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Translocation of plasminogen activator inhibitor-1 during serum stimulated growth of mouse embryo fibroblasts

S. SRINIVAS, T. NAGASHUNMUGAM and G. SHANMUGAM
Cancer Biology Division, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

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Abstract. Serum-stimulated mouse embryo fibroblasts specifically secrete two proteins of molecular weights 48,000 and 26,000. The 48 kDa protein showed affinity to concanavalin A and was precipitated by antibody to plasminogen activator inhibitor. Immunoflow-cytometry using anti plasminogen activator inhibitor-1 serum indicate the presence of the 48 kDa protein in quiescent cells; this protein was virtually absent in serum-stimulated cells. The presence of the plasminogen activator inhibitor-1 related protein in quiescent cells and its absence in serum-stimulated cells in combination with the observation on the absence of this protein, in the medium of quiescent cells and its presence in the medium of stimulated cells indicate that the 48 kDa protein was transferred from the cells into the medium upon serum-stimulation. The serum-mediated transfer of plasminogen activator inhibitor-1 from the cells into the medium was inhibited by actinomycin-D suggesting that the transfer process required actinomycin-D sensitive events. Treatment of pre-labelled quiescent cells with medium containing 20% fetal calf serum resulted in the gradual transfer of the labelled 48 kDa protein to the extra cellular matrix. These studies indicate that exposure of quiescent cells to fetal calf serum results in the transfer of plasminogen activator inhibitor-1 from the cells to the growth medium *via* extracellular matrix. The translocation of the protease inhibitor from the cells to the matrix and medium may enable the cellular and possibly the membrane proteases to act on growth factors or their receptors thereby initiating the mitogenic response.

Keywords. Plasminogen activator inhibitor; deposition; fibroblasts.

Introduction

The mechanisms controlling eukaryotic cell growth and the role of proteases and their inhibitors in growth control remain poorly elucidated. Its understanding requires detailed knowledge of the biochemical events that occur when a proliferative response is induced by physiological stimuli. The interaction of growth factors with specific surface receptors on resting or G_o phase cells rapidly induces a cascade of biochemical events including the formation of phosphoinositide metabolites, phosphorylation of proteins and a transient increase of intracellular pH and free Ca^{2+} levels (Rozengurt, 1986). One or more of these biochemical events are thought to generate signals within the nucleus that activate a set of specific growth related genes resulting at the onset of DNA replication (Lau and Nathans, 1985; Denhardt *et al.,* 1986).

Secreted proteins play an important role in the regulation of cell proliferation. The secreted proteins fibronectin and collagens are extracellular matrix proteins

Abbreviations used: M_r, Molecular weight; ConA, concanavalin A; PAI-1, plasminogen activator inhibitor-1; MEM, minimum essential medium; FCS, fetal calf serum; ECM, extra cellular matrix; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate.

involved in the attachment of the cells to the substratum (Yamada *et al.,* 1985), while secreted proteases like collagenase and plasminogen activators and their inhibitors are involved in the turnover of matrix components and in tumor cell invasion (Bauer *et al.,* 1985; Hart and Rehemtulla, 1988; Moscateli and Rifkin 1988). In addition, secretion of growth inhibitors and growth factors were observed at different stages of cell growth and development (Nilsen–Hamilton and Hamilton, 1982; Harel *et al.,* 1985; Hsu and Wang 1986; Nagashunmugam and Shanmugam, 1987). Cell surface proteinases are shown to be important in growth and division of normal and transformed cells and the role of proteinases and their inhibitors in eukaryotic cell growth has been reviewed by Scott (1987). The secretion and deposition of proteases such as plasminogen activators (PA) and their inhibitors such as protease nexins and plasminogen activator inhibitors in the extracellular matrix suggest the possibility of cellular self regulation by growth related proteinases and their inhibitors (Laiho and Keski-Oja, 1989).

In the studies reported here, we have identified and characterized a serum-induced secreted protein of molecular weight (M_r) 48,000. This protein is specifically present in the growth medium of serum-stimulated cells but not in the medium of quiescent cells. Immunoprecipitation and concanavalin A (Con A) binding studies indicate that this protein is related to endothelial cell type plasminogen activator inhibitor (PAI-1). Evidence is provided for the translocation of the 48 kDa protein from the quiescent cells to the growth medium upon serum-stimulation.

Materials and methods

Cell culture

Secondary cultures of Swiss mouse embryo fibroblasts were maintained at 37°C in minimum essential medium (MEM) of Eagle (Flow Labs, UK) containing 10% bovine serum (Flow Labs, UK) and gentamycin 50 μg/ml. Cells from the fourth passage and above were used for experiments. Cells were synchronized at quiescence by maintaining sub-confluent monolayers in 0·5% serum containing medium for 72 h. Cells were released from quiescence by the addition of 20% fetal calf serum (FCS) containing medium. Serum-stimulation period was 6 h unless otherwise mentioned.

Radiolabelling of proteins

Quiescent and stimulated monolayers (120 cm^2) were pulse-labelled with 50 μCi of [^{35}S] methionine (Amersham, specific activity 10·7 Ci/μmol) for 30 min in 5 ml of Hank's balanced salt solution. At the end of labelling, the medium was removed and the monolayer was washed thrice with MEM. The radioactivity was chased by incubating the monolayers for 30 min in serum-free medium. After the chase, the medium was saved for the analysis of secreted proteins. The extracellular matrix (ECM) proteins were isolated as described by Laiho *et al.* (1986). The secreted and ECM proteins were processed for electrophoresis as described earlier (Nagashunmugam *et al.,* 1989). Briefly, the conditioned medium containing equal amounts of radioactivity were precipitated with 10% trichloroacetic acid in the

presence of 50 μg of bovine serum albumin (BSA) and kept at 4°C for 30 min. The samples were centrifuged at 15,000 *g* for 20 min and the protein precipitate was air dried, dissolved in 0·1 N NaOH and then mixed with equal volume of 2 × electrophoresis sample buffer.

Analysis of cellular fluorescence

The intracellular content of PAI-1 in quiescent, and serum-stimulated cells was quantitated using PAI-1 antiserum obtained from Dr. David Loskutoff, Scripps Clinic, San Diego, USA. FITC conjugated anti-rabbit IgG was used as second antibody and the fluorescence was quantitated in a fluorescence activated cell scanner (Becton Dickinson) as described earlier (Nagashunmugam *et al.,* 1989).

Immunoprecipitation

Immunoprecipitation of secreted and ECM proteins were carried out following the procedure of Chackalaparampil *et al.* (1985). Labelled medium (10 ml) containing secreted proteins from serum stimulated cells were concentrated to 0·5 ml by ultrafiltration and made up to 1 ml in RlPA buffer [0·1 % Triton X-100, 1% sodium deoxy cholate, 0·1 % sodium dodecyl sulphate (SDS), 0·15 M NaCl, 2mM phenylmethylsulphonyl fluoride and 0·05 M Tris-HCl pH 7·2] and centrifuged at 10,000 *g* for 20 min. The extracellular matrix proteins were collected in R1PA buffer and centrifuged at 10,000 *g* for 20 min. To the 10,000 g supernatants 10μl of anti PAI-1 serum was added and the mixtures were incubated at 37°C for 30 min and then kept at 4°C overnight. The antigen-antibody complexes were treated with an excess of protein A sepharose (30 μl of 50% slurry in Rl PA buffer) at 4°C for 6–8 h. The absorbed complex was washed once with RIPA buffer thrice with washing buffer (50 mM Tris-HCl pH 7·4, 5% sucrose, 1% Nonidet P-40, 0·5 M NaCl and 5 mM EDTA) and finally with distilled water. The immune complexes along with protein A sepharose were subsequently suspended in 50 μl of electrophoresis sample buffer and heated at 100°C for 3 min. The sepharose beads were pelleted and the proteins in the supernatant were electrophoresed in 5–18% SDS-polyacrylamide gradient gels.

Gel electrophoresis and autoradiography

Intracellular and secreted proteins of quiescent and serum-stimulated cells were separated on high resolution SDS polyacrylamide gradient (5–18%) gels containing 6% stacking gels (Laemmeli and Favre, 1973). The protein samples were prepared in electrophoresis sample buffer and samples derived from equal amounts of cells were electrophoresed. Following electrophoresis, gels were stained with Coomassie blue and fluorographed (Bonner and Laskey, 1974). For the determination of molecular weights of the labelled polypeptides, molecular weight markers (Rajakumar and Shanmugam, 1983) were electrophoresed in parallel lanes and stained. The fluorogram was super-imposed over the dried gel containing stained marker proteins to determine the relative mobilities of the proteins. The molecular weights of the proteins were subsequently determined as described earlier (Rajakumar and Shanmugam, 1983).

Results

Secreted proteins of serum-stimulated and starved cells

Two proteins of M_r 48,000 and 26,000 were specifically secreted by quiescent cells in response to serum-stimulation (figure 1A). The 48 kDa secreted protein was absent in the conditioned medium of quiescent cells. However, high levels of this protein were present in the medium of stimulated cells. During the reverse process (serum-starvation), the level of the 48 kDa protein in the growth medium slowly declined (figure 1B). The low levels of the 48 kDa protein in the medium of serum-starved cells may be due to lack of transfer of this protein from the starved cells into the medium. This conclusion is supported by the immunofluorescence studies described below.

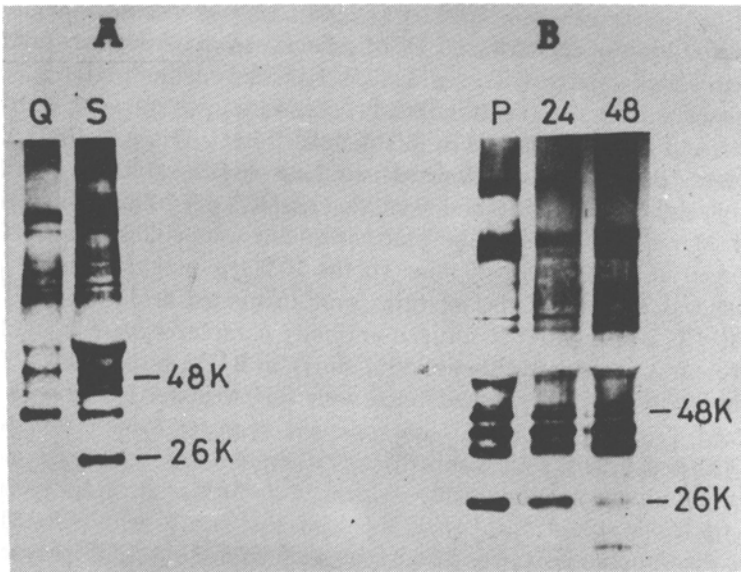

Figure 1. Effect of serum-stimulation **(A)** and serum-starvation **(B)** on the levels of secreted proteins. Q, quiescent cells; S, serum-stimulated cells; P, proliferating cells in 10% serum-containing medium. The numbers 24 and 48 indicate hours after shift of proliferating cells into serum-limiting (0·5% serum) medium. Cells were pulse labelled for 30 min with [^{35}S]methionine and then the radioactivity was chased for 30 min in serum-free chase medium. The proteins from the chase medium were electrophoresed in 5–18% polyacrylamide gradient gels containing SDS and fluorographed.

Another secreted protein of M_r 26,000 was also present in reduced levels in the medium of serum-starved cells in comparision to its amount in serum-stimulated cells (figure 1). Further studies on this protein are in progress. The 40 kDa secreted protein was shown to be a quiescent cell specific protein; the level of this protein declined in the growth medium of serum-stimulated cells (Subramaniam and Shanmugam, 1988).

To know whether the 48 kDa secreted protein is a glycosylated protein, ConA

binding studies were done. Figure 2 lane B shows that the 48 kDa secreted protein and the 240 kDa fibronectin (Subramaniam and Shanmugam, 1985) were the predominant ConA binding proteins secreted by serum-stimulated cells. The 48 kDa protein was also found to be a major component of the ECM (lane D). Since a ConA binding ECM protein of M_r 48,000 was identified as PAI-1 (van Mourik *et al.,* 1984), it was of interest to know whether the 48 kDa protein with similar properties reported here is related to PAI-1. Immunoprecipitation assays using antiserum to endothelial cell type plasminogen activator inhibitor indicate that the 48 kDa ECM and secreted proteins are related to PAI-1 (figure 2, lanes C and E).

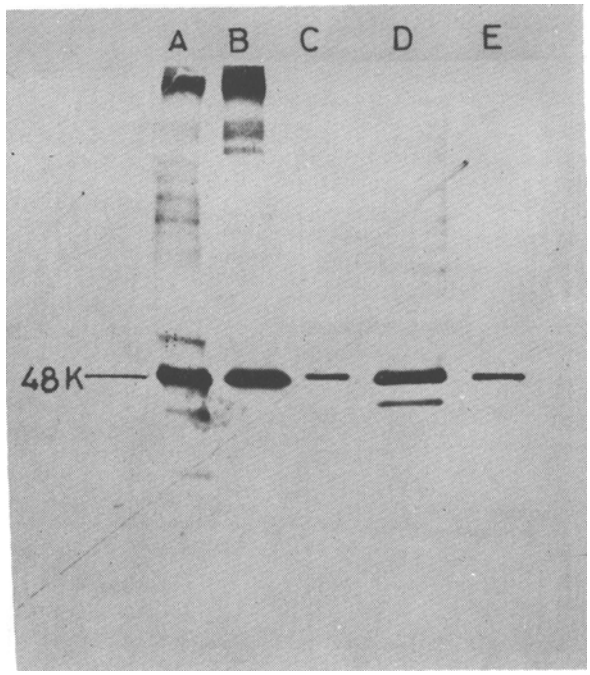

Figure 2. ConA binding and immunoprecipitation. Conditioned medium of serum-stimulated [^{35}S]methionine labelled cells were concentrated by ultrafiltration and aliquots were used for ConA binding and immunoprecipitation studies. For ConA binding, 50 μl of 50% suspension of ConA-sepharose 4B and 0·5 ml of concentrated conditioned medium were mixed and left at 4^0C over night with intermittant shaking. The ConA bound proteins were isolated as described by Laiho *et al.* (1986) and electrophoresed in 5–18% polyacrylamide gradient gels. Details of immunoprecipitation are given in the 'materials and methods'. **A.** Secreted proteins of serum-stimulated cells. **B.** Proteins of serum-stimulated cells bound to ConA-sepharose. **C.** Immunoprecipitate obtained using anti PAI-1 serum and concentrated conditioned medium from serum-stimulated cells. **D.** ECM proteins of serum-stimulated cells. E. Immunoprecipitate obtained using anti PAI-1 serum and ECM proteins of serum-stimulated cells. The proteins were electrophoresed in 5–18% polyacrylamide gradient gels containing SDS and autoradiographed.

Quantitation of intracellular PAI

The cellular levels of PAI-1 was quantitated by immunoflow-cytometry. Cells were

permeabilized with 0·1% Triton X-100 and reacted first with rabbit antiserum to PAI-1 and then with FITC conjugated anti-rabbit IgG. The cellular fluorescence was monitored by a FAC scanner (Nagashunmugam *et al.*, 1989). Quiescent cells treated with normal serum showed a single peak of low fluorescence (figure 3A). In contrast, quiescent cells reacted with an anti PAI-1 serum showed an additional (second) peak with higher fluorescence (figure 3B). Serum-stimulated cells treated with PAI-1 antibody did not show the high fluorescence (second) peak (figure 3C). However, serum-stimulated cells that were treated with actinomycin D (1 μg/ml) showed the high fluorescence peak when reacted with anti PAI-1 antibody. These results indicate the presence of PAI-1 in quiescent cells, its absence in serum-stimulated cells and its persistence in serum-stimulated cells maintained in actinomycin D containing medium.

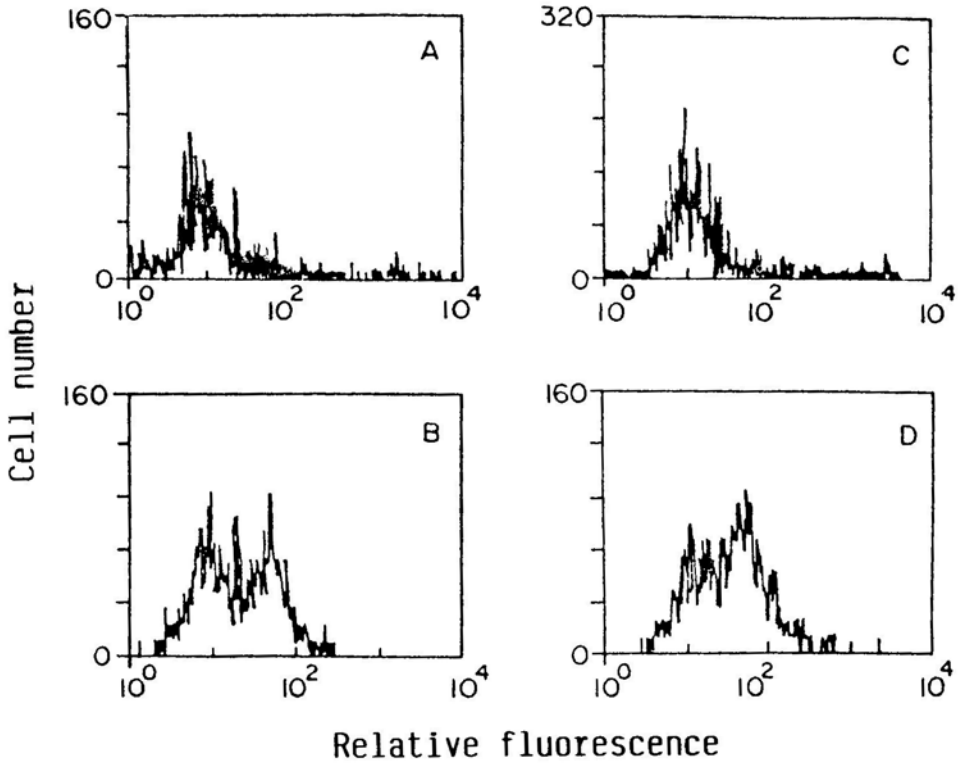

Figure 3. Quantitation of intracellular levels of PAI-1. Quiescent and serum-stimulated cells were collected, fixed with formaldehyde and permeabilized by Triton X-100 treatment. The permeabilized cells were reacted with either rabbit PAI-1 antibody **(B, C, D)** or normal rabbit serum (A). After reacting with FITC conjugated sheep anti rabbit γ-globulin, the fluorescence was quantitated in a FAC scanner. **A, B.** Quiescent cells. **C.** Serum-stimulated cells. D. Serum-stimulated cells treated with actinomycin **D.**

Effect of serum on prelabelled 48 kDa protein

The absence of the 48 kDa PAI related protein in the stimulated cells while being present in the quiescent cells and its appearance in the medium of serum-stimulated

cells implies that this protein may be translocated from the quiescent cells to the matrix and growth medium upon mitogenic stimuli. Studies on the fate of pre-labelled proteins provide evidence for this hypothesis. For these studies, 4 monolayers of MEF, each with equal number of cells were made quiescent by serum-starvation for 72 h. When these quiescent cells were pre-labelled for 30 min with [^{35}S] methionine and then stimulated with 20 % FCS in non-radioactive chase medium, substantial quantities of the labelled 48 kDa protein was found to be deposited in the extra cellular matrix of the serum-stimulated cells (figure 4). Laser

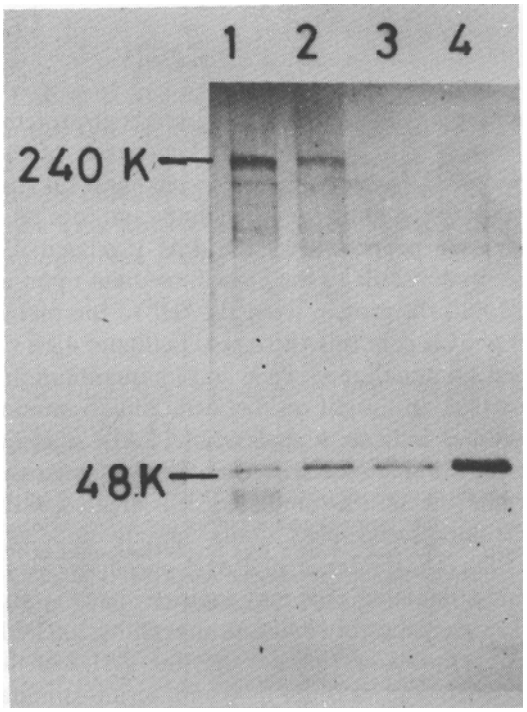

Figure 4. Fate of prelabelled 48 kDa protein. Quiescent mouse embryo fibroblasts were labelled with [^{35}S]methionine. After 30 min, the radioactive medium was removed, the monolayers were washed thrice with PBS and incubated for an hour in the conditioned medium obtained from quiescent cultures (Q medium). Lane 1, matrix proteins from cells that were maintained for an additional hour in Q medium. Lane 2, matrix proteins of cells that were maintained in 20% FCS containing medium for an additional hour. Lane 3, matrix proteins of cells that were maintained for an additional 2 h in Q medium. Lane 4, matrix proteins of cells that were maintained for an additional 2 h in 20% FCS containing medium. The matrix proteins were resolved by electrophoresis in 5-18% polyacrylamide gradient gels containing SDS and fluorographed.

densitometric quantitation of the radioactive 48 kDa protein bands shown in figure 4 indicates an increase of about 39 % in the intensity of the band after 1 h exposure of pre-labelled cells to 20% serum-containing medium (lane 2). This increase was much higher (2·6-fold) in the matrix derived from pre-labelled cells that were treated for 2 h with 20% serum-containing medium (lane 4). Under the same conditions, the level of the pre-labelled 240 kDa protein [previously identified as fibronectin

(Subramaniam and Shanmugam, 1985)] in the matrix declined in the first hour of exposure of quiescent cells with serum and disappeared at the end of 2 h treatment of pre-labelled cells with serum-containing medium. While FCS treatment had a spectacular effect on the time dependent transfer of the 48 kDa protein, the disappearance of the 240 kDa protein was not influenced by treatment of quiescent cells with FCS (lanes 3 and 4). These studies imply that mitogenic stimulation triggered the transfer of PAI-1 from the cells into the matrix. Previous studies indicate that PAI-1 is transferred into the medium *via* the extracellular matrix (Levin and Santell, 1987).

Discussion

In this study, we have identified a serum-induced secreted protein of M_r 48,000 as PAI-1. Based on the results of the presence of high levels of this protein in the quiescent cells and low levels in the stimulated cells, and on the absence of this protein in the quiescent cell conditioned medium and on its presence in the stimulated cell medium, we propose that the PAI-1 related 48 kDa protein is translocated from the quiescent cells to the growth medium upon mitogenic stimuli. Transfer of pre-labelled 48 kDa protein from the cell to the matrix in response to exposure of cells to 20% FCS confirms these results (figure 4).

We have used indirect immunofluorescence and its quantitation by fluorescence-activated cell analyser to get an insight on the intracellular amounts of PAI-1. The results presented in figure 3 indicate high levels of PAI-1 specific fluorescence in quiescent cells. The serum-stimulated cells did not show significant levels of PAI-1 specific fluorescence while the serum-stimulated cells treated with actinomycin D showed a PAI-1 specific fluorescence peak similar in magnitude as that observed in quiescent cells (figure 3D). The presence of PAI-1 specific peak in quiescent cells and its absence in serum-stimulated cells may indicate that the protein was lost by the stimulated cells. However, the concomitant appearance of PAI-1 in the matrix and medium upon serum-stimulation implies that the PAI-1 of the stimulated cell was transferred to the matrix and medium following serum-stimulated growth. The persistence of intracellular PAI-1 in serum-stimulated cells that were treated with actinomycin D implies that the transfer process of this protein required serum-stimulated actinomycin D sensitive proteins.

Exogenous and endogenous proteinases were shown to be involved in the release of membrane-bound mitogenic proteins (autocrine growth effectors) into the medium (Lieberman, 1983). The presence of these protease inhibitors at discrete sites may inhibit the action of proteases from triggering the mitogenic signal. Trypsin, thrombin and some serine proteinases initiate DNA synthesis in cultured cells by their action on the cell surface (Scott, 1987). Several peptide mitogens were found in association with esterolytic or proteolytic activities. Gospodarowicz and Moran (1976) have suggested that proteinases could potentiate the effect of growth factors by activation, or by conditioning the target cells, enabling them more sensitive to growth factors.

Since proteases elicit mitogenic response, removal or translocation of protease inhibitors from the cell may enable the action of cell associated proteases in the activation of peptide growth factors and their receptors. Our results on the translocation of PAI-1 is interesting in the light of the suggested role of PAI in the

inhibition of cleavage of pre-TGF-*β* (Keski–Oja and Moses, 1987). Thalacker and Nilsen–Hamilton (1987) have shown that TGF-*β* and TP A induced the secretion of PAI-1 in a variety of cell lines. In certain cells, TGF-*β* is implicated in the induction of cellular oncogenes like *c-sis,* which presumably activate *c-fos* and *c-myc* genes culminating in DNA synthesis (Leof *et al.* 1986). PAI-1 may inhibit the cleavage and subsequent activation of TGF-*β* and other growth factors by its presence in the quiescent cell. Translocation or removal of PAI from the quiescent cell may trigger the activation of growth related proteases in the quiescent cell enabling it to enter into the proliferating phase.

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