

# Correlation between a Specific Molecular Weight Form of Plasminogen Activator and Metabolic Activity of 3T3 Cells

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**ABSTRACT** In quiescent cultures of 3T3 cells, plasminogen activator (PA) is found predominantly as a 75,000 dalton species. When quiescent cells are exposed to mitogenic agents such as phorbol myristate acetate,  $Ca^{++}$ , or 25% serum, the absolute levels of PA in cell lysates may either increase or decrease. However, a consistent observation is that in the stimulated cultures PA is found predominantly as a 49,000 dalton species. This also is the predominant form of PA in growing and transformed cells. Concomitant with the mitogen-induced stimulation of the 49,000 dalton PA in quiescent cultures is a change in morphology to one that is characteristic of growing and transformed cells. The data suggest that PA is not operative in causing the morphological change that occurs with activation; however, the 49,000 dalton PA in particular is closely related to the pleiotypic response accompanying growth stimulation and transformation.

Plasminogen activator (PA) is a serine protease that catalyzes the conversion of the serum zymogen, plasminogen, to the active fibrinolytic protease, plasmin. The exact role(s) of the enzyme(s) in cellular processes is not known, but several inferences can be drawn from the regulation of the expression of the activity in specific cell types. PA levels have been shown to fluctuate with the developmental stage and/or the hormonal environment of cells. For example, the enzyme is induced in ovarian granulosa cells exposed to follicle-stimulating hormone, thus providing conditions leading to follicle rupture (1), in mammary tissue explants exposed to a hormonal environment favoring involution (2), and in trophoblasts at the time of implantation (3). This has led to the suggestion that PA is involved in the general process of tissue remodeling by providing localized proteolytic activity at defined times for specific cell types. In addition to its role in normal cellular processes, there are many examples of increased PA in transformed cells or malignant tissues when compared with their normal counterparts (see references 4–7, and 33). Because addition of exogenous proteases can transiently induce several of the properties of the malignant phenotype such as alterations in growth rate, agglutinability with lectins, adhesiveness, and morphology (7), it has been suggested that the loss of PA regulation accompanying transformation may be related to some of the characteristics of the malignant cell such as the ability to invade and metastasize. The importance of proteolytic activity in carcinogenesis is demonstrated by experiments using the two-stage

mouse skin assay in which protease (including PA) inhibitors were shown to suppress the production of tumors by the potent tumor promoter, phorbol myristate acetate (PMA) (8, 9). The correlation of PA with tumorigenesis and tissue remodeling is strengthened by observations that compounds affecting these processes, such as PMA and retinoic acid, are potent inducers in cultured fibroblasts (10–14). The biological effects of PA are not necessarily restricted to its ability to produce plasmin and subsequent fibrinolytic activity. The potential involvement of PA in modification of cell surface structures has been recognized. Quigley (15) has suggested a plasminogen-independent effect of PA on cell-to-cell interactions of transformed chick embryo fibroblasts, and Miskin et al. (16, 17) have recognized the potential role for PA-mediated regulation of cell surface receptor turnover.

All of these proposed biological effects of PA depend upon the generation of extracellular enzymatic activity. The enzyme is found in a cell-associated form, which is membrane-bound, and in the extracellular medium in a soluble form (18, 19). Three studies concerning subcellular distribution have demonstrated that most of the activity is associated with a plasma membrane-enriched fraction in cells that release the enzyme extracellularly (18–20). A predominantly intracellular location was found in cells that do not release the enzyme extracellularly (20). These observations leave open the possibility of potential PA action either as a cell surface-associated ectoenzyme or as an extracellular enzyme, and suggest that differences in sub-

cellular location of the enzyme may reflect different enzymatic functions. Several molecular weight forms of the enzyme have been identified, but the relationship, if any, among them is not clear (10, 21, 22). It is possible that different forms of PA have distinct biological activities. The studies to be presented in this and the accompanying paper address the question of regulation of the expression of particular molecular weight forms of the enzyme in 3T3 cells under different growth conditions and the subcellular distribution of the activity in quiescent and metabolically active cells.

## MATERIALS AND METHODS

### Materials

Materials were obtained from the following sources: [<sup>3</sup>H]thymidine from New England Nuclear, Boston, Mass.; SDS from BDH, British Drug Houses, Ltd., Poole, England; tissue culture materials from GIBCO, Animal Resources Laboratories, Madison, Wis.; PAGE (polyacrylamide gel electrophoresis) reagents from Bio-Rad Laboratories, Richmond, Calif.; all others from Sigma Chemical Co., St. Louis, Mo.

### Methods

**CELL CULTURE CONDITIONS:** Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Typically, the cells reached confluence 3 d after plating and were used 5–7 d after plating for experiments involving stimulation of quiescent cultures. Serum-arrested cultures were prepared by changing 2-d cultures to medium containing 0.25% fetal bovine serum for 24 h.

**[<sup>3</sup>H]THYMIDINE INCORPORATION:** Cell monolayers, grown to confluence in 60 mm petri dishes with 2 ml of medium, were incubated with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml) for 30 min at 37°C. The incorporation of radioactivity into acid-insoluble materials was measured as described by Kaplan et al. (23).

**PA ASSAY:** PA activity was estimated by the fibrinolytic assay as described by Unkless et al. (24) and modified as described (20). Briefly, the plasminogen-dependent hydrolysis of a <sup>125</sup>I-fibrin monolayer was measured. The results are reported as the percentage of the total available <sup>125</sup>I-fibrin hydrolyzed by a given sample. Cell lysates were prepared by dissolving rinsed monolayers in 0.1 M Tris (pH 8.1) containing 0.1% Triton X-100 (Tris-Triton). Protein was measured by the method of Lowry et al. (25).

**SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE):** Cell lysates for electrophoresis were prepared by dissolving cell monolayers in sample preparation buffer (0.06 M Tris, pH 6.8, containing 5.0% glycerol, 0.04% bromophenol blue, and 0.1% SDS). The viscosity of the lysate was reduced by forcing the solution through a 22-G needle three times. (Identical results were obtained with cell lysates prepared in Tris-Triton to which SDS was added 30 min before electrophoresis.) There was no difference in PA activity of SDS vs. Triton lysates if a ratio of  $1 \times 10^6$  cells/0.5 ml sample preparation buffer was maintained.

The samples (200  $\mu$ l) were electrophoresed through a 10% separating gel with a 4% stacking gel according to the method of Laemmli (26). The gels were sliced into 2-mm sections and eluted overnight in 0.3 ml of water. The eluates were assayed the next day for PA. There was no loss in activity during storage for up to 7 d at 4°C. The percentage of the activity recovered in the eluates was determined by adding together the activity in the peak fractions and dividing by the total activity applied to the gel. This averaged  $25 \pm 10\%$  (absolute range). The data from each gel have been normalized by dividing the activity of each slice by the activity of the most active slice so that the graphs can all be drawn to the same scale (percentage of maximum activity). Therefore, the data show changes in the relative amounts of the two molecular weight species and not the absolute amounts. The peak activity for each gel is indicated in the figure legends.

**CELL FIXATION AND PHOTOMICROSCOPY:** Cells were fixed with a combination of glutaraldehyde and carbodiimide without permeabilization as described by Willingham et al. (27). Fixed cells were stained with Giemsa stain. Photomicrographs were taken using a 20X objective and phase contrast illumination on Kodak Tri-X film.

## RESULTS

### Effect of Mitogenic Stimulation on Intracellular PA Activity

The level of cell-associated PA in postconfluent 3T3 cells was measured following stimulation by several mitogens (Fig.

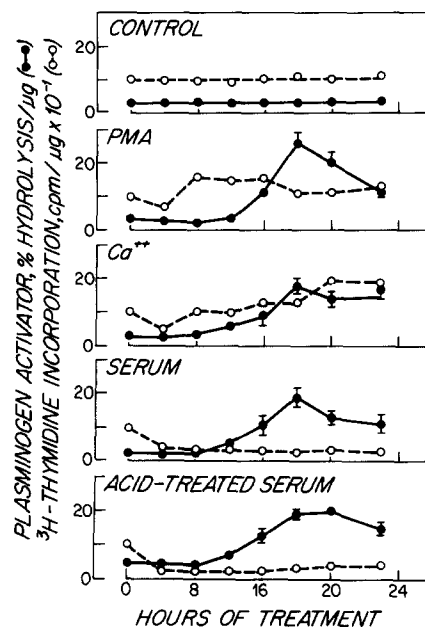


FIGURE 1 Effect of mitogenic stimulation on intracellular PA activity. Postconfluent monolayers were treated with PMA (100 ng/ml), calcium (5.4 mM), serum (25%), or acid-treated serum (25%). Cell lysates were collected at various times following treatment for measurement of PA. At those times, the amount of [<sup>3</sup>H]thymidine incorporation into acid-insoluble materials was also determined. Cell number per dish was also determined at 30 h post-treatment and was as follows: control,  $5.6 \times 10^5$ ; PMA-treated,  $8.4 \times 10^5$ ;  $Ca^{++}$ -treated,  $9.0 \times 10^5$ ; serum-treated,  $12.6 \times 10^5$ ; acid- and serum-treated,  $14.0 \times 10^5$ . Error is included within the symbol except where indicated otherwise.

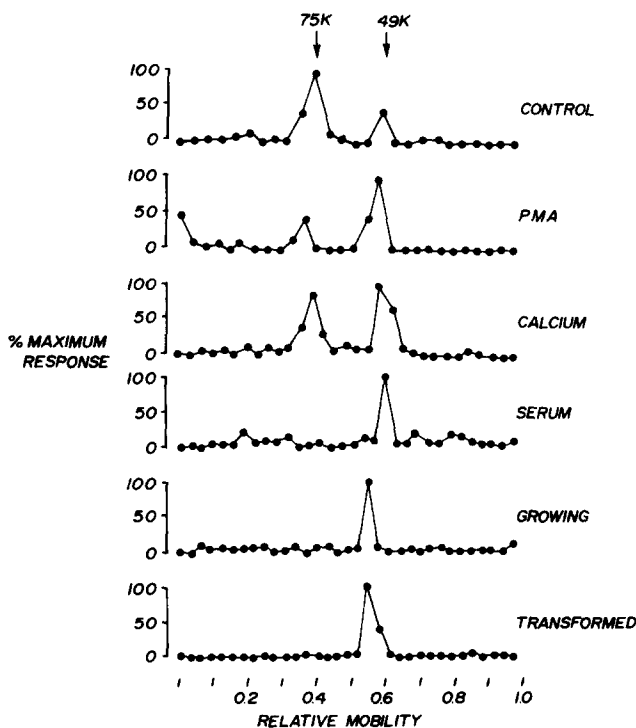


FIGURE 2 Effect of mitogenic stimulation on the molecular weight of PA. Postconfluent monolayers were treated with PMA (100 ng/ml), calcium (5.4 mM), or serum (25%) for 14 h. Cell lysates were then prepared as described in Methods. Data from growing and SV40-3T3 cells are included for comparison. Maximum percentage of hydrolysis for each gel was as follows: control, 10%; PMA, 10%; calcium, 7%; serum, 14%; growing, 10%; transformed, 22%.

1). In all cases, an increase in [ $^3\text{H}$ ]thymidine incorporation was observed 16–20 h after addition of the mitogen. However, the effect on PA lysate activity varied. Both PMA and  $\text{Ca}^{++}$  caused a decrease in PA activity at 4 h after treatment and a subsequent increase in activity of up to two times that of the initial value. In contrast, the mitogenic stimulation due to serum was accompanied by decreased levels of PA that did not return to control levels for as long as 30 h after treatment. Acid treatment of the serum to remove potential acid-labile inhibitors of the fibrinolytic assay did not alleviate this effect. Therefore, the changes in intracellular levels of PA following mitogenic stimulation were not directly related to the metabolic activity of the cell population.

### *Effect of Mitogenic Stimulation on the Molecular Weight of PA and Cell Morphology*

The molecular weight of PA in the lysates of control and mitogen-stimulated cells was estimated by SDS-PAGE. The results (Fig. 2) are expressed as percentage of maximum activity in each gel and are therefore indicative of the ratio of the two molecular weight forms, not the absolute activity. In confluent, quiescent (control) cultures, most of the activity was associated with a 75,000 dalton species. In each of the mitogen-stimulated cultures, the ratio of 49,000:75,000 dalton PA was increased. (This is due to an increase in 49,000 dalton PA; see accompanying paper, 28.) In addition, the 49,000 dalton form was

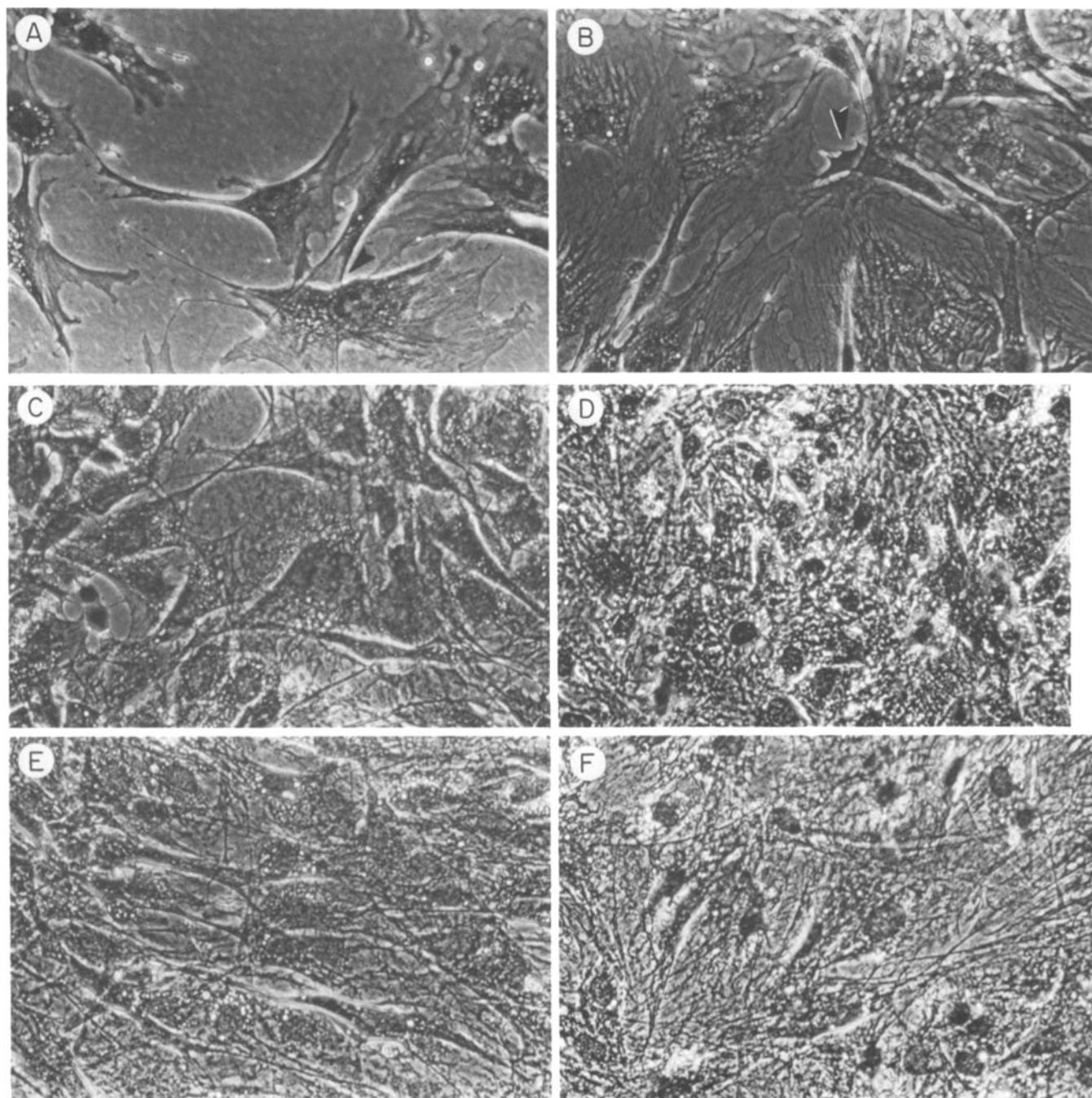


FIGURE 3 Effect of cell density on the morphology of 3T3 cells. Duplicate plates from the experiment described in Fig. 4 were fixed and stained as described in Methods. (a) day 2, (b) day 3, (c) day 4, (d) day 5, (e) confluent monolayer treated with PMA for 12 h, (f) confluent monolayer treated with 9.0 mM  $\text{Ca}^{++}$  for 14 h. Magnification:  $\times 300$  for all micrographs.

the predominant species in growing and SV40-transformed cells (Fig. 2). Therefore, even in cases in which total lysate activity is less than in quiescent control cells (serum-stimulated and growing; 20, 29, 30), the 49,000 dalton was the predominant species. Although no correlation between total lysate PA and the metabolic activity of the cells could be made, the 75,000 dalton PA was the predominant form in quiescent cells and the 49,000 dalton PA was the predominant form in active cells.

The predominance of the 49,000 dalton species correlated with a cellular morphology characteristic of active cells (see, for examples, Fig. 3). In confluent, quiescent monolayers, the nuclei were evenly distributed and did not overlap one another, but it was difficult to distinguish individual cell boundaries. This appears to be due to superimposition of cells, particularly at the peripheries, such that the transparent nature of the cytoplasm obscured the detection of individual cell boundaries (31). Under careful examination, the cells could be seen to have regular outlines. Cells treated with PMA,  $Ca^{++}$ , or 25% serum had more distinct outlines (Fig. 3e and f). The cells contracted, leading to elongated forms with long cytoplasmic processes. The multipolar shapes and spiky processes also were characteristic of growing untransformed cells (Fig. 3a and b) and transformed cells (not shown). The correlation between the predominance of the 49,000 dalton PA species, the metabolic activity of the cells, and this characteristic morphology was investigated further.

#### Correlation between Predominance of Molecular Weight Species and Morphology in Growing vs. Confluent Cells

The molecular weight profiles of lysates collected from monolayers grown for 2–7 d in culture, together with the corresponding growth curve, are shown in Fig. 4. During the logarithmic phase of growth on days 2 and 3, almost all the PA activity was found as 49,000 dalton species. The migratory activity of the day 2 and 3 cells is suggested by underlapping cells (arrows, Fig. 3a and b) with the characteristic leading ruffles and trailing retraction fibers. On day 4, when the cells reached confluence, there was a dramatic change in the molecular weight profile due to the presence of 75,000 dalton species PA. This corresponded to a morphological change in the cultures (Fig. 3c) from the active morphology on day 3 to the less distinct morphology characteristic of quiescent cells. Beyond day 4, no increase in cell number occurred, the 75,000 dalton species was the predominant molecular weight species, and the cellular morphology of the entire population was that of quiescent, stationary cells.

#### Correlation between Induction of 49,000 PA and Morphological Change with Time of PMA Treatment

The time-course of PMA-induced change in ratios of the molecular weight species is shown in Fig. 5. Between 1 and 3 h after exposure to PMA, the change in ratios was evident. By this time, well before the increase in DNA synthesis (Fig. 1), the cells had begun to contract such that the cell boundaries became distinct. The cells continued to contract to elongated forms with long processes (Fig. 3e). After 48 h the molecular weight profile returned to that of the quiescent control. The cells spread out again such that cell boundaries were indistinct, although evidence of the long cellular processes remained.

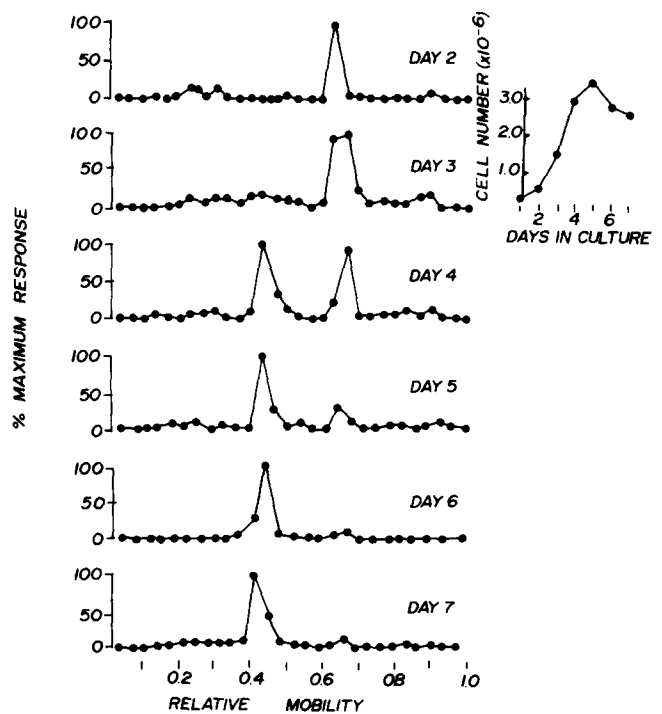


FIGURE 4 Effect of cell density on the molecular weight of PA. Cell lysates were prepared ( $1 \times 10^6$  cells/0.5 ml sample preparation buffer) from monolayers on days 2–7 following plating for determining the molecular weight profile of lysate PA. Cells were removed from duplicate dishes by trypsin for determining cell number (displayed in the upper right-hand corner). Maximum percentage of hydrolysis for each gel was as follows: day 2, 4%; day 3, 12%; day 4, 19%; day 5, 26%; day 6, 41%; day 7, 58%.

#### Correlation between Induction of 49,000 Dalton Species PA and Morphological Changes with $Ca^{++}$

The induction of 49,000 dalton PA species by  $Ca^{++}$  could be titrated by increasing the concentration of  $Ca^{++}$  in the medium from 1.8 (untreated controls) to 9.0 mM. Thus, at 1.8 mM  $Ca^{++}$ , the 49,000 dalton species accounted for only ~20% of the total recovered PA activity; at 5.5 mM  $Ca^{++}$ , it accounted for ~50% of the total recovered PA activity, and, at 9.0 mM  $Ca^{++}$ , it accounted for ~80% of the total recovered PA activity. This may be related to the increased mitogenic effect of increasing concentrations of  $Ca^{++}$  as reported by Dulbecco and Elkington (32). The induction of 49,000 dalton species could be correlated with a morphological change. With 5.5–9.0 mM  $Ca^{++}$ , this was the predominant molecular weight form and most of the population consisted of elongated cells with spiky processes (Fig. 3f and reference 32).

#### Effect of Serum Starvation on Molecular Weight of PA and Cell Morphology

Subconfluent cultures growing in medium containing 10% serum had predominantly 49,000 dalton PA species (Fig. 4). These cultures became quiescent when their environment was changed to medium containing 0.25% serum for 24 h. The induced quiescence resulted in a change in the molecular weight profile such that the 75,000 dalton species became the predominant species (Fig. 6). This was accompanied by a morphological change from cells with ruffles and irregular

PMA (HRS)	% TOTAL ACTIVITY +	
	75K	49K
0	80	20
1	74	26
3	54	46
6	56	44
16	26	74
48	76	24

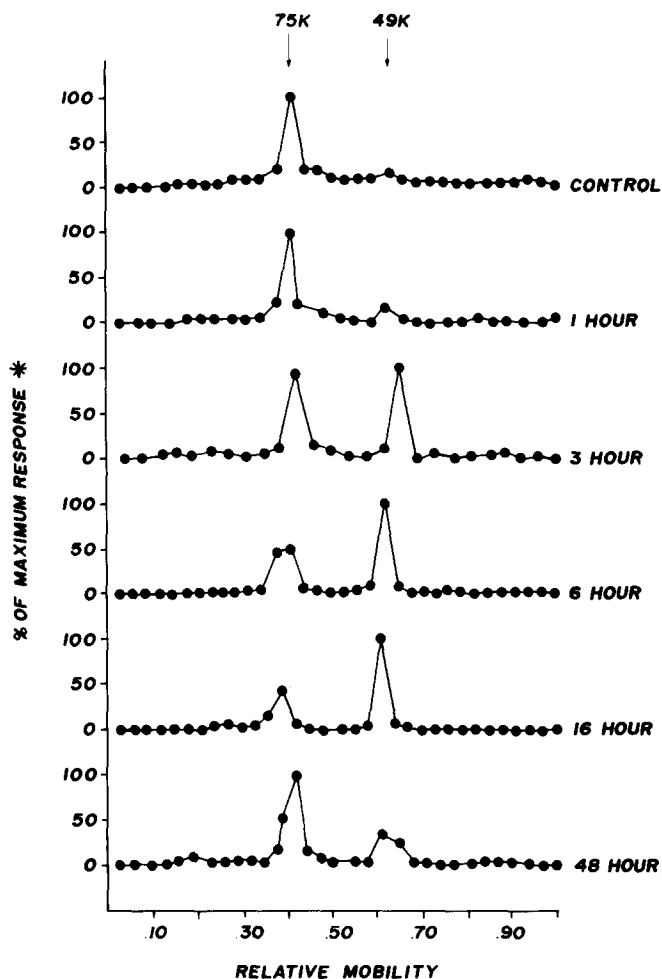


FIGURE 5 Time-course of PMA-effect on molecular weight of PA. Postconfluent monolayers were treated with PMA (100 ng/ml) for the indicated times. Cell lysates were then collected for subsequent analysis with SDS-PAGE. Maximum percentage of hydrolysis for each gel was as follows: control (no PMA treatment), 23%, 1-h exposure to PMA, 22%; 3-h exposure to PMA, 18%; 6-h exposure to PMA, 50%; 16-h exposure to PMA, 38%; 48-h exposure to PMA, 43%.

shapes to larger cells without ruffles and with very regular shapes (Fig. 7).

#### Effect of Protease Inhibitors on Morphological Change

The effect of several protease inhibitors on the PMA-induced morphological change was examined in order to test the possibility of a causal relationship between increased cell surface and extracellular PA and the morphological change. None of the following compounds (which are known to inhibit PA and/or plasmin) prevented the morphological change: soybean trypsin inhibitor (10 mg/ml), *p*-aminobenzamidine ( $1 \times 10^{-3}$  M), *p*-nitrophenyl guanidinobenzoate ( $1 \times 10^{-6}$  M), *N*- $\alpha$ -tosyl-

L-lysyl chloromethylketone (300  $\mu$ g/ml) or phenylmethylsulfonyl fluoride ( $1 \times 10^{-3}$  M). These results agree with those reported by Quigley (15) for a similar PMA-induced morphological change (elongation but not clustering) in chick embryo fibroblasts. Therefore, it seems unlikely that increased proteolytic activity due to PA is the cause of the change in morphology unless the PA is in a protected microenvironment and inaccessible to the inhibitors.

#### DISCUSSION

In this paper we have presented data that demonstrate the correlation between a particular molecular weight species of PA and the metabolic activity of the cells. Mitogenic stimulation of quiescent monolayers led to either an increase or a decrease in measurable lysate activity of PA and could not be used as a reliable indicator of the effects of compounds on the metabolic activity of 3T3 cells. However, in each case of demonstrated mitogenic stimulation, the ratio of 49,000:75,000 dalton PA increased with respect to quiescent control cells. The increased ratio of 49,000:75,000 dalton PA is also a characteristic of growing and transformed cells. The increase in 49,000:75,000 dalton PA ratio correlated with the PMA-induced change in morphology and the concentration-dependent  $Ca^{++}$ -induced change in morphology. When growing cells were rendered quiescent by serum deprivation, a reversion from predominance of 49,000 to 75,000 dalton PA occurred. Therefore, the ratio of 49,000:75,000 dalton PA was a sensitive indicator of the metabolic activity of the cells. The increased ratio of 49,000:75,000 dalton PA did not appear to cause the morphological change because inhibitors of PA and/or plasmin did not inhibit the change; however, the increased PA ratio did closely parallel the morphological change and may be considered part of the pleiotypic response characteristic of growth and transformation (see reference 33 for review).

Previous studies concerned with levels of cell-associated PA in mouse fibroblasts under different growth conditions have shown that as the cells achieve confluence, or after serum deprivation, there is an increase in cell-associated PA (29, 30). Our results agree with those reports, and extend the observations to indicate that the increased activity is due to the presence of a 75,000 dalton species that is present in only very low levels in growing cells.

Although we have used fixed cultures to study cellular

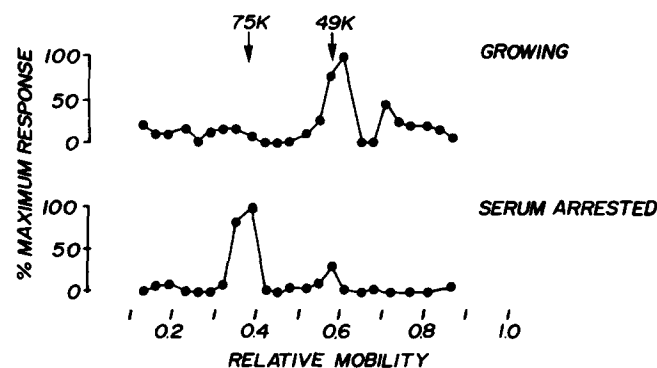


FIGURE 6 Effect of serum deprivation on molecular weight PA. Growing cultures were serum arrested by incubating them in Dulbecco's medium containing 0.25% serum for 24 h. Cell lysates were collected for subsequent analysis with SDS-PAGE. Maximum percentage of hydrolysis for each gel was as follows: growing, 6%; serum-arrested, 14%.

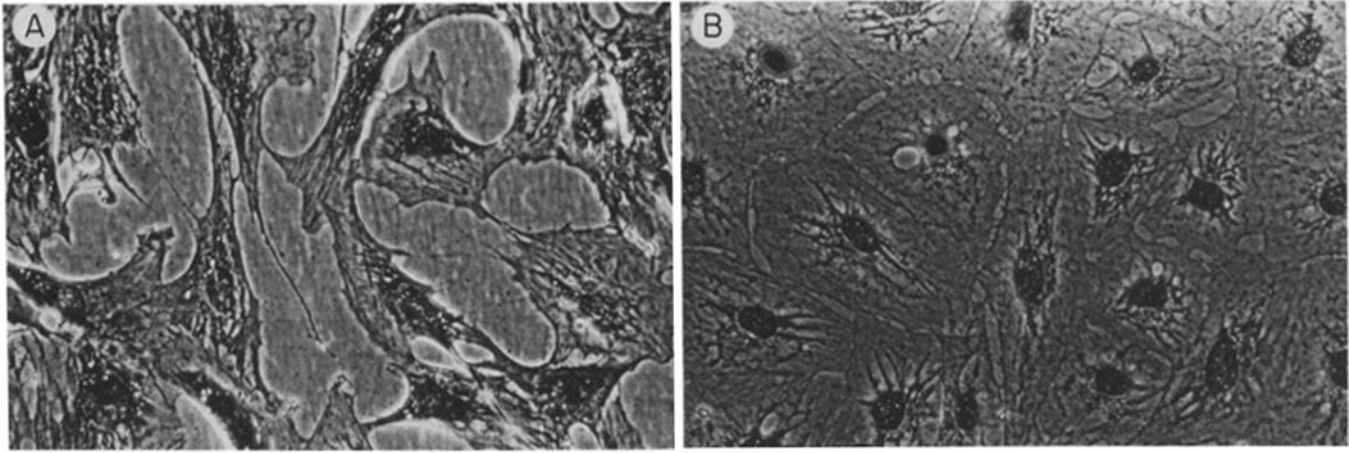


FIGURE 7 Effect of serum deprivation on cell morphology. Duplicate plates from the experiment described in Fig. 6 were fixed and stained as described in Methods: (a) growing, (b) serum-arrested. Cell density in both cultures was  $3 \times 10^5$  cells per dish.

morphology, cells with predominantly 49,000 dalton PA show indications of a migratory behavior such as ruffles, underlapping, and retraction fibers. This morphology, along with decreased substrate adhesiveness, increases the probability that cell contact between lamellipodia will result in underlapping rather than inhibition of cell movement (31). Although the 49,000 dalton species PA does not appear to be a cause of the morphological change, it may well play a role in the locomotory process or substrate adhesion of cells. Further examination of the role of PA in these processes may help to explain a PA role in tissue remodeling and cell transformation.

It is important to understand the relationship between 75,000 and 49,000 dalton PA species. They may be separate gene products or the same gene product altered through post-translational modification. The relationship between the two molecular weight species and their subcellular distribution was investigated further in the accompanying paper (28).

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