Regulation of Plasminogen Activator in 3T3 Cells: Effect of Phorbol Myristate Acetate on Subcellular Distribution and Molecular Weight

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ABSTRACT The tumor promoter, phorbol myristate acetate (PMA), stimulates plasminogen activator production and extracellular release in confluent Swiss 3T3 cells. Coordinated with the increased extracellular release is a redistribution of the activity into plasma membrane-enriched fractions and a shift in the predominant molecular weight species from 75,000 to 49,000 daltons. The evidence suggests that PMA induces the formation of the 49,000 dalton species which is preferentially located in plasma membrane-enriched fractions.

Plasminogen activator (PA) is a serine protease that catalyzes the conversion of the serum zymogen, plasminogen, to plasmin, the main source of serum fibrinolytic activity. Thus, the presence of PA allows for potential production of large amounts of extracellular proteolytic activity via plasmin. In addition to its role in generating plasmin, the possibility that PA acts independently of plasmin, in localized cell surface proteolytic events, remains open (1). The close correlation between the presence of PA activity and cellular processes involving cell migration, tissue remodeling, or malignant transformation has led to the suggestion that PA, through generation of extracellular proteolytic activity, is an important component of these processes, although the exact role is unknown. Part of the difficulty in assigning an exact role for PA in cellular processes may be related to the observation of the existence of several molecular weight forms. The different molecular weight forms have been identified only in terms of fibrinolytic activity, and, because several proteases may be capable of activating plasminogen, it is possible that the PA activity being measured in the fibrinolytic activity is actually a class of otherwise unrelated enzymes. These different molecular weight forms may or may not share similar biological functions.

In the accompanying paper (2), we have demonstrated that upon mitogenic stimulation of quiescent monolayers of 3T3 cells, there is an apparent increase in the level of a particular molecular weight species of PA. In addition, this is the predominant species in growing and transformed cells (2). In previous work, we have demonstrated a difference in subcellular distribution of PA between quiescent and growing or transformed 3T3 cells (3). We have extended those observations in the present work by examining the subcellular distribution of each PA species in cells exposed to the potent tumor promoter, phorbol myristate acetate (PMA).

PMA has been shown to temporarily and reversibly cause acquisition of several of the properties of the transformed phenotype, including increased saturation density, decreased collagen production, decreased cell surface fibronectin, altered cell morphology, increased ornithine decarboxylase, and increased PA (for review, see reference 4). We, therefore, used PMA to activate cells in order to study those aspects of regulation of PA that may be important in relation to the transformed phenotype. Specifically, we have studied the effect of PMA on the association of PA with a plasma membrane-enriched fraction, thus placing PA in an environment in which it could contribute to extracellular proteolytic activity that may be important in mediating several properties of the transformed phenotype.

MATERIALS AND METHODS

Materials

Materials were purchased from the following sources: acrylamide from Bio-Rad Laboratories, Richmond, Calif.; dextran (40,000 daltons) and sucrose from Schwarz-Mann Div. of Becton, Dickson and Co., Orangeburg, N.Y.); sodium dodecyl sulfate from British Drug Houses, Ltd., Poole, England; fibrinogen from The American Red Cross; wheat germ agglutinin from Calbiochem-Bohring Corp., San Diego, Calif.; all other compounds were from Sigma Chemical Co.,

St. Louis, Mo. Plasminogen was prepared from human serum as described by Deutsch and Mertz (5). Fibrinogen was iodinated as described by Helkamp et al. (6). Wheat germ agglutinin (WGA) was iodinated as described by Stanley and Carver (7).

Subcellular Fractions

Subcellular fractionation was accomplished using a discontinuous sucrose-dextran gradient system developed by Graham et al. (8) as described (3). Recoveries of protein, WGA, enzymatic markers and PA were always 100 ± 20%. No more than 15% of the PA was lost to the crude nuclear pellet in preparation of the postnuclear supernate.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The methods are described in the accompanying paper (2).

RESULTS

Stimulation of PA Production with PMA

PMA (100 ng/ml) was added to postconfluent cultures for the indicated times as shown in Fig. 1. Although lower concentrations of PMA were effective, this concentration of PMA was used in all experiments because it produced consistent, maximal effects and was shown not to be toxic to these cells (9). The results show that, after an initial decrease, PA activity began to increase at 5 h after exposure. The increase in lysate activity resulted in increased elaboration of extracellular activity. These results indicate that PMA mediates increased cell-associated PA which is followed temporally by increased extracellular activity.

Effect of PMA on Intracellular Distribution of PA

We have previously shown that growing and transformed cells, which release more PA extracellularly than quiescent cells, contain PA predominantly in a plasma membrane-enriched fraction compared with their quiescent counterparts (3). Therefore, we examined whether induction of extracellular release by PMA was accompanied by a shift in intracellular distribution.

Control and PMA-treated cells (6 h) were processed for subcellular fractionation (Fig. 2). The distributions of protein, ^{125}I-WGA (plasma membrane marker), NADH-oxidase (endoplasmic reticulum marker), and succinate-cytochrome *c* reductase (mitochondrial marker) were similar for the control and PMA-treated cultures. Recovery of protein, all the

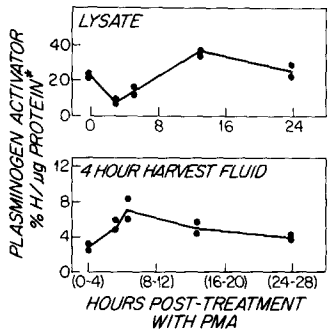

FIGURE 1 Kinetics of effect of PMA on intracellular and extracellular PA. Confluent monolayers were treated with PMA (100 ng/ml). At the indicated times, lysates were collected and parallel cultures were switched to serum-free medium without PMA. The harvest fluids were collected after a 4-h incubation period. Samples were collected from two separate plates and assayed in triplicate. * Percentage of hydrolysis per microgram cell protein.

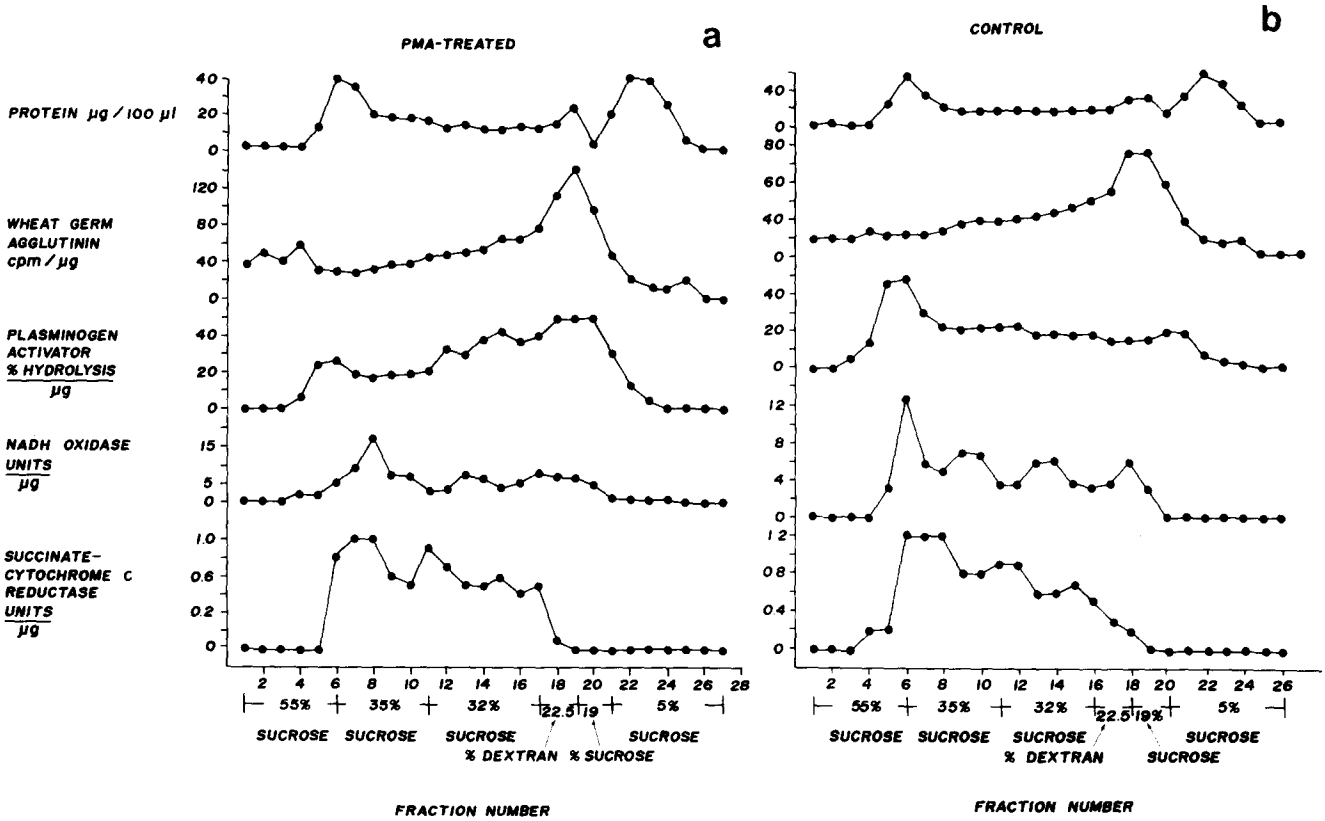

FIGURE 2 Subcellular distribution of PA in confluent and PMA-treated cultures. Confluent monolayers were exposed to PMA (100 ng/ml) for 6 h. At this time, treated (*a*) and untreated (*b*) cultures were processed for subcellular fractionation as described in Methods. The specific activity of several marker proteins is included.

markers, and PA was 100 ± 20% for each gradient. Recovery of PA in the lower portion of the gradient (fractions 1–11) was 69 ± 4% for quiescent cells and 42 ± 6% for PMA-treated cells (n = 8, $P < 0.001$, paired Student's t test). Thus, there is a significant redistribution of PA from the intracellular membrane fractions (55%/35% sucrose interface) to the plasma membrane-enriched fractions (22.5% dextran/19% sucrose interface) in the PMA-treated cultures. In 13 experiments, there was a 1.5- to 4.0-fold increase in specific activity in the plasma membrane-enriched fractions from PMA-treated relative to control cultures. The decrease in specific activity in the intracellular membrane fraction shown in Fig. 2 did not always occur (see Fig. 3, for example).

Time of PMA Exposure for Induction of the Redistribution of PA

To examine whether there was a temporal correlation between the increase in specific activity of PA in the plasma membrane-enriched fractions and the increase in total cellular PA, confluent monolayers were treated with PMA for various times and then processed for subcellular fractionation. Each of the gradient fractions was assayed for protein, ^{125}I-WGA, and PA. NADH-oxidase and succinate-cytochrome c reductase activities were not measured. The protein and ^{125}I-WGA profiles for each gradient were similar; therefore, only the distribution of PA is reported (Fig. 3). Within 3 h after exposure to PMA, there was a decrease in the specific activity of PA in the postnuclear supernatant fluid (PNsup) and throughout the gradient fractions. Between 3 and 6 h, the specific activity in the PNsup increased. This was accompanied by an increase in the specific activity in the plasma membrane-enriched fraction. This increase was maintained, although not maximally, for 24 h, as was the increase in the PNsup. Therefore, between 3 and 6 h, during the time of increasing intracellular activity, there was an increase in PA in the plasma membrane-enriched fractions.

Effect of PMA on Molecular Weight of PA

The effect of PMA on the relative amounts of the molecular weight forms of PA is shown in Fig. 5 of the accompanying paper (2). These data indicate that during the period 3–6 h after exposure to PMA, which corresponded with increasing lysate activity (Fig. 1) and increasing plasma membrane-enriched gradient fraction activity (Fig. 3), there was an increase in the 49,000:75,000 dalton PA ratio. In five experiments, the percentage of the total activity recovered as 75,000 dalton PA in control vs. PMA-treated samples was 84 ± 10% (n = 15) vs. 24 ± 8% (n = 13). Within 3 h after exposure to PMA, the 49,000 dalton species became more prominent. Because the data are expressed in relative terms, the change in ratios could be explained by a decrease in 75,000 dalton PA or an increase in 49,000 dalton PA. This point was addressed directly by comparing the absolute amount of activity recovered as each species after various times of exposure to PMA (Table I). The initial decrease in PA lysate activity (3 h, Fig. 1) correlated with a decrease in 75,000 dalton PA to 59% of control levels.

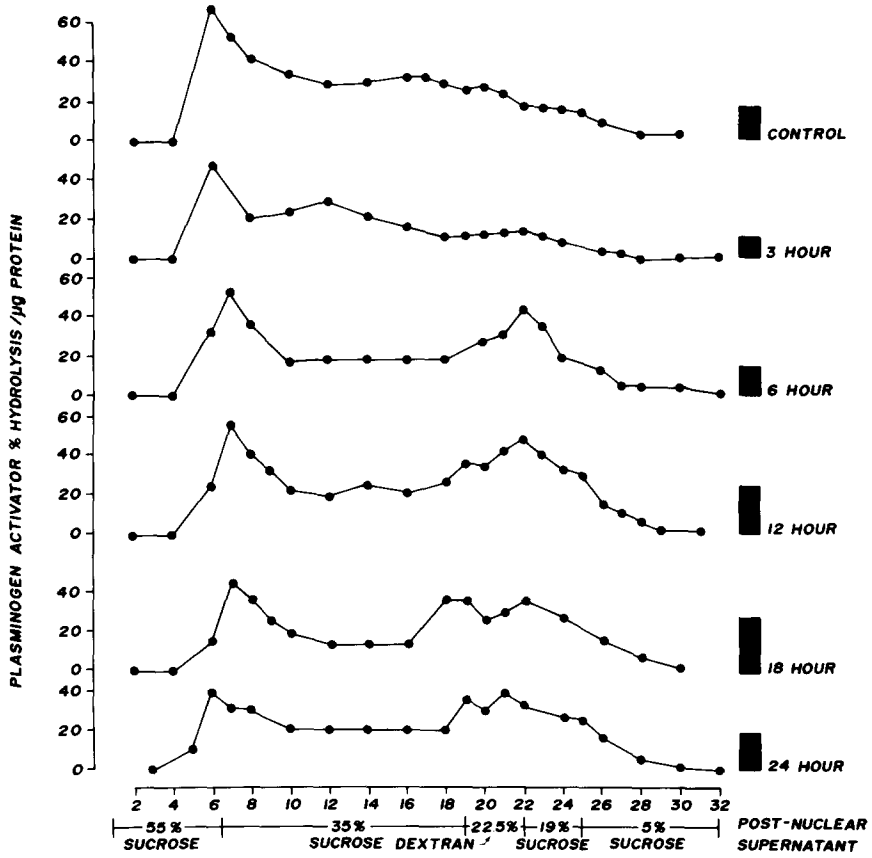

FIGURE 3 Kinetics of PMA induction of subcellular redistribution of PA. Confluent monolayers were exposed to PMA (100 ng/ml) for various times. At the indicated times, cells were processed for subcellular fractionation as described in Material and Methods. Only the results for the distribution of PA are shown. The solid rectangles represent the specific activity of PA in the PNsup applied to the gradient.

TABLE I

Effect of PMA on Absolute Amounts of 75,000 and 49,000 Dalton Species PA Activity

Time of PMA treatment	Lysate	75,000 dalton	49,000 dalton
h			
0	100	100	100
3	59 ± 6*	60 ± 16*	269 ± 47*
5–8	173 ± 18*	82 ± 22	432 ± 103*
24	128 ± 20	111 ± 19	332 ± 59*

Confluent monolayers were treated with PMA for the indicated times. Lysates were collected and processed for SDS-PAGE as described. The amounts of 75,000 and 49,000 dalton species PA activity recovered from each gel were determined by the fibrinolytic assay. The data are reported as a percentage of control lysate, 75,000 or 49,000 dalton species PA activity measured at each time (mean ± SE, $n = 12$).

* Difference from untreated control value significant at $P < 0.05$ level according to analysis of variance (19).

During the period of increased lysate activity (5–8 h, Fig. 1) and increased activity in the plasma membrane-enriched fractions (Fig. 3), the 49,000 dalton PA increased to 432% of control levels, whereas the 75,000 dalton PA showed only gradual increase. After 24 h, lysate and 75,000 dalton PA activity were not significantly different from control levels. The amount of 49,000 dalton PA declined, yet remained 332% of control. Therefore, PMA modulates the expression of activity associated with both molecular weight forms.

Distribution of Molecular Weight Species in Subcellular Fractions

Because PMA induced an increase in the PA in plasma membrane-enriched fractions and also induced a specific molecular weight species, an experiment was designed to determine whether there was a specificity for the 49,000 dalton species in the plasma membrane-enriched fractions. The molecular weights of PA in the intracellular and plasma membrane-enriched fractions, from control and PMA-treated cultures, was determined (Fig. 4).

The results indicate that in control, 3-, and 16-h PMA-treated cultures, there was virtually none of the 49,000 dalton species present in the intracellular membrane fraction. The majority of the 49,000 dalton species was found in the plasma membrane-enriched fraction, whereas the majority of the 75,000 dalton activity was in the intracellular membrane fraction. These results indicate a specificity for the subcellular location of each form of PA.

DISCUSSION

There are several correlations between extracellular release of PA and aggressive cell behavior (for review, see reference 10). This has led to the suggestion that extracellular proteolytic activity mediated through PA is an important component of the processes of tissue remodeling, cell migration, and metastatic growth. Very little is known about the regulation of PA. In fibroblasts, the cell-associated enzyme has the solubility characteristics of a membrane-associated protein, but the extracellular activity is soluble (3, 11, 12). The goal of the present study was to extend our original observations on the correlation between the predominance of plasma membrane-associated vs. intracellular activity and the metabolic activity of the cells. We have shown that the increase in activity after PMA treatment is accompanied by an increase in PA in plasma membrane-enriched fractions. This correlation leaves open the possibility that PA mediates its effect on cellular behavior either as a plasma membrane-associated ectoenzyme or as a soluble, extracellular enzyme.

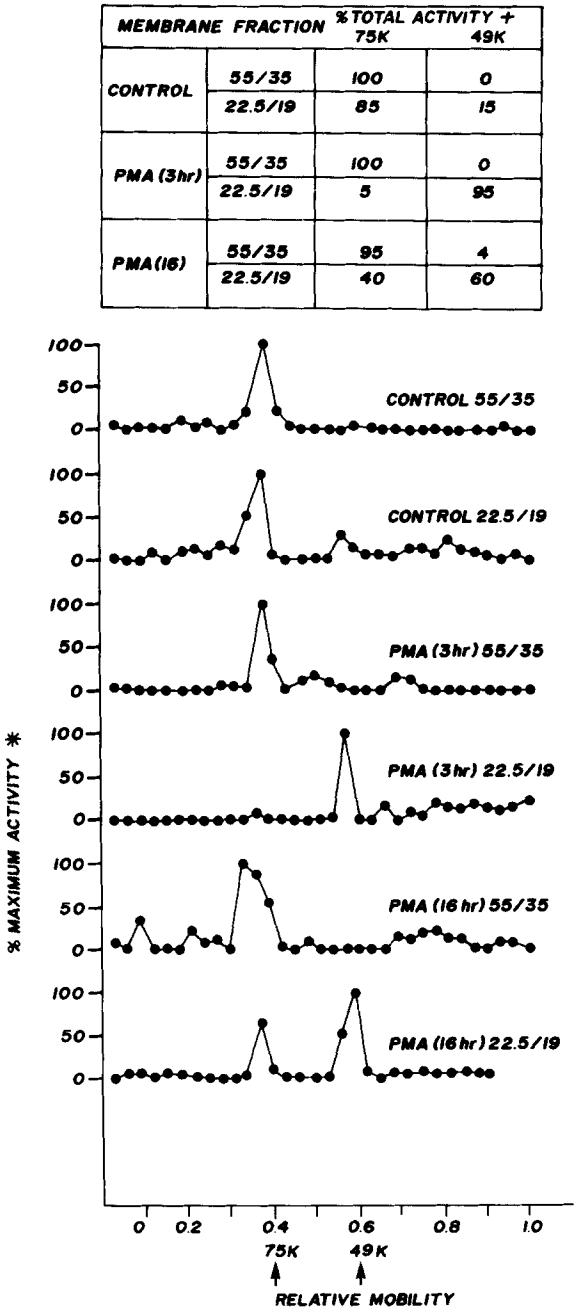

FIGURE 4 Distribution of molecular weight species in subcellular fractions. Confluent monolayers were treated with PMA for 3 or 16 h. Control and PMA-treated cells were then processed for subcellular fractionation. The molecular weights of PA in the intracellular membrane fractions (55%/35% sucrose interface) and the plasma membrane-enriched fractions (22.5% dextran/19% sucrose interface) of each gradient were then determined by SDS-PAGE. The maximum percentage of hydrolysis for each gel was as follows: control 55/35, 38%; control 22.5/19, 14%; PMA (3 h) 55/35, 17%; PMA (3 h) 22.5/19, 13%; PMA (16 h), 55/35, 8%; PMA (16 h) 22.5/19, 16%.

The effects of PMA on cultured cells have been extensively examined. The reported effects include decreased cell surface fibronectin, increased sugar transport, increased saturation density, altered morphology, and alterations in cell-to-cell re-

lations and orientation that suggest that PMA causes temporary changes including several aspects of the transformed or growing phenotype (for a review, see reference 4). Under the conditions used in these experiments, PMA is mitogenic and caused a change in morphology to that characteristic of growing or activated 3T3 cells (2). The results described here indicate that PMA caused an increase in cell-associated PA that is due to the induction of a 49,000 dalton species of PA which was found mostly in plasma membrane-enriched fractions. This may then be the source of increased extracellular (either plasma membrane-associated or noncell-associated) proteolytic activity, an action that may result in cellular changes related to the morphological change and increased activity of PMA-treated cells. This is strengthened by the fact that other mitogens such as Ca^{++} and serum also induced the 49,000 dalton species. In addition, it was the major PA species in growing and transformed 3T3 cells (3).

We cannot exclude the possibility that the increase in plasma membrane-associated activity is due to readsorption of extracellularly released PA. If this does occur, then the readsorbed activity must be tightly associated with membrane components as throughout the several washing steps of the gradient procedure it resists being washed away and <10% of the total activity is recovered in the soluble fractions. Therefore, we feel that nonspecific readsorption of PA to the cell surface cannot explain the increased plasma membrane localization, but we cannot eliminate the possibility of a very tight, perhaps specific, interaction.

In view of the current interest in the regulation of PA production in transformed and untransformed cells, it is important to understand the relationship between the different molecular weight forms. There are three possible alternatives to consider: (*a*) The 75,000 and 49,000 dalton species are separate gene products. Support for this hypothesis can be found from the observation of Christman et al. (15) on the effects of PMA on PA from hamster fibroblasts. They also observed two molecular weight forms (~75,000 and 50,000 daltons) and induction of only the 50,000 dalton species with PMA. The 75,000 and 50,000 daltons were found to be immunochemically distinct and to differ in subunit size, which may suggest that they are different polypeptides. (*b*) The 75,000 dalton is a precursor of the 49,000 dalton species. Another alternative is that these two forms of PA are linked in a precursor-product relationship and that PMA increases the rate of conversion of 75,000 to 49,000 dalton PA. (*c*) The 49,000 dalton is a precursor of the 75,000 dalton species. The last alternative is that the 49,000 dalton represents the initial gene product which, after insertion into the plasma membrane, would be available for association with a 26,000 dalton species. It should be pointed out that an SDS-stable complex between one form of PA (urokinase), and a cell-secreted factor has been demonstrated (16). There is not enough evidence to distinguish among the possibilities at the present time.

The observations presented in this paper may help to clarify the relationship between the levels of PA in transformed cells vs. their untransformed counterparts. Whereas previous studies have demonstrated a strong correlation between increased levels of PA and the expression of the transformed phenotype, the current literature contains exceptions (13, 14, 17, see reference 18 for review). Thus, the increased PA levels in postconfluent fibroblast monolayers compared with growing or transformed cells should not be interpreted as providing evidence for the lack of importance of PA in transformation. In quiescent cells, the activity is probably intracellular and unable to exert influence on the cell surface. The level of 49,000 dalton plasma membrane-associated activity is related to the metabolic activity of the cells. It is the presence of this form and not the level of total lysate activity that may be related to some of the cell surface characteristics of growing and transformed cells which can be influenced by proteolytic activity such as decreased fibronectin, decreased adhesiveness, and increased cell migration.

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