Biochem. J. (1996) 314, 1041–1046 (Printed in Great Britain)

Complex regulation of plasminogen activator inhibitor type-1 (PAI-1) gene expression by serum and substrate adhesion

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Expression of plasminogen activator inhibitor type-1 (PAI-1), a member of the serine protease inhibitor (SERPIN) superfamily that functions to negatively regulate the plasmin-based pericellular proteolytic cascade, was induced early after exposure of growth-arrested normal rat kidney (NRK) cells to serum-containing medium. Increased PAI-1 transcription was rapid (evident within 10 min of serum addition) and involved immediate-early response kinetics. [^{3}H]Thymidine autoradiography was used to map the time frame of PAI-1 expression during a synchronous growth cycle. PAI-1 transcript accumulation peaked in mid-G_1 phase (approx. 4–6 h post-stimulation) and declined prior to, or concomitant with, the onset of DNA synthetic phase. Serum increased PAI-1 expression in NRK cells in agarose suspension, as well as monolayer, culture; induction in suspended cells (similar to monolayer culture conditions) also occurred in the presence of cyclohexamide or puromycin. The serum-inductive pathway leading to PAI-1 gene activation is thus functional regardless of adhesive conditions or capacity for *de novo* protein synthesis. The amplitude of induction and maintenance of expression in later stages of G_1, however, were subject to adhesive influences. PAI-1 transcript accumulation at 4 and 8 h post-stimulation in newly adherent cells, moreover, was blocked by puromycin, indicating that both immediate-early and secondary mechanisms regulate PAI-1 mRNA levels during progression of NRK cells through an 'activated' G_1 growth phase.

INTRODUCTION

In mammalian cells, degradation of the extracellular matrix (ECM) is regulated largely by the plasmin-based proteolytic cascade in which plasminogen is converted into the active broad-spectrum protease plasmin by urokinase plasminogen activator (uPA) [1–3]. Plasmin, in turn, can degrade the ECM directly as well as indirectly by activating latent metalloproteinases [1,2,4,5]. uPA is found in particular abundance on the extracellular face of cell-to-ECM adhesion complexes (i.e. focal contacts) [6,7]. Such adhesion-site focalization of matrix-active proteases [6] provides a mechanism to rapidly alter the pericellular proteolytic micro-environment, as well as the nature and/or stability of focal-contact structures, in response to specific stimuli (e.g. induced cell proliferation or motility) [7]. Plasminogen activator inhibitor type-1 (PAI-1), a member of the serine protease inhibitor (SERPIN) superfamily, functions as a negative modulator of this pericellular proteolytic cascade by complexing with and inhibiting the catalytic activity of uPA [1,6]. Recent results suggest that PAI-1 transcript levels, similar to those encoding components of the adhesive complex proper (i.e. vinculin, β_1-integrin, fibronectin, actin, uPA) [6–10], may be growth-state-dependent [11]. In fact significant and rapid undersurface PAI-1 protein deposition accompanies serum-induced entry of normal rat kidney (NRK) cells into the division cycle, with maximal accumulations evident in regions immediately juxtaposed to contact site structures [11,12]. This has important ramifications with regard to cell growth regulation. Indeed, the contention that anchorage-dependent control of cell proliferation resides within G_1 phase [13,14] highlights the potential significance of adhesion-modulating proteins (such as PAI-1) in proliferative-state transitions [11].

NRK cells provide an *in vitro* paradigm to evaluate molecular mechanisms associated with proliferative control since they require both growth factors and substrate adhesion to progress through the division cycle [11,13,14]. When deprived of serum mitogenic factors, NRK cells enter a G_o condition; PAI-1 mRNA and protein expression is low in quiescent cells but both are rapidly induced following serum stimulation commensurate with transit into a cycling G_1 condition [11]. To define the relationship between PAI-1 expression and re-entry into proliferative-phase growth, molecular events involved in PAI-1 gene regulation were examined in serum-stimulated NRK cells. The PAI-1 gene was found to be subject to a complex combination of both immediate-early and secondary influences. A novel, adhesion-dependent mechanism of PAI-1 gene control was identified which modulates the extent and duration of the serum-initiated inductive response.

MATERIALS AND METHODS

Cell culture

NRK-52E proximal tubular cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; 1000 mg/l D-glucose) supplemented with 10 % (v/v) fetal bovine serum (FBS), L-glutamine (20 mM), penicillin (1000 units/ml) and streptomycin (1000 μg/ml) (GIBCO, Grand Island, NY, U.S.A.). Monolayer cultures were trypsinized, resuspended in DMEM/10 % (v/v) FBS and seeded into 150-mm-diam. tissue-culture dishes. After reaching 70–80 % confluency, cells were washed twice with Hanks' balanced salt solution (HBSS), incubated in medium

Abbreviations used: SERPIN, serine protease inhibitor; NRK, normal rat kidney; PAI-1, plasminogen activator inhibitor type-1; ECM, extracellular matrix; uPA, urokinase plasminogen activator; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; CX, cyclohexamide; ACT-D, actinomycin D; ssDNA, salmon sperm DNA; GAPD, glyceraldehyde-3-phosphate dehydrogenase; SPARC, osteonectin; FBS, fetal bovine serum; NP-40, Nonidet P-40.

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containing 0.5% FBS for 3 days to achieve quiescence, then stimulated with 20% (v/v) FBS-supplemented DMEM for specified times [11]. Alternatively, quiescent cells were trypsinized, washed with serum-free DMEM, replated in suspension culture (i.e. by overlaying cells onto 100-mm-diam. tissue-culture dishes previously coated with 15 ml of 0.9% agarose in DMEM), and maintained under serum-free conditions for 15 h [13]. Suspended cells were either exposed to serum (by direct addition to a final concentration of 20%) or harvested and allowed to attach to tissue-culture dishes in 20% (v/v) serum-supplemented medium. Controls were maintained in suspension or allowed to attach to tissue-culture dishes under serum-free conditions for various times. For metabolic inhibitor studies, growth-arrested, quiescent-adherent or suspension cultures were pretreated with cyclohexamide (CX; 5×10^{-5} M), puromycin (20 μg/ml), or actinomycin D (ACT-D; 5 μg/ml) for 15 min in serum-free medium; effective concentrations of CX, puromycin and ACT-D were determined previously [12].

RNA isolation and Northern-blot analysis

Suspended cells, collected by centrifugation at 500 ***g*** for 10 min, and adherent monolayers were washed with ice-cold PBS and total RNA isolated using RNAzol B (Cinna/Biotecx Laboratories International Inc., Friendswood, TX, U.S.A.). For Northern-blot analysis, 10 μg of total RNA was separated on 1.2% agarose/formaldehyde gels then transferred to Nytran maximum-strength nylon membranes (Schleicher and Schuell, Keene, NH, U.S.A.) by capillary action in 10 × SSPE (1.8 M NaCl, 100 mM NaH_2PO_4, pH 7.4, 10 mM EDTA). RNA was immobilized by UV cross-linking and blots prehybridized at 42 °C for 2 h in buffer containing 50% formamide, 5 × Denhardt's reagent, 1% (w/v) SDS, 100 μg/ml sheared/heat-denatured salmon sperm DNA (ssDNA) and 5 × SSPE. Hybridization with random-primed [^{32}P]dCTP-labelled cDNA probes [(2–5) × 10^6 c.p.m.] to rat PAI-1 [15,16], actin, glyceraldehyde-3-phosphate dehydrogenase (GAPD), osteonectin (SPARC; gift from E. Helene Sage, University of Washington, Seattle, WA, U.S.A.), and A50 (gift from J. Stavnezer, University of Massachusetts, Worcester, MA, U.S.A.) was for 16 h at 42 °C in buffer consisting of 50% formamide, 2.5 × Denhardt's reagent, 1% SDS, 100 μg/ml ssDNA, 5 × SSPE, and 10% dextran sulphate. Membranes were washed twice in 6 × SSPE/0.1% SDS at room temperature for 15 min followed by two 15 min washes in 1 × SSPE/0.1% SDS at 37 °C. Probe was removed from membranes by incubation in 55% formamide/2 × SSPE/1% SDS for 1 h at 65 °C and rinsing in 0.1 × SSPE/0.1% SDS prior to rehybridization. Linearized pTRI-GAPD (containing rat GAPD, exons 5–8), pTRI-c-fos (containing mouse c-*fos* exon 2) and pT7 18 S RNA (containing an 80-bp fragment of the human 18 S ribosomal RNA gene) served to generate T7 RNA polymerase-directed ^{32}P-labelled antisense riboprobes using the MAXIscript T7/T3 *in vitro* transcription kit (Ambion, Austin, TX, U.S.A.). For riboprobes, prehybridization and hybridization were carried out at 60 °C using the buffers described above after which membranes were washed three times in 1 × SSPE/0.5% SDS for 15 min at 65 °C followed by one 15 min wash in 0.1 × SSPE/0.1% SDS at 60 °C. Blots were exposed to X-OMAT AR-5 film (Kodak, Rochester, NY, U.S.A.) using intensifying screens.

Run-off transcription assay

Linearized PAI-1, actin, A50, pfos-1 (containing a v-*fos* specific sequence; A. T. C. C., Rockville, MD, U.S.A.) and pBluescript (SK^-) plasmids were denatured in 0.3 M NaOH at 65 °C for 30 min; 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) was added to achieve a final concentration of 6 × and 5 μg of each DNA was slot-blotted on to nitrocellulose. Membranes were baked at 80 °C *in vacuo* for 1 h. Monolayer cultures of quiescent and serum-stimulated NRK cells were washed twice, scraped into ice-cold PBS, and collected by centrifugation at 500 ***g*** for 5 min at 4 °C. Nonidet P-40 (NP-40) lysis buffer (10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) was added while gently vortexing cell pellets; lysed cells were incubated on ice for 5 min. Nuclei, harvested by centrifugation (as described above), were washed in lysis buffer and resuspended in 50 mM Tris/HCl (pH 8.3), 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA for storage in liquid nitrogen. For labelling of initiated transcripts, (5–10) × 10^6 nuclei (in storage buffer) were mixed with an equal volume of 2 × reaction buffer {10 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 5 mM dithiothreitol, 1 mM each ATP, CTP, GTP, and 100 μCi of [^{32}P]CTP (Dupont/New England Nuclear, Boston, MA, U.S.A.)}, and incubated at 30 °C for 30 min with gentle shaking. Nuclear RNA was isolated using the RNAzol B procedure and resuspended in 1 mM EDTA, pH 7.4. For analysis, 10^6 c.p.m. labelled nascent RNA were hybridized in 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, pH 7.4, 10 mM EDTA, 0.2% SDS, 0.6 M NaCl at 65 °C for 40 h to plasmid DNA immobilized on to nitrocellulose. Membranes were washed twice for 1 h at 65 °C in 2 × SSC, digested with 10 μg/ml RNase A in 2 × SSC at 37 °C for 30 min, and washed for 30 min at 37 °C in 2 × SSC. Membranes were exposed to X-OMAT AR-5 film using intensifying screens.

Autoradiography

NRK cultures, grown on glass coverslips in 35-mm-diam. tissue-culture dishes, were growth-arrested by incubation in DMEM/0.5% FBS for 3 days prior to the addition of [^{3}H]thymidine (0.1 μCi/ml) in medium containing 0% or 20% FBS. At 2 h intervals, duplicate coverslips were processed for autoradiography; three sets of 300 cells each were counted per coverslip to calculate the percentage labelled nuclei (i.e. > 15 grains/nucleus).

RESULTS

PAI-1 expression is induced in an immediate-early response manner in serum-stimulated NRK cells

The previous finding that PAI-1 expression was growth-state-regulated in NRK cells [11] necessitated further definition of both the kinetics and metabolic requirements for serum-mediated induction. To establish these parameters within the time frame of re-entry into the division cycle, a functional marker of cell-cycle progression (i.e. incorporation of [^{3}H]thymidine) was utilized to topographically map the course of induced PAI-1 expression as a function of growth state. PAI-1 mRNA levels increased significantly within 2 h of addition of serum to quiescent NRK cells, were maximal at 4 h, and declined with the onset of DNA synthesis at 12 h; by 24 h after serum-stimulation, PAI-1 transcript abundance approximated to that of growth-arrested cultures (Figure 1). SPARC expression appeared restricted to G_1 while c-*fos* mRNA induction was confined, as expected, to the early stages of cell activation. GAPD mRNA increased in late G_1 and remained elevated throughout the remainder of the growth cycle, whereas actin transcripts paralleled those of PAI-1 but at reduced levels. A50 and 18S ribosomal RNAs were approximately unchanged throughout the 24 h post-stimulation period. PAI-1 transcript induction required ongoing RNA synthesis and

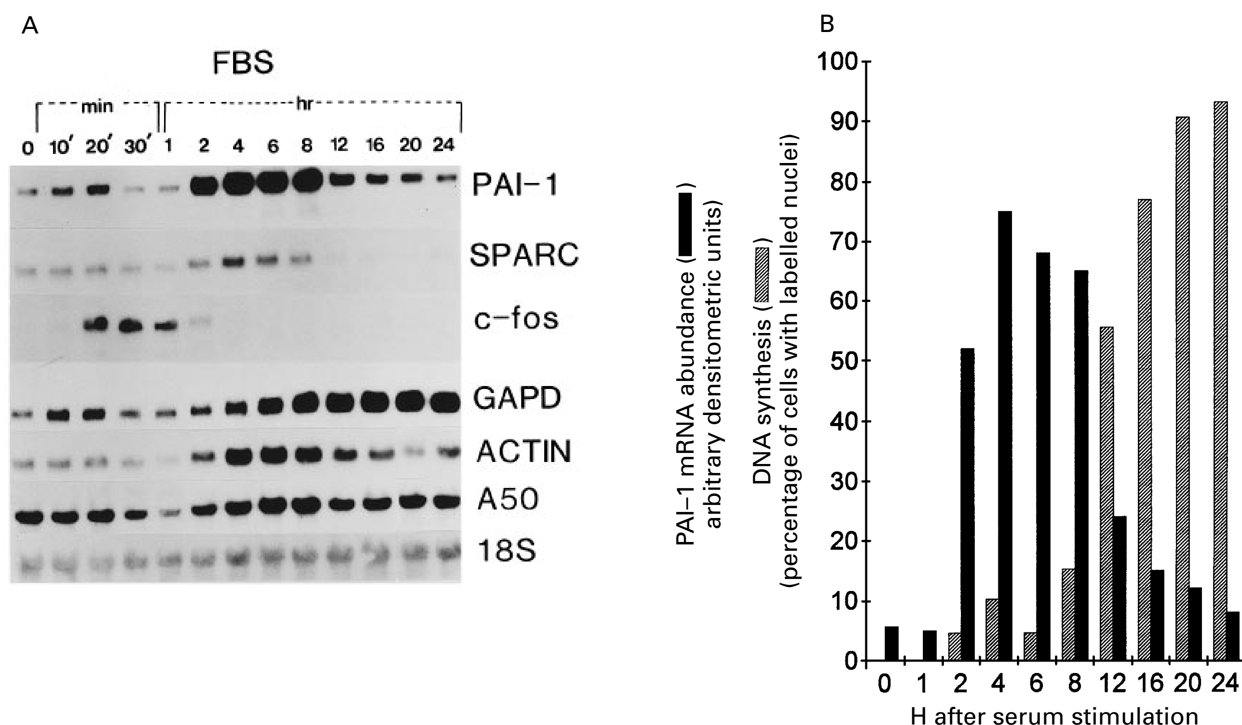

Figure 1 PAI-1 mRNA inductive kinetics as a function of time after addition of serum to quiescent NRK cells

(**A**) The medium in subconfluent NRK cell cultures was changed to DMEM/0.5% FBS; after 3 days, growth-arrested populations were stimulated with 20% (v/v) FBS-containing medium (FBS) and total cellular RNA isolated at the times shown (10 min to 24 h). Quiescent cultures harvested prior to stimulation served as time 0 controls. Each lane was loaded with 10 μg of RNA and Northern blots hybridized with the indicated ^{32}P-labelled probes. (**B**) Temporal relationship between induced PAI-1 transcript levels and onset of DNA synthesis as a function of time after addition of 20% FBS-containing medium to growth-arrested NRK cell cultures. PAI-1 mRNA expression was maximal in mid-G_1 (4 to 6 h post-stimulation); transcript abundance declined by 12 h after serum addition correlating with entrance into S phase.

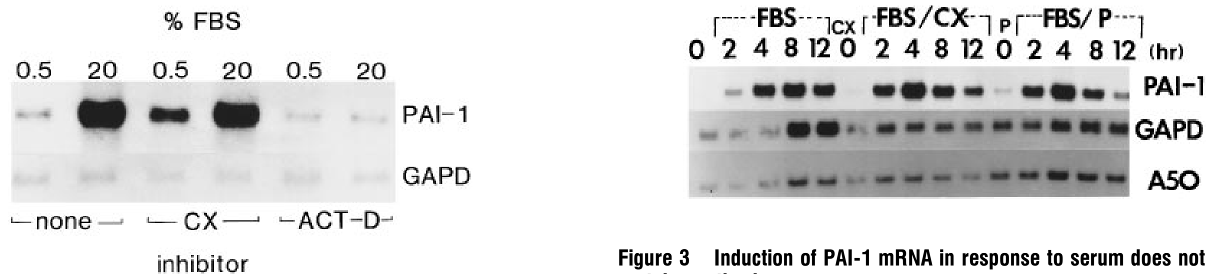

Figure 2 Metabolic requirements for PAI-1 mRNA induction in response to serum-stimulation

RNA was isolated from quiescent (0.5% FBS) and stimulated (20% FBS for 2 h) NRK cells as well as from quiescent cells exposed to CX or ACT-D in the presence or absence of 20% FBS. Blots were hybridized with ^{32}P-labelled cDNA probes to PAI-1 and GAPD.

Figure 3 Induction of PAI-1 mRNA in response to serum does not require protein synthesis

Quiescent NRK cells were stimulated with 20% FBS-containing medium in the absence (FBS) and presence of CX (FBS/CX) or puromycin (FBS/P) for 2 to 12 h. Cells maintained under growth-arrested conditions in the absence of inhibitors (time 0) as well as quiescent cultures pretreated with CX (time 0/CX) or puromycin (time 0/P) alone served as unstimulated controls. RNA was isolated at the indicated times and Northern blots hybridized with ^{32}P-labelled cDNA probes to PAI-1, GAPD and A50.

was not blocked by inhibition of *de novo* protein synthesis (Figure 2), although the temporal pattern of PAI-1 expression was different between control and CX- or puromycin-treated cultures (Figure 3). Inhibition of *de novo* protein synthesis did not dramatically influence steady-state levels of A50 transcripts; the serum-dependent increases in GAPD mRNA at the 8–12 h time points, however, were completely blocked in the presence of CX (Figures 1 and 3). PAI-1-inductive characteristics in response to serum, therefore, appear to be consistent with an immediate-early mode of transcriptional activation [17,18]. Indeed, increased PAI-1 transcription, evident within 10 min of serum addition (correlating with c-*fos* gene activation), occurred in the presence of either CX or puromycin, and attained maximal levels 2–8 h post-serum addition before declining at 12 h (Figure 4). PAI-1 transcriptional activity thus parallels the mRNA abundance profile (Figure 1) and defines a window of induced expression during transit of stimulated cells through the G_1 phase of the growth cycle.

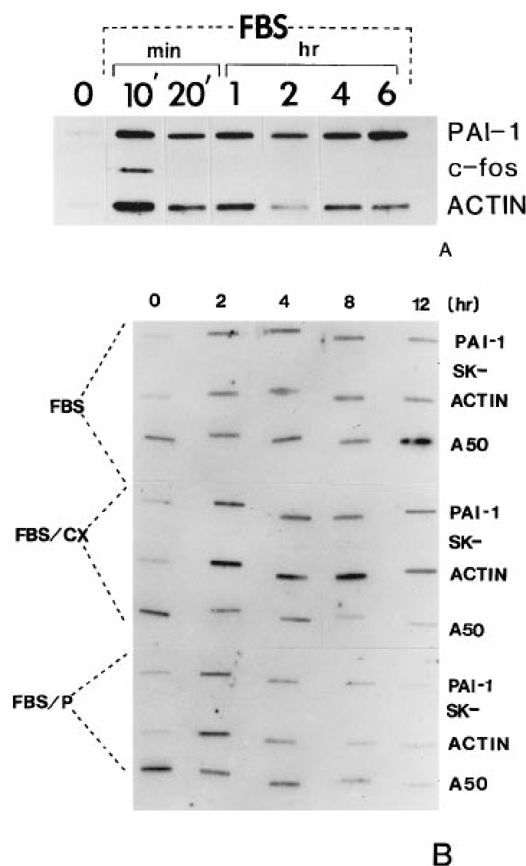

Figure 4 Transcriptional activation of the PAI-1 gene occurs in the absence of *de novo* protein synthesis

(**A**) Quiescent NRK cultures (time 0) were stimulated with 20% serum-containing medium (FBS) for 10 min to 6 h. Alternatively (**B**), quiescent cells as well as quiescent cells pretreated with CX or puromycin were stimulated with DMEM/20% FBS (FBS) or with the same medium containing CX (FBS/CX) or puromycin (FBS/P) for 2 to 12 h. Nuclei were isolated at the indicated times. For run-off assays, nascent PAI-1, c-fos, actin and A50 [^{32}P]UTP-labelled transcripts were detected by hybridization to the indicated immobilized probes. pBluescript (SK^-) DNA served as a control for non-specific hybridization.

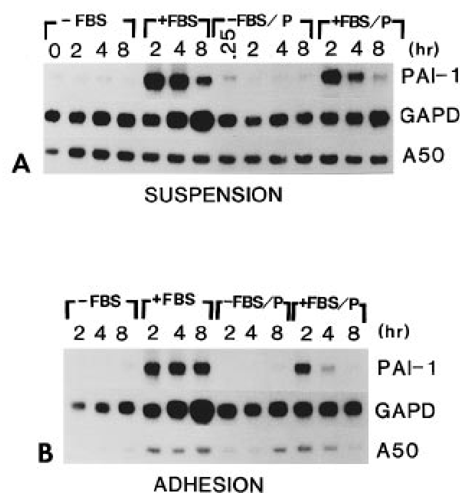

Figure 5 Effects of serum and adhesion on PAI-1 mRNA levels

(**A**) Quiescent NRK cells were cultured in suspension for 15 h in serum-free medium (time 0; −FBS). Cells were then maintained in suspension under either serum-free conditions (−FBS) or in the presence of serum at a final concentration of 20% (+FBS) for 2 to 8 h. For inhibitor studies, suspended cells were pretreated with puromycin for 15 min (0.25 h; −FBS/P). RNA was harvested at 2, 4, and 8 h from puromycin-treated cells cultured in the presence (+FBS/P) or absence (−FBS/P) of 20% FBS. In adhesion experiments (**B**), time 0 suspended cells were allowed to attach to tissue-culture plastic in the absence (−FBS) or presence (+FBS) of 20% FBS-containing medium. Alternatively, puromycin-pretreated suspended cells (maintained in puromycin) were allowed to attach in the absence (−FBS/P) or presence (+FBS) of 20% FBS-containing medium. After 2 to 8 h, RNA was isolated from adherent populations and hybridized with ^{32}P-labelled cDNA probes to PAI-1, GAPD and A50.

Serum and adhesion differentially regulate PAI-1 mRNA levels in NRK cells

Assessment of G_1/S transit by [^{3}H]thymidine autoradiography confirmed that the decrease in PAI-1 mRNA content in late G_1 phase correlated with onset of DNA-synthetic phase (at approx. 12-h post-serum stimulation in the NRK cell system) (Figure 1). NRK cells have a stringent anchorage requirement for entry into S phase; this adhesion-dependent growth restriction point also maps to late G_1 [13,14], suggesting that PAI-1 down-regulation and attachment-dependent metabolic events may be related. In fact, serum and adhesion independently induce the immediate-early response genes c-*fos* and c-*myc* in cultured fibroblasts [14,19]. To evaluate potential cooperative influences between serum and adhesion on PAI-1 expression, quiescent NRK cells in monolayer culture were trypsinized and seeded on to agarose-coated surfaces (where they remained in an unattached state). After 15 h, serum was added to a final concentration of 20% while controls were maintained in serum-free medium. Serum effectively induced PAI-1 mRNA expression in suspended cells (Figure 5A) although transcript levels rapidly declined between 2 and 8 h post-serum addition. Unlike for c-*fos* and c-*myc* expression [19], adhesion of quiescent NRK cells to culture-grade plastic in the absence of serum did not elicit a PAI-1-inductive response (Figure 5B). Attachment, however, significantly modulated the extent and kinetics of serum-induced PAI-1 expression. Relative to the collateral suspension culture, adhesion attenuated PAI-1 mRNA accumulation in response to serum at the earliest time point examined (2 h). Expression in newly adherent cells, moreover, remained stably elevated for up to 8 h, unlike stimulated suspension cultures in which PAI-1 mRNA levels progressively declined over the same time period. In contrast, induction of GAPD mRNA by serum in suspension culture was similar to that observed in newly adherent cells and, thus, was not influenced by adhesion. A50 transcripts, however, were regulated similarly to PAI-1 under newly adherent, but not suspension, culture conditions.

Adhesion-dependent maintenance of PAI-1 expression in late G_1 requires *de novo* protein synthesis

The time course of PAI-1 expression in suspension cultures stimulated with serum in the presence of puromycin was virtually identical to kinetics evident in cells stimulated in the absence of the inhibitor (Figure 5A). Puromycin also failed to inhibit PAI-1 induction by serum in newly adherent populations. Expression kinetics in such adherent, inhibitor-treated cells were similar to serum-stimulated suspension cultures, indicating that the serum response pathway is functional regardless of adhesive influences or capacity for *de novo* protein synthesis (Figure 5B). PAI-1 mRNA abundance in cells allowed to adhere in the presence of serum and puromycin for 2 h was reduced, however, compared with suspension cultures similarly treated for the same period of time. Adhesion-mediated attenuation of PAI-1 induction thus

also does not require *de novo* protein synthesis. In contrast, maintenance of steady-state PAI-1 transcripts at comparable levels throughout the 2–8 h post-serum addition period required both attachment and *de novo* protein synthesis as mid-G_1 PAI-1 expression markedly declined in serum-stimulated cultures allowed to adhere in the presence of puromycin (Figure 5B). Similarly, PAI-1 mRNA levels at the 8 to 12 h time points in serum-stimulated monolayer cultures were reduced in the presence of CX or puromycin (Figure 3), suggesting that the adhesion-dependent secondary (i.e. protein synthesis-requiring) mechanism associated with maintenance of PAI-1 expression in late G_1 is operative under 'normal' monolayer culture conditions.

DISCUSSION

In serum-stimulated monolayer cultures of NRK cells, PAI-1 transcription and mRNA accumulation occur within a restricted window in G_1 phase. Assessment of the duration of this 'activated' G_1 period, using [^{3}H]thymidine autoradiography to delineate onset of S phase, indicated that maximal transcript levels are restricted to approximately mid-G_1 and decline prior to, or upon entrance into, the DNA-synthetic phase (i.e. approx. 8–12 h after serum stimulation). Although protein synthesis inhibitors did not block transcriptional induction, PAI-1 mRNA abundance in CX- and puromycin-treated cultures 8 to 12 h after serum addition (i.e. in late-G_1/early-S phase) was reduced compared with cultures stimulated in the absence of the drugs. A protein synthesis-dependent event thus appears to be required for late-G_1 maintenance of PAI-1 transcripts in stimulated monolayer cultures. Puromycin reduced PAI-1 transcription between 2 and 4 h, unlike the continued transcription evident at 4 and 8 h in FBS and FBS/CX populations. Transcriptional suppression was not coincident with an abrupt reduction in PAI-1 mRNA levels but rather with continued accumulation reaching levels (at 4 h) greater than that observed in cultures serum-stimulated in the absence of the inhibitor. PAI-1 mRNA content in CX-treated, serum-stimulated cultures was also elevated at the 2 and 4 h time points relative to untreated controls. Inhibition of translation with CX or puromycin increases stability of mRNAs containing AU-rich destabilizing elements within the 3′ untranslated region (e.g. c-*fos* and c-*myc*) [20–22]. AU-rich sequences within the 3′ untranslated region [23,24], AU-binding factors [25,26] and other destabilizing elements (e.g. in the coding region of c-*fos*) [24] have been identified in rapidly degraded mRNAs which appear to mediate deadenylation and decay when recognized by the translational machinery. The capacity of CX and puromycin to stabilize mRNA is thought to be associated with translational inhibition and/or the loss of ancillary proteins that mediate message degradation as a consequence of protein synthesis inhibition. In addition, CX promotes message stabilization by trapping mRNA on polysomes thus protecting them from cytoplasmic nucleases [27,28]. Since AU-rich destabilizing elements are present in the 3′ untranslated region of the 3.1-kb rat PAI-1 mRNA transcript [29], PAI-1 mRNA stability changes may contribute to enhanced accumulation of PAI-1 mRNA in cultures serum-stimulated in the presence of CX and puromycin at 2 and 4 h even though nascent PAI-1 transcript levels are reduced in puromycin-treated cultures within the same time period. Suppression of later-stage PAI-1 gene transcription in serum-stimulated cultures as a consequence of puromycin treatment, however, was ultimately reflected in the reduced accumulation of PAI-1 mRNA at 8 and 12 h. These results suggest the existence of a novel, albeit complex, mechanism of PAI-1 gene regulation in serum-stimulated monolayer cultures of NRK cells and support a model of induced PAI-1 expression which incorporates both immediate-early (protein synthesis-independent) inductive and secondary (protein synthesis-dependent) modulatory events. Both inductive mechanisms, in fact, have been implicated separately in the control of PAI-1 synthesis in several cell types [30–34]. In the NRK cell system, serum induces PAI-1 transcription in an immediate-early response fashion, whereas adhesion constitutes the modulatory component in this response.

Under appropriate circumstances, adhesion-dependent signal transduction events regulate both G_o/G_1 and G_1/S progression (e.g. [14,35–37]) as well as the expression of growth-related gene products associated with these transition states. Adhesion or integrin occupancy alone under culture conditions that abrogate cell spreading appears sufficient to elicit Na^+/H^+ exchange [38–40], influence inositol lipid turnover [41], and induce expression of certain immediate-early response genes (c-*fos*, c-*myc* and actin) [19], all of which are characteristic of G_o/G_1 transit. Transcripts which typically accumulate at the G_1/S border (i.e. cyclin A, thymidine kinase and histone H4) are also dependent upon substrate attachment for expression in 3T3 and NRK cells [13,14]; such delayed-early or late G_1 genes usually exhibit secondary inductive characteristics (e.g. [34,42,43]). Data obtained in the suspension culture system demonstrate that serum effectively induced PAI-1 mRNA in an adhesion- and protein synthesis-independent manner. Simple re-attachment of suspended cells (i.e. in the absence of serum) did not elicit PAI-1 mRNA accumulation while replating and spreading on to plastic effectively attenuated induced levels of PAI-1 expression. Indeed, reduced PAI-1 transcripts were evident in newly adherent, serum-stimulated NRK cells (at the 2 h time point) relative to the collateral suspension culture. While PAI-1 may be regulated differently from other immediate-early genes which respond positively to adhesion, the ability of cell-to-substrate attachment to down-modulate gene expression may not be unique to PAI-1. Both c-*myc* and actin transcript levels appear attenuated in serum-stimulated monolayer cultures, albeit with differing kinetics, as compared with stimulated suspended cells [13]. PAI-1 mRNA levels were maintained and, therefore, considerably greater at later time points (8 h) in newly adherent as compared with serum-stimulated suspension cells, implicating adhesive influences in maintenance of PAI-1 expression. While puromycin did not block the attenuating effects of adhesion in the early stages of PAI-1 induction (in the presence or absence of serum), inhibition of protein synthesis blocked adhesion-mediated secondary mechanisms associated with continued PAI-1 expression.

Induced renal cell proliferation *in vivo* (during compensatory regeneration of the tubular epithelium) as well as *in vitro* is characterized by specific changes in cellular shape and substrate adhesion [11,44,45]. Such cytoarchitectural concomitants (i.e. increased cell flattening and substrate attachment) [44,45] of the growth process appear necessary for progression into DNA synthetic phase [14,46] and initiate signals which modulate the expression of particular genes (e.g. PAI-1). In this regard, the attachment requirement for maintenance of PAI-1 expression in mid-G_1 may represent one aspect of the complex adhesive changes necessary for proper G_1/S transit within the overall programme of anchorage-dependent cell growth control.

This work was supported by NIH grant DK46272. We thank X. Mu, J. Slack, and L. White for valuable discussions during the course of this work.

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Received 25 September 1995/13 November 1995; accepted 16 November 1995