that proteases are involved in cellular alterations that are related to growth control and malignant transformation (see reference 49). Although the exact function and mechanism of the protease(s) with respect to specific cellular alterations are unclear, many of the effects of the proteolytic activity appear to reside at the cell surface. Thus, when exogenous proteases are added to cultures of normal cells, alterations in the growth rate, adhesiveness, and morphology of the cells are observed (5, 9, 13, 29, 37, 53, 58); conversely, when specific protease inhibitors are added to cultures of transformed cells, the opposite effects on the cells' growth rate, agglutinability, adhesiveness, and morphology are observed (21, 22, 52, 64); and finally, specific polypeptide alterations, temporally related to the onset of malignant transformation, have been detected on the surface of transformed cells (10, 25, 27, 28, 62, 65). It has been inferred from these studies that proteolytic activity may be enhanced in cultures of transformed cells and may be involved in both the initiation and the uninterrupted maintenance of characteristic changes on the surface membrane of the malignant cell. If transformation-dependent proteases have such a function, they must either be secreted by the transformed cells and have catalytic access to the cell surface and/or be an intrinsic part of the surface membrane and catalytically function within the cell surface domain.

The secretion of a specific protease that can function as a plasminogen activator (PA) has been shown to be substantially increased in cultures of transformed cells (41, 48, 60). There are many characteristics of this protease which make it an attractive candidate as a malignant cell-specific protease: (a) its appearance is closely correlated with, and is an early event in malignant transformation (16, 23, 41, 44, 60, 61); (b) total activity is substantially elevated (10-100-fold) in tumor virus-transformed primary cultures, in primary cultures of tumors, in chemically induced tumors, and in tumor cell lines including human tumor cell lines (32, 41, 50, 60, 67); (c) the PA also functions at neutral pH under physiological conditions and is actively released by transformed cells (41, 60, 61) and therefore has catalytic access to the cell surface; and (d) it activates serum plasminogen to plasmin, thereby generating additional proteolytic activity in serum-supplemented cultures (39, 47, 61). The generated plasmin is also a neutral protease that is capable of stimulating cell division (5), modifying cell surfaces (5, 27), and enhancing cell migration (40). A correlation between PA activity, plasmin generation, and malignant transformation has been demonstrated by a number of laboratories (15, 23, 32, 44, 48, 67).

Much of the work on the characterization of transformation-dependent PA, however, has dealt with the extracellular, secreted form of the enzyme. The nature of the cell-associated form of the enzyme has been examined in only a few studies. Unkeless et al. (61) have reported that cell-associated PA was granule bound in a crude lysosomal fraction. Christman et al. (16) reported that PA is membrane-associated, possibly surface membrane associated. The finding of a surface membrane-associated form of the enzyme would have far-reaching implications regarding the cell surface alterations that accompany malignant transformation and the possible role of an intrinsic membrane protease in mediating these alterations, either as a protease itself or as a zymogen activator.

The purpose of this report is to examine quantitatively the subcellular distribution of cell-associated PA in both transformed and normal cells under various conditions in order to determine directly whether a membrane-associated form of the protease does exist in cultured cells. A preliminary report of this work has appeared in abstract form (45).

MATERIALS AND METHODS

Cell Cultures

Primary cultures of chick embryo fibroblasts (CEF) were prepared from 11-12-day embryos (57). The cultures were maintained and propagated in Eagle's minimal medium supplemented with 10% (vol/vol) fetal bovine serum. Hamster SV40 cells were obtained from L. Ossowski (The Rockefeller University, New York) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Fresh media and sera were added to cell cultures every 2 days.

Virus Infections

Chick embryo fibroblast secondary cultures were infected with Schmidt-Ruppin Rous sarcoma virus (RSV)
Subcellular Fractionation and Isolation of Membranes

Subconfluent to confluent cultures of RSV-CEF (5–15 × 10^6 cells/100-mm petri dish) were washed twice with ice-cold phosphate-buffered saline. The cells (1–5 × 10^9) were removed from the culture dishes by scraping in phosphate-buffered saline minus Ca^{2+} and Mg^{2+} containing 1 mM EDTA. The scraped cells were washed twice by centrifugation in isotonic saline-EDTA (1 mM). The washed cell pellet was resuspended in 10 vol homogenizing medium (0.25 M sucrose-10 mM Tris-1 mM EDTA, pH 7.4) and homogenized in a tight-fitting Dounce homogenizer. Homogenization was stopped when 80–100% of the cells appeared broken as judged by phase microscopy (approximately 25–50 strokes). An aliquot of the homogenate was removed and saved for enzymatic analysis, and the remainder was centrifuged at 600 g for 8 min. The supernate was set aside and the pellet was resuspended in 5 vol homogenizing medium and was referred to as the nuclear fraction. The washing steps are essential to remove significant amounts of cytoplasmic and membranous elements which are present in the first low-speed pellet. The combined post-nuclear supernates were centrifuged at 100,000 g for 90 min at 37°C and terminated by the addition of 0.1 ml of 100 μM NaN_3 and 5–25 μg protein. The reaction was carried out at 37°C in a 0.5-ml mixture containing 4 mM ATP, 5 mM MgCl_2, 120 mM NaCl, 20 mM KCl, 25 mM HEPES buffer (pH 7.4), 0.1 mM EGTA, 5 mM NaN_3 and 5–25 μg protein. The reaction was averaged and inorganic phosphate was measured as described above. Na^+, K^+-ATPase was calculated as the difference in ATPase activity in the presence and absence of 1 mM ouabain. Mg^{2+}-ATPase was calculated as the ATPase activity that was insensitive to 1 mM ouabain.

β-N-Acetylglucosaminidase (E.C. 3.2.1.30) was employed as a lysosomal marker and was assayed according to Sellinger et al. (54) as modified by Unkeless et al. (60) by using p-nitrophenyl-N-acetyl β-d glucosaminide as the substrate. The release of p-nitrophenol was measured at 410 nm. Activity units are micromoles of p-nitrophenol liberated per hour at 37°C.

Thiamine pyrophosphatase (TPPase) was used as a marker for Golgi membranes (7) and was assayed by the method of Meldolesi et al. (35). Cytochrome oxidase was used to monitor subcellular fractions for the presence of mitochondria and was assayed according to the procedure of Cooperstein and Lazarow (17) as modified by Castle et al. (11). NADH diaphorase (NADH oxidase E.C. 1.69.9.3) was used as a marker for endoplasmic reticulum (2, 24) and was assayed by the method of Avruch and Wallach (2). Lactic dehydrogenase was used to monitor subcellular fractions for the presence of soluble cytoplasmic enzymes and was assayed in a 2.0-ml incubation mixture containing 100 μM NADH, 500 μM sodium pyruvate, and 0.03 M sodium phosphate buffer, pH 7.4. Changes in absorbancy at 340 nm were recorded at 30 s intervals after the addition of sample (10–50 μg protein).

Plasminogen activator assay was based on the fibrinolysis of 125I-fibrin-coated petri dishes. Plasminogen-free fibrinogen was prepared according to the method of Mosesson (36) and iodinated according to the method of Helmkkamp et al. (26) as described by Unkeless et al. (60). The 125I-fibrinogen was coated on petri dishes (10 μg/cm², 500–700 cpm/μg) and dried at 46°C for 48 h. The fibrinogen-coated dishes were incubated at 37°C for 2.5 h in Eagle’s MEM supplemented with 5% plasminogen-free fetal bovine serum (47) in order to convert the 125I-fibrinogen to insoluble fibrin (the serum served as a source of thrombin). The petri dishes were washed twice with phosphate-buffered saline and were used within 72 h. Just before assay, the plates were washed an additional time with 0.1 M Tris (pH 8.1). Plasminogen was purified from fetal bovine serum or chicken serum by two passages over a lysine sepharose affinity column (47). The incubation mixture (1.0 ml) contained 5–10
μg of purified plasminogen, 0.1 M Tris (pH 8.1), 0.02% Triton, and an aliquot of an RSV-CEF subcellular fraction (1–10 μg protein). Aliquots were removed from duplicate petri dishes at 1, 2, and 3 h during incubation at 37°C and the radioactivity released was determined directly in an Intertechnique gamma counter (Intertechnique, Fairfield, N.J.). Activity units are cpm [3H] released per hour at 37°C. Plasminogen activator activity in CEF subcellular fractions was determined by incubating for longer periods of time (3–8 h) with greater amounts of protein (10–50 μg).

All of the above enzymes were assayed at different protein concentrations and different substrate and cofactor concentrations, and were incubated for various periods of time. The calculations of percent enzyme distribution and specific activity were based on assays carried out under conditions in which the enzyme activities were linear with time and protein concentration and independent of substrate and cofactor concentration. In addition, the enzymes were assayed at various times after the initial preparation of the cellular homogenate in order to determine the stability of enzymes upon storage at 0°C or at -20°C. The enzymes in the isolated subcellular fractions were assayed before their respective activities had significantly changed upon storage.

Protein was determined by the method of Lowry et al. (34) with bovine serum albumin as the standard. The distribution of DNA in subcellular fractions was determined in preparations from cultures grown for 2 days in [methyl-3H]thymidine (1.0 μCi/ml, 5 Ci/mmol). An aliquot of each fraction was added to ice-cold 10% trichloroacetic acid (TCA) and filtered by suction on a Millipore filter apparatus (Millipore Corp., Bedford, Mass.). The filter was washed twice with ice-cold 10% TCA and air-dried, and the radioactivity remaining on the filter was determined. The distribution of fucose-labeled glycoproteins was determined in subcellular fractions isolated from cultures grown for 16–20 h in L-[3H]fucose (1.0 μCi/ml, 3.3 Ci/mmol). The TCA-insoluble radioactivity was determined as described above.

**Electron Microscopy**

Fractions for electron microscopy were prepared according to the method of Ishikawa et al. (30). Sections were cut on a Sorvall Porter-Blum MT-2B microtome (DuPont Instruments, Sorvall Operations, Newtown, Conn.) and specimens were observed with a Siemens Elmiskop 1A electron microscope.

**Materials**

ATP, AMP, NADH, thiamine pyrophosphate, N-acetyl β-D-glucosamine, pyruvate, and cytochrome c (type III horse heart) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bovine fibrinogen was obtained from Miles Laboratories (Kankakee, Ill.), and chick embryos from Cofal-negative eggs (Spafas Inc., Norwich, Conn.). All media and sera were obtained from Grand Island Biological Co. (Grand Island, N. Y.) and all plasticware from Falcon Co. (Oxnard, Calif.). Sucrose was density gradient ultrapure from Schwarz/Mann Corp., Div. of Becton, Dickinson & Co. (Orangeburg, N. Y.). [3H]Thymidine and [3H]fucose were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). All other materials and reagents were of the best commercially available grades.

**RESULTS**

**Differential Centrifugation and Sucrose Gradient Centrifugation**

Homogenates of RSV-transformed CEF were subjected to differential centrifugation. A nuclear fraction, a total membrane plus granule fraction, and a cytoplasmic fraction were isolated. The specific activity of PA and a number of biochemical markers was determined in each isolated fraction. Table I demonstrates that >80% of the PA activity is recovered in the membrane plus granule fraction. This fraction contains approximately 30% of the total cellular protein and the bulk of the membrane- and granule-associated enzyme activities. (The enzyme markers examined in this study are listed in Materials and Methods.) Less than 10% of the PA activity is found in the nuclear fraction. This fraction contains 87% of the DNA ([3H]thymidine) and >90% of the morphologically identifiable nuclei, as determined by phase-contrast microscopy. Only 10% of the PA activity is found in a soluble cytoplasmic fraction that contains 50–60% of total cellular protein and 90% of the lactic dehydrogenase activity. The cytoplasmic fraction also contains significant amounts of TPPase and β-glucosaminidase, indicating that these enzymes are either released from membranes and granules during isolation or exist in two forms, a soluble and a particulate.

The membrane plus granule fraction (Table I) was further purified by centrifugation on a discontinuous sucrose gradient. A number of discrete bands and pellets were separated as schematically illustrated in Fig. 1. Analysis of the gradient fractions (Table II) indicates that most of the PA activity is found associated with a plasma membrane-enriched fraction (fraction B). This fraction contains 26% of the protein present in the initial membrane plus granule fraction, or approximately 8% of the total cellular protein. Of the enzymes examined, only the plasma membrane markers, 5'-nucleotidase and Na+, K+-ATPase, are significantly enriched in this fraction (>50% of the total...
**TABLE I**

*Distribution of PA Activity and Subcellular Markers after Differential Centrifugation of a Total Cellular Homogenate From RSV-CEF*

| Marker               | Nuclear fraction | Membrane + granule fraction | Cytoplasmic fraction |
|----------------------|------------------|-----------------------------|----------------------|
| Protein              | 10 ± 3           | 31 ± 2                      | 58 ± 3               |
| [3H]Thymidine        | 87 ± 5           | 13 ± 4                      | 2 ± 1                |
| Lactic dehydrogenase | 6 ± 2 (0.4)      | 3 ± 1 (0.1)                 | 91 ± 5 (1.8)         |
| 5'-Nucleotidase      | 9 ± 2 (0.7)      | 86 ± 4 (2.4)                | 9 ± 1 (0.1)          |
| Na⁺⁺-K⁺⁺-ATPase      | 15 ± 4 (0.9)     | 85 ± 5 (2.3)                | 1 ± 1 (<0.1)         |
| Mg⁺⁺-ATPase          | 4 ± 2 (0.6)      | 84 ± 7 (2.8)                | 12 ± 6 (0.4)         |
| TPase                | 6 ± 1 (0.9)      | 51 ± 3 (2.0)                | 43 ± 2 (0.9)         |
| β-D-Glucosaminidase  | 4 ± 1 (0.6)      | 76 ± 3 (2.4)                | 20 ± 3 (0.4)         |
| NADH diaphorase      | 4 ± 1 (0.8)      | 83 ± 2 (2.5)                | 8 ± 3 (0.1)          |
| Cytochrome oxidase   | 8 ± 2 (0.6)      | 92 ± 2 (2.8)                | 1 ± 1 (<0.1)         |
| PA                   | 8 ± 2 (1.0)      | 83 ± 4 (3.1)                | 10 ± 3 (0.1)         |

Fractions were isolated by differential centrifugation as described in Materials and Methods. Each fraction was assayed for protein, TCA-insoluble [3H]thymidine radioactivity, and the indicated enzymes. The values represent the percent distribution in each of the fractions of the total recovered activity. The actual recoveries, based on the original homogenate, ranged from 81% to 112% for the 11 enzymes and markers tested. The values in parentheses represent the ratio of the specific activity of the enzyme in the isolated fraction to that in the original homogenate and serve as an indicator of enzyme enrichment. The specific activities of the original homogenate were as follows: lactic dehydrogenase, 9.0 μmol/min/mg protein; 5'-nucleotidase, 0.37 μmol/h/mg protein; Na⁺⁺-K⁺⁺-ATPase, 0.30 μmol/h/mg protein; Mg⁺⁺-ATPase, 1.89 μmol/h/mg protein; TPase 0.47 μmol/h/mg protein; β-D-glucosaminidase, 0.37 μmol/h/mg protein; NADH diaphorase, 0.22 μmol/min/mg protein; cytochrome oxidase, 0.017 μmol/min/mg protein; PA, 12.7 × 10⁴ cpm/h/mg protein. The latter value is equal to 102 U/h/mg as defined originally by Unkeless et al. (61). The values in the Table are the average from three to five separate experiments in which all enzyme assays were carried out in duplicate or triplicate.

A relatively high portion (43%) of the putative Golgi marker TPase is also present in fraction B but the specific activity ratio (2.8) is significantly less than that of 5'-nucleotidase, Na⁺⁺-K⁺⁺-ATPase and PA. The mitochondrial and endoplasmic reticulum markers, cytochrome oxidase and NADH diaphorase, respectively, are enriched in fraction C. This fraction has less than 20% the PA activity of fraction B. Fraction A contains <10% of the total membrane protein and <20% of any of the marker enzyme activities. However, fraction A does contain two- to threefold more 5'-nucleotidase and Na⁺⁺-K⁺⁺-ATPase activity than PA activity. The lysosomal marker β-D-glucosaminidase is present throughout the gradient but does not exhibit a significant enrichment in any one fraction. The soluble fraction contains those components that are released from their initial membrane and granule association during gradient centrifugation and subsequent washing of the gradient fractions. This fraction exhibits a significant increase in both the lysosomal marker (β-D-glucosaminidase) and overall protein. This result suggests that breakage of lysosomal granules and release of loosely bound protein occurs during the isolation. Very little PA activity is found in the soluble fraction.

Since the use of enzymatic activities as specific markers for subcellular fractions presents a num-
### Table II

Distribution of PA and Membrane and Granule-Associated Enzymes after Sucrose Gradient Centrifugation of the Membrane + Granule Fraction

| Enzyme                  | A Band | B Band | C Band | D Pellet | Soluble |
|-------------------------|--------|--------|--------|----------|---------|
| Protein                 | 9      | 26     | 23     | 6        | 34      |
| 5'-Nucleotidase         | 12 (2.9) | 57 (5.2) | 21 (1.8) | 4 (0.6) | 6 (0.3) |
| Na⁺, K⁺-ATPase          | 16 (2.4) | 60 (5.4) | 18 (1.4) | 4 (0.8) | 3 (0.1) |
| Mg⁺⁺-ATPase             | 17 (4.5) | 55 (4.9) | 19 (2.0) | 2 (0.6) | 6 (0.4) |
| TPPase                  | 9 (1.2) | 43 (2.8) | 24 (1.4) | 4 (0.8) | 20 (0.7) |
| β-D-Glucosaminidase     | 10 (2.1) | 22 (1.5) | 17 (1.4) | 4 (1.2) | 47 (2.5) |
| NADH Diaphorase         | 5 (0.9) | 15 (1.0) | 69 (5.7) | 8 (3.0) | 3 (0.2) |
| Cytochrome oxidase      | 1 (0.1) | 14 (0.7) | 68 (4.3) | 11 (2.6) | 6 (0.2) |
| PA                      | 5 (1.9) | 73 (9.9) | 15 (2.5) | 4 (1.8) | 4 (0.5) |

The fractions were isolated from a discontinuous sucrose gradient (Fig. 1) and washed by centrifugation as described in Materials and Methods. The soluble fraction is the pooled washings of the gradient fractions. The values represent the percent distribution in each of the gradient fractions of the total enzyme activity recovered from the gradient. The actual recoveries, based on the initial membrane + granule fraction (Table I), ranged from 78% to 109%. The values in parenthesis represent the ratio of the specific activity of the enzyme in the isolated fraction to that in the original homogenate.

### Table III

Comparison of the Percent Distribution of PA and [³H]Fucose after Sucrose Gradient Centrifugation of the Membrane and Granule Fraction

| Fraction | PA       | [³H]Fucose |
|----------|----------|-----------|
| A        | 6 ± 1 (3.1) | 11 ± 1 (3.6) |
| B        | 56 ± 3 (5.0) | 59 ± 4 (5.3) |
| C        | 27 ± 1 (2.5) | 22 ± 2 (2.0) |
| D        | 6 ± 1 (2.1) | 3 ± 1 (1.4) |
| Soluble  | 5 ± 2 (0.1) | 4 ± 2 (0.3) |

Growing cultures of RSV-CEF were labeled with [³H]fucose for 16–20 h in media supplemented with 10% fetal bovine serum. The cells were harvested and subcellular fractions were isolated by differential centrifugation as described in Table I and Methods and Materials. Approximately 70–80% of the trichloroacetic acid (TCA)-insoluble [³H]fucose activity and PA activity was sedimented in the total membrane and granule fraction (8–19% of both activities was found in the nuclear fraction and 5–11% of both activities was found in the soluble cytoplasmic fraction). The total membrane and granule fraction was separated on a discontinuous sucrose gradient as described in Table II and Fig. 1. The fractions were analyzed for TCA-insoluble [³H]fucose and PA activity. The values represent percent distribution and specific activity ratio as in Table II, and are the averages from three separate experiments in which all determinations were carried out in duplicate or triplicate.

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**Isolation of Membranes under Various Conditions**

Since the above-described isolation scheme results in a selective enrichment of cell-associated PA activity in a specific membrane fraction, it was important to determine whether the association of PA activity with membranes (a) was simply due to the method of cell homogenization, or (b) unique to RSV-transformed CEF. Table IV shows the subcellular distribution of PA activity after homogenization of RSV-CEF in two distinctly different homogenizing media. Fractionation of cells homogenized in either iso-osmotic sucrose plus 477
EDTA or hypotonic Tris buffer plus MgCl₂ yields a similar enrichment of PA activity in fraction B. The subcellular fractionation of SV40-transformed hamster fibroblasts also results in selective enrichment of PA activity in fraction B (Table IV). In addition, the isolation of a majority of the cell-associated PA activity in fraction B is not altered by the growth state of the cells nor by the presence or absence of plasminogen in the growth media (results not shown). Also, when PA is assayed by the method of Troll and colleagues (8) using a fluorescent-labeled substrate, the distribution of PA is similar to the distribution data obtained when PA is assayed by the standard ¹²⁵I-fibrin plate assay (results not shown).

Electron Microscopy

Electron micrographs of the fractions obtained by sucrose gradient centrifugation are shown in Fig. 2. Fraction B (Fig. 2b) is composed of a relatively homogeneous population of distinct membrane structures, mostly in the form of vesicles, and is free of identifiable nuclei, mitochondria, and rough endoplasmic reticulum. Although some dense-staining granules are occasionally observed, fraction B does not appear to be enriched in secretory or zymogen granules. Fraction B appears morphologically similar to a plasma membrane fraction isolated from CEF by Perdue and co-workers (42, 43). Fraction C (Fig. 2c) is a heterogeneous membrane fraction composed of broken and swollen mitochondria (EDTA is present throughout the isolation) and some endoplasmic reticulum elements. This fraction does contain significant amounts of dense-staining granules. Fraction D (Fig. 2d) is also a heterogeneous membrane fraction containing fragmented and swollen mitochondria, fibrous material, and some nuclei. Fraction A (Fig. 2a), which represents less than 3% of the total cellular protein, is composed of membrane vesicles with interspersed amorphous material.

Equilibrium Centrifugation

Since fraction B is isolated from a 20–40% sucrose interface on a discontinuous gradient, it is therefore composed of membrane structures varying in buoyant density from 1.06 to 1.18 g/cm³. Further purification of fraction B on a shallow continuous sucrose gradient (Fig. 3) indicates that the equilibrium distribution of PA activity closely parallels the distribution of the membrane enzyme, 5'-nucleotidase. The two peaks of 5'-nucleotidase and PA activity are similar in buoyant density to two peaks of CEF plasma-membrane-enriched fractions previously reported by Perdue and co-workers (42, 43). The peak of enzyme activities near the bottom of the gradient may

| Table IV |
| --- | --- | --- |
| Subcellular Distribution of PA – Different Preparations of Cellular Homogenates |
| Cell source (homogenizing media) | RSV-CEF (0.25 M sucrose 1 mM EDTA) | RSV-CEF (0.01 M Tris 0.2 mM MgCl₂) | SV40 Hamster (0.25 M sucrose 1 mM EDTA) |
| --- | --- | --- | --- |
| Differential centrifugation | | | |
| Nuclear | 17 | 18 | 8 (0.7) |
| Membrane + granule | 73 | 79 | 86 (2.3) |
| Cytoplasmic | 10 | 2 | 6 (0.1) |
| Discontinuous gradient | | | |
| A | 4 | 2 | 6 (0.8) |
| B | 70 | 60 | 61 (6.8) |
| C | 23 | 25 | 10 (0.8) |
| D | 1 | 2 | 12 (0.7) |
| Soluble | 3 | 12 | 12 (0.3) |

Companion cultures of RSV-CEF were processed in parallel and the isolated cells were homogenized in the indicated medium. Subcellular fractionation was carried out as described for Tables I and II, except that each fraction was washed and resuspended in the respective homogenizing media. Values represent the percent distribution of PA activity. Fractionation of hamster SV40 cells was carried out in a separate experiment. The values in parenthesis for the hamster SV40 fractionation scheme represent the specific activity ratio as defined in Tables I and II. The specific activity of the hamster SV40 cellular homogenate is 17.2 × 10⁴ cpm/h/mg protein.
Figure 2  Electron micrographs of discontinuous gradient fractions. Fractions A–D (Fig. 2, Table II) were prepared for electron microscopy as described in Materials and Methods. a–d Represent fractions A–D, respectively. Calibration bar = 1 μm; × 25,000.
represent elements of the plasma membrane which are either aggregated with or adsorbed to nonplasma membrane structures since the activities of the mitochondrial and endoplasmic reticulum enzymes, which initially contaminated fraction B, are found in this area of the gradient (results not shown). The larger amount of PA activity, relative to 5'-nucleotidase activity, at the bottom of the gradient also indicates that PA may be associated to some extent with other membranous organelles or substructures. The lysosomal marker, β-glucosaminidase, initially present in the low amounts in fraction B, is now found in fraction 2 and fractions 5–7 (results not shown). Similar equilibrium distribution data are obtained when Ficoll or dextran gradients are employed.1

Treatment of Fraction B

In order to examine the nature of the association of PA with membranes, fraction B was subjected to a number of chemical and physical treatments and centrifuged at 100,000 g for 1 h, and the distribution of the PA and other marker enzymes was followed. Table V indicates that after hypotonic EDTA extraction, high salt extraction, or vigorous sonication, the PA activity remains sedimentable and rebands in a sucrose gradient at the initial density. The plasma membrane marker, 5'-nucleotidase, behaves similarly under the same conditions. The small amount of the lysosomal enzyme β-glucosaminidase present in fraction B serves as an internal control for the behavior of granule-bound enzymes after such treatments. Table V demonstrates that all three procedures result in a substantial solubilization of the β-glucosaminidase activity, indicating that granule-bound enzymes can be released by these treatments. In addition, 41% and 45% of the protein in fraction B are solubilized by sonication and high salt extraction, respectively, indicating that substantial amounts of protein are loosely bound or adsorbed to the isolated membranes. The sedimentation of 80–90% of the PA activity after these treatments and its subsequent rebanding on a sucrose gradient indicate that PA is firmly associated with cellular membrane components.

PA in Normal Cells

The subcellular distribution of PA activity in normal cells has also been examined. Homogenates of normal cells have only small amounts of detectable PA activity compared with homogenates of transformed cells (61). This might be due to decreased synthesis of PA, to a latent, unexpressed activity in normal cells, or to the presence of a PA inhibitor in normal cells. In the latter case, one might expect that subcellular fractionation of normal cell homogenates might yield a partially purified fraction, relatively free of inhibitors, in which the PA activity approached that of a comparable fraction from transformed cells. Therefore, the specific activity of PA in all normal cell fractions was determined and compared directly to the specific activity of PA observed in similar fractions isolated in parallel from transformed cells. Normal and transformed cells were grown under identical

1 J. P. Quigley. Unpublished observations.
TABLE V
Distribution of PA, and Membrane and Granule-Associated Enzymes in a High-Speed Supernate and Pellet after Various Treatments and Extractions of Fraction B

| Treatment                | Fraction | Protein | PA  | 5'-Nucleotidase | β-D-Glucosaminidase |
|--------------------------|----------|---------|-----|-----------------|---------------------|
| Control-homogenizing medium | pellet   | 72      | 90  | 91              | 83                  |
|                          | supernate| 28      | 10  | 9               | 17                  |
| Sonication in homogenizing medium | pellet* | 59      | 81  | 83              | 34                  |
|                          | supernate| 41      | 19  | 17              | 66                  |
| Extraction in 0.001 M EDTA | pellet   | 71      | 85  | 87              | 57                  |
|                          | supernate| 29      | 15  | 13              | 43                  |
| Extraction in 0.5 M NaCl   | pellet   | 55      | 84  | 88              | 40                  |
|                          | supernate| 45      | 16  | 12              | 60                  |

Fraction B, containing 4.8 mg protein, 390 U PA, 7.4 U 5'-nucleotidase, and 2.6 U glucosaminidase, was divided into four equal aliquots. One aliquot was diluted with homogenizing medium and served as a control. Another aliquot was diluted with homogenizing medium and was sonicated three times for 10-s duration each time. A third aliquot was diluted with distilled water containing 1 mM EDTA and homogenized in a Dounce homogenizer. The fourth aliquot was diluted with NaCl to a final concentration of 0.5 M and homogenized in a Dounce homogenizer. All four samples were centrifuged at 50,000 rpm for 1 h in an SW50.1 rotor. The resulting pellets and supernates were analyzed for protein and the indicated enzymes. The values represent the percent distribution of protein and enzyme activities between the supernate and the pellet. The recoveries based on the original fraction B were: protein (86-102%); PA (90-99%); glucosaminidase (101-137%); 5'-nucleotidase (85-90%).

* Pellet was rebanded on a discontinuous sucrose gradient, and 87% of the PA activity and 81% of the 5'-nucleotidase activity were recovered at the 20–40% sucrose interface.

conditions and harvested at near-confluent densities. Both cell types had doubled in cell number during the 20 h before harvesting. Table VI indicates that, although the PA activity in normal cells is approximately 40–50-fold lower than that of transformed cells, its subcellular distribution is nearly identical. In addition, the ratio of the specific activity of normal cell PA to that of transformed cell PA (last column, Table VI) in the isolated fractions is essentially the same as the ratio of the original homogenates.

DISCUSSION
A membrane fraction fraction has been isolated from RSV-transformed CEF that contains approximately 60% of the total cell-associated PA activity but less than 8% of the total cellular protein. The specific activity of PA in this fraction (Fraction B) is 40-fold higher than that of a comparable fraction isolated from companion cultures of normal cells grown under identical conditions. The pattern of PA distribution in isolated RSV-CEF subcellular fractions and the enrichment of PA activity in fraction B are not significantly affected by the growth state of the cells or the ionic composition of the homogenization medium. In addition, an enrichment of PA activity in a similar fraction obtained from mammalian cells transformed by a DNA tumor virus demonstrates that a membrane-associated form of PA is not unique to RSV-CEF. Fraction B contains little or no nuclear and soluble cytoplasmic material and is contaminated to a relatively small degree with mitochondria, lysosomes, and endoplasmic reticulum (Table II). The fraction contains a majority of the putative plasma membrane markers, 5'-nucleotidase, Na⁺-K⁺-ATPase, and [³H]fucose, and significant amounts of the putative Golgi marker, TPPase. Although 43% of the TPPase activity present in the initial membrane and granule fraction is recovered in fraction B, the initial membrane and granule fraction contains only 51% of the total cellular TPPase activity (Table I). Thus, only 22% of the total TPPase activity is present in fraction B.
TABLE VI
Specific Activity and Intracellular Distribution of PA in Normal Cells—Comparison with Transformed Cells

| Fraction            | Normal CEF | RSV-transformed CEF | Ratio sp act normal sp act transformed |
|---------------------|------------|---------------------|---------------------------------------|
|                     | Protein    | PA sp act* % dist     | Protein    | PA sp act* % dist     |                                             |
| I. Nuclear          |            |                      |            |                      |                                             |
| Membrane + granule  |            |                      |            |                      |                                             |
| Cytoplasmic         |            |                      |            |                      |                                             |
| % distribution      | 14 7 0.18  | 18 11 11.2 0.02      | 51 13 0.09 | 52 9 3.2 0.03       |                                             |
| sp act*             |            |                      |            |                      |                                             |
| % distribution      | 35 80 0.85 | 30 80 46.6 0.02      | 41 68 1.59 | 42 77 65.7 0.02      |                                             |
| sp act*             |            |                      |            |                      |                                             |
| % distribution      | 51 13 0.09 | 52 9 3.2 0.03       | 32 25 0.79 | 34 19 27.4 0.03      |                                             |
| sp act*             |            |                      |            |                      |                                             |
| % distribution      | 9 6 0.34  | 5 2 14.7 0.02       | 0.31       | 13.1 0.02             |                                             |
| sp act*             |            |                      |            |                      |                                             |

Subcellular fractionation was performed on parallel cultures of CEF and RSV-CEF as described in Materials and Methods and Tables I and II, except that the gradient fractions were not washed.
* Specific activity is expressed as cpm \(25^1/h/mg\text{ protein} \times 10^{-4}\).

compared with 50-60% of the total 5'-nucleotidase, Na\(^+\), K\(^+\)-ATPase, \[^3\H\]fucose, and PA activities. The relatively high specific activities of Na\(^+\), K\(^+\)-ATPase, 5'-nucleotidase, and \[^3\H\]fucose further indicate that fraction B is enriched in plasma membrane. The fraction also appears to be enriched in Golgi membranes but the specific activity enrichment of TPPase is not so great as that obtained for 5'-nucleotidase, Na\(^+\), K\(^+\)-ATPase, \[^3\H\]fucose, and PA (Tables II and III). It should also be noted that TPPase has been shown to be a marker for Golgi membrane only in noncultured cells (7) and that its use as a Golgi marker in this study is based on inference. On the other hand, 5'-nucleotidase and Na\(^+\), K\(^+\)-ATPase have been reported to be plasma membrane markers in cultured CEF (4, 43). Mg\(^++\)-ATPase is also enriched in fraction B, but the ubiquitous nature of this enzyme activity in many membrane fractions precludes the use of it as a marker for specific membranes. The activity of this enzyme is measured only as a necessary part of the Na\(^+\), K\(^+\)-ATPase assay.

Further purification of the PA activity in the B band by equilibrium gradient centrifugation (Fig. 3) results in a PA distribution that closely parallels the distribution of the membrane enzyme, 5'-nucleotidase. The equilibrium centrifugation also separates the remaining contaminating activities of NADH diaphorase and cytochrome oxidase from the bulk of the PA and 5'-nucleotidase activities. Although the lysosomal marker \(\beta\)-D-glucosaminidase can be only partially separated from the PA activity by equilibrium centrifugation, the pattern of distribution in the gradient is distinctly different for the two enzymes. These data in conjunction with data on the distribution of the lysosomal marker after differential centrifugation (Table I), its distribution after discontinuous sucrose gradient centrifugation (Table II), the low incidence of morphologically identifiable lysosomes in fraction B (Fig. 2), the lack of a significant enrichment of \(\beta\)-D-glucosaminidase in fraction B compared to PA (Table II), and the different behavior of the two enzymes after various treatments (Table V) indicate that PA is not a lysosomal enzyme.

These data indicate that a transformation-dependent, neutral serine protease is firmly associated with specific cellular membranes. Although the data do not completely distinguish between plasma membranes and Golgi membranes or heretofore undefined membranes as the specific PA-associated membrane, the marker enzyme analysis and the observed behavior of the membrane-associated PA do indicate that PA is not associated to any great extent with the nucleus, mitochondria, endoplasmic reticulum, or lysosomes. The distribution and behavior of PA activity do appear closely to parallel those of the putative plasma membrane marker, 5'-nucleotidase. This observation, however, could be subject to a number of interpretations. The use of 5'-nucleotidase as a definitive plasma membrane marker has been questioned by a number of workers (12,
It appears that in some cell types 5'-nucleotidase is not necessarily unique to the plasma membrane and does not exactly parallel the distribution of true plasma membrane markers such as specific surface receptors (12). In fact, in some cell types it has been shown that 5'-nucleotidase is found on Golgi membranes (19, 33). However, all these studies have dealt with the 5'-nucleotidase from fat cells, liver cells, and HeLa cells. In the case of cultured CEF, which is the cell type used in the present study, 5'-nucleotidase has been used effectively to monitor the purification of the surface membrane (4, 43).

Although PA activity does closely parallel 5'-nucleotidase activity, its distribution is not identical to that of 5'-nucleotidase. Fraction A (Table II) contains more 5'-nucleotidase activity (and Na⁺, K⁺-ATPase activity) than PA activity relative to the major plasma membrane-enriched fraction, fraction B. In addition, the bottom of the equilibrium gradient (Fig. 3) contains more PA activity than 5'-nucleotidase activity. These slight but significant differences may indicate that PA is not solely associated with plasma membrane and is a part of other membranous organelles that are "plasma membrane-like" in their biophysical behavior, such as Golgi membranes or precursors of plasma membranes. On the other hand, these differences may also indicate that there exists a heterogeneity within the plasma membrane. Distinct domains of plasma membrane that contain different ratios of lipid, carbohydrate, protein, and enzymatic activities may exist and be separable under conditions of sucrose gradient fractionation. It has been suggested that a lack of exact continuity between the subcellular distribution of individual surface membrane markers indicates that a molecular heterogeneity exists within a cell's surface membrane (18, 51, 63). Thus, the lack of exact continuity between PA and membrane markers in fraction A and in the equilibrium gradient may reflect this heterogeneity. It should be noted, however, that fraction A and the bottom of the equilibrium gradient represent only minor portions of the total PA and membrane marker activities. The bulk of the PA activity closely parallels the bulk of the 5'-nucleotidase, Na⁺, K⁺-ATPase, and [3H]fucose activities.

The exact nature of the association of PA with membranes in fraction B is not clear at the present time. The results do not distinguish between PA being an "integral" membrane protein (55) and PA being a protein firmly bound to subcellular membranes, possibly through specific receptors on the membrane. The data in Table V do indicate, however, that PA is not a "peripheral" membrane protein (55), i.e. one that is loosely bound to cellular membranes. The data in Table V also indicate that PA, as isolated, is not enclosed or trapped within membrane vesicles or granules that are disruptable upon hypotonic extraction, hypertonic extraction, or vigorous sonication.

Since PA is closely associated with plasma membrane-like or Golgi membrane-like elements of the cell, one can speculate that a protease which has catalytic access to the cell surface and is dramatically enhanced upon malignant transformation may be an intrinsic modifier of cell surface proteins after malignant transformation. PA is a neutral, serine protease (61) and therefore it can function catalytically within the neutral pH environment of the cell surface. In addition, since nanogram quantities of exogenously added, neutral serine proteases, such as thrombin, can have dramatic effects on the surface properties of normal cells (13, 58), an endogenous, membrane-associated protease could have pronounced intrinsic effects on the surface properties of transformed cells. Although plasminogen is the only known substrate of PA, this does not preclude the possibility that other proteins within the microenvironment of the cell surface may also be substrates for this enzyme or substrates for the plasmin that might be generated at the cell surface by the action of PA. Cellular behavior thus could be profoundly influenced by the enhanced activity (greater than 40-fold over normal cells) of a membrane-associated enzyme that has the potential to catalytically modify or hydrolytically eliminate cell surface regulatory molecules.

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REFERENCES

1. ATKINSON, P. H., and D. F. SUMMERS. 1971. Purification and properties of HeLa cell plasma membranes. J. Biol. Chem. 246:5162–5175.

2. AVRUCH, J., and D. F. H. WALLACH. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. Biochim. Biophys. Acta. 235:334.

3. BENNETT, G., and C. P. LEBLOND. 1970. Formation of cell coat material for the whole surface of columnar cells in the rat small intestine, as visualized by radioautography with 1-fucose-2H. J. Cell. Biol. 46:409–416.

4. BINGHAM, R. W., and D. C. BURKE. 1972. Isolation of plasma membrane and endoplasmic reticulum fragments from chick embryo fibroblasts. Biochem. Biophys. Acta. 274:348–352.

5. BLUMBERG, P. M., and P. W. ROBBINS. 1975. Membrane receptors as general markers for cell division and escape from contact inhibition of growth. Nature (Lond.). 250:488–500.

6. BOUTRY, J. M., and A. B. NOVIKOFF. 1975. Cytochemical studies on Golgi apparatus, GERL, and lysosomes in neurons of dorsal root ganglia in mice. Proc. Natl. Acad. Sci. U. S. A. 72:508–512.

7. BROWN, F., M. L. FREEDMAN, and W. TROLL. 1973. Sensitive fluorescent determination of trypsin-like proteases. Biochem. Biophys. Res. Commun. 53:75–81.

8. CASTLE, J. D., J. D. JAMIESON, and G. E. PALADE. 1974. Cytochemistry of Golgi fractions and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. Biochim. Biophys. Acta. 235:334–352.

9. CHANG, J. K., and G. ACS. 1974. Purification and characterization of a cellular fibrinolytic factor associated with oncogenic transformation: The plasminogen activator from SV-40 transformed hamster cells. Biochim. Biophys. Acta. 340:339–347.

10. CHRISTMAN, J. K., G. ACS, S. SILAGI, and S. C. SILVERSTEIN. 1975. Plasminogen activator: Biochemical characterization and correlation with tumorigenicity. In Proteases and Biological Control. E. Reich, D. B. Rifkin and E. Shaw, editors. 827–839. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

11. CHRISTMAN, J. K., and G. ACS. 1974. Purification and characterization of a cellular fibrinolytic factor associated with oncogenic transformation: The plasminogen activator from SV-40 transformed hamster cells. Biochim. Biophys. Acta. 340:339–347.

12. DE PIERRE, J. W., and M. L. KARNOVSKY. 1973. Plasma membranes of mammalian cells. A review of methods for their characterization and isolation. J. Cell. Biol. 60:275–303.

13. FARQUHAR, M. G., J. J. M. BERGERON, and G. E. PALADE. 1974. Cytochemistry of Golgi fractions prepared from rat liver. J. Cell. Biol. 60:8–25.

14. FISHER, A. 1946. Mechanism of the proteolytic activity of malignant tissue cells. Nature (Lond.). 157:442.

15. GOETZ, I. E., C. WEINSTEIN, and E. ROBERTS. 1972. Effects of protease inhibitors on growth of hamster tumor cells in culture. Cancer Res. 32:2469–2474.

16. GREENBERG, C. S., and M. C. GICK. 1972. Isolation and characterization of membranes from normal and transformed tissue-culture cells. Biochim. J. 130:1113.

17. GREENBERG, C. S., and M. C. GICK. 1972. Electrophoretic study of the polypeptides from surface membranes of mammalian cells. Biochemistry. 11:3680–3685.

18. HELMKAMP, R. W., R. L. GOODLAND, W. F. BALE, I. L. SPAR, and L. E. MUTCHEL. 1960. High specific activity iodination of globulin with iodine-131monochloride. Cancer Res. 20:1945.

19. HYNE, O. 1973. Alteration of cell-surface proteins by viral transformation and by proteolysis. Proc. Natl. Acad. Sci. U. S. A. 71:3710–3714.

20. HYNE, R. O., J. A. WYKE, J. M. BYE, K. C. HEMPHRY, and E. S. PEARLESTEIN. 1975. Are proteases involved in altering surface proteins during viral transformation? In Proteases and Biological Control. E. Reich, D. B. Rifkin, and E. Shaw, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. 931–944.

21. INBAR, M., and L. SACHS. 1969. Structural differ-
ence in sites on the surface membrane of normal and transformed cells. Nature (Lond.). 223:710–712.

30. Ishikawa, H., R. Bischoff, and H. Holtzer. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. J. Cell. Biol. 38:538.

31. Johnsen, S., T. Stokke, and H. Prvoz. 1974. HeLa cell plasma membranes. I. 5'-nucleotidase and ouabain-sensitive ATPase as markers for plasma membranes. J. Cell. Biol. 63:357–363.

32. Lang, W. E., P. A. Jones, and W. F. Benedict. 1975. Evidence for the translocation of 5'-nucleotidase across hepatic membranes in vivo. Proc. Natl. Acad. Sci. U. S. A. 72:4013–4017.

33. LFittle, J. S., and C. C. Widnell. 1975. Evidence for the translocation of 5'-nucleotidase across hepatic membranes in vivo. Proc. Natl. Acad. Sci. U. S. A. 72:4013–4017.

34. Lang, W. E., P. A. Jones, and W. F. Benedict. 1975. Evidence for the translocation of 5'-nucleotidase across hepatic membranes in vivo. Proc. Natl. Acad. Sci. U. S. A. 72:4013–4017.

35. Meldolesi, J., J. D. Jameson, and G. E. Palade. 1971. Composition of cellular membranes in the pancreas of the guinea pig. III. Enzymatic activities. J. Cell. Biol. 49:150.

36. Moseson, M. W. 1962. The preparation of human fibrinogen free of plasminogen. Biochim. Biophys. Acta. 57:204–213.

37. Nicolson, G. L. 1972. Topography of membrane concanavalin A sites modified by proteolysis. Nat. New Biol. 239:192–197.

38. Ossowska, L., J. P. Quigley, and E. Reich. 1974. Plasminogen, the serum proenzyme activated by factors from cells transformed by oncogenic viruses. J. Biol. Chem. 249:4306-4311.

39. Ossowska, L., J. P. Quigley, G. M. Kellerman, and E. Reich. 1973. Properties of plasminogen activators from transformed cells. J. Cell. Biol. 67(2, Pt. 2):347a. (Abstr.).

40. Ossowska, L., J. P. Quigley, and E. Reich. 1973. Fibrinolysis associated with transformation of primary rat embryo cells by simian virus 40. Proc. Natl. Acad. Sci. U. S. A. 71:4792–4796.

41. Ossowska, L., J. C. Unkeless, A. Tobia, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. II. Mammalian fibroblast cultures transformed by DNA and RNA tumor viruses. J. Exp. Med. 138:1056–1064.

42. Perdue, J. F., R. Kletzien, and K. Miller. 1971. The isolation and characterization of plasma membrane from cultured cells. I. The chemical composition of membrane isolated from uninfected and oncopgenic RNA virus-converted chick embryo fibroblasts. Biochim. Biophys. Acta. 249:419–434.

43. Perdue, J. F., and J. Snider. 1970. The isolation and characterization of the plasma membrane from chick embryo fibroblasts. Biochim. Biophys. Acta. 196:125–140.

44. Pollack, R., R. Risser, S. Conlon, and D. Rifkin. 1974. Plasminogen activator production accompanies loss of anchorage regulation in transformation of primary rat embryo cells by simian virus 40. Proc. Natl. Acad. Sci. U. S. A. 71:4792–4796.

45. Quigley, J. P. 1975. Intracellular distribution of plasminogen activator from transformed cells. J. Cell. Biol. 67(2, Pt. 2):347a. (Abstr.).

46. Quigley, J. P., and G. S. Gotterer. 1969. Distribution of (Na+K+)-stimulated ATPase activity in rat intestinal mucosa. Biochim. Biophys. Acta. 173:456–468.

47. Quigley, J. P., L. Ossowska, and E. Reich. 1974. Plasminogen, the serum proenzyme activated by factors from cells transformed by oncogenic viruses. J. Biol. Chem. 249:4306-4311.

48. Reich, E. 1973. Tumor-associated fibrinolysis. Fed. Proc. 32:2174–2175.

49. Reich, E., D. B. Rifkin, and E. Shaw, editors 1975. Proteases and Biological Control. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. 777–977.

50. Rifkin, D. B., J. N. Loeb, G. Moore, and E. Reich. 1974. Properties of plasminogen activators formed by neoplastic human cell cultures. J. Exp. Med. 139:1317–1328.

51. Schimmel, S. D., C. Kent, R. Bischoff, and P. R. Vagelos. 1975. Plasma membranes from cultured muscle cells: Isolation procedure and separation of putative plasma membrane marker enzymes. Proc. Natl. Acad. Sci. U. S. A. 70:3195.

52. Schernbi, H. P., and M. Burger. 1972. Selective inhibition of growth of transformed cells by protease inhibitors. Proc. Natl. Acad. Sci. U. S. A. 69:3825–3827.

53. Sefton, B. M., and H. Rubin. 1970. Release from density dependent growth inhibition by prolylolytic enzymes. Nature (Lond.). 227:843–845.

54. Sellinger, O. Z., H. Beaupay, P. Jacques, A. Dozen, and C. de Duve. 1960. Intradcellular distribution and properties of β-N-acetylglucosaminidase and β-galactosidase in rat liver. Biochem. J. 74:450.

55. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. Science (Wash. D. C.). 175:720–731.

56. Sylven, B., and I. Bois-Svenssen. 1965. On the chemical pathology of interstitial fluid. I. Proteolytic activities in transplanted mouse tumors. Cancer Res. 25:485.

57. Temin, H. 1960. The control of cellular morphology in embryonic cells infected with Rous sarcoma
virus in vitro. Virology. 10:182-197.

58. TENG, N. N. H., and L. B. CHEN. 1975. The role of surface proteins in cell proliferation as studied with thrombin and other proteases. Proc. Natl. Acad. Sci. U. S. A. 72:413-417.

59. TROLL, W., A. KLASSEN, and A. JANOFF. 1970. Tumorigenesis in mouse skin: Inhibitions by synthetic inhibitors of proteases. Science (Wash. D. C.). 169:1211.

60. UNKELESS, J. C., A. TOBIA, L. OSSOWSKI, J. P. QUIGLEY, D. B. RIFKIN, and E. REICH. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. 1. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. J. Exp. Med. 137:85-111.

61. Unkeless, J., K. DANO, G. M. KELLERMAN, and E. REICH. 1974. Fibrinolysis associated with oncogenic transformation. Partial purification and characterization of the cell factor, a plasminogen activator. J. Biol. Chem. 249:4295-4305.

62. VAHERI, A., and E. RUOSKLAKTI. 1974. Disappearance of a major cell-type specific surface glycoprotein antigen (SF) after transformation of fibroblasts by Rous sarcoma virus. Int. J. Cancer. 13:579-586.

63. Wallach, D. F. H., and P. S. LIN. 1973. A critical evaluation of plasma membrane fractionation. Biochim. Biophys. Acta. 300:211-254.

64. WEBER, M. J. 1975. Inhibition of protease activity in cultures of Rous sarcoma virus-transformed cells: Effect on the transformed phenotype. Cell. 5:253-261.

65. WICKUS, G. C., and P. W. ROBBINS. 1973. Plasma membrane proteins of normal and Rous sarcoma virus-transformed chick embryo fibroblasts. Nat. New Biol. 245:65-67.

66. YAMANISHI, F., M. K. DARBOUS, and K. Hashenoto. 1972. Effect of collagenolytic activity in basal cell epithelioma of the skin on reconstituted collagen and physical properties and kinetics of the crude enzyme. Cancer Res. 32:2551.

67. Yunis, A. A., D. R. SCHULTZ, and G. H. SATO. 1973. The secretion of fibrinolysin by cultured rat ovarian tumor cells. Biochem. Biophys. Res. Commun. 52:1003-1012.

68. ZamecniK, P. C., and M. L. STEPHENSON. 1947. Activity of catheptic enzymes in p-dimethylaminoazobenzene hepatomas. Cancer Res. 7:326.