Glucocorticoid Modulation of Plasminogen Activators and of One of Their Inhibitors in the Human Mammary Carcinoma Cell Line MDA-MB-231

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ABSTRACT

In cultures of the human mammary carcinoma-derived cell line MDA-MB-231, plasminogen activator (PA) activity was reduced substantially following treatment with the glucocorticoid dexamethasone. These cells produced urokinase-type PA (u-PA) and tissue-type PA (t-PA), and both enzymes were decreased in dexamethasone-treated cultures. The drop in u-PA activity was associated with a decrease in the synthesis of single-chain pro-u-PA and in the concentration of u-PA messenger RNA; however, the decrease in u-PA activity was more extensive than could be accounted for by inhibition of enzyme synthesis only, suggesting that posttranslational events were also involved. The comparatively small dexamethasone-induced decrease in t-PA activity was not associated with a change in the concentration of t-PA messenger RNA. Hence, the two PA genes are differentially regulated by the same hormone. MDA-MB-231 cells also produced a PA-specific inhibitor related to that produced by bovine aortic endothelial cells (PAI-1). This inhibitor was present in two forms: one functionally active, and the other which required activation by sodium dodecyl sulfate; both forms were increased in cultures exposed to dexamethasone. Thus, glucocorticoid-induced inhibition of PA activity in these cells results from a decrease in u-PA synthesis and a concomitant increase in the production of a PA inhibitor.

INTRODUCTION

Two mammalian serine proteases function as specific activators of the plasma zymogen, plasminogen; they are known as u-PA and t-PA, respectively. These enzymes are the products of two distinct genes (1, 2). Functional studies have implicated t-PA in maintenance of the fluidity of the extracellular milieu, and in particular in thrombolytic activity, while u-PA is thought to be preferentially involved in the proteolytic events that accompany tissue remodeling and cell migration in both normal and pathological processes (for a recent review, see Ref. 3).

The association of elevated u-PA levels with certain types of neoplastic alterations has been repeatedly documented: transformation of cells with oncogenic viruses has been correlated with enhanced u-PA activity (3, 4); various experimental and human tumors have been shown to express more u-PA activity than the corresponding normal tissues (5–8); furthermore, in an experimental system, specific antibodies which block the enzymatic activity of human u-PA drastically reduced the formation of metastasis of a human tumor (9).

Production of PAs is subject to modulation by a variety of agents, including several hormones. Among these, glucocorticoids have been shown to cause a decrease in the u-PA activity of different cells (10–16). Interestingly, the inhibition of u-PA production by hydrocortisone in mouse mammary carcinomas has been correlated with a reduction of tumor growth (17). The response to glucocorticoids of t-PA-producing cell types is variable: in some cells t-PA activity was not affected by dexamethasone (12, 15, 18), whereas in others t-PA activity was either suppressed (14, 19) or enhanced (13, 16) by the drug.

In view of the substantial body of information concerning PAs in mammary gland physiology (5, 6, 8, 17), this organ appears to be a target of choice for further studies on the control of a function associated with the malignant phenotype. We have explored here the molecular mechanisms involved in the glucocorticoid regulation of PAs in the human mammary carcinoma-derived cell line MDA-MB-231.

MATERIALS AND METHODS

Materials. Rabbit antibodies to human u-PA and t-PA were gifts of Dr. W. D. Schleuning and Dr. E. Dowdle, respectively. Rabbit antisera to a human monocyte-derived PAI was kindly provided by Dr. E. K. O. Kruithof; this monocytic inhibitor is functionally and immunologically indistinguishable from PAI-2 (20). Rabbit antisera to a PAI purified from bovine aortic endothelial cells (PAI-1) (21) was the generous gift of Dr. D. J. Loskutoff. Human u-PA and t-PA cDNA probes were provided by Dr. F. Biasi and Dr. E. K. Waller, respectively. Plasminogen was purified from human plasma (22). L-15 medium (210102) was from Boehringer (Mannheim, Federal Republic of Germany). All chemicals were of the best commercial grade available and have been described elsewhere (16).

Cells and Medium. The MDA-MB-231 human breast tumor cell line, established from a pleural effusion (23), was obtained at the 24th passage from the American Type Culture Collection (Rockville, MD). The cells were grown in monolayer in L-15 medium supplemented with 10% fetal calf serum, pyruvate (110 μg/ml), streptomycin (200 μg/ml), and penicillin (5 × 10⁶ units/ml).

Preparation of Culture Media and Cell Extracts. Cultures were seeded at a density of 10⁴ cells/ml of medium at 37°C in culture dishes. After 24 h, the confluent cultures were washed 3 times in phosphate-buffered saline and incubated in 1 ml serum-free L-15 containing 100 μg/ml BSA with or without 10⁻⁶ m dexamethasone (a 10⁻⁸ m stock solution in 0.1 HNaO was diluted 10⁻⁵-fold in L-15 containing 100 μg/ml BSA). Control medium contained the same amount of ethanol. After 24 h of incubation, the culture media were collected, centrifuged for 10 min at 500 × g, and used immediately or stored at -20°C. Cell extracts were prepared by resuspension of the monolayers in 0.1 m Tris-HCl, pH 8.1-0.4% Triton X-100. The samples were centrifuged for 10 min at 500 × g, and the supernatants were used immediately or stored at -20°C. Protein content of the cell extracts was 0.8–1 mg/35-mm dish as measured by the method of Bradford (24) using BSA as a standard.

Caseinolytic Assay of PA. Ten μl undiluted or diluted samples of culture media or cell extracts were incubated in 96-well microtest plates at 37°C with 100 μl 0.1 m Tris-HCl, pH 8.1 containing 1.3% Carnation instant nonfat dry milk and 12 μg/ml plasminogen. Ten μl of urokinase standards (0.3–10 units/ml) were assayed in parallel. Caseinolysis was monitored by measuring the absorbance at 410 nm of undigested casein using a microplate reader (Dynatech MR600; Kloten, Switzerland). For each sample, the absorbance was plotted as a function of incubation time. The plasminogen-dependent proteolytic activity was calculated by comparing the slopes of substrate lysis with those obtained with

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2 The abbreviations used are: u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; PA, plasminogen activator; PAI, plasminogen activator inhibitor; PAI-1, endothelial-type plasminogen activator inhibitor; PAI-2, placental-type plasminogen activator inhibitor; L-15 medium, Leibovitz ‘medium; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; cDNA, complementary DNA.

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**RESULTS**

Characterization and Glucocorticoid Regulation of PAs Produced by MDA-MB-231 Cells. The PA activity of MDA-MB-231 cell cultures was first measured by a caseinolytic assay (Table 1). Approximately two-thirds of the total PA activity was recovered in the cell extract. Upon culture in the presence of 10^{-7} M dexamethasone, both cell-associated and secreted PA activities were markedly reduced.

To assess the respective contributions of u-PA and t-PA to the plasminogen-dependent proteolytic activity of MDA-MB-231 cultures, samples of cell extracts and conditioned media were subjected to a zymographic assay after selective removal of either PA type (Fig. 1). In the cell extract of control cultures (Fig. 1, tracks 1–3), the prominent activity was that of Mr 55,000, as evidenced by its absence after immunoprecipitation with anti-u-PA IgG. In the culture medium of control cells (tracks 7–9), both u-PA (Mr, 55,000) and t-PA (Mr, 72,000 and >100,000) related activities were present.

In agreement with the caseinolytic assay (see Table 1), very low or no activity was revealed by zymography of samples from dexamethasone-treated cultures (Fig. 1, tracks 4–6 and 10–12). In particular, u-PA activity was nearly undetectable in these samples; comparison of the total amount of u-PA in cultures exposed for 24 h to dexamethasone with the enzyme content of cell extracts prepared at the onset of the experiment indicated a net loss of u-PA activity during culture in presence of the drug (not shown). A decrease in both t-PA-related activities was also noted.

To determine whether these effects could be accounted for by a dexamethasone-induced decrease in the synthesis of the enzymes, we incubated control and dexamethasone-treated MDA-MB-231 cells in presence of [35S]cysteine. Samples of culture media were incubated with anti-u-PA, anti-t-PA, or irrelevant IgG; the immune complexes were analyzed by SDS-PAGE under reducing conditions (Fig. 2). One-chain u-PA (Mr, 55,000) and a large amount of a Mr, 84,000 u-PA-related antigen, probably a complex between the heavy chain (Mr, 30,000) of u-PA and a PA inhibitor produced by these cells (see below), were present in the culture medium (Fig. 2, track 1); the u-PA light chain (Mr, 23,000) was recovered at the bottom of the gel. t-PA was also synthesized in the cultures: a radiolabeled band of Mr, 72,000, corresponding to the Mr, of one-chain t-PA, was selectively immunoprecipitated from the culture medium of control cells (Fig. 2, track 2); in addition a large amount of a high Mr, complex (>100,000), formed between t-PA and a PA

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**Table 1** Dexamethasone modulation of PA activity in cultures of MDA-MB-231 cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>With dexamethasone</th>
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<tr>
<td>Cell extract</td>
<td>2.15 ± 0.50</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td>Culture medium</td>
<td>1.10 ± 0.34</td>
<td>Not detectable</td>
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**Fig. 1.** Zymographic analysis of the PAs produced by control and dexamethasone (DEX)-treated MDA-MB-231 cells. Samples of cell extracts and culture media were subjected to SDS-PAGE and analyzed by zymography.

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**Fig. 2.** Zymographic analysis of the PAs produced by control and dexamethasone (DEX)-treated MDA-MB-231 cells. Samples of cell extracts and culture media were subjected to SDS-PAGE and analyzed by zymography.
inhibitor as demonstrated below, was also detected. The trailing of radioactivity between the position of the complex and that of the free t-PA suggests that a partial dissociation of the complex occurs during electrophoresis; hence, we do not know what proportion of the M, 72,000 band represents free enzyme present in the samples prior to electrophoresis.

Dexamethasone treatment of MDA-MB-231 cells led to a decrease in free u-PA (M, 55,000), with no significant alteration in the amount of M, 84,000 and 23,000 u-PA-related antigens (compare Fig. 2, track 1 with 4). In addition, both free and complexed t-PA forms were slightly decreased in presence of the hormone (compare Fig. 2, track 2 with 5); in particular, less free t-PA was consistently recovered from dexamethasone-treated cultures. The effects of dexamethasone on PA synthesis were selective, since total accumulation of newly synthesized proteins was similar in control and dexamethasone-treated cultures (data not shown). In conclusion, there is a striking discrepancy between the near-complete disappearance of PA enzymatic activity and the limited changes in the autoradiographic pattern of biosynthetically labeled PAs.

Analysis of u-PA and t-PA mRNA Content. To further assess the effect of dexamethasone on the expression of u-PA and t-PA genes, we extracted total cellular RNAs from control and dexamethasone-treated cultures and hybridized them to specific single-stranded cRNA probes. We first analyzed, by dot-blot hybridization, the concentration of u-PA and t-PA mRNAs in control and dexamethasone-treated cultures. Taking into account the length and the specific activity of the probes, we estimated that both u-PA and t-PA mRNAs were present at concentrations of 0.4–1.2 pg/µg of total cellular RNA. No difference in u-PA mRNA content was detectable within the first 3 h following addition of dexamethasone (Fig. 3A); after 7 h, however, the u-PA mRNA level was reduced 3- to 4-fold and this decrease was maintained for at least 20 h. Under the same conditions, t-PA mRNA content was unaffected by dexamethasone (Fig. 3B). In order to assess possible dexamethasone-induced changes (36) in the size of u-PA and t-PA mRNAs, total cellular RNAs were subjected to Northern-blot hybridization (Fig. 4); u-PA and t-PA mRNA levels following exposure of MDA-MB-231 cells to dexamethasone. Total RNA from confluent cultures maintained in serum-free medium or serum-free medium containing 10^{-7} M dexamethasone was prepared at different times after addition of the drug. The RNA was dotted on nitrocellulose and hybridized to u-PA (4) or t-PA (5) [32P]-labeled complementary RNA probes. The dots were excised and counted. Values, mean ± SE (bars) of three separate determinations.

To determine whether the decreased u-PA mRNA content resulted from a direct effect of the glucocorticoid on the u-PA gene or whether it required the synthesis of a regulatory protein, we exposed the cells to cycloheximide before addition of dexamethasone. The presence of cycloheximide itself markedly increased the amount of u-PA mRNA (Fig. 4A, compare track 3 to 1). By quantitative dot-blot hybridization we found that cycloheximide-treated cells contained 5-fold more u-PA mRNA than did control cells (Fig. 5). Inhibition of protein synthesis did not cause an accumulation of all cellular mRNAs, since the level of t-PA mRNA was not increased upon addition of cycloheximide (see Fig. 4B, track 3 and Fig. 5, the corresponding bar graph). Addition of dexamethasone to cycloheximide-treated cultures resulted in a slight decrease in u-PA mRNA content (to 80% of control); since this decrease was only a fraction of that observed in absence of cycloheximide (to 25% of control), the effect of the glucocorticoid may require the synthesis of a regulatory protein.

In order to assess the stability of u-PA and t-PA mRNAs and a possible effect of dexamethasone on u-PA mRNA stability, we also analyzed mRNA from actinomycin D-treated cells: both PA mRNAs were stable, and dexamethasone had no effect on the half-life of u-PA mRNA (Fig. 5). Similar results have been obtained with another human mammary cell line (16).

Taken together, our results show that u-PA gene expression is reduced in dexamethasone-treated cultures; however, this reduction is not sufficient to account for the dramatic decrease in u-PA enzymatic activity. Hence, posttranslational events must play a significant role in the glucocorticoid modulation of enzymatic activity.

Characterization and Glucocorticoid Regulation of a PA Inhibitor Produced by MDA-MB-231 Cells. Among the posttranslational events that may lead to decreased PA activity in glucocorticoid-treated cultures is the inactivation of the enzymes by specific inhibitors. Increased production of a PAI was previously reported in rat hepatoma (19), human fibrosarcoma HT1080 (15), and human fibroblastic (37) cells exposed to dexamethasone. Different high-affinity PAs have been characterized in mammalian cells: protease-nexin (38); PAI-2, an
Fig. 4. Effects of inhibition of protein synthesis on glucocorticoid regulation of u-PA and t-PA mRNAs. Cells were incubated for 7 h in serum-free medium (track 1), serum-free medium supplemented with $10^{-7}$ M dexamethasone (track 2), or with cycloheximide (track 3), or with cycloheximide plus dexamethasone (track 4). Dexamethasone was added 30 min after addition of cycloheximide. Total cellular RNA was extracted from these cultures as well as from t-PA-rich Bowes melanoma cells (track 5) and u-PA-rich epidermoid carcinoma Hep-3 cells (track 6). Northern hybridization analysis was performed using 12 pg of RNA per tissue. The filter was hybridized first to a u-PA 32P-labeled complementary RNA probe (A); the u-PA probe hybridized to mRNA from Hep-3 cells but not to mRNA from Bowes melanoma cells. The Northern blot was rehybridized to a t-PA 32P-labeled complementary RNA probe (B); as expected, the t-PA probe hybridized to Bowes melanoma cells mRNA but not to Hep-3 cells mRNA.

Fig. 5. Quantitation of the effects of protein and RNA synthesis inhibitors on glucocorticoid regulation of u-PA and t-PA mRNA. Total cellular RNA was extracted from MDA-MB-231 cells incubated for 7 h in serum-free medium (C) or serum-free medium supplemented with dexamethasone (DEX) ($10^{-7}$ M in bar graphs 2, 3, and 4 and $10^{-5}$ M in bar graph 1) with cycloheximide (Cyc) (10 μg/ml), with cycloheximide plus dexamethasone (the hormone being added 30 min after addition of cycloheximide), with actinomycin D (Act) (5 μg/ml), or with actinomycin D plus dexamethasone. Quantitative analysis of the corresponding samples was done by dot-blot hybridization. Results, mean ± SE (bars) of three separate determinations.

Fig. 6. Zymographic characterization of the t-PA-inhibitor complexes. Samples (20 μl) from culture media of control and dexamethasone-treated cells were immunoprecipitated with antisera to PAI-2 (tracks 1 and 4), to PAI-1 (tracks 2 and 5), or with nonimmune serum (tracks 3 and 6). The immunosupernatants were subjected to SDS-PAGE and analyzed by zymography.

To identify the putative inhibitor present in the $M_r > 100,000$ t-PA-related complex detected by zymography (Fig. 1), we immunoprecipitated samples from control and dexamethasone-treated cultures with specific antisera against PAI-1 and PAI-2. The supernatants were analyzed by zymography (Fig. 6). In the culture media of control and dexamethasone-treated cells, the high molecular weight t-PA-related form was immunoprecipitated by anti-PAI-1 antisera (Fig. 6, tracks 2 and 5); together with the results of Fig. 1, these data demonstrate that the $M_r > 100,000$ PA form revealed by zymography consists of a complex between t-PA and PAI-1.

To detect, characterize, and quantify PA inhibitors, we used an assay that takes advantage of the capacity of free PAIs to form covalent complexes with purified u-PA. 125I-labeled u-PA was added to samples from MDA-MB-231 cultures; the enzyme-inhibitor complexes formed were then immunoprecipitated with the two anti-PAI sera described above, and the supernatants subjected to SDS-PAGE. The corresponding autoradiography (Fig. 7) revealed, in addition to unreacted u-PA ($M_r 33,000$ form), u-PA-PAI complexes with an apparent molecular weight of 83,000. Immunodepletion with antiserum against PAI-1 resulted in the loss of the complex (see Fig. 7, tracks 2, 5, 8, and 11); thus, unreacted PAI-1 could be detected in cultures of MDA-MB-231 cells. Dexamethasone treatment of the cultures markedly increased the amount of the inhibitor recovered both in cell extract and culture medium (compare
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Fig. 7. Glucocorticoid modulation of PAIs produced by MDA-MB-231 cells. Samples (10 μl) of cell extracts and conditioned media from dexamethasone (DEX)-treated and untreated cultures were incubated for 1 h at 4°C after addition of 2 ng ¹²⁵I-labeled u-PA (M₄, 33,000). The radiolabeled u-PA-inhibitor complexes formed were immunoprecipitated with antisera to PAI-2 (tracks 1, 4, 7, and 10), to PAI-1 (tracks 2, 3, 8, and 11), or with nonimmune serum (tracks 3, 6, 9, and 12). The immunosupernatants were analyzed by autoradiography after SDS-PAGE.

DISCUSSION

In this report, we have analyzed the glucocorticoid modulation of the PA system in the human mammary carcinoma-derived cell line MDA-MB-231. Both t-PA and u-PA were produced by MDA-MB-231 cells. As shown with other cell types, t-PA was preferentially secreted (16, 40), whereas a substantial fraction of total u-PA in the cultures remained cell associated (27, 41) presumably through interaction with a u-PA-specific plasma membrane binding site (42). In presence of dexamethasone, both cell-associated and secreted u-PA activity was decreased at least 20-fold. The level of u-PA mRNA was also decreased in steroid-treated cultures; however, this decrease was only 3- to 4-fold and therefore insufficient to entirely account for the reduced enzymatic activity. A possible effect of dexamethasone on u-PA mRNA translation was explored by biosynthetic labeling and immunoprecipitation. The interpretation of these experiments was complicated by the presence of both free and inhibitor-complexed u-PA: as expected, free u-PA was markedly decreased in dexamethasone-treated cultures, but radiolabeling of the enzyme-inhibitor complex was similar in control and treated cultures. In the absence of information on possible effects of glucocorticoids on the formation and fate of such complexes, it appears difficult to evaluate the relative rates of u-PA synthesis in the different cultures. In this context, it is of interest to recall that protease-protease-nexin (38) and t-PA-inhibitor complexes (43) can be rapidly cleared from cultures by intracellular catabolism. Taken together, our results indicate that the effect of dexamethasone u-PA mRNA level and synthetic rate is dramatically amplified by postsynthetic events. A similar discrepancy between u-PA mRNA level and enzymatic activity has already been described (44): upon culture of Hep-3 human carcinoma cells in the presence of dimethyl sulfoxide, a 3- to 4-fold decrease in the level of u-PA mRNA was shown to accompany a 10- to 20-fold decrease in u-PA activity. Thus, in both cases...
it appears that the stability of u-PA can vary according to culture conditions.

Posttranslational events are known targets of glucocorticoids (45). The role of such events in the modulation of PA activity in MDA-MB-231 cultures was further illustrated by our analysis of t-PA gene expression. Following exposure of the cells to dexamethasone, the level of t-PA mRNA remained unchanged; however, the accumulation of newly synthesized t-PA-related forms and the amount of t-PA-related enzymatic activities were both decreased. Although we cannot exclude a possible effect of dexamethasone on the translational efficiency of t-PA mRNA, our results, together with those describing a cell-associated t-PA catabolism (43), suggest that the stability of t-PA protein was decreased in presence of the glucocorticoid.

An exhaustive analysis of the mechanisms underlying glucocorticoid regulation of PA activity in MDA-MB-231 cells must involve the characterization and quantification of the PAIs produced by these cells. We have demonstrated the presence in culture media and cell lysates of a PAI immunologically and functionally related to that originally described in bovine aortic endothelial cells (21). This PAI-1 was present in part as a functionally active antiprotease and in part in a latent form, functionally related to that originally described in bovine aortic endothelial cells (21). This effect of the glucocorticoid could be due to an increase in total inhibitor production, or to a combination of these mechanisms. The second possibility appears less likely, since the increase in inhibitor clearly exceeded the decrease in both u-PA and t-PA. Thus, as already reported for other cell types (15, 19, 37), dexamethasone may increase the synthesis of PAI-1 by MDA-MB-231 cells.

We have previously studied the glucocorticoid regulation of PAs in HBL-100 cells, a mammary cell line established from the milk of an apparently healthy woman (16). The constituents of the PA system and their response to glucocorticoid modulation in the HBL-100 and MDA-MB-231 cell lines are summarized in Table 2. The only consistent effect of glucocorticoids on the PA system in these cell lines is an inhibition of u-PA production. This result is in agreement with findings already reported in other cell types (10–16).

In both mammary cell lines explored to date (HBL-100 and MDA-MB-231) as well as in murine macrophages, the decrease in u-PA mRNA level elicited by dexamethasone did not take place in the absence of ongoing protein synthesis. It has also been observed in these and other cell types (46, 47), that inhibition of protein synthesis alone results in an increase in the level of u-PA mRNA. The increase is gene specific since, for instance, t-PA mRNA levels remained unchanged in both HBL-100 and MDA-MB-231 cells. This suggests the existence of a protein modulator(s) of u-PA mRNA transcription and/or stability and raises the possibility that the effect of glucocorticoids on u-PA mRNA is mediated through an increase in the level or activity of such a modulator.

Whatever the precise mechanism of action of glucocorticoids on the PA system may be, the present work illustrates the difficulties in interpreting modulation experiments based only on measurements of PA activity. In view of the stimulatory effect of glucocorticoids on the synthesis of a PA inhibitor in various cell lines including MDA-MB-231 cells (15, 19, 37), it is clear that assays based exclusively on the catalytic activity of the enzymes cannot provide a comprehensive picture of the regulation of this proteolytic system by steroids. Analyses of the synthesis of PA proteins are difficult to carry out and interpret: rather long labeling periods are necessary to ensure accumulation of detectable amounts of such low abundance proteins, and there is no good way to explore the fate of the enzymes synthesized during these long periods. Thus, evaluation of PA mRNA levels is at present the best available test to measure the synthetic potential of a given tissue for these enzymes. This approach may be valid since there is at present no evidence for a translational control of PA expression in somatic cells.

PA activity may be required for the invasive and metastatic behavior of malignant tumors (9). We have recently performed an analysis of PA mRNA levels in a series of human mammary and lung carcinomas. Our results support the proposed association of elevated levels of u-PA with the malignant phenotype. Glucocorticoids have been shown to modulate the PA production and invasive growth of certain experimental mammary tumors (17). The modulation of the PA system by these hormones can now be dissected at the molecular level. This may provide a better understanding of the mechanisms which control PA activity in normal and neoplastic tissues.

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Note Added in Proof

For all hybridizations, filters were prehybridized for 3 h at 58°C with 20 µg/ml of hybridization mixture and then hybridized for 15–20 h in fresh hybridization mixture (50 µl/cm²) containing the indicated 32P-complementary RNA probes at 5 ng/ml.

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