Cell cycle-dependent fluctuation of urokinase-type plasminogen activator, its receptor, and inhibitors in cultured bovine mammary epithelial and myoepithelial cells

Boris Zavizion *, Jeffrey H. White, Andrew J. Bramley

Department of Animal and Food Sciences, University of Vermont, Burlington, VT 05405, USA

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Abstract

Bovine mammary epithelial (BME-UV1, clone E-T and BME-UV, clone E-T2) and myoepithelial (BMM-UV, clone m-T2) cell lines were used to study the modulation of cell-associated activity of urokinase-type plasminogen activator (u-PA), as well as mRNA transcripts of u-PA, its receptor (u-PAR), and inhibitors (PAI-1 and PAI-2) during the cell cycle. After release from a growth arrest accomplished by growth factor deprivation, the length of the cell cycle was determined as 19–21 h, with G1, S, and G2+M phases of 6–7, 7–9, and 5–6 h respectively. As the cell cycle progressed, accumulated cell-associated u-PA activity increased. Maximal activity occurred at the S/G2 boundary and decreased during the G2/M phases. All cell lines tested produced plasmin-specific inhibitor(s). Accumulation of u-PA mRNA peaked 3 h after stimulation into the growth cycle for m-T2 and E-T and during 3–6 h for E-T2 cells. Maximum levels of u-PAR mRNA were observed at 3 h for the E-T cell line, 6–9 h for E-T2 cells, and 3–9 h for m-T2 cells. The cell cycle distribution of the PAI-1 mRNA was similar to that of u-PA for both epithelial cell lines, while for m-T2 cells maximal accumulation of PAI-1 mRNA was detected at 3–9 h after growth initiation. The increase of PAI-2 mRNA transcription for m-T2 and E-T cells was detected at 3–6 h. The PAI-2 mRNA in E-T2 cells was under detectable levels. The data indicate that the expression of the constituents of the PA system in bovine mammary epithelial and myoepithelial cells is not cell type-dependent but is tightly connected to the phase of the cell cycle. © 1998 Elsevier Science B.V.

Keywords: Cell cycle; Mammary cell; Plasminogen activator

1. Introduction

Urokinase-type plasminogen activator (u-PA) is a specific serine protease expressed by many mammalian cells [1]. While its major feature is to convert inactive plasminogen into the active enzyme, plasmin [2,3], u-PA can also directly degrade some extracellular matrix proteins [4]. Plasmin, which is also a serine protease with a broader spectrum of specificity than that of u-PA, can degrade the extracellular matrix directly or indirectly by activating a broad range of latent metalloproteinases [5]. Significant attention has been paid to plasminogen activators because of their importance in a wide variety of biological processes including tissue remodeling, wound healing, cell migration, tumor invasion and metastasis, and extracellular matrix turnover (for review see [2,3,5,6]). It is widely accepted that the production of u-PA is...
regulated by growth factors [7,8], hormones [9,10], extracellular matrix proteins [10], retinoic acid [11], and even some bacterial products [12,13]. The activity of u-PA in vivo is directed and controlled by a specific u-PA receptor (u-PAR) and by a network of PA-specific inhibitors including PAI-1, PAI-2, protease nexin (PN-1), and PAI-3 (protein C inhibitor). u-PAR expression in different cell types is also highly regulated in a manner similar to that of u-PA [14]. As is the case with PA and u-PAR, the PAI are secreted by specific cell types in a highly regulated manner and were found to be expressed at invasive foci in many types of tumors as well as by different normal and established cell types [15]. Finally, plasmin activity is also modulated by numerous plasmin-specific inhibitors (α2-antiplasmin, α2-macroglobulin, protease nexin, and urinary trypsin inhibitor/bikunin) synthesized by the liver and other tissues [1,5,6,16]. Since the PA/plasminogen system is implicated in a variety of basic biological processes, the physiological, biochemical, enzymological, and biological characteristics of the different components of this system have been extensively studied during the last decade.

Less attention has been given to the role of the plasminogen activator system during the cell cycle. Recently, Ryan et al. [16] have reported that in synchronized normal rat kidney cells, accumulation of PAI-1 transcript peaked in mid-G1 phase and declined during the S phase of the cell cycle. Similar regulation of PAI-1 mRNA transcription has been shown for synchronized human epidermal keratinocytes [17]. Orfanoudakis et al. [18] and Aggeler et al. [19], using different cell models, found that the highest level of cell-associated PA activity occurred during S/G2 transition and was greatly reduced during G2/M. This suggests that PA may be involved in DNA replication and/or cell division. To our knowledge, no comprehensive studies have investigated the kinetics of cell cycle-dependent changes in PA activity along with modulation of corresponding mRNA transcripts.

We have previously reported that bovine mammary cells in culture express u-PA, u-PAR, and PAI-1 [20,21]. Here we demonstrate that the modulation of the expression of the components of the PA system in bovine mammary epithelial and myoepithelial cells is independent of cell type but is closely related to a specific stage of the cell cycle. We also provide data indicating that as the cell cycle progresses, plasmin-specific inhibitor(s) may be as critically involved in the regulation of the PA/plasminogen system as are PA inhibitors.

2. Materials and methods

2.1. Reagents

Cell culture media (DME-F12, RPMI 1640, NCTC-135, DMEM), HBSS, lactose, lactalbumin enzymatic hydrolysate, glutathione, l-ascorbic acid, hydrocortisone, progesterone, gentamicin, penicillin/streptomycin/neomycin, kanamycin, tetracyclin, cell dissociation solution, Triton X-100, amiloride, d-Val-Leu-Lys p-nitroanilide, human and bovine plasminogen were purchased from Sigma. Bovine insulin, transferrin, fetal bovine, newborn, and iron-supplemented calf sera (all heat-inactivated) were from Life Technologies (Gibco BRL). Elastase (porcine pancreas) was purchased from Calbiochem-Novabiochem. Chromogenic substrate, Spectrozyme UK, and u-PA standards were from American Diagnostica. Zeta-Probe GT nylon membranes were purchased from BioRad. Bovine u-PA and u-PAR cDNA probes were provided by Dr. J. Krätzschmar (Berlin, Germany). Bovine PAI-1 cDNA was a kind gift from Dr. M.S. Pepper (Geneva, Switzerland). Human PAI-2 cDNA and genomic proliferating cell nuclear antigen (PCNA) DNA were obtained from ATCC (Rockville, MD; ATCC(R) 61352 and 61054). [α-32P]dCTP and [methyl-3H]thymidine were purchased from Amersham. Prime IT RmT Random Primer kit was purchased from Stratagene.

2.2. Cells and culture conditions

Bovine mammary epithelial (BME-UV1, clone E-T and BME-UV, clone E-T2) and myoepithelial (BMM-UV, clone m-T2) cell lines were established in our laboratory from primary culture developed from a lactating mammary gland [22,23]. Cells within 200–250 population doublings (100–125 passages) were employed. Epithelial cells were maintained in combined DME-F12, RPMI 1640, and NCTC-135 (2:1:1) medium and myoepithelial cells were cultured...
in DM-F12, RPMI 1640, NCTC-135, and DMEM (1:1:1:1) medium with different supplements as described [23–25]. Myoepithelial cells were routinely passaged using standard trypsin-EDTA (0.25% and 0.02% respectively) treatment while epithelial cells were first treated with elastase (2.5 U/ml in non-enzymatic cell dissociation solution) for 5 min at 37°C followed by regular trypsin-EDTA treatment [24].

2.3. Determination of cell cycle phases

Growth of a subconfluent cell layer in 24-well plates was arrested by incubation in serum- and growth factor/hormone-depleted medium for 24 h. At the end of this period, cells were washed 3 times with HBSS and renewed growth was stimulated by addition of complete growth medium. Growth-stimulated cells were pulse-labeled with [methyl-³H]thymidine (5 W Ci/ml) for 30 min. At indicated time points cells were detached. Half of the cells were used to determine [³H]thymidine incorporation into DNA in terms of TCA precipitable radioactivity as described elsewhere [25]. The other half were counted using a hemocytometer. Each determination was done in triplicate.

2.4. Preparation of cell lysate

In experiments performed to determine cell-associated PA activity, bovine mammary cells were grown in 60 mm petri dishes and synchronized as described above. At specific times after the initiation of synchronous growth, the cells were washed 3 times with HBSS and harvested with a rubber policeman in the presence of 0.1% Triton X-100, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4. Cell lysates were vortexed vigorously and stored at −20°C. After centrifugation at 5000 × g for 10 min, the supernatants were used for the assays of PA and plasmin inhibitor activity. Additionally, the number of viable cells at each time point was determined by hemocytometry in the presence of Trypan blue.

2.5. Plasmin and plasminogen activator assay

PA activity in cell lysates was measured in triplicate aliquots by an assay that uses different chromogenic substrates to discriminate between u-PA-specific plasminogen activation and direct u-PA proteolytic (amidolytic) activity according to manufacturer recommendations. Plasmin activity generated in the presence of u-PA was monitored using the substrate Val-Leu-Lys p-nitroanilide while Spectrozyme was used to detect u-PA activity. To explore the possible presence of plasin inhibitor(s), cell lysates were preincubated with plasin and the assay was performed in the presence of plasin-specific substrate. All assays were conducted at 37°C in 96-well plates as described [10]. u-PA activity was expressed as the increase in absorbance per 60 min at λ₄05 nm and finally was normalized to the corresponding cell number.

2.6. RNA extraction and Northern blot analysis

Subconfluent monolayers of bovine mammary cells grown in 100 mm petri dishes were treated as described above. At specific times after the initiation of synchronous growth, total RNA extraction was performed using a guanidinium thiocyanate isolation procedure. Briefly, culture medium was aspirated and cells were lysed by the addition of 0.6 ml of solution D. Cellular lysates were collected with a rubber policeman and processed as described [26]. Total RNA (15 μg) was fractionated on 1% agarose gel in the presence of 2.0 M formaldehyde and blot transferred to the membrane in 10×SSC (10×SSC, 1.5 M NaCl, 0.15 M Na₃citrate, pH 7.0). Membranes were baked for 1 h at 80°C and subsequently UV-cross-linked. Blots were probed with u-PA, u-PAR, PAI-1, PAI-2, and PCNA specific probes labeled with [α-³²P]dCTP by the random priming technique as recommended by the manufacturer. Ethidium bromide staining and 18S cDNA re-probing were used as an internal loading and transfer controls.

3. Results

3.1. Duration of cell cycle

Monolayer cultures of bovine mammary myoepithelial (clone m-T2) and epithelial (clones E-T and ET2) cells were grown to approx. 25% confluence, after which the cells were synchronized in G₀/G₁ by
incubation in medium without serum and growth promoter supplements for 24 h. Preliminary experiments (data not shown) have confirmed that under these conditions approx. 95% of the cells are quiescent. After growth re-initiation, cells were pulse-labeled with \(^{3}H\)thymidine, and thymidine incorporation and the corresponding cell numbers were determined (Fig. 1). The classic pattern of the progression of all three cell lines through the cell cycle is apparent, with clearly distinguishable G1, S, and G2/M phases of the cycle. The length of the complete cell cycle is approx. 18 h (m-T2), 19.5 h (E-T), and 21 h (E-T2), with G1, S and G2/M phases comprising 6–7, 7–9, and 5–6 h respectively.

3.2. Accumulation of u-PA activity during cell cycle progression

The lysates of cells synchronized throughout the cell cycle showed a consistent increase in the level of cell-associated u-PA proteolytic (amidolytic) activity, beginning immediately after growth stimulation (Fig. 2). For all cell lines, the highest levels of activity occur at the late S/early G2 stage of the cell cycle followed by a decrease, primarily during the late M phase. The relative accumulation of u-PA activity increases from 100% in growth-arrested cells to 170% for m-T2 cell line (Fig. 2A), to 205% for E-T (Fig. 2B), and to 255% for E-T2 (Fig. 2C) and then decreases to 129%, 190%, and 186% correspondingly. As expected, the relative accumulation increases when the cells enter a new cell cycle. Despite the distinct differences in the absolute values (both maximal and minimal levels) of activity detected in different cell lines, these data clearly indicate that the distribution of cell-associated u-PA activity within cell cycle phases is not dependent on if it is epithelial or myoepithelial cells but rather is closely associated with the order of cell cycle progression. To eliminate...
underestimation of detected u-PA activity (in order to activate presumably existing scu-PA), cellular lysates were treated with trace amounts (0.01 U/ml) of plasmin followed by the direct assay in the presence or absence of the plasmin inhibitor aprotinin. The data (not presented) revealed that there is little or no inactive u-PA in the cell lysates we used.

3.3. Detection of plasminogen activation activity

To further characterize the modulation of u-PA activity, we tested the same cell lysates using an indirect PA assay which employs exogenously added plasminogen and the plasmin-specific chromogenic substrate. Surprisingly, the time course of u-PA-specific plasminogen activation activity did not reflect the dynamics of the u-PA amidolytic activity described above. Furthermore, as observed in Fig. 3, the appearance of u-PA-generated plasmin activity is greatly reduced as cells traverse the G1 phase of the cell cycle remaining, for the m-T2 myoepithelial cell line (Fig. 3A), undetectable in S and G2 segments with a rapid increase in the M phase and thereafter. For the E-T2 epithelial cell line (Fig. 3C), the level of plasmin activity detected was approximately halved during S and G2/M phases as compared to quiescent cells with a slight increase at the beginning of the next cell cycle. Finally, the E-T epithelial cell line lysates showed a 2-fold decrease in generated plasmin activity during only the G1 phase of the cell cycle.

Fig. 3. Cell cycle regulation of PA activity in m-T2 (A), E-T (B), and E-T2 (C) cell lysates. The same cell lysates as in Fig. 1 were analyzed for PA activity using indirect chromogenic substrate assay in the presence of plasminogen and plasmin-specific substrate. The data were obtained and processed as in Fig. 2 and are expressed as percentage of the activity presented in cell extracts at time 0.

Fig. 4. Kinetics of induction of plasmin inhibitors in growth-stimulated m-T2 (A), E-T (B), and E-T2 (C) cells during cell cycle progression. Samples of the same cell extracts as in Figs. 2 and 3 were preincubated with plasmin (0.25 U/ml) for 30 min at 37°C followed by chromogenic substrate assay with plasmin-specific substrate. The data are expressed as percentage of recovered activity from the activity of plasmin preincubated with lysis buffer.
3.4. Detection of plasmin inhibitor activity

In order to learn more about the involvement of plasmin inhibitor(s) activity in the regulation of the PA/plasminogen system during cell cycle progression, we employed direct plasmin assay using the same samples. This allows us to rule out any possible influence of PA inhibitors. In Fig. 4, it can be clearly seen that even quiescent cell lysates contain substances capable of plasmin inhibition. Further decline in plasmin activity detected occurs for all cell lines during the G1 phase of the cell cycle. For the m-T2 cell line (Fig. 4A), the 2-fold decrease persists during G1 and S phases followed by a 1.5-fold increase in G2/M, and finally approaches a steady-state level. Similar results were observed for both epithelial cell lines except a 4-fold inhibition of plasmin activity in E-T cell lysates (Fig. 4B) persisted into early G2 phase while for the E-T2 cell line it was achieved at late G1 phase of the cell cycle (Fig. 4C). These results indicate that the inhibitory activity(ies) observed in both plasminogen activator and plasmin assays is mainly due to the presence of plasmin inhibitor(s) rather than PA inhibitor(s).

3.5. Northern blot hybridization

Northern blot analysis was performed to evaluate the modulation of u-PA, u-PAR, PAI-1 and PAI-2 mRNA transcripts during the cell cycle. Consistent with the results of the u-PA-specific amidolytic assay, low u-PA mRNA levels were detected in quiescent cells (Fig. 5A,B,C, lane 1). Increases in u-PA transcription were rapid, attained maximal level in mid-G1 phase (3 h post stimulation for m-T2 and E-T cells and 3–6 h for the E-T2 cell line), and declined with the onset of DNA synthesis. During G2/M (Fig. 3B) with progressive increase during all subsequent stages.
phases, the u-PA mRNA abundance approximated that of growth-arrested cells.

Similar to u-PA transcripts, the u-PAR mRNA levels were low in quiescent cells (Fig. 6A,B,C, lane 1) for all cell lines and increased significantly within 3 h of growth stimulation (Fig. 6, lane 2). Subsequent kinetics of the u-PAR mRNA transcript accumulation during the cell cycle were different from that of u-PA and were unique for each cell line. For the E-T epithelial cells, the u-PAR transcripts peaked at 3 h but, unlike those of u-PA mRNA, continued to be elevated at 6 and even 9 h after growth stimulation (Fig. 6B). For the E-T2 epithelial cell line, the u-PAR mRNA abundance peaked during 6 and 9 h, and remained at the increased level even at 12 h (mid-S phase) (Fig. 6C). For the m-T2 myoepithelial cells, the maximal increase in u-PAR transcript levels seen at 3 h (mid-G1) was maintained at the same levels through 6 h (G1/S boundary) and 9 h (early S phase) of the cell cycle (Fig. 6A). These results suggest that as the cell cycle progresses, the increase in u-PAR mRNA transcription is one step behind the corresponding increase in u-PA mRNA transcription.

As can be seen from Fig. 7, the cell cycle regulation of PAI-1 mRNA transcription is very similar to that of u-PA. Again, maximal accumulation of PAI-1 mRNA in E-T and E-T2 cell lines occurred at 3 h and 3–6 h after growth stimulation respectively (Fig. 7B,C). The m-T2 myoepithelial cells differed somewhat in that the PAI-1 mRNA levels were equally elevated from 3 to 9 h after initiation of growth cycle (Fig. 7A) while u-PA transcript clearly peaked at 3 h (Fig. 5A). The PAI-2 mRNA transcripts in quiescent cells were at very low levels (Fig. 8). As expected, growth stimulation triggered the increase in PAI-2 mRNA transcription. Maximal induction was detected in early G1 phase of the cell cycle for m-T2 cells and during early/late G1 for E-T cells. We did not find any detectable levels of PAI-2 transcripts in the E-T2 cell line.

In order to ascertain cell cycle position, the membranes were hybridized with a PCNA (a co-factor of DNA polymerase δ also known as cyclin) probe. In agreement with a previous report [27], cyclin mRNA expression was undetectable in growth-arrested cells. Appearance of PCNA transcripts was first observed during G1/S transition (Fig. 9), reached a maximum during the S phase, and rapidly decreased at the end of the S phase.

Taken collectively, these results raised the possibility that during the cell cycle the different components
of the PA system are regulated in a concerted manner.

4. Discussion

In this study we investigated the cell cycle-specific variation of cell-associated u-PA activity and, in parallel, the expression of transcripts of u-PA and other components of the PA system in established bovine mammary epithelial and myoepithelial cell lines. Our results demonstrate that the biosynthesis and appearance of u-PA-specific activity are highly regulated by the stage of the cell cycle. Direct u-PA amidolytic assay reveals that quiescent bovine mammary cells start to increase steady-state levels of cell-associated u-PA activity immediately after mitogenic stimulation (Fig. 2) with maximal accumulation within the late S/early G2 stage, falling during G2/M phase. While the magnitude varied somewhat, all cell lines showed a very similar pattern of the distribution of PA activity in corresponding phases of the cell cycle. Aggeler et al. [19] using fibrinolytic assay found that cell-associated PA peaked at the late S phase in Chinese hamster ovary cells (CHO) with a great reduction during G2/M. Human alveolar epithelial cells (A549) also produce high levels of PA at S/G2 transition [18], while rat hepatoma cells (ZHC) show maximum cell-associated PA activity at the very beginning of the S phase [19]. In these studies, however, the accumulation of PA during the G1 phase was not recorded.

Since the indirect PA assay in the presence of plasminogen is estimated to be 10–1000-fold more sensitive than the direct assay, we used this test to reprobe the same samples. Surprisingly, the results obtained with indirect PA assay conflicted with the data from direct PA assay. No increase in plasminogen activation activity was observed during the G1 phase of the cell cycle that we anticipated because of the increase in PA activity. On the contrary, we detected a strong (40–100%) decrease of plasminogen activation as compared to quiescent cell lysates. In addition, the overall profile of plasminogen activation was unique for each cell line. Namely, the m-T2 myoepithelial cells showed the greatest decrease during early G1, falling to undetectable levels during S and G2 stages with subsequent increase in M phase. The E-T epithelial cells showed reduced (50% decrease) plasminogen activation activity only during G1 phase followed by a constant increase thereafter. Finally, E-T2 epithelial cells showed a decrease of plasminogen activation through all phases of the cell cycle with only a small increase as the next cycle began. These results can only be explained by the presence of increasing amounts of PA and/or plasmin inhibitors. In this regard, the fact that the amidolytic activity of u-PA detected in the direct PA assay was unaffected may indicate that the corresponding inhibitors are present in non-active, or latent forms. Indeed, recent structural studies on PAI-1 [28,29], PAI-2 [30,31], and protein C inhibitor (PAI-3) [32] have shown that these inhibitors occur in both active and latent forms.

Fig. 9. Appearance of 1.3 kb PCNA mRNA in growth-stimulated cells. The same membranes as in Figs 5–8 were stripped and reprobed with labeled human PCNA probe. The lane indications and autorad letters are the same as in Fig. 5. Bottom panel represents control hybridization with 18S RNA-specific probe.
conformations which may be activated by plasmin generated from plasminogen in the presence of u-PA. Furthermore, PAI-2 has been characterized as a predominantly intracellular inhibitor whose physiological intracellular function is as yet uncertain [30,33]. Latent conformations of plasmin-specific inhibitors which can be proteolytically activated have also been described for α2-macroglobulin [34], inter-α-trypsin inhibitor [35], and urinary trypsin inhibitor/bikunin [36].

To separate the effect of PAIs and plasmin inhibitors, we included a colorimetric assay in which active plasmin was preincubated with the cell lysate samples. If the activation of latent PAIs was a key influence on the results of the indirect PA assay, the direct plasmin assay would reveal no differences in recorded activity. However, plasmin assay showed that for all cell lines tested the role of plasmin inhibitors is important at least during the G1 and S phases (Fig. 4). According to our preliminary data (not shown) one of these inhibitors is urinary trypsin inhibitor/bikunin. Further evaluation and quantitation of the data obtained with all three cell lines reveal that plasmin inhibitors play a central role in the PA/plasminogen system regulation during the cell cycle. This conclusion raises the question of the role of u-PA in the progression through the cell cycle. If the role is solely to activate plasminogen, then it would be logical to control the very early steps of the amplification loop, i.e., plasminogen activation. This seems not to be the case since, during G1 and S phases of the cell cycle, cell-associated u-PA activity progressively increases while u-PA-generated plasmin activity is strongly suppressed. It is tempting to speculate that during cell cycle progression u-PA may have targets other than plasminogen activation. These might include the activation of other latent proteases, plasmin-independent proteolytic processing of other proteins including growth factor activation [37,38], or a direct function as a growth factor due to the presence within the u-PA molecule of a growth factor-like domain [39]. Interestingly, the decrease in plasmin inhibitory activity detected during G2/M phases coincides with reduced levels of cell-associated u-PA activity. Since others have described an increased appearance of secreted PA in cell-conditioned medium during this stage of the cell cycle [19,40], it may be that mitotic cells use the PA/plasminogen system in order to disrupt their association with the extracellular matrix allowing cell division. On the basis of these results, it is constructive to speculate that extracellular proteolysis during mitotic division is mainly governed by secreted free u-PA while focalized cell surface-associated u-PA is more involved in pericellular proteolysis (or other activities) during the S phase of the cell cycle.

A second group of experiments provides intriguing data. Based upon Northern blot hybridization, it can be concluded that the maximum transcription of the u-PA gene is restricted to a short ‘window’ in mid- to late G1 phase (3–6 h after growth stimulation) of the cell cycle (Fig. 5). The highest levels of cell-associated u-PA activity, however, are found at S/G2 boundary or early G2 stage (9–12 h later) when the u-PA mRNA level is approximately that of quiescent cells. The physiological significance of such a delay between maximal transcription and the appearance of active protein is as yet unclear. In contrast, the present study and earlier reports [27,41] showed that the maximal expression of cell cycle-dependent PCNA (both at mRNA and protein levels) occurs during the S phase. Resembling the pattern of u-PA mRNA appearance, the u-PAR, PAI-1, and PAI-2 mRNA transcripts also peaked early in the G1 phase. Similar cell cycle-dependent accumulation of PAI-1 and PAI-2 transcripts (mid-G1 phase) has been described in previous reports [16,17,42,43]. None of these studies, however, described the cell cycle regulation of u-PA and u-PAR transcripts or the modulation of u-PA activity. Since the majority of total cell-associated u-PA in bovine mammary cells is presented as membrane receptor-bound activity [21], the concurrent modulation of u-PA, u-PAR, PAI-1, and PAI-2 should lead to the modulation of detected extracellular u-PA activity during the cell cycle. The delay between maximum transcription and the maximum activity of cell-associated u-PA may be due to posttranscriptional regulation. Alternatively, during initial stages of the cell cycle u-PA (alone or complexed with PAIs and/or u-PAR) may have non-proteolytic functions.

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