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Endothelial cells must express plasminogen activator inhibitor type-1 (PAI-1) in order to undergo an angiogenic switch during tumor progression. The PAI-1 gene, therefore, has emerged as an important candidate target for gene therapy of human breast cancer. During the period of this grant, we carried out studies to confirm that targeted ablation of PAI-1 gene expression resulted in a marked attenuation of endothelial cell migration as well as an inability to form and maintain capillary network structures on Matrigel-coated surfaces. Addition of PAI-1 protein or transfection of PAI-1 expression vectors rescued the migratory phenotype. Confirmatory results were obtained with human microvessel endothelial cells. Breast cancer-derived factors (TGF-β, EGF)were found to be important contributors of continued PAI-1 expression and long-term maintenance of capillary structures. PAI-1 synthesis and/or protease inhibitory activity, even in mature tubular networks, was required for a invasive growth and a stabilized angiogenic response. The work carried out under this award support the "balanced proteolysis" concept of angiogenesis, identified critical molecular mechanisms underlying the angiogenic response and support our hypothesis that the PAI-1 gene is an accessible anti-angiogenic target.

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INTRODUCTION

Continued growth of a malignant tumor beyond a certain critical size is dependent on the development of a network of feeder blood vessels (1,2). This "angiogenic switch" is highly-dependent on the temporally-regulated and focalized activity of several extracellular proteases and protease inhibitors involving members of both the plasmin-based and metalloproteinase cascades (3,4). *In vitro* analysis of the requirements for the formation of endothelial tubular networks in various culture model systems implicated both urokinase plasminogen activator (uPA) and its fast-acting type-1 inhibitor (PAI-1) as necessary to achieve complete angiogenesis, consistent with the "balanced proteolysis" concept of endothelial cell migration (5-7). Recent data in mice genetically-engineered to be deficient in expression of genes that encode specific elements of the plasmin activation system has confirmed, in fact, the critical importance of PAI-1 synthesis in tumor-induced angiogenesis (5-7). Indeed, the absence of host PAI-1 completely inhibited local invasion and vascularization of transplanted malignant tumors in PAI-1 null mice (5,6). This inability to mount an angiogenic response, moreover, prevented invasive growth by a highly aggressive and metastatic tumor type (5,6).

Breast tumors with elevated PAI-1 levels are particularly fast-growing carcinomas with a well-developed angiogenic network, a significant incidence of metastatic spread, early recurrence and poor prognosis (8, reviewed in 9). PAI-1 as a determinant in aggressive growth behavior is specifically important in the progression of mammary carcinoma. Most recently, PAI-1 has been shown to be markedly promigratory for invasive breast cancer cells, an effect attenuated by direct inhibition of PAI-1 function (10). Targeted knockout/reconstitution studies, moreover, identified PAI-1 as essential for, and an efficient modulator of, the neovascularization process. Recent findings, in fact, support a multifunctional role for PAI-1 in angiogenesis (e.g., this uPA inhibitor regulates stromal barrier proteolysis, facilitates endothelial cell migration by focalized exposure of cryptic matrix-binding sites, stabilizes nascent vessel structure and controls cell-to-matrix adhesion/de-adhesion). Analyses of systems that model specific stages in angiogenesis have disclosed that PAI-1 can exert potent pro- and anti-angiogenic effects depending on the context and the concentration (as well as specific activity) of this SERPIN (serine protease inhibitor) in the tissue microenvironment. These data highlight the potential relevance of PAI-1 modulation as a means to treat human breast cancer and complement our own work on genetic targeting of PAI-1 mRNA transcripts with subsequent attenuation of cellular motile traits (11-13).

The goals of this investigation were to determine, for the first time, the level of endothelial cell PAI-1 expression necessary for development and maintenance of the angiogenic phenotype in a 3-D culture model of breast tumor-stromal-endothelial cell interactions that mimics the *in vivo* disease state. Gene therapy approaches using antisense vector constructs as well as homologous recombination methods previously developed in this laboratory were utilized to directly disrupt PAI-1 gene expression in cultured endothelial cells. The consequences of this targeted disruption on the ability of endothelial cells to form branching angiogenic networks in response to co-culture with human breast cancer cells or identified breast cancer-derived factors was evaluated. This study constitutes the first comprehensive assessment of PAI-1 genetic therapy as an approach to inhibit growth of human breast cancers by targeting a gene essential for the angiogenic process. We also took this opportunity to assess mechanisms of PAI-1 gene expression control under culture conditions that closely mimic the optimal angiogenic response in vivo.

BODY

The work carried out during the period of this grant focused on three specific aims:

Task 1. To quantify the consequences of PAI-1 down-regulation (by antisense targeting) on the angiogenic response and the effects of vector-driven PAI-1 synthesis on human breast tumor-induced angiogenesis in endothelial cells (including PAI-1-null cells, a clone in which the endogenous PAI-1 gene was disrupted by molecular targeting). This phase of the work was necessary to determine the threshold level of PAI-1 expression required for cultured endothelial cells to undergo a switch to an angiogenic phenotype and involved the following:

a. development of a panel of endogenous PAI-1-null endothelial cells which synthesize differing levels of exogenous (vector-driven) PAI-1 mRNA and protein upon transfection with a constitutively active (CMV promoter-based) PAI-1 expression construct. This panel complements cells in which PAI-1 synthesis was disrupted using antisense expression vectors.

b. correlate levels of PAI-1 expression for each transfectant PAI-1-null-derived endothelial cell line with the extent of the angiogenic response (i.e., ability to form branched tubular networks and migrate over planar surfaces) induced by co-culture with human breast tumor cells and/or tumor-derived angiogenic stimuli (i.e., secreted growth factors).

Task 2. To determine the consequences of molecular genetic down-regulation of endogenous PAI-1 gene expression on the ability of endothelial cells to form branching angiogenic networks upon co-culture with human breast carcinoma cells as well as to migrate through matrix barriers in response to tumor-derived angiogenic stimuli. This is necessary to assess the extent to which endogenous PAI-1 gene expression is susceptible to genetic manipulation and the window of down-regulation required to achieve a defined and quantifiable therapeutic result (i.e., a reduction in, or loss of, the angiogenic response). The mechanism of PAI-1 gene regulation was also addressed using culture conditions and identified growth factors that predispose to development of an optimal angiogenic response.

Task 3. To evaluate the therapeutic usefulness of endothelial cell-specific targeting of anti-angiogenic expression vector constructs in the human breast carcinoma-stimulated co-culture model of induced angiogenesis.

Experimental findings.

Endothelial cell migration and capillary sprouting requires proteolysis. Studies in mice deficient in elements in the plasmin activation cascade confirmed the importance of uPA, PAI-1 and plasmin in cell migration (13). Excessive protease activity as typically evident in chronic wounds, however, prevents the coordinated assembly of endothelial cells into capillary structures highlighting the requirement for an appropriate proteolytic "balance" in tubular network formation. Genetic studies *in vivo*, moreover, have implicated PAI-1 as an important regulator of this balance. These data have been reviewed in our recent publication (13) **(appended)**. Indeed, orienting experiments confirmed that PAI-1 is expressed specifically in angiogenic "cords" and

migrating endothelial cells **(Figure 1)**.

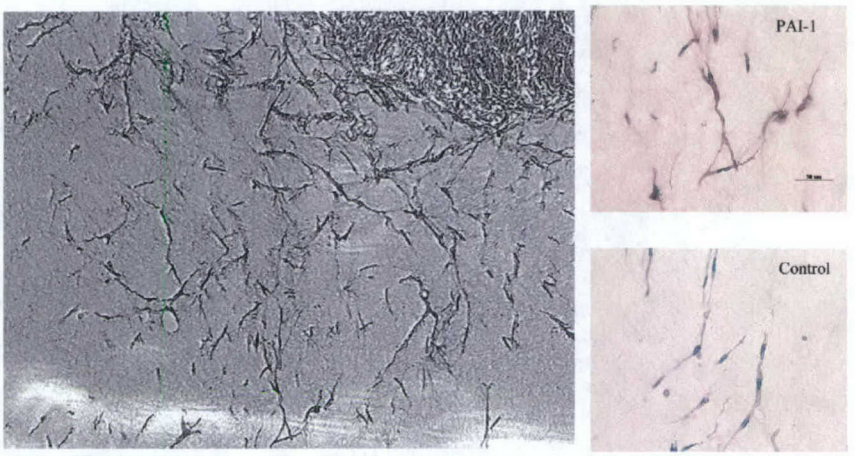

Figure 1. Identification of PAI-1-expressing endothelial cells in outgrowth culture. Human saphenous vein endothelial cells migrate into a fibrin gel within 2 weeks after placement of vein "rings" into a fibrin matrix (left panel). The original ring is at the upper right. Cells that migrate out of the vein segment express PAI-1 (upper right panel). Substitution of pre-immune rabbit serum in place of PAI-1 antibodies resulted in loss of immunoreactivity (Control, lower right panel). Unlike the obvious expression of PAI-1 by endothelial elements locomoting through the fibrin gel, the residual endothelium in the explant was PAI-1-negative.

Once it was established that PAI-1 synthesis was part of the angiogenic program, we created functional PAI-1 "knockout" cells using T2 cells as the parental strain by transfection of a PAI-1 antisense expression vector (10,11) to assess the role of this serine protease inhibitor in the angiogenic process. While wild-type T2 cells formed tubular networks when placed in culture over a Matrigel substrate and expressed high levels of PAI-1 mRNA, antisense vector-transfectants did not exhibit an angiogenic response (**Figure 2**). Northern blot analysis confirmed that the T2/IAP antisense cell line did not express PAI-1 transcripts (**Figure 2**) and that the down-regulation of expression achieved was, at the protein level, specific for PAI-1 **(Figure 3)**. A similar approach was used to create the PAI-1 functionally-null cell line 4HH (12). While wild-type T2 cells were capable of forming extensively branched capillary networks in a complex 3-D gel consisting of a 3:1 mixture of Vitrogen-Matrigel, 4HH cells were incapable of lattice formation and effectively degraded the gel scaffold **(Figure 3)**.

Since these data provided proof of principle (i.e., that inhibition of PAI-1 ablated *in vitro* angiogenesis), we derived a stable PAI-1-null T2 cell line by molecularly-disrupting the endogenous gene with a targeting vector. The resulting cell line (T2-null) similarly failed to form branched angiogenic networks *in vitro*. T2-null cells were subsequently transfected with the PAI-1 sense expression vector Rc/CMVPAI (8,9) and four neomycin (G418)-resistant cell lines were derived that varied in the level of vector-driven PAI-1 transcript expression and migratory ability (**Table 1**). Unlike T2 cells (or the related 4HH cell line) where PAI-1 expression is ablated by antisense PAI-1 (9), antisense c-*fos* (10) constructs or by targeted gene disruption and which are poorly motile and non-angiogenic (**Table 1**), vector-mediated PAI-1 "rescue" restored (to varying extents) cellular motile ability. The data presented in **Figures 2** and **3** as well as in **Table 1** implicate PAI-1 as an important element in the cellular motile process. To further evaluate the role of PAI-1 in various endothelial cell functions (i.e., matrix adhesion, motility, tubulogenesis), two endothelial cell lines (T2-WT, HMEC-1) were used in experiments to: (a) specifically attenuate

PAI-1 synthesis by transfection with the Rc/CMVIAP vector (as above), followed by attempts to "rescue" endothelial function by addition of exogenous active PAI-1 protein, or (b) inhibit PAI-1 activity with a neutralizing antibody. Genetically-targeted PAI-1 down-regulation with the antisense approach effectively inhibited T2 cell migration **(as in Table 1)** and this motile deficit could be rescued by addition of active PAI-1 protein; a similar rescue was evident in the PAI-1-

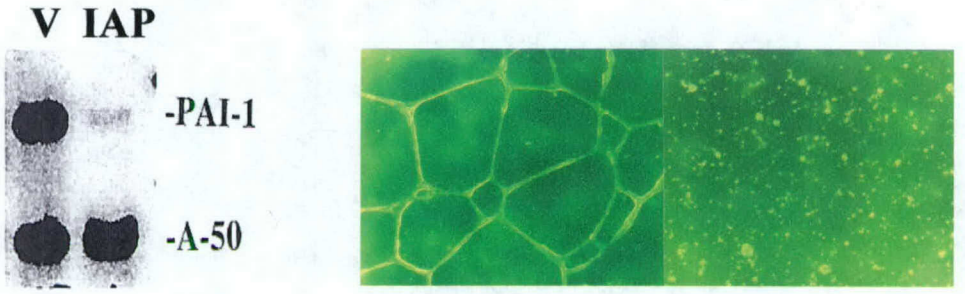

Figure 2. Capillary formation on Matrigel requires migration. T2 cells were stably transfected with the "empty" Rc/CMV vector (V) or vector bearing a full-length PAI-1 cDNA insert in antisense configuration (IAP) (as in Figure 2). Northern analysis indicated that control T2 cells or T2/V cells expressed abundant PAI-1 transcripts (left panel) and formed well-developed highly-branched tubular structures with luminal spaces within 48 hours after plating onto hydrated Matrigel-coated surfaces (middle panel). T2/IAP transfectants, in contrast, had significantly reduced PAI-1 mRNA levels (left panel) and failed migrate and coalesce into a defined capillary network. T2/IAP cells remained either as single cells (without any evidence of an associated migratory track) or formed small multicellular aggregates (right panel).

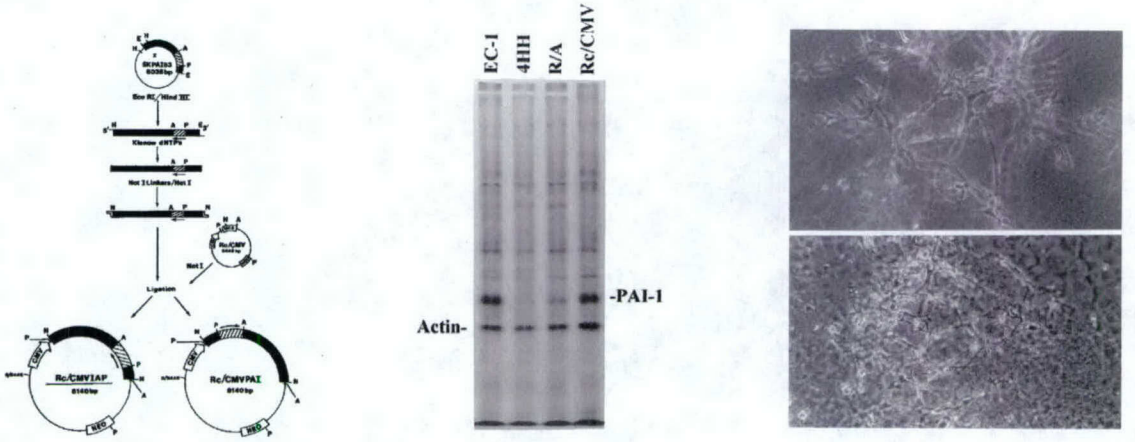

Figure 3. Targeted down-regulation of PAI-1 synthesis inhibits *in vitro* tubulogenesis. Construction of Rc/CMV plasmid vectors that drive expression of a full-length PAI-1 cDNA insert, cloned in sense (PAI) and antisense (IAP) orientations, under control of a CMV promoter (left panel). The black fill-in region corresponds to the PAI-1 cDNA inset. EC-1 parental cells were transfected with the Rc/CMVIAP vector and stable clones derived. To assess the success of Rc/CMVIAP driven down-regulation of PAI-1 synthesis and matrix accumulation, saponin-extracts of ^{35}S-methionine-labeled cells were separated by gel electrophoresis and proteins visualized by fluorography (middle panel). One derivative (4HH) did not express detectable PAI-1 protein nor accumulate PAI-1 in the matrix (middle panel). Cells transfected with Rc/CMV vector without insert expressed levels of PAI-1 similar to that of EC-1 controls. Wild-type T2 cells (top right panel) formed highly-branched and anastomizing capillary networks when suspended in a complex support matrix consisting of a 3:1 mixture of Vitrogen-Matrigel. Many of these tubular processes had clearly evident lumens. Extensive sprout formation was evident at the tips of T2 branches, moreover, indicative of both invasive and differentiated compartments. PAI-1$^{-/-}$ 4HH cells (bottom right panel), in contrast, failed to construct stable tubular structures and extensively degraded the gel matrix.

Table 1. Effect of PAI-1 expression targeting and vector "rescue" on cell motility using a quantifiable assay of planar locomotion[a]

Cell Line	Method of PAI-1 Expression Regulation	Relative Motility[b]
T2	None (wild-type)	100
T2/IAP	Rc/CMVIAP transfection	40 ± 8
T2-null	Targeted disruption vector	37 ± 5
T2-nullR1	Rc/CMVPAI	56 ± 4
T2-nullR2	Rc/CMVPAI	78 ± 9
T2-nullR3	Rc/CMVPAI	93 ± 7
T2-nullR4	Rc/CMVPAI	49 ± 3

[a] Wounds were created by pushing the narrow end of a sterile P1000 plastic pipette tip (Continental Laboratory Products, San Diego, CA) through the monolayer. Cultures were incubated in the existing media for times indicated in the text. Wound closure was assessed by time-lapse photomicroscopy and injury repair rates calculated, as a function of time, from measurements made utilizing an inverted microscope fitted with a calibrated ocular grid.

[b] Relative motility = distance migrated in 24 hours compared to wild-type T2 cells.

functionally-null 4HH cell line **(Figure 4)**. Use of PAI-1 neutralizing antibodies similarly attenuated T2 cell locomotion and also promoted substrate detachment of both T2 and HMEC-1 cells. PAI-1 activity was required for endothelial tubulogenesis on Matrigel surfaces as addition of neutralizing antibodies blocked cellular coalescence into tubular networks. This effect is consistent with the block in tubulogenesis attained by Rc/CMVIAP transfection **(Figures 2 and 3)**

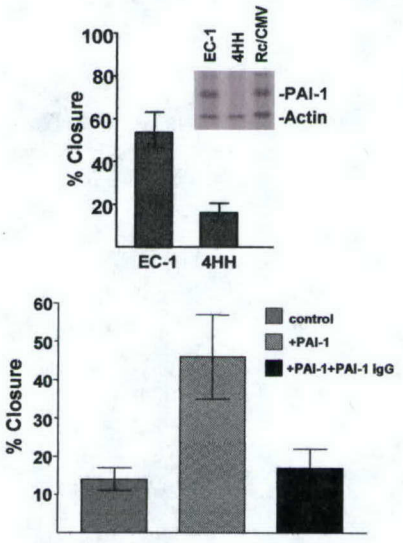

Figure 4. Effect of targeted PAI-1 ablation on migration of EC-1 cells and the PAI-1 functionally-null, EC-1-derived, 4HH cell line **(top panel)**. The deficient motility typical of 4HH cells reflected a failure to synthesize *de novo* PAI-1 protein, as a consequence of constitutive expression of PAI-1 antisense transcripts, compared to parental EC-1 or Rc/CMV empty vector-transfected EC-1 contols **(insert in top panel)**. Wound closure in 4HH cultures increased significantly following addition of active PAI-1 (1μg/ml) **(bottom panel)**. PAI-1-augmented motility was effectively ablated by co-incubation of PAI-1 with neutralizing PAI-1 antibodies prior to addition to wounded 4HH monolayers. Data plotted is the mean±s.d. of 15 individual measurements on duplicate cell cultures.

but, more specifically, implicate PAI-1 activity as required for tube formation. Importantly, the integrity of capillary networks formed by both WT-T2 and HMEC-1 endothelial cells was disrupted by addition of neutralizing PAI-1 antibodies suggesting that continued PAI-1 synthesis, even in "mature" tubes, was required for network stability. Indeed, recent findings (14) support a model whereby continued PAI-1 synthesis by "mature" tubular networks is required to prevent plasmin-dependent capillary regression and our results are confirmatory of this suggestion. These data are reviewed in the appended paper by Kutz and Higgins (2004). Collectively, these data indicate that PAI-1 expression is an essential and targetable aspect of a successful angiogenic response in vitro. The results presented in this Final Report, therefore, support our hypothesis that therapies directed at perturbation of PAI-1 synthesis or function in developing as well as mature angiogenic network structures may have significant benefit in the management of human breast tumors.

While T2 cells formed tubular structures in Matrigel culture, these networks were generally unstable and regressed after 2-3 days. The long-term (i.e., > 1 week) maintenance of the angiogenic response (complete with gel invasion, sprout formation from the lateral surface of the tubular structures, formation of complex branching patterns in 3-D orientation and, most importantly, maintenance of capillary network integrity) required co-culture with MDA-MB-231 human breast cells (as monolayers under the Matrigel overlays). A similar effect could be achieved (albeit with varying efficiency) using MDA-MB-231 conditioned medium in place of cells in the Matrigel culture. More importantly, the collagen-induced reorganization of endothelial cell cultures into "pre-capillary" structures (i.e., the earliest recognizable tubular response) was evident not only in T2 cells but also in primary rat aortic, bovine microvessel, human microvessel, and human umbilical endothelial cells and was similarly augmented by conditioned medium from human breast cancer cells. A variety of approaches (neutralizing antibodies to cytokines, pharmacologic inhibitors of signal transduction pathways) were used to identify the "active" angiogenic component elaborated by the human breast cancer cells. TGF-β1 was found to be essential to long-term maintenance of the tubular structures in both T2 and HMEC-1 cells since addition of neutralizing antibodies to MDA-MB-231 conditioned medium before addition to Matrigel cultures attenuated network stability and promoted tubular regression. More importantly, use of the EGF receptor inhibitor AG1478 also significantly reduced network stability suggesting that TGF-β1 and EGF ligands cooperate to maintain the long-term integrity of angiogenic networks that form in response to stimuli produced by human breast cancer cells. Since our data indicate that PAI-1 was a critical element in angiogenesis, it was important to determine if TGF-β1 actually induced EGF receptor activation (i.e., phosphorylation) and whether receptor activation was required for TGF-β1-induced PAI-1 expression. Indeed, TGF-β1 effectively stimulated EGF receptor activation **(Figure 5)** and pharmacologic inhibition of this receptor completely blocked PAI-1 gene expression **(Figure 6)** as well as growth factor- induced cell migration **(Figure 7)**. We are currently evaluating whether this same requirement governs the angiogenic response in primary endothelial cells.

We had established that cell migration (i.e., one essential aspect of the angiogenic switch) was a sufficient stimulus to activate PAI-1 expression in previously quiescent monolayers and that induced expression required binding of the bHLH-LZ transcription factor USF-1 to an E box motif in the PAI-1 promoter (these data are detailed in the three appended papers: Providence et al, 2002; Qi and Higgins, 2003; Allen et al, 2004). Most recently, culture of endothelial cells under optimal angiogenic conditions (i.e., in Matrigel using medium supplemented with FBS and TGF-β1), resulting in long-term maintenance of the formed capillary network, was determined to require

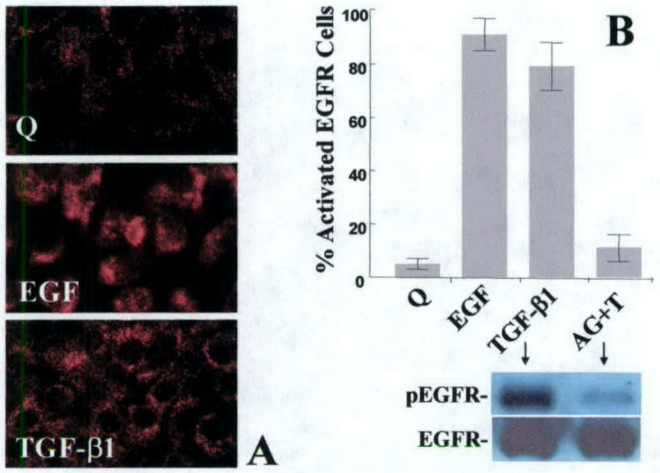

Figure 5. Activation of the EGFR by TGF-β1. Immunocytochemistry using antibodies specific for the activated form of the EGFR (clone 74) confirmed a relatively low level of receptor activation in quiescent (Q) cells **(A)**. Exposure to individual growth factors (EGF, TGF-β1) resulted in a significant increase in fluorescence intensity and perinuclear EGFR accumulation (indicative of increased receptor internalization). The fraction of TGF-β1-treated cells that exhibited increased EGFR internalization (i.e., "activated" EGFR) was approximately comparable to that of EGF-stimulated cells; this induction was inhibited by AG1478 pretreatment **(B)**. Data plotted **(B)** represent the mean±s.d. of triplicate experiments. Western analysis of EGFR activation (by immunoreactivity with clone 74 antibodies) indicated that phosphorylation of the 170 kDa EGFR in TGF-β1-stimulated cultures was AG1478-sensitive **(B)**. AG = AG1478 (2.5 μM); T = TGF-β1.

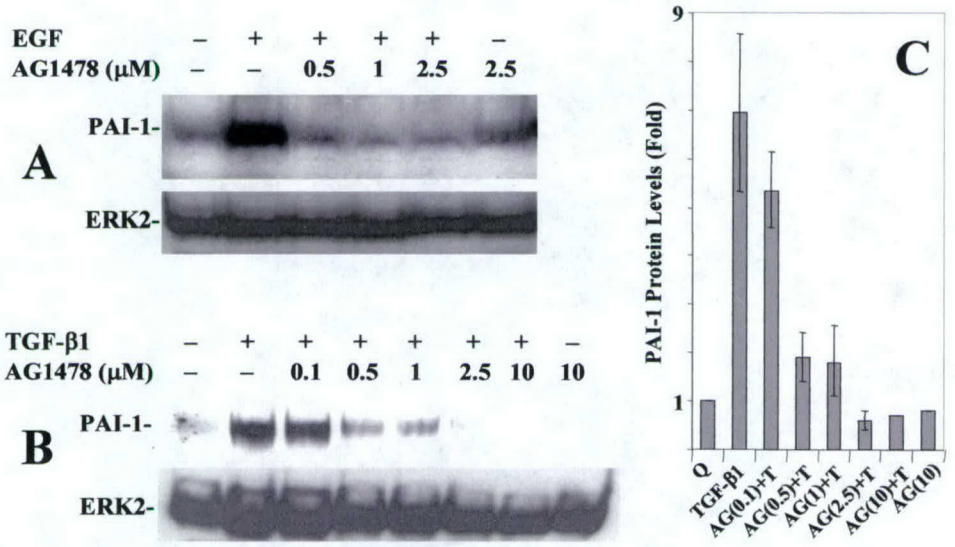

Figure 6. EGF- and TGF-β1-induced PAI-1 protein expression is attenuated by pretreatment with the EGFR inhibitor AG1478. Following serum-deprivation, near-confluent quiescent R22 cell cultures were exposed to increasing concentrations of AG1478 (as indicated) for 30 minutes prior to a 6-hour incubation with either EGF (10 ng/ml) or TGF-β1 (1 ng/ml). Cell extracts were separated by electrophoresis and PAI-1 protein levels assessed by western blotting. As expected, AG1478 effectively suppressed EGF-induced PAI-1 expression **(A)**. TGF-β1-stimulated PAI-1 expression was similarly sensitive to AG1478 pretreatment (with complete ablation of PAI-1 induction at AG1478 concentrations >1 μM), suggesting the involvement of EGFR signaling in TGF-β1-mediated PAI-1 synthesis **(B)**. Histogram **(C)** represents the summary (mean±s.d.) of triplicate experiments normalized to cellular ERK2 levels. AG = AG1478 (numbers indicate concentration in μM); T = TGF-β1.

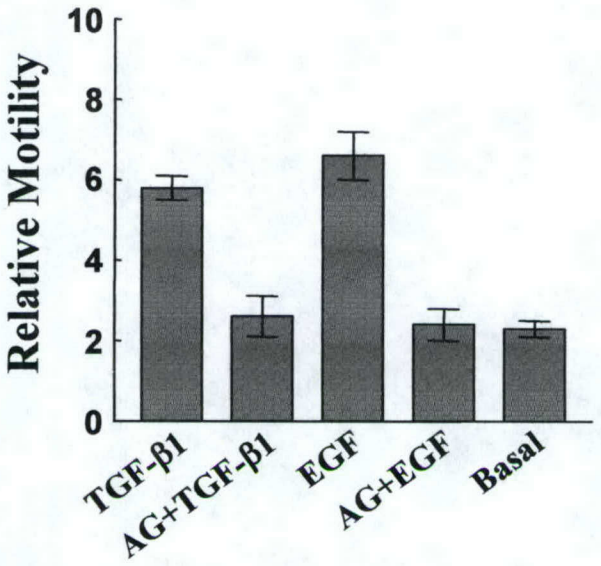

Figure 7. TGF-β1-stimulated cell motility is attenuated by pretreatment with AG1478. Pretreatment with the EGFR inhibitor AG1478 reduced scrape-stimulated cell migration to that approximating basal motility. Histogram represents mean±s.d. of triplicate experiments. AG = AG1478.

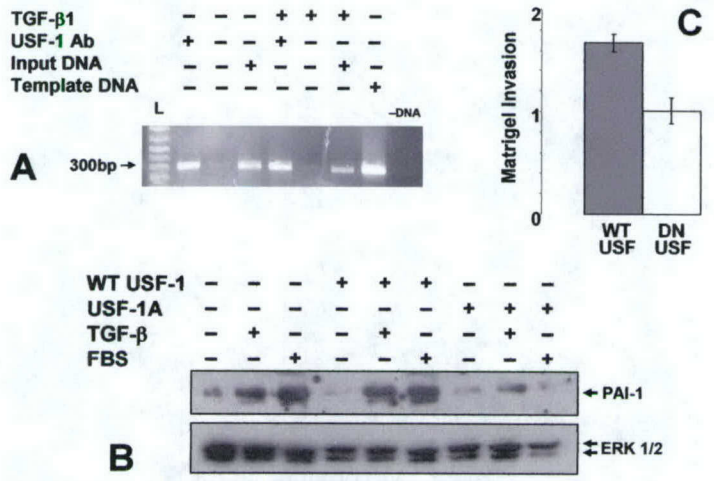

Figure 8. Dominant-negative USF-1 (USF-1A) attenuates TGF-β1-induced PAI-1 protein expression and barrier invasion. USF-1 was confirmed to be an endogenous PAI-1 promoter PE2 region DNA-binding factor by chromatin immunoprecipitation using antibodies to USF-1 **(A)**. Controls included addition of sonicated cellular DNA but without immunoprecipitation (input DNA), addition of PAI-1p806-Luc DNA as a control template for PCR (template DNA) and PCR reaction mixtures with H_2O in place of DNA (–DNA). L = sizing ladder. To evaluate the effects of molecular genetic interference with USF function, RK cells were untransfected or transfected with CMV-driven WT USF-1 or CMV-driven dominant-negative USF-1A. Whole cell lysates from quiescent cultures or cells stimulated with 20% FBS or 1 ng/ml TGF-β1 were collected, separated on 9% SDS-PAGE, proteins transferred, and blots probed with anti-rat PAI-1 antibody **(B)**. Transfers were reprobed with antibodies to ERK1/2 to assess protein loading. Invasion of RK cells expressing either WT (USF-1) or dominant-negative (USF-1A) expression constructs was compared ± TGF-β1 **(C)**. Data in **(C)** specifically represent TGF-β1-induced fold-invasion of Matrigel-coated barriers (i.e., TGF-β1-associated invasion/untreated controls); histogram represents mean±standard error from 8 migration evaluations. Approximately 60% of the cells used in invasion assays were anti-HA positive (i.e., 60% transfection efficiency). Only 5% of cells that invaded the Matrigel barrier, in contrast, stained positively with the HA antibody indicating that the majority of USF-1A-expressing cells were non-invasive.

continued PAI-1 synthesis (Kutz and Higgins, in preparation) likely to prevent tubular regression (14). Such PAI-1 expression was both USF-1-dependent and inhibited by exposure to AG1478. Most importantly, and a critical aspect of the work directed to Task 2, we demonstrated that targeted down-regulation of TGF-β1-induced PAI-1 expression using a dominant-negative USF-1A construct effectively attenuated matrix barrier invasion in response to TGF-β1 **(Figure 8)**. We are presently evaluating the ability of both dominant-negative USF-1A and PAI-1 antisense expression vectors to similarly inhibit TGF-β1-induced barrier invasion by transfected primary

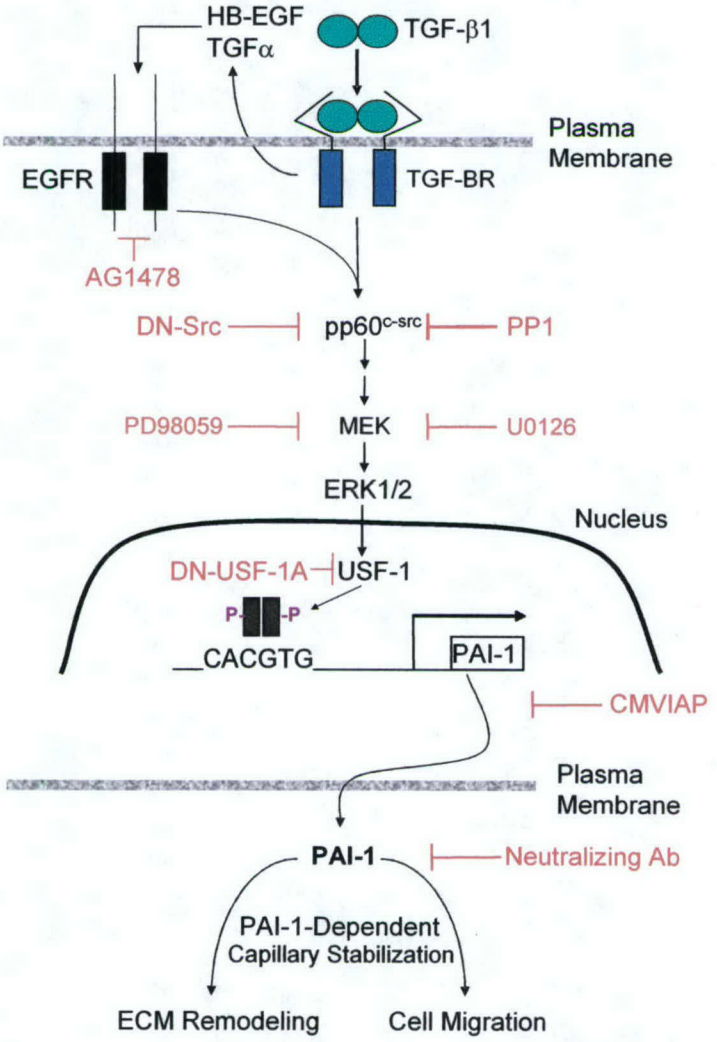

Figure 9. Schematic illustration of factors that regulate PAI-1 expression and function in response to TGF-β1 stimulation. TGF-β1 initiates a kinase cascade, at least partly as a function of EGFR activation (either through release of appropriate ligands or direct receptor transactivation), that involves participation of MEK and ERK1/2. Pharmacologic intervention, use of dominant-negative constructs and kinase assays suggest that pp60$^{c\text{-}src}$ is upstream of MEK and ERK1/2 in this model. Activated ERKs are thought to target nuclear bHLH-LZ transcription factors including members of the USF family that, once phosphorylated, bind as dimers to E box (5'-CACGTG-3') motifs in the PAI-1 promoter. E box site occupancy relieves PAI-1 transcriptional repression characteristic of the quiescent state. Expression attenuation and inhibition of PAI-1 synthesis can be achieved by transfection of DN-USF-1 or PAI-1 antisense (CMVIAP) expression vectors. Addition of PAI-1 neutralizing antibodies, in turn, effectively modulates PAI-1-dependent, uPA- and/or plasmin-mediated, matrix remodeling as well as cellular motility.

endothelial cells of human and rodent origin. Collectively, the work carried out during the tenure of this grant have led us to construct an overall model of PAI-1 gene expression control by breast cancer-derived promigratory growth factors such as TGF-β1 that involves an EGF inductive loop **(Figure 9)**. These data are consistent with known molecular events associated with tumor progression in the breast and other organ sites **(Figure 10)**.

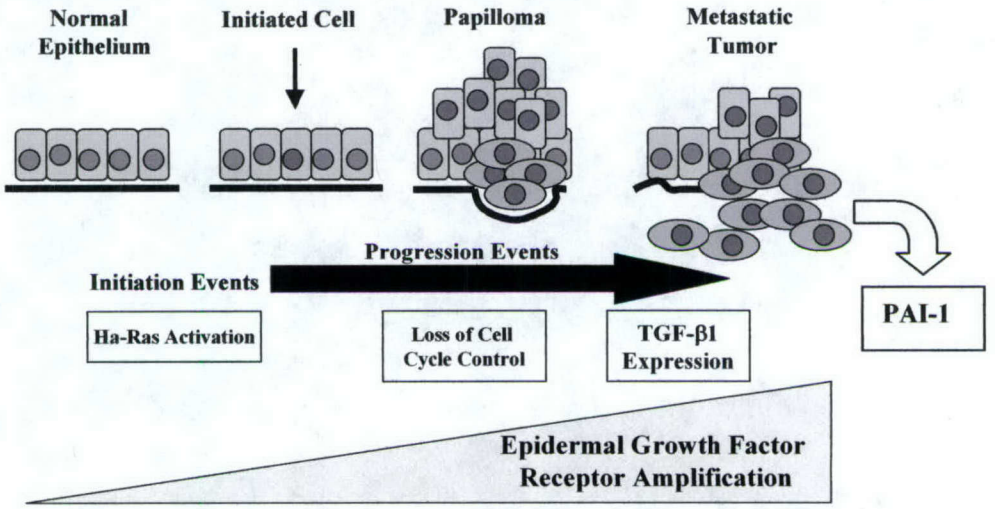

Figure 10. Molecular events associated with breast tumor progression. In experimental models of breast cancer, Ha-*ras* activation results in the emergence of initiated epithelial cells that ultimately progress (through loss of cell cycle control elements) to benign lesions. Elevated autocrine/paracrine expression of TGF-β1, and/or amplification of the epidermal growth factor receptor, is frequently associated with progression to an aggressive, high-level PAI-1 synthesizing, metastatic phenotype.

Experiments were initiated to evaluate if replacement of the CMV promoter element in the current PAI-1 antisense Rc/CMVIAP construct with endothelial cell-specific transcriptional control elements (derived from the flt-1 and VE-cadherin promoters) conferred endothelial-specific PAI-1 gene control. Primers were designed to amplify regions of both the flt-1 and VE-cadherin promoter regions for substitution cloning into Rc/CMVIAP. One initial VE-cadherin-IAP candidate clone was selected for transfection into both endothelial (HMEC-1) an non-endothelial (HaCaT) cells. Preliminary findings suggest that a VE-cadherin-driven IAP construct was expressed at high levels in HMEC-1 cells but not (or at least at very low levels relatively) HaCaT transfectants. We plan to continue these studies with one goal being to submit a new USAMRMC research grant utilizing these preliminary data as supporting evidence that endothelial-specific targeting of antisense PAI-1 transcripts will have therapeutic application.

KEY RESEARCH ACCOMPLISHMENTS

The key accomplishments achieved during the grant period are as follows:

1. Confirmed that targeted ablation of endothelial cell PAI-1 gene expression, using antisense expression vectors (Rc/CMVIAP) resulted in marked inhibition of cell motility and an inability to form angiogenic networks on Matrigel-coated surfaces.

2. Developed transfection techniques to introduce sense PAI-1 expression vectors (Rc/CMVPAI) into endothelial cells in which the PAI-1 gene was disrupted by homologous recombination (T2-null) to assess the effects of expression "rescue" on cell motility.

3. Created 4 such rescued cell lines (T2-nullR1-4); each line exhibited unique patterns of locomotion in the monolayer denudation injury model of induced cell motility that was statistically different (Student's t-test) from the rate of migration characteristic of the parental T2-null cells.

4. Addition of recombinant PAI-1 to cultures of T2 endothelial cells with genetically-attenuated PAI-1 levels restored their migratory rate to approximate that of wild-type cells. Similar positive effects on the migration of the PAI-1 functionally-null 4HH cell line was achieved by exogenous PAI-1 supplementation. This approach to motility assessments confirmed that PAI-1 is a critical element in the cellular migratory program. These finding support the likelihood that the overall experimental strategy to target PAI-1 expression in tumor angiogenesis will result in the design of genetic approaches that will have a defined therapeutic applicability.

5. Confirmatory results were obtained in the human microvessel endothelial cell line HMEC-1 which validates findings in the well-characterized T2 and 4HH cell systems in the context of human endothelial cells and tubulogenic differentiation.

6. Importantly, the integrity of capillary networks formed by both WT-T2 and HMEC-1 endothelial cells was disrupted by addition of neutralizing PAI-1 antibodies suggesting that continued PAI-1 synthesis, even in "mature" tubes, was required for network stability. Collectively, these data indicate that PAI-1 expression is an essential and targetable aspect of a successful angiogenic response in vitro.

7. TGF-β1 and EGF ligands were determined to be important breast cancer-derived factors that contributed to the long-term maintenance of formed angiogenic networks, induced PAI-1 gene expression and cell migratory ability.

1. Down-regulation of TGF-β1-induced PAI-1 expression using a dominant-negative USF-1A construct effectively attenuated growth factor-stimulated matrix barrier invasion confirming our hypothesis that the PAI-1 transcriptional control network represents a potential therapeutic target for breast cancer treatment.

2. We have created a model that integrates our findings within the general context of human breast cancer progression that details molecular events which contribute to PAI-1 over-expression resulting in matrix remodeling, induced cell invasivity, and angiogenic capillary stabilization. Collectively, these effects foster breast tumor progression and metastatic spread.

3. Studies were initiated, and are presently ongoing, to develop vectors designed for endothelial cell-specific expression of potentially therapeutically-useful constructs

(dominant-negative USF-1A; antisense PAI-1 sequences) identified during the course of this study. Current strategy involves use of endothelial-specific gene promoters (flt-1, VE-cadherin) to drive expression of cloned inserts.

REPORTABLE OUTCOMES

1. The following manuscripts have been published or are in press and cite support from grant DAMD17-00-1-0124.

Kutz, S.M., Hordines, J., McKeown-Longo, P.J., and Higgins, P.J. (2001) TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. Journal of Cell Science 114:3905-3914.

Samarakoon, R. and Higgins, P.J. (2002) MEK/ERK pathway mediates cell-shape-dependent plasminogen activator inhibitor type-1 gene expression upon drug-induced disruption of the microfilament and microtubule networks. Journal of Cell Science 115:3093-3103.

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Samarakoon, R. and Higgins, P.J. (2003) $pp60^{c\text{-}src}$ mediates ERK activation/nuclear localization and PAI-1 gene expression in response to cellular deformation. Journal of Cellular Physiology 195:411-420.

Qi, L. and Higgins, P.J. (2003) Use of chromatin immunoprecipitation to identify E box-binding transcription factors in the promoter of the growth state-regulated human PAI-1 gene. Recent Research Developments in Molecular Biology 1:1-12.

Providence, K.M., Staiano-Coico, L., and Higgins, P.J. (2003) A quantifiable *in vitro* model to assess the effects of PAI-1 gene targeting on epithelial cell motility. In: ;Wound Healing: Methods and Protocols (DiPietra, L., Editor), Methods in Molecular Medicine 78:293-303.

Providence, K.P and Higgins, P.J. (2004) PAI-1 expression is required epithelial cell migration in two distinct phases of in vitro wound repair. Journal of Cellular Physiology 200:297-308.

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targeting: new therapeutic approaches to regulate tumor growth and angiogenesis. Recent Research Developments in Cell Science 1:1-10.

Allen, R.R., Qi, L. and Higgins, P.J. (2004) Upstream stimulatory factor regulates E box-dependent PAI-1 transcription in human epidermal keratinocytes. Journal of Cellular Physiology (accepted for publication).

2. Abstracts published during funding of this grant.

Higgins, P.J. (2000) PAI-1 gene expression in breast carcinoma cells: implications for cellular migratory activity. Proceedings of the Department of Defense Breast Cancer Research Meeting, I, 85.

Higgins, P.J. (2000) Antisense targeting of PAI-1 expression inhibits TGF-β1-induced migration in premalignant epidermal epithelial cells. "New Molecular Targets for Cancer Therapy" Conference Proceedings.

Kutz, S.M. and Higgins, P.J. (2001) TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. Molecular Biology of the Cell 12, 281a.

Samarakoon, R. and Higgins, P.J. (2001) Cytoskeletal controls on PAI-1 gene regulation. Molecular Biology of the Cell 12, 353a-354a.

Higgins, P.J. and Tang, J. (2002) PAI-1 promoter-driven PAI-1-GFP expression vectors for direct assessment of transcriptional activity in individual cells *in vivo*. Proceedings of the American Association for Cancer Research Special Conference on "Molecular Imaging in Cancer: Linking Biology, Function, and Clinical Applications *In Vivo*", p. A-8.

Higgins, P.J. (2002) PAI-1 is an important regulator of substrate adhesion and migration in human breast carcinoma cells. Proceedings of the Department of Defense Breast Cancer Research Meeting, P26-14.

Higgins, P.J. (2002) PAI-1 expression is required for endothelial cell migration and *in vitro* angiogenesis. Proceedings of the Department of Defense Breast Cancer Research Meeting, P26-15.

Higgins, P.J. (2002) PAI-1 promoter-driven PAI-1-GFP expression vectors: tools for assessment of transcriptional activity and cellular invasive traits. Proceedings of the XXXth Meeting of the International Society for Oncodevelopmental Biology and Medicine "Translational Cancer Research".

Higgins, P.J. (2002) PAI-1 promoter-driven PAI-1/GFP expression vectors: probes for PAI-1 transcriptional activity and induced angiogenesis. Proceedings of the First Annual Meeting of the Society for Molecular Imaging.

Allen, R.R. and Higgins, P.J. (2002) Transcriptional regulation of type 1 plasminogen activator inhibitor (PAI-1) by transforming growth factor β (TGFβ) in human epithelial cells. Proceedings of the American Association for Cancer Research Special Conference on "Proteases, Extracellular Matrix, and Cancer", A43.

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Higgins, P.J. (2003) $pp60^{c\text{-}src}$/MEK activation are required for TGF-β1-stimulated PAI-1 gene transcription in microvessel endothelial cells. Proceedings of the American Association for Cancer Research Conference on the TGF-β Superfamily: Roles in thePathogenesis of Cancer and Other Diseases, 2281.

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Higgins, P.J. (2003) Disruption of PAI-1 gene expression inhibits endothelial cell migration and *in vitro* angiogenesis. Proceedings of the Joint Cancer Conference of the Florida Universities.

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Higgins, P.J. (2004) Transforming growth factor-β1-induced plasminogen activator inhibitor type-1 transcription requires $pp60^{c\text{-}src}$/mitogen-activated protein kinase activity and utilizes a

proximal promoter E box motif. Proceedings of the Institute Pasteur Euroconference, "Cancer: New Insights in Molecular Diagnosis and Therapy".

Higgins, P.J. (2004) pp60$^{c\text{-}src}$, MEK-ERK and EGFR signaling are required for TGF-β1-induced transcription of the epithelial-to-mesenchymal transition (EMT)-associated PAI-1 gene. Proceedings of the Third Biennial Meeting: Molecular Targets for Cancer Therapy.

3. A new Idea proposal entitled "Inducible Anti-Angiogenic Gene Therapy" was funded by the DOD Breast Cancer Program.

4. Vectors have been created (Rc/CMVPAI, Rc/CMVIAP, USF-1A, endothelial-specific PAI-1 antisense) that can be distributed to DOD investigators involved in angiogenesis research.

5. Several cell lines have been developed on the T2 genetic background that will be made available to DOD investigators interested in angiogenesis and breast cancer research. The T2 line is particularly adaptable for basic studies into the mechanisms underlying the tubulogenic process as well as for drug discovery anti-angiogenic investigations (pharmacologic and genetic based). T2 was originally isolated from a mass culture of normal rat kidney cells and determined to have an "epitheloid" phenotype. These cells form branching tubulogenic networks in Matrigel culture, organize into pre-capillary-like formations in collagen I, have VEGF receptors, use VEGF as a mitogen, have von Willebrand-like inclusions and, when transformed, form tumors with a hemangiosarcoma-like pathology. T2 cells, therefore, appear to be a primitive immortalized microvessel endothelial-like cell type. Since it is specifically the microvasculature that contributes to the tumor-dependent angiogenic response, these cells represent a unique resource well-adapted for studies on tubular differentiation and angiogenesis.

CONCLUSIONS

Several important conclusions were derived as a result of work completed during the period covered by this report.

- PAI-1 expression is required for optimal endothelial cell migration *in vitro*.

- Endothelial cell motile deficits, produced as a consequence of PAI-1 expression targeting, can be restored to approximately wild-type levels by addition of exogenous "active" PAI-1 protein.

- Confirmatory results were obtained in the human microvessel endothelial cell line HMEC-1 which validates findings in the well-characterized T2 and 4HH cell systems in the context of human endothelial cells and tubulogenic differentiation.

- The integrity of capillary networks formed by both WT-T2 and HMEC-1 endothelial cells was disrupted by addition of neutralizing PAI-1 antibodies suggesting that continued PAI-1 synthesis, even in "mature" tubes, was required for network stability.

- Stimulation of angiogenesis observed clinically during the progression of human breast cancer is likely due to PAI-1 transcription induced in response to particular growth factors (i.e., TGF-β and EGF family members) elaborated by the tumor cells.

- Down-regulation of TGF-β1-induced PAI-1 expression using a dominant-negative USF-1A construct effectively attenuated growth factor-stimulated matrix barrier invasion confirming our hypothesis that the PAI-1 transcriptional control network represents a potential and accessible therapeutic target for breast cancer treatment.

- It appears possible to develop vectors designed for endothelial cell-specific expression of potentially therapeutically-useful constructs (dominant-negative USF-1A; antisense PAI-1 sequences) identified during the course of this study. Although it is appreciated that important confirmatory studies will have to be carried out in primary microvessel endothelial cells, this finding will facilitate the creation of endothelial-targeted genetic therapies important for Phase I clinical trials.

- These data are inconsistent with the emerging realization that "balanced proteolysis", in general, is an essential aspect of a successful angiogenic response and that PAI-1, in particular, is a major regulator of tumor-dependent angiogenesis.

- It is possible to design targeted genetic therapies to manipulate expression of an important pro-angiogenic gene (PAI-1) under defined *in vitro* conditions.

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APPENDED INFORMATION

Publications not previously submitted and now appended.

Kutz, S.M., Hordines, J., McKeown-Longo, P.J. and Higgins, P.J. (2001) TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. J. Cell Sci. 114, 3905-3914.

Providence, K.M., White, L.A., Tang, J., Gonclaves, J., Staiano-Coico, L., and Higgins, P.J. (2002) Epithelial monolayer wounding stimulates binding of USF-1 to an E-box motif in the plasminogen activator inhibitor type 1 gene. J. Cell Sci. 115, 3767-3777.

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Allen, R.R., Qi, L. and Higgins, P.J. (2004) Upstream stimulatory factor regulates E

box-dependent PAI-1 transcription in human epidermal keratinocytes. J. Cell. Physiol. (accepted for publication).

Kutz, S.M. and Higgins, P.J. (2004) Plasminogen activator inhibitor type-1 expression targeting: new therapeutic approaches to regulate tumor growth and angiogenesis. Recent Res. Devel. Cell Sci. 1, 1-10.

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Paul J. Higgins, Ph.D., Principal Investigator
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TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion

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SUMMARY

The type-1 inhibitor of plasminogen activator (PAI-1) is an important physiological regulator of extracellular matrix (ECM) homeostasis and cell motility. Various growth factors mediate temporal changes in the expression and/or focalization of PAI-1 and its protease target PAs, thereby influencing cell migration by barrier proteolysis and/or ECM adhesion modulation. TGF-β1, in particular, is an effective inducer of matrix deposition/turnover, cell locomotion and PAI-1 expression. Therefore, the relationship between motility and PAI-1 induction was assessed in TGF-β1-sensitive T2 renal epithelial cells. PAI-1 synthesis and its matrix deposition in response to TGF-β1 correlated with a significant increase in cell motility. PAI-1 expression was an important aspect in cellular movement as PAI-1-deficient cells had significantly impaired basal locomotion and were unresponsive to TGF-β1. However, the induced migratory response to this growth factor was complex. TGF-β1 concentrations of 1-2 ng/ml were significantly promigratory, whereas lower levels (0.2-0.6 ng/ml) were ineffective and final concentrations ≥5 ng/ml inhibited T2 cell motility. This same growth factor range progressively increased PAI-1 transcript levels in T2 cells consistent with a bifunctional role for PAI-1 in cell migration. TGF-β1 induced PAI-1 mRNA transcripts in quiescent T2 cells via an immediate-early response mechanism. Full TGF-β1-stimulated expression required tyrosine kinase activity and involved MAPK/ERK kinase (MEK). MEK appeared to be a major mediator of TGF-β1-dependent PAI-1 expression and T2 cell motility since PD98059 effectively attenuated both TGF-β1-induced ERK1/2 activation and PAI-1 transcription as well as basal and growth factor-stimulated planar migration. Since MEK activation in response to growth factors is adhesion-dependent, it was important to determine whether cellular adhesive state influenced TGF-β1-mediated PAI-1 expression in the T2 cell system. Cells maintained in suspension culture (i.e., over agarose underlays) in growth factor-free medium or treated with TGF-β1 in suspension expressed relatively low levels of PAI-1 transcripts compared with the significant induction of PAI-1 mRNA evident in T2 cells upon stimulation with TGF-β1 during adhesion to a fibronectin-coated substrate. Attachment to fibronectin alone (i.e., in the absence of added growth factor) was sufficient to initiate PAI-1 transcription, albeit at levels considerably lower than that induced by the combination of cell adhesion in the presence of TGF-β1. T2 cells allowed to attach to vitronectin-coated surfaces also expressed PAI-1 transcripts but to a significantly reduced extent relative to cells adherent to fibronectin. Moreover, newly vitronectin-attached cells did not exhibit a PAI-1 inductive response to TGF-β1, at least during the short 2 hour period of combined treatment. PAI-1 mRNA synthesis in response to substrate attachment, like TGF-β1-mediated induction in adherent cultures, also required MEK activity as fibronectin-stimulated PAI-1 expression was effectively attenuated by the MEK inhibitor PD98059. These data indicate that cellular adhesive state modulates TGF-β1 signaling to particular target genes (i.e., PAI-1) and that MEK is a critical mediator of the PAI-1$^+$/promigratory phenotype switch induced by TGF-β1 in T2 cells.

Key words: PAI-1, Gene expression, Signal transduction, TGF-β1

INTRODUCTION

Genetic analysis and adaptation of physiologically-relevant in vitro models of wound repair have clarified basic mechanisms involved in the tissue response to injury (Garlick and Taichman, 1994; Romer et al., 1996; Creemers et al., 2000; Providence et al., 2000). Fundamental to this process is the conversion of normally sedentary cells to an actively migrating, invasive phenotype (Martin, 1997). However, stimulated cell movement and locomotion through the provisional extracellular matrix (ECM) requires cycles of adhesion-deadhesion and precise control of the pericellular proteolytic environment (Yamada and Clark, 1996; Greenwood and Murphy-Ullrich, 1998; Xie et al., 1998; Pilcher et al., 1999). Efficient wound re-epithelialization involves several protease systems with repair outcome highly dependent on the generation of plasmin by urokinase plasminogen activator (uPA). uPA activity is regulated, in turn, by its fast-acting type-1 inhibitor (PAI-1) (Andreasen et al., 1997; Lund et al., 1999; Zhou et al., 2000; Legrand et al., 2001). This cascade directly influences the overall injury site proteolytic balance and is a critical determinant in wound resolution (Mazzieri et al., 1997; Wysocki et al., 1999) as well as directed cell movement (Pepper et al., 1987; Pepper et al., 1992; Okedon et al., 1992; Providence et al., 2000).

Injury-induced cell motility is orchestrated by various

autocrine/paracrine-acting growth factors (Martin, 1997). Most prominent are members of the transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), and epidermal growth factor (EGF) families (Boland et al., 1996; Sato and Rifkin, 1988; Song et al., 2000; Ellis et al., 2001; Goke et al., 2001). TGF-β1 and activin A, in particular, integrate the complex processes of tissue repair and cell migration (Zambruno et al., 1995; Munz et al., 1999) largely through control of genes that encode matrix components (fibronectin, type I collagen), regulators of ECM homeostasis (e.g., uPA, PAI-1) and the cellular adhesive apparatus (e.g., PAI-1, integrin subunits) (Cajot et al., 1989; Cajot et al., 1990; Wrana et al., 1991; Munz et al., 1999; Lai et al., 2000; Providence et al., 2000). Therefore, growth factor-initiated changes in the expression, focalization and/or relative activity of uPA/PAI-1 may stimulate or inhibit cell migration via ECM barrier proteolysis or by altering cellular adhesive interactions with the ECM (Stefansson and Lawrence, 1996; Mignatti and Rifkin, 2000). Variances in PAI-1 synthesis (Providence et al., 2000) and/or site-localization (Kutz et al., 1997) would specifically impact on cellular migration by affecting uPA activity as well as uPAR/vitronectin- or integrin/vitronectin-dependent cell attachment (Ciambrone and McKeown-Longo, 1990; Deng et al., 1996; Chapman, 1997; Stefansson and Lawrence, 1996; Loskutoff et al., 1999).

Since TGF-β1 stimulates cell motility (Kutz et al., 2001), PAI-1 induction (Boehm et al., 1999) in response to TGF-β1 is probably critical to the motile process and the acquisition of epithelial cell 'plasticity' (Akiyoshi et al., 2001; Zavadil et al., 2001). This was confirmed in the present study using the PAI-1-deficient 4HH cell line (Providence et al., 2000) in a quantitative model of induced cell locomotion. Therefore, it was important to define mechanisms involved in TGF-β1-dependent PAI-1 gene expression. PAI-1 transcription in TGF-β1-responsive T2 epithelial cells used an immediate-early, tyrosine kinase-mediated, signaling pathway. Moreover, PAI-1 induction and basal as well as TGF-β1-stimulated T2 cell locomotion was MEK-dependent. The involvement of MEK in TGF-β1-initiated PAI-1 expression and the adhesion-dependency of MEK activation (Renshaw et al., 1997) suggested that substrate attachment may influence TGF-β1-induced PAI-1 gene regulation. TGF-β1, in fact, poorly induced PAI-1 transcription in cells maintained in suspension culture but significantly increased PAI-1 expression during attachment to fibronectin-coated surfaces. Cellular adherence to fibronectin alone (i.e., in the absence of TGF-β1), and to a lesser extent vitronectin, also stimulated PAI-1 mRNA synthesis indicating that adhesive state can modulate TGF-β1 signaling to particular target genes (i.e., PAI-1).

MATERIALS AND METHODS

Cell culture

T2 and EC-1 renal epithelial cells and the PAI-1-deficient 4HH line (Providence et al., 2000) were cultured in DMEM containing 10% fetal bovine serum (FBS) and antibiotics. Cells were washed twice with Hanks' balanced salt solution (HBSS; 1.3 mM $CaCl_2$, 5 mM KC1, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$-$6H_20$, 0.4 mM $MgSO_4$-$7H_20$, 0.14 m NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose) then incubated in serum-free DMEM for 3 days to initiate a quiescent state (Kutz et al., 1997). Cells were either maintained in quiescence medium or stimulated by the direct addition of FBS or TGF-β1 (to final concentrations of 20% and 0.2-10.0 ng/ml, respectively). Puromycin (100 μg/ml), actinomycin D (5 μg/ml), genistein (100 μM), PD98059 (5-50 nM) or wortmanin (50 nM) were added 30 minutes prior to stimulation with FBS or TGF-β1. For culture under non-adherent conditions, quiescent T2 cells were harvested by trypsinization, washed with soybean trypsin inhibitor, and plated on 1% agarose underlays in serum-free DMEM for 4 hours (Ryan et al., 1996). Agarose-suspended cells were maintained (for an additional 2 hours) under non-adherent conditions (in the presence or absence of TGF-β1) or seeded onto fibronectin-, vitronectin- or bovine serum albumin (BSA)-coated 100 mm plastic dishes (coating concentration 10 μg protein/ml) and allowed to re-attach for 2-6 hours (in the presence or absence of TGF-β1) prior to RNA isolation. Directional migration assays used methods developed previously (Providence et al., 2000). Three-day post-confluent T2 cell cultures were washed twice with HBSS and incubated for 3 days in serum-free DMEM. Monolayers were maintained under serum-free or TGF-β1-/serum-supplemented conditions prior to scrape-wounding using the small end of a 1000 μl pipette tip. Initial wound size was determined for each culture dish and extent of injury closure assessed 24 hours later with an inverted microscope fitted with a calibrated ocular grid.

Northern blot analysis

Total cellular RNA was isolated and denatured at 55°C for 15 minutes in 1× MOPS, 6.5% formaldehyde and 50% formamide prior to electrophoresis on agarose/formaldehyde gels (1.2% agarose, 1.1% formaldehyde, 1× MOPS, 50 mM sodium acetate, 1 mM EDTA, pH 8.0). RNA was transferred to Nytran membranes by capillary action in 10× SSC (3M NaCl, 0.3 M Na citrate, pH 7.0), UV crosslinked and incubated for 2 hours at 42°C in 50% formamide, 5× Denhardt's solution, 1% SDS, 100 μg/ml sheared/heat-denatured salmon sperm DNA (ssDNA) and 5× SSC. RNA blots were hybridized with ^{32}P-labeled cDNA probes for PAI-1 and A-50 (Ryan and Higgins, 1993) for 24 hours at 42°C in 50% formamide, 2.5× Denhardt's solution, 1% SDS, 100 μg/ml ssDNA, 5× SSC and 10% dextran sulfate. Membranes were washed 3 times in 0.1× SSC/0.1% SDS for 15 minutes each at 42°C followed by 3 washes at 55°C prior to exposure to film.

Microscopy

Cells were washed twice with Ca^{2+}/Mg^{2+} free phosphate-buffered saline (PBS-CMF; 2.7 mM KC1, 1.2 mM KH_2PO_4, 0.14 M NaC1, 8.1 mM Na_2HPO_4-$7H_2O$) and fixed in 10% formalin/PBS-CMF for 10 minutes. Following permeabilization with cold 0.5% Triton X-100/PBS-CMF (for PAI-1 immunolocalization) or 1% NP-40/PBS-CMF (for phalloidin-actin binding) for 10 minutes at 4°C, cells were washed 3 times (5 minutes each) with PBS-CMF then overlayed with rabbit antibodies to PAI-1 (Kutz et al., 1997) in BSA (3 mg/ml)/PBS-CMF. After three PBS-CMF washes, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG (1:20 in BSA/PBS-CMF) for 30 minutes at 37°C, washed, and coverslips mounted with 100 mM *n*-propylgalate in 50% glycerol/PBS-CMF. Rhodamine-phalloidin was used to visualize actin microfilament structures (Ryan and Higgins, 1993).

MAP kinase assays

Cells were extracted for 30 minutes in cold lysis buffer (0.5% deoxycholate, 50 mM Hepes [pH 7.5], 1% Triton X-100, 1% NP-40, 150 mM NaCl, 50 mM NaF, 1 mM Na-orthovanadate, 0.1% aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF) and lysates clarified by centrifugation at 14,000 ***g*** for 15 minutes at 4°C. For immunoprecipitation, aliquots containing 500 μg protein were incubated with 2 μg ERK1/2 antibody for 2 hours with gentle rocking. Protein A/G Plus-agarose (30 μl) was added for 2 hours, immune complexes collected by centrifugation, washed twice with lysis buffer and twice with 100 mM NaCl in 50 mM Hepes (pH 8.0) and then

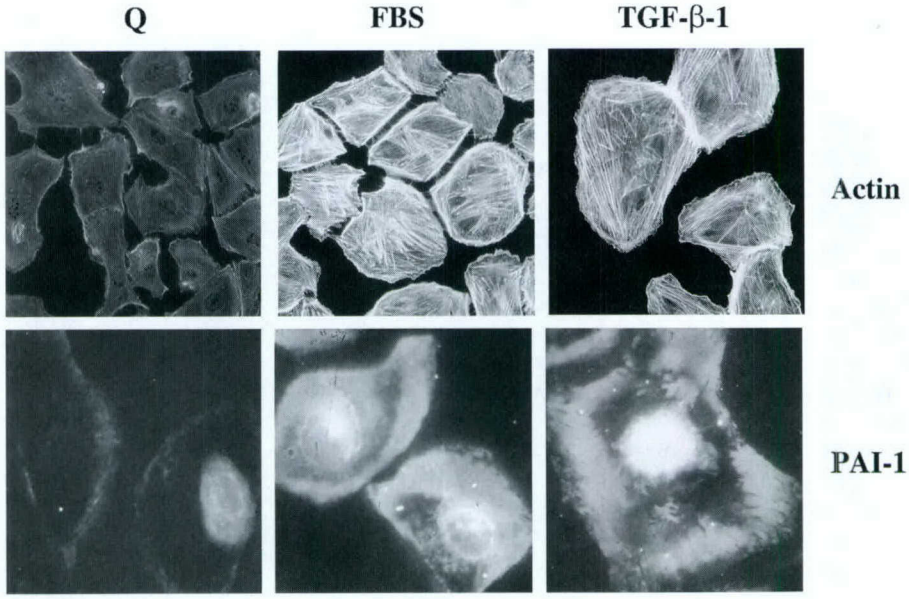

Fig. 1. PAI-1 deposition in T2 cells after stimulation with serum or TGF-β1. Quiescent (Q) cell cultures were stimulated by addition of FBS or TGF-β1 (to final concentrations of 20% and 1 ng/ml, respectively). After 4 hours, cells were fixed and processed (see Materials and Methods) for visualization of microfilament organization (Actin) and PAI-1 immunolocalization (PAI-1).

incubated at 37°C for 15 minutes in kinase reaction buffer (10 μCi ^{32}P-ATP, 50 μM ATP, 20 mM Hepes (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine, 0.3 mg/ml myelin basic protein (MBP)). Electrophoresis buffer (50 mM Tris (pH 6.0), 10% glycerol, 1% SDS, 1% β-mercaptoethanol) was added, samples boiled for 10 minutes and 15 μl aliquots separated on SDS/15% polyacrylamide gels. Proteins were transferred to nitrocellulose in 25 mM Tris, 190 mM glycine, 20% methanol and membranes exposed to film for visualization of phosphorylated MBP. Western blotting for ERK2 and total MBP detection by Ponceau S staining were used to confirm equivalent loading per lane. For detection of phosphorylated ERK1/2, membranes were washed for 10 minutes in 0.05% Triton X-100/PBS-CMF followed by 2 hours in wash solution containing 3% milk. Phospho-ERK monoclonal antibody (1:1000) was added for an overnight incubation in blocking solution at room temperature. Following 3 washes for 20 minutes, horseradish peroxidase (HRP)-labeled anti-mouse secondary antibody (1:3000 in blocking solution) was added and incubated for an additional 1 hour. Membranes were washed 5 times for 10 minutes each in wash solution, incubated with ECL substrate solution (Amersham, Piscataway, NJ) for 2 minutes with gentle rocking and exposed to film. Membranes were stripped for 90 minutes at room temperature using the Western Stripper Kit (Bioworld, Dublin, Ohio), neutralized then incubated in a ERK1/2 primary antibody mixture (each diluted 1:3000 in blocking solution) followed by HRP-anti-rabbit secondary antibody and ECL reagent as described above.

RESULTS

TGF-β1 stimulation of PAI-1 expression and directional motility is MEK-dependent

PAI-1 synthesis and accumulation in the ventral undersurface region in response to TGF-β1 (Fig. 1) correlated with a significant increase in T2 cell motility (relative to the basal rate of movement in the scrape-wound assay) approximating that of serum-stimulated cells (Fig. 2). This rather dramatic effect of TGF-β1 on cell migration and cytoarchitecture is consistent with involvement of TGF-β1 target genes (e.g., PAI-1) in cellular 'plasticity' and invasive behavior (Zavadil et al., 2001). Antibodies to PAI-1, in fact, inhibit cell attachment (Higgins et al., 1991), promote substrate detachment (Rheinwald et al., 1987) and attenuate cell activation (Kutz et al., 1997). Furthermore, recent findings have highlighted the functional linkage between induced PAI-1 synthesis and cellular motility (Mignatti and Rifkin, 2000; Providence et al., 2000; Kutz et al., 2001). To directly assess the role of PAI-1 in growth factor-stimulated cell locomotion, TGF-β1-dependent wound closure was compared in EC-1 and 4HH cells. 4HH is a derivative of EC-1 in which PAI-1 synthesis is specifically ablated by stable constitutive expression of PAI-1 antisense transcripts under

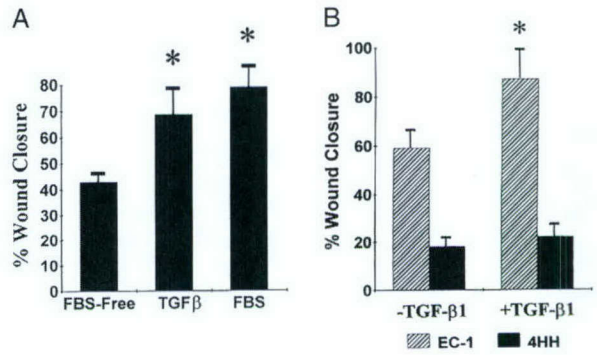

Fig. 2. Stimulation of cell motility by serum or TGF-β1. Confluent cultures of T2 cells were incubated in serum-free DMEM for 3 days prior to scrape-wounding. Cells were maintained in the FBS-free medium to assess basal migration (A). The percent (%) wound closure was determined 24 hours later. Data plotted are the means±s.d. of 20 individual measurements made on each of triplicate cultures for each treatment condition. Asterisks indicate a statistically significant difference (Student's *t*-test, $P>0.01$) in cell migration for TGF-β1- and FBS-supplemented cultures compared with basal (FBS-free) motility. The effect of targeted PAI-1 ablation on basal (–TGF-β1) as well as TGF-β1-induced (+TGF-β1) cell locomotion was assessed in EC-1 and 4HH cell cultures by evaluation of the extent (%) of wound closure in the absence and presence of growth factor (1 ng/ml) (B). Data plotted are the means±s.d. of 15 individual measurements on duplicate cultures/treatment group. Asterisk indicates a statistically significant difference ($P<0.01$) between motility under growth factor-free and supplemented conditions for EC-1 cells. By contrast, 4HH cells were unresponsive to TGF-β1 in this assay.

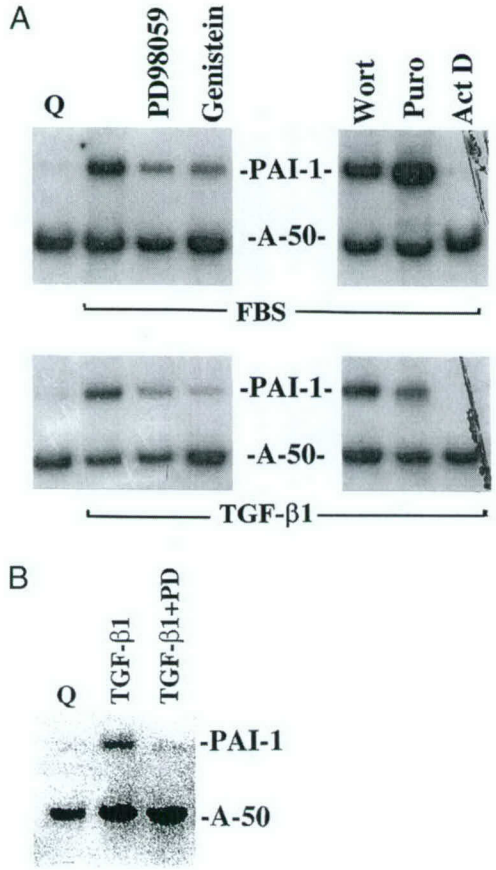

Fig. 3. Metabolic requirements for TGF-β1-induced PAI-1 expression. To assess pathways underlying induced PAI-1 expression, quiescent (Q) T2 cells were stimulated with serum (20%) or TGF-β1 (1 ng/ml) for 2 hours, in the presence or absence of a 30 minute pretreatment with the indicated inhibitors, prior to RNA isolation (A). Northern blots were hybridized with ^{32}P-labeled cDNA probes for PAI-1 and A-50 simultaneously. The inability of puromycin to attenuate either serum or TGF-β1-induced PAI-1 transcripts and the sensitivity of expression to actinomycin D indicated that PAI-1 induction by both stimuli was an immediate-early (i.e., primary) response. TGF-β1-induced PAI-1 expression in T2 cells is MEK-dependent (B). Quiescent (Q) T2 cells were stimulated with TGF-β1 (1 ng/ml) for 2 hours in the absence or presence of a 30 minute pretreatment with PD98059 (50 nM) prior to isolation of RNA. Northern blots were hybridized with ^{32}P-labeled cDNA probes to PAI-1 and A-50.

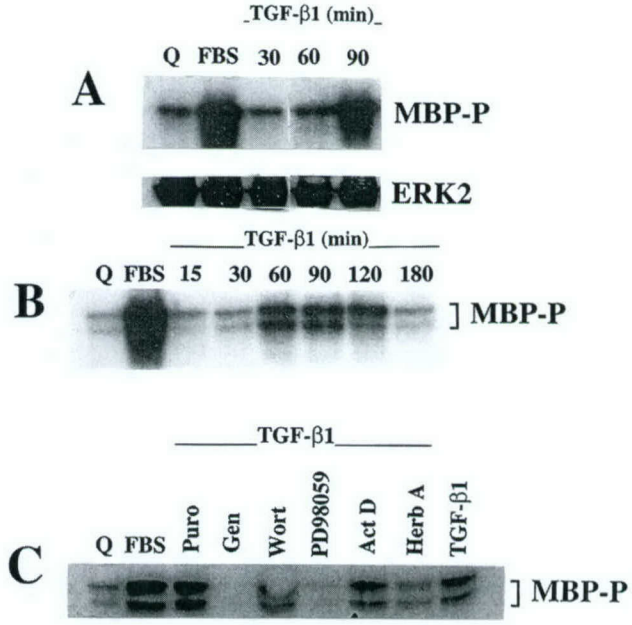

Fig. 4. Coupled ERK immunoprecipitation/MBP kinase assay for assessment of TGF-β1-induced ERK activation. ERK1/2 were immunoprecipitated from lysates of quiescent (Q), FBS- and TGF-β1-stimulated T2 cells. Exposure of quiescent cells to FBS (20%) was for 15 minutes and stimulation with TGF-β1 (1 ng/ml) was for 30, 60 and 90 minutes prior to cell disruption and ERK1/2 immunoprecipitation. MBP phosphorylation reaction products (MBP-P) were separated by gel electrophoresis; equivalent loading of MBP and ERK per lane was confirmed by Ponceau S staining (not shown) and ERK2 western blotting, respectively (A). In contrast to the relatively rapid rate of ERK activation by serum (15 minutes), TGF-β1-induced changes in ERK activity were not evident until 60 minutes after growth factor addition (A), remained elevated for approximately 2 hours and then rapidly declined (B). Coupled ERK immunoprecipitation/MBP phosphorylation (MBP-P) assays (C) confirmed that ERK activation in TGF-β1-stimulated T2 cells is sensitive to the same pharmacologic inhibitors that attenuate growth factor-induced PAI-1 expression. The more pathway restrictive inhibitor herbimycin A (250 nM) (Fukazawa et al., 1994) attenuated MBP phosphorylation but not to the same extent as genistein or PD98059.

control of a strong CMV promoter (Higgins et al., 1997). These cells do not produce detectable PAI-1 protein under growth factor-supplemented culture conditions (Providence et al., 2000), thus, providing a tool to assess the relationship between PAI-1 expression and cell motility. The 4HH basal migration rate (i.e., locomotion in serum-/growth factor-free medium) was ≤30% that of parental controls. Moreover, relative to EC-1 cells in which wound closure is significantly enhanced by TGF-β1, 4HH cells were unresponsive and failed to increase substantially their rate of movement in TGF-β1-supplemented medium (Fig. 2).

Since PAI-1 appears to be a critical element in cellular migration, it was important to clarify pathways by which TGF-β1 influenced PAI-1 gene expression and the motile phenotype. TGF-β1-mediated PAI-1 transcription, similar to induction by serum, was an immediate-early response (i.e., resistant to protein synthesis inhibitors) and significantly reduced by prior exposure to genistein (Fig. 3). The MEK-specific compound PD98059 effectively attenuated (at the 5-20 nM range) (Fig. 3A) and completely ablated (at 50 nM) (Fig. 3B) TGF-β1-induced PAI-1 expression. In agreement with previous studies (Hartsough and Mulder, 1995; Yonekura et al., 1999), and the PD98059-sensitivity of PAI-1 induction (Fig. 3), addition of TGF-β1 to quiescent T2 cells stimulated ERK1/2 activity. However, the time course of ERK activation by TGF-β1 (as assessed by the ability of ERK1/2 to phosphorylate the target substrate MBP in a linked immunoprecipitation-in vitro kinase assay), was delayed (by approximately 60 minutes) compared with the rapid induction (within 15 minutes) typical of serum-treated cells (Fig. 4). Similarly, increases in phospho-ERK1/2 levels (sixfold those of unstimulated cells) were not evident until 1 hour after addition of TGF-β1 to quiescent cultures (not shown). Consistent with

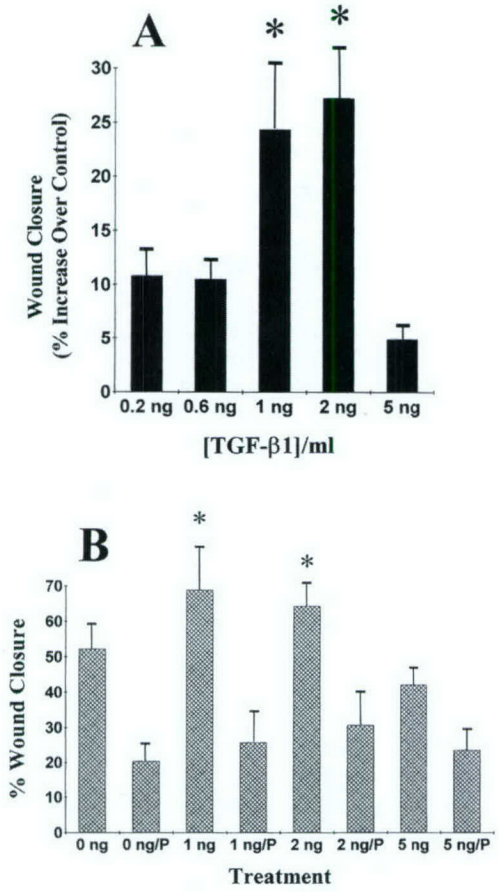

Fig. 5. The MEK inhibitor PD98059 attenuates both basal and TGF-β1-stimulated T2 cell migration. Initial experiments were designed to determine the optimal concentration of TGF-β1 on wound-induced motility (A). After scrape-injury, TGF-β1 was added (in the concentrations indicated) and extent of migration determined 24 hours later. Data plotted is % increase in wound closure relative to non-supplemented (FBS-free) cultures. Asterisks indicate those concentrations for which motility was significantly different from basal migration (Student's *t*-test, $P>0.0005$). To assess the requirement for MEK activity in stimulated cell movement, monolayer scrape wound-closure assays were carried out in TGF-β1-supplemented (concentration range 0, 1, 2 and 5 ng/ml) serum-free medium in the presence (P) or absence of PD98059 (50 μM) (B). TGF-β1 at 1 and 2 ng/ml significantly increased T2 cell directional motility (Student's *t*-test, $P>0.001$; asterisks) relative to basal mobility (0 ng). In this series of experiments, cells exposed to 5 ng/ml of the growth factor actually had a decreased rate of locomotion relative to unsupplemented controls. At each concentration of TGF-β1, PD98059 effectively reduced wound closure; there was no significant difference in the % closure rate among any of the treatment groups in the presence of PD98059.

the metabolic requirements for PAI-1 expression in response to TGF-β1 (Fig. 3), genistein as well as PD98059 effectively blocked TGF-β1-mediated ERK1/2 activation in coupled immunoprecipitation/MBP-phosphorylation assays (Fig. 4).

Although MEK was an important intermediate in TGF-β1-dependent PAI-1 transcription, the role of this signaling pathway in TGF-β1-stimulated T2 cell motility was unclear (Kutz et al., 2001). Since the migration-promoting effects of TGF-β1 are often concentration, as well as context-dependent,

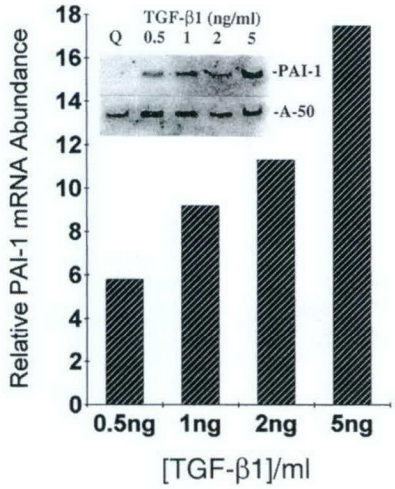

Fig. 6. TGF-β1 concentration-dependent increase in relative PAI-1 mRNA transcripts. Quiescent T2 cells (Q) were stimulated with TGF-β1 at the indicated concentrations and RNA isolated 2 hours later. Northern blots (insert for example) were scanned and the average PAI-1 transcript abundance, normalized to A-50 signal, calculated for 2 separate experiments.

it was necessary to define the optimal level of growth factor required to maximally stimulate directional T2 cell locomotion. Dose-assessments indicated that the migratory response to TGF-β1 was complex. Motility rates in cultures supplemented with low (0.2-0.6 ng/ml) as well as high (5 ng/ml) TGF-β1 concentrations were not significantly different from control values compared with the obvious promigratory effect associated with exposure to 1 and 2 ng/ml (Fig. 5A). TGF-β1 levels >5 μg/ml actually inhibited wound-induced T2 cell locomotion (when used alone or in the presence of serum) (not shown). PAI-1 transcripts progressively increased over the same growth factor concentration range (0.5-5.0 ng/ml) (Fig. 6). Collectively, these data are consistent with the suggestion that PAI-1 is a bifunctional regulator of cellular motility with positive effects likely restricted to a relatively narrow expression level 'window' (Mignatti and Rifkin, 2000). Using the determined optimal TGF-β1 concentration (1-2 ng/ml), as well as a subeffective 5 ng/ml dose, MEK activity appeared to be important to both TGF-β1-stimulated PAI-1 gene expression and planar motility, as PD98059 effectively attenuated both responses (Fig. 3; Fig. 5B).

Adhesive controls on TGF-β1-induced PAI-1 expression in T2 cells

PD98059 blockade implicated MEK as a critical intermediate in the TGF-β1-initiated pathway of PAI-1 gene expression (Fig. 3). Growth factor- and integrin-activated signaling pathways are interdependent, often converging on the MAPK cascade (Zhu and Assoian, 1995; Lin et al., 1997; Roovers et al., 1999), and MEK activation in response to growth factors requires substrate adhesion (Renshaw et al., 1997). Therefore, experiments were designed to assess whether cellular adhesive state modulated TGF-β1-induced PAI-1 expression. Quiescent T2 cells were trypsinized and plated over agarose underlays in serum-/TGF-β1-free DMEM where they remained in suspension as single cells. After 3 hours, agarose-cultured cells

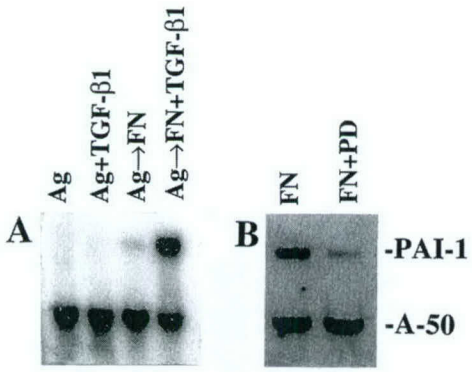

Fig. 7. Optimum response of the PAI-1 gene to TGF-β1 stimulation in T2 cells requires substrate adhesion. TGF-β1-induced PAI-1 transcripts requires adhesion (to a fibronectin (FN) substrate) since cells cultured in suspension (agarose, Ag) or stimulated with TGF-β1 (1 ng/ml) in suspension (Ag+TGF-β1) did not express PAI-1 mRNA (A). Cells plated onto FN from suspension culture (Ag→FN) did produce low levels of PAI-1 transcripts, whereas plating onto FN in the presence of TGF-β1 (Ag→FN+TGF-β1) yielded optimal induction. PAI-1 induction as a consequence of FN attachment alone was also attenuated by addition of PD98059 during the 2 hour period of adhesion suggesting that MEK activity was also required for adhesion-dependent expression under growth factor-free conditions (B).

were maintained in non-supplemented medium, stimulated with TGF-β1 (1 ng/ml) in suspension, transferred to fibronectin-coated dishes in serum-/TGF-β1-free medium, or stimulated with TGF-β1 during adhesion to fibronectin (all treatments were for 2 hours). Cells in agarose culture under supplement-free conditions or treated with TGF-β1 in suspension expressed relatively low levels of PAI-1 mRNA compared with the robust expression evident upon stimulation with TGF-β1 during attachment to fibronectin (Fig. 7A). Adhesion to fibronectin matrices alone, in the absence of added TGF-β1, was sufficient to initiate modest PAI-1 transcription (Fig. 7A,B). This adhesion-dependent induction reflected a similarly conservative increase (i.e. threefold) in phospho-ERK1/2 levels (not shown) and, like TGF-β1-mediated expression in normally anchored cells (Fig. 3), also required MEK activity as it was effectively inhibited by PD98059 (Fig. 7B). To further assess if this adhesion-related PAI-1 induction (in either TGF-β1-stimulated or unstimulated cells) was dependent on the nature of the 'matrix' encountered, T2 cells were plated onto dishes coated with 10 μg/ml fibronectin, vitronectin or BSA. TGF-β1 did not induce PAI-1 under non-adherent conditions (i.e., culture on BSA-coated surfaces). Attachment to fibronectin for 4 hours (in the absence of TGF-β1) was an effective inducer of PAI-1 transcripts (by three to fivefold) relative to adhesion to vitronectin (Fig. 8). Moreover, preliminary kinetic determinations indicated that PAI-1 mRNA levels increased as a function of time of attachment suggesting that subsequent cell spreading may be a factor in expression control. This was not unique to T2 cells as a similar response was evident in human dermal fibroblasts and microvessel endothelial cells (data not shown). However, no difference in either the attachment and/or spreading of T2 cells plated on fibronectin- compared with vitronectin-coated surfaces was evident within the same time (Fig. 9). More importantly, TGF-β1 significantly enhanced PAI-1 expression only in T2 cells

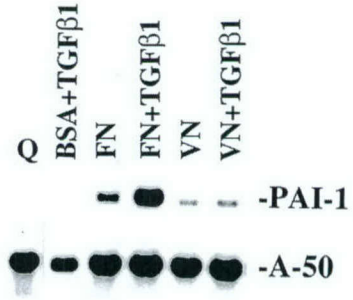

Fig. 8. Matrix-type dependency of basal and TGF-β1-induced PAI-1 expression. Quiescent (Q) T2 cells were trypsinized and replated on plastic dishes coated with BSA, fibronectin (FN) or vitronectin (VN) in the presence or absence of TGF-β1 (1 ng/ml) for a 2 hour period. While both FN and VN induced PAI-1 mRNA transcripts, the amplitude of induction was significantly greater on FN-coated surfaces; TGF-β1 stimulated expression only on T2 cells adhering to FN.

during attachment to fibronectin (Fig. 8). PAI-1 mRNA levels in cells seeded on vitronectin in the presence of TGF-β1 were not different from that expressed during adhesion to vitronectin alone.

DISCUSSION

Several important aspects of in vivo injury repair (i.e. regional uPA/PAI-1 expression, spatial/temporal distinctions between motile and proliferative phenotypes) (Reidy et al., 1995; Romer et al., 1991; Romer et al., 1994) are recapitulated during cell migration into the denuded areas of a scrape-injured monolayer (Pepper et al., 1987; Pepper et al., 1992; Garlick and Taichman, 1994; Zahm et al., 1997; Providence et al., 2000). PAI-1 is rapidly synthesized by cells immediately adjacent to experimentally-created wounds (Pepper et al., 1992; Pawar et al., 1995; Providence et al., 2000). PAI-1 synthesis and deposition into cellular migration tracks are characteristics of a mobile cohort (Seebacher et al., 1992; Pepper et al., 1992) and an essential component of the migratory program (Providence et al., 2000; Kutz et al., 2001). The in situ distribution of this protein is consistent with a function in cell locomotion. De novo synthesized PAI-1 protein accumulates in the cellular undersurface region, probably in a complex with matrix vitronectin (Seiffert et al., 1994; Lawrence et al., 1997) although it appears that PAI-1 may also associate with fibronectin and/or laminin deposits in migration tracks (Seebacher et al., 1992). Therefore, this SERPIN is well-positioned to modulate integrin-ECM or uPA/uPAR-ECM interactions as well as ECM barrier proteolysis. In vitro studies suggest that PAI-1 may dissociate bound vitronectin from the uPAR, detaching cells that use this receptor as a vitronectin anchor (Deng et al., 1996; Kjoller et al., 1997; Loskutoff et al., 1999). Alternatively, PAI-1 may directly inhibit αv integrin-mediated attachment to vitronectin by blocking accessibility to the RGD sequence located proximal to the uPAR binding site (Stefansson and Lawrence, 1996; Loskutoff et al., 1999), although this inhibition is subject to spatial-temporal constraints (Germer et al., 1998). Furthermore, uPAR-associated uPA/PAI-1 complexes are internalized by endocytosis, which promotes uPA receptor recycling (Andreasen et al., 1997) and thereby

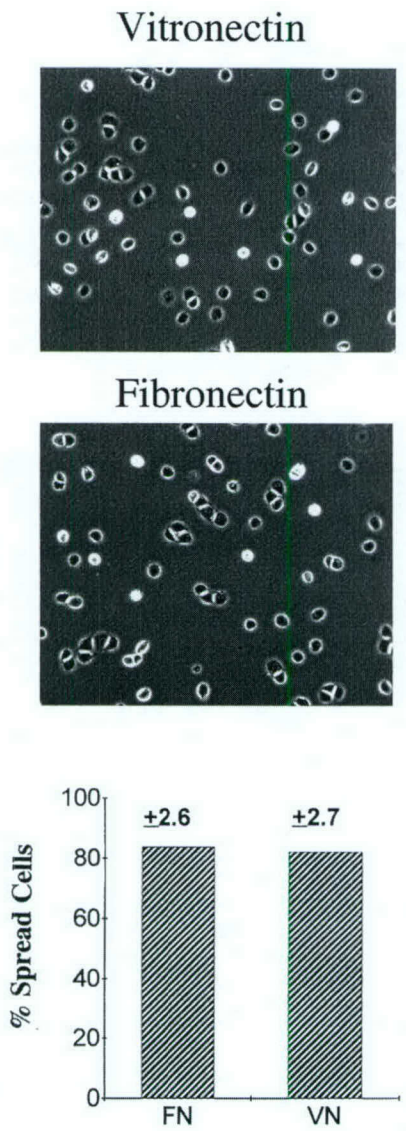

Fig. 9. Relative spreading of T2 cells on fibronectin and vitronectin. Suspended T2 cells were seeded in serum-free medium to dishes previously coated with fibronectin or vitronectin (10 μg/ml). After 4 hours, random fields were photographed (representative examples shown) and the percent spread cells (i.e. non-refractive) calculated. Data plotted are the means±s.d. for assessments on three separate dishes/substrate. There was no difference in either T2 cell attachment or spreading on fibronectin or vitronectin.

vitronectin-dependent cell movement. However, transgenic approaches have suggested that PAI-1 promotes vitronectin-independent angiogenesis specifically by inhibition of plasmin proteolysis, thus preserving an appropriate matrix scaffold or providing required neovessel stability (Bajou et al., 2001). Although in vivo compensatory mechanisms may partly explain this discrepancy between animal and culture models, motility controls clearly vary and depend on the level of expression of participating elements, the nature of the provisional 'matrix' encountered, the context of the system studied and the growth factor environment.

TGF-β1 exerts concentration-dependent effects on cellular locomotion in 3D culture systems (Gajdusek et al., 1993) as well as in the more spatially restricted planar model of denudation injury (this study) (Gajdusek et al., 1993; Zicha et al., 1999). Wound repair analysis of the PAI-1-deficient 4HH cell line, in which PAI-1 synthesis is specifically ablated by antisense targeting (Higgins et al., 1997; Providence et al., 2000), supports the contention that PAI-1 is an important component in the motile program in this model. Since TGF-β1 stimulates PAI-1 synthesis and PAI-1 impacts directly on cell motility (this study) (Deng et al., 1999; Providence et al., 2000), it was important to assess TGF-β1-dependent controls on PAI-1 expression as well as on cellular migration. TGF-β1 initiates PAI-1 transcription in quiescent T2 cells via an immediate-early response, tyrosine kinase-dependent pathway that involves MEK, an upstream activator of ERK1/2. However, unlike the typical rapid ERK phosphorylation associated with serum-stimulation (i.e., within 15 minutes), TGF-β1-mediated ERK activation (as assessed by phosphorylation of the target substrate MBP) was delayed by 30-60 minutes. The MEK dependency for PAI-1 expression and TGF-β1-stimulated as well as basal migration in T2 cells suggests that these events are related. Although MEK blockade probably interferes with cell movement at several levels (Klemke et al., 1997; Rikitake et al., 2000), genetic targeting approaches confirmed that both basal and TGF-β1-stimulated cell migration (over a 24-36 hour period) requires PAI-1 expression (Providence et al., 2000; Kutz et al., 2001). Clearly, MEK inhibition may not affect basal locomotion in all cell types (Nguyen et al., 1998) but results may depend on the specific system studied. For example, in the monolayer denudation model (unlike random motility assays), 'basal' migration is most probably growth factor-mediated. Indeed, monolayer wounding in various cell types, in and of itself, is a sufficient stimulus to initiate autocrine growth factor expression (e.g., TGF-β1, basic FGF, heparin-binding EGF) (Sato and Rifkin, 1988) and activate MAP kinases (Dieckgraefe et al., 1997). Moreover, growth factor synthesis and ERK phosphorylation/nuclear translocation occurs specifically in cells adjacent to the injury site (Dieckgraefe et al., 1997; Song et al., 2000; Ellis et al., 2001) similar to the distribution of locomoting PAI-1-expressing cells (Providence et al., 2000).

Matrix attachment, perhaps as part of the motile response, also stimulates PAI-1 expression. This has particular physiologic relevance. Although the present data suggest that not all matrices have equivalent inductive capability, during the process of wound healing cells 'switch' their integrin complement to accommodate the composition of the provisional ECM (Yamada et al., 1996). In certain instances, TGF-β1 directly mediates changes in integrin availability and, therefore, cellular adhesive traits (Collo and Pepper, 1999; Dalton et al., 1999; Lai et al., 2000). Engagement of particular integrins (i.e. αvβ3, α3β1) by immobilized antibodies or ligands has been implicated in PAI-1 gene control (Ghosh et al., 2000; Khatib et al., 2001). The α3β1 ligands laminin-5 and collagen I, when presented immobilized on beads, were also effective inducers of uPA synthesis (Ghosh et al., 2000). Similar to data reported in this study with regard to adhesive controls on PAI-1 expression, β1 integrin aggregation-induced uPA synthesis was also MEK-dependent as PD98059 inhibited ERK activation and uPA expression. Perhaps not coincidentally, both uPA and PAI-1 are induced by

pharmacologic disorganization of the actin-based microfilament system as is ERK activation (Higgins et al., 1992; Irigoyen et al., 1997). Since integrin ligation/clustering and cell adhesion result in various levels of cytoskeletal reorganization and recruitment of signaling intermediates (Zhu and Assoian, 1995; Lin et al., 1997; Miyamoto et al., 1998; Renshaw et al., 1997), the control of specific protease/protease inhibitors may be a common event in 'outside-in' signaling initiated by adhesive state and integrin engagement. Several matrices (i.e., fibronectin vs vitronectin) clearly differ in relative capacity to induce PAI-1 expression in T2 cells allowed to adhere under growth factor-free conditions. Most novel, however, is the observation that only certain matrix attachments synergize with TGF-β1 to achieve maximal PAI-1 expression. Whether matrix-type variations in the amplitude and duration of ERK signaling underlies this differential response in T2 epithelial cells is currently under study. Nevertheless, the present findings indicate that adhesive influences also modulate TGF-β1 signaling to target genes (i.e. PAI-1).

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Epithelial monolayer wounding stimulates binding of USF-1 to an E-box motif in the plasminogen activator inhibitor type 1 gene

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Summary

Several proteases and their co-expressed inhibitors modulate the interdependent processes of cell migration and matrix proteolysis during wound repair. Transcription of the gene encoding plasminogen activator inhibitor type 1 (PAI-1), a serine protease inhibitor important in the control of barrier proteolysis and cell-to-matrix adhesion, is spatially-temporally regulated following epithelial denudation injury in vitro as well as in vivo. Using a well-defined culture model of acute epidermal wounding and reepithelialization, PAI-1 mRNA/protein synthesis was induced early after monolayer scraping and restricted to cells comprising the motile cohort. PAI-1 levels in locomoting cells remained elevated (relative to the distal, contact-inhibited monolayer regions) throughout the time course of trauma repair. Targeted PAI-1 downregulation by transfection of antisense PAI-1 expression constructs significantly impaired keratinocyte migration and monolayer scrape wound closure. Injury-induced PAI-1 transcription closely paralleled growth state-dependent controls on the PAI-1 gene. An E-box motif (CACGTG) in the PAI-1 proximal promoter (located at nucleotides –160 to –165), previously shown to be necessary for serum-induced PAI-1 expression, was bound by nuclear factors from wound-stimulated but not quiescent, contact-inhibited, keratinocytes. UV crosslinking approaches to identify E-box-binding factors coupled with deoxyoligonucleotide affinity chromatography and gel retardation assays confirmed at least one major E-box-binding protein in both serum- and wound-activated cells to be USF-1, a member of the helix-loop-helix family of transcription factors. An intact hexanucleotide E-box motif was necessary and sufficient for USF-1 binding using nuclear extracts from both serum- and wound-simulated cells. Two species of immunoreactive USF-1 were identified by western blotting of total cellular lysates that corresponded to the previously characterized phosphorylated and non-phosphorylated forms of the protein. USF-1 isolated by PAI-1 promoter-DNA affinity chromatography was almost exclusively phosphorylated. Only a fraction of the total cellular USF-1 in proliferating cultures, by comparison, was phosphorylated at any given time. PAI-1 E-box binding activity, assessed by probe mobility shift criteria, increased within 2 hours of monolayer scrape injury, a time frame consistent with wound-stimulated increases in PAI-1 transcription. Relative to intact cultures, scrape site-juxtaposed cells had significantly greater cytoplasmic and nuclear USF-1 immunoreactivity correlating with the specific in situ-restricted expression of PAI-1 transcripts/protein in the wound-edge cohort. USF-1 immunocytochemical staining declined significantly with increasing distance from the denudation site. These data are the first to indicate that binding of USF-1 to its target motif can be induced by 'tissue' injury in vitro and implicate USF-1 as a transcriptional regulator of genes (e.g. PAI-1) involved in wound repair.

Key words: Keratinocytes, PAI-1, Reepithelialization, Gene targeting

Introduction

Cellular locomotion across planar surfaces requires lamellipodium extension, creation of new adhesive contacts, cell body contraction and trailing edge detachment (Lauffenburger and Horwitz, 1996; Friedl and Brocker, 2000; Wells, 2000; Ridley, 2001). Stimulated cell movement through 3D matrices, by contrast, imposes a resistance to cell migration that necessitates both shape adaptations and proteolytic activity (Murphy and Gavrilovic, 1999; Friedl and Brocker, 2000). Indeed, growth factor-induced cell migration during wound re-epithelization, tumor metastasis and in normal as well as pathologic angiogenesis involves participation of several, frequently interacting, protease systems. Stromal invasion in these contexts is often highly dependent on the generation of plasmin by urokinase plasminogen activator (uPA); uPA activity is regulated, in turn, by its fast-acting type-1 inhibitor (PAI-1) (Andreasen et al., 1997; Bajou et al., 1998; Farina et al., 1998; Lund et al., 1999; Zhou et al., 2000; Legrand et al., 2001). This cascade directly influences the overall tissue site proteolytic balance and is a critical determinant in directed cell

movement, provisional matrix remodeling and extracellular matrix (ECM) invasion (Pepper et al., 1987; Pepper et al., 1992; Okedon et al., 1992; Jeffers et al., 1996; Mazzieri et al., 1997; Wysocki et al., 1999; Providence et al., 2000; Providence et al., 2002; Reijerkerk et al., 2000; Brooks et al., 2001). Recent studies in PAI-$1^{-/-}$ mice confirmed the importance of this SERPIN in tumor spread and the associated angiogenic response (Bajou et al., 1998; Bajou et al., 2001; Gutierrez et al., 2000; McMahon et al., 2001; Stefansson et al., 2001). A critical balance between proteases and their specific inhibitors, thus, has been suggested as necessary to maintain an ECM scaffold structure compatible with efficient cellular locomotion (Bajou et al., 2001).

Growth factor-initiated changes in the expression, focalization and/or relative activity of uPA/PAI-1 may modulate cell migration either by controlling the rate and extent of ECM barrier proteolysis or altering cellular adhesive interactions with the ECM (Pepper et al., 1992; Seebacher et al., 1992; Stefansson and Lawrence, 1996; Mignatti and Rifkin, 2000). Co-expression of uPA, its surface-anchored receptor (uPAR) and PAI-1, for example, are required for optimal Matrigel invasion by lung tumor cells (Liu et al., 1995). Peptides that inhibit binding of uPA to its receptor ablated transforming growth factor-β1 (TGF-β1)-induced planar motility by transformed keratinocytes and significantly attenuated invasion across Matrigel barriers (Santibanez et al., 1999). PAI-1 levels are consistently elevated, moreover, in aggressive tumor phenotypes and PAI-1 expression is a major molecular feature of the TGF-β1-initiated epithelial-to-mesenchymal transition in various cell systems (Santibanez et al., 1999; Akiyoshi et al., 2001; Zavadil et al., 2001). Variances in PAI-1 synthesis (Providence et al., 2000) and/or site-localization (Kutz et al., 1997), therefore, would be expected to specifically impact on cellular migration by affecting uPA activity as well as uPAR/vitronectin- or integrin/vitronectin-dependent contacts (Ciambrone and McKeown-Longo, 1990; Deng et al., 1996; Chapman, 1997; Stefansson and Lawrence, 1996; Loskutoff et al., 1999). Targeted downregulation of PAI-1 synthesis with antisense expression vectors and use of function-blocking antibodies, in fact, inhibited basal as well as TGF-β1-stimulated epithelial cell motility in both 2- and 3-D model systems (Providence et al., 2000; Providence et al., 2002; Brooks et al., 2001; Kutz et al., 2001; Chazaud et al., 2002).

PAI-1 gene expression under conditions of induced migration is predominantly transcriptional (Pawar et al., 1995; Providence et al., 2000; Kutz et al., 2001). Multiple promoter elements mediate stimulus-specific controls on PAI-1 transcription (e.g. Westerhausen et al., 1991; Ryan et al., 1996; Slack and Higgins, 1999). One prominent regulatory sequence is the hexanucleotide E-box motif (CACGTG) that is recognized by several members of the helix-loop-helix family of transcription factors (e.g. MYC, MAX, TFE3, USF-1, USF-2, HIF-1) (Riccio et al., 1992; Hua et al., 1998; Hua et al., 1999; Dennler et al., 1998; White et al., 2000) and the snail zinc-finger superfamily (Nieto, 2002; Hajra et al., 2002). A consensus E-box, located at nucleotides –160 to –165 upstream of the transcription start site in the PAI-1 gene, is required for PAI-1 promoter-directed reporter gene activation in growing EC-1 cells as well as in hepatocytes subjected to mild hypoxia (Kietzmann et al., 1999; White et al., 2000). Using an in vitro model of the epidermal response to injury, we now report that monolayer wounding stimulates nuclear protein binding to a PAI-1 E-box-specific probe in the same cohort of cells induced to express PAI-1 mRNA transcripts/protein. Deoxyolignucleotide affinity chromatography revealed USF-1 to be a major PAI-1 E-box-binding factor. Monolayer wounding, moreover, stimulated USF-1 nuclear translocation and PAI-1 E-box occupancy in wound-proximal cells. These data suggest that the E-box may function as a major transcriptional control element for migration-associated genes in much the same manner as it has been implicated in the regulation of cell cycle progression genes (e.g. Cogswell et al., 1995; White et al., 2000).

Materials and Methods

Cell culture

HaCaT (Li et al., 2000), RK (Boehm et al., 1999) and EC-1 (Kutz et al., 2001) epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). Confluent cultures were washed with HBSS and maintained in serum-free DMEM for 3 days to initiate a state of growth-arrest (Ryan et al., 1996). Cells were stimulated globally by addition of FBS (to a final concentration of 20%) directly to the quiescence maintenance medium (Ryan et al., 1996) or more focally by scrape injury (Providence et al., 2000).

Motility assessments and collection of cell subpopulations

Denudation zones were created by pushing the narrow end of a sterile P1000 plastic pipette tip through the quiescent, contact-inhibited, epithelial monolayer. Wound closure rates, a function of planar motility in this directional 2D migration assay (Kutz et al., 2001), were calculated from measurements made using an inverted microscope fitted with a calibrated ocular grid (Providence et al., 2000). 'Activated' cells (i.e. those immediately adjacent to the denudation site, including cells that locomoted into the wound 'bed') were harvested by pushing the wide end of a P1000 pipette tip along the original injury tract, displacing cells directly at, and 5 mm from, the migratory edge. Scrape-released cells were aspirated and collected by centrifugation at 1400 ***g***. Cells located 40 mm from the original wound border (i.e. in the intact distal monolayer) were similarly harvested. To assess the effect of targeted perturbation of PAI-1 synthesis on wound-induced motility, RK cells were transfected with PAI-1 antisense and sense constructs created in the Rc/CMV expression vector (Higgins et al., 1997).

Northern blot analysis

Total cellular RNA was isolated and denatured at 55°C for 15 minutes in 1× MOPS, 6.5% formaldehyde, and 50% formamide prior to electrophoresis on agarose/formaldehyde gels (1.2% agarose, 1.1% formaldehyde, 1× MOPS). RNA was transferred to Nytran membranes by capillary action in 10× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), UV crosslinked and incubated for 2 hours at 42°C in 50% formamide, 5× Denhardt's solution, 1% SDS, 100 μg/ml sheared/heat-denatured salmon sperm DNA (ssDNA) and 5× SSC. RNA blots were hybridized simultaneously with ^{32}P-labeled cDNA probes to PAI-1 and A-50 (RK and EC-1 cells) or PAI-1 and GAPD (HaCaT cells) for 24 hours at 42°C in 50% formamide, 2.5× Denhardt's solution, 1% SDS, 100 μg/ml ssDNA, 5× SSC and 10% dextran sulfate. Membranes were washed three times in 0.1× SSC/0.1% SDS for 15 minutes each at 42°C followed by three washes at 55°C prior to exposure to film.

Nuclear extracts

Cells were trypsinized, harvested by centrifugation, resuspended in 400 μl of cold buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT), placed on ice for 15 minutes, then vortexed for 10 seconds after addition of 25 μl 10% Nonidet NP-40. Nuclei were collected by centrifugation for 30 seconds at 14,000 ***g***, resuspended in 50 μl of cold lysis buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) containing leupeptin, aprotinin, chymostatin, pepstatin A, antipain (each at a final concentration of 10 μg/ml), rocked at 4°C for 15 minutes and extracts clarified at 10,000 ***g*** for 5 minutes. For phosphatase treatments, isolated nuclei were lysed (Cheung et al., 1999) and 5 μg nuclear extract protein incubated with potato acid phosphatase in PIPES/KOH digestion buffer, pH 6.5, for 2 hours at 37°C prior to electrophoresis on SDS/12% acrylamide gels and western blotting for USF-1 (as indicated below).

Mobility shift assay

Double-stranded deoxyoligonucleotides (3-5 pM) were incubated at 37°C for 10 minutes with T4 polynucleotide kinase (5-10 units/μl) in 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM $MgCl_2$, 5 mM DTT and [γ-^{32}P]dATP (3000 Ci/mmol). Probes were purified by filtration through 10 kDa cellulose spin columns. Constructs used were as follows (only the coding strand is indicated):

PAI-1 E box, 5′-TACACA***CACGTG***TCCCAG-3′;

PAI-1 mutant E-box #1, 5′-TACACA***CACGGA***TCCCAG-3′;

PAI-1 mutant E-box #2, 5′-TACACA***TCCGTG***TCCCAG-3′;

standard consensus E-box, 5′-GGAAGCAGAC***CACGTG***GTCT-GTGCTTCC-3′;

AP-1 consensus sequence, 5′-CGCTTGA***TGACTCA***GCCGGAA-3′.

Nuclear extracts were incubated with 50,000-100,000 cpm ^{32}P end-labeled target deoxyoligonucleotides in 5× gel shift buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.4 mg/ml dIdC). Following room temperature incubation for 20 minutes, gel loading buffer (25 mM Tris-HCl, pH 7.5, 0.02% bromophenol blue, 0.02% xylene cyanol, 4% glycerol) was added. Complexes were separated on Tris/glycine gels (Tris/glycine buffer: 5 mM Tris-HCl, 2 mM EDTA, 100 mM glycine) containing 4% acrylamide, 0.5% bisacrylamide, 2.5% glycerol, 0.75% ammonium persulfate and 0.085% TEMED). Antibodies (1-2 μg per reaction) were added to the formed extract protein/^{32}P-labeled DNA probe complexes and maintained at room temperature for 20 minutes prior to electrophoresis for supershift assays (White et al., 2000).

UV crosslinking

A PAI-1 wild-type (WT) E-box probe body-labeled with ^{32}P for use as a probe in mobility shift assays was generated by PCR using a primer set corresponding to promoter region nucleotides –171 to –166 and –159 to –154 and purified on a 10 kDa spin column. Nuclear extract-probe binding reactions were incubated in a 96-well microtiter plate for 20 minutes at room temperature prior to UV irradiation (4.8 to 24.0 μJoules/cm^2) followed by DNase-1 treatment (2 μg/ml). Sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol, 0.01% bromophenol blue) was added, the complexes boiled and resolved on SDS/polyacrylamide slab gels (9% acrylamide, 0.24% bis-acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.03% ammonium persulfate, 0.025% TEMED).

Immunocytochemistry

Media were aspirated and the cells washed 3× in PBS prior to fixation for 10 minutes in 3% formaldehyde. After three PBS washes (5 minutes each), fixed cells were permeabilized with 0.5% Triton X-100/PBS for 10 minutes at 4°C, washed three times in PBS, incubated in glycine (10 mg/ml) for 15 minutes, and incubated with antibodies to USF-1 (or preimmune IgG) followed by fluorescein isothiocynate (FITC)-labeled secondary antibodies. Cells were visualized by incubation in propidium iodide which yields red nuclear fluorescence under UV light. Coverslips were mounted in anti-fade reagent for confocal microscopy. For immunodetection of PAI-1, cultures were fixed in 100% methanol for 20 minutes at –20°C then rehydrated by rinsing in PBS prior to sequential incubations with PAI-1 antibody followed by FITC-conjugated secondary antibody.

Tethered deoxyoligonucleotide protein binding and western blotting

The PAI-1 18 bp wild-type E-box deoxyoligonucleotide was ligated to a biotinylated 16-mer target sequence tethered to streptavidin-coated magnetic particles using the Boehringer Mannheim DNA-Binding Protein Purification kit. Nuclear protein binding to the PAI-1 'bait' construct was done in the presence of poly dIdC and poly L-lysine, the mixture vortexed, particles harvested with a magnetic separator and washed, and bound proteins eluted by boiling in electrophoresis sample buffer (50 mM Tris/HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol) prior to separation on SDS/12% acrylamide slab gels. Protein transfers were probed with antibodies to USF-1 (c-20) or c-FOS (4-1D-G) (Santa Cruz Biotechnology) and antigen:antibody complexes visualized by horseradish peroxidase-conjugated secondary antibodies and ECL detection reagent (Amersham Pharmacia) using X-OMAT AR-5 film (Li et al., 2000).

PAI-1-GFP chimera expression constructs

The human PAI-1 promoter sequence from –800 to +71 was PCR-amplified for 30 cycles using the p800-Luc reporter plasmid as a template and Platinum *Taq* polymerase. The promoter fragment was gel-purified for subsequent cloning into the *Sac*I/*Kpn*I sites of the promoter-less expression vector pEGFP-1 (Clontech, Palo Alto, CA). The full-length human PAI-1 coding sequence (approximately 1.3 kb) was derived by RT-PCR from total RNA isolated from human foreskin fibroblasts, amplified by PCR using Platinum *Taq* polymerase, gel-purified and expressed as a GFP fusion protein by T4 ligase insertion into the *Kpn*I/*Eco*RI sites of pEGFP-N3. The same PAI-1 coding region was also transferred into the *Bam*HI/*Age*I site of the PAI-1 promoter-derived pEGFP-1 vector. All constructs were sequence verified. RK cells were seeded into 35 mm dishes and allowed to reach a density of 1×10^5 cells/cm^2 prior to transfection with 1-2 μg DNA using Lipofectamine-Plus. Transfected cells were trypinsized and plated at low cell density in EGF-supplemented (1 ng/ml) growth medium. In some cases, transfected cells were removed from the culture dish prior to microscopy by incubation in 0.2% saponin in Ca^{2+}/Mg^{2+}-free PBS leaving a substrate-attached PAI-1-rich matrix (Higgins et al., 1997).

Results

PAI-1 synthesis is induced in migrating epithelial cells and required for optimal in vitro wound repair

Time-lapse videomicroscopy of the epithelial response to in vitro wounding resulted in the identification of two spatially distinct subpopulations of cells that are activated by scrape-injury and function in concert to re-establish a confluent monolayer (Providence et al., 2000). The 'leading edge' cohort (i.e. cells immediately adjacent to the wound border) quickly transition from a cuboidal to flattened phenotype, become polarized (i.e. extend membrane ruffles and lamellipodia along the cellular 'face' juxtaposed to the denuded zone) and begin

to migrate within 1-2 hours after initial injury. Mitotic bodies were not evident in the motile front during the time course of wound coverage. Addition of the thymidine analogue BrdU at the time of scraping and immunochemical visualization of DNA-synthesizing cells at various times post-trauma confirmed that migrating cells did not enter S phase (not shown). Significant nuclear labeling was evident, however, in a band of cells located approximately 1-2 mm from, and parallel to, the long axis of the injury site (for details, see Providence et al., 2000). This margin-displaced 'proliferative' cohort provides a reservoir of new cells to support the continued progression and cohesiveness of the migrating front prior to the increase in S phase activity in the neoepidermis. Functional compartmentalization in this in vitro model, therefore, closely recapitulates specific early events associated with acute epidermal injury repair in vivo (Coulombe, 1997).

PAI-1 is highly expressed in migratory cells in vitro as well as in vivo (Pepper et al., 1992; Romer et al., 1991; Providence et al., 2000). Similarly, elevated de novo synthesis of PAI-1 by two keratinocyte cell lines, RK (determined immunocytochemically) and HaCaT (by western blotting), in the in vitro monolayer wound model is restricted to cells immediately juxtaposed to the denudation site and which have acquired morphologic characteristics of an early motile phenotype (Fig. 1). Leading-edge cells extending cytoplasmic projections into the denuded area were particularly immunoreactive with PAI-1 antibodies. Differential harvest of locomoting RK, HaCaT and EC-1 epithelial cells indicated, moreover, that PAI-1 transcripts were significantly increased in cells harvested along the site of the original wound track (13- to 27-fold relative to the distal quiescent, unperturbed, monolayer cells) and remained elevated throughout the repair process (Fig. 1). The difference in PAI-1 mRNA expression kinetics between RK/EC-1 and HaCaT populations was attributable to the relatively protracted time course of wound resolution (24-36 hours vs. 48-72 hours) by HaCaT cells. Visual examination of the motile front population suggested that the newly synthesized PAI-1 was particularly abundant at a plane of focus corresponding to the cellular undersurface-culture substrate region. Transfection of RK cells with a GFP-tagged PAI-1 expression vector, in which transcription of the chimeric PAI-1-GFP insert is under the control of PAI-1 promoter sequences, provided for the direct visualization of PAI-1-GFP protein in the 'matrix' of saponin-dislodged keratinocytes as well as in the migratory tracks of growth factor-stimulated cells (Fig. 2). This approach to insert expression control (i.e. using PAI-1 upstream elements to drive PAI-1-GFP transcription) was selected since the time course of PAI-1-GFP chimera induction closely approximated that of the endogenous PAI-1 gene (e.g. Ryan et al., 1996). The PAI-1 promoter-PAI-1 coding-GFP construct was transfected into confluent RK monolayers followed by incubation in serum-free medium prior to monolayer scraping. By 5 hours, scrape-activated cells had migrated well into the wound 'bed' exhibiting PAI-1-GFP-decorated migration tracks perpendicular to the long axis of the original scrape injury (not shown). These findings (e.g. Fig. 2) suggested that PAI-1 might function as a component of the basal epidermal cell motile apparatus as it appears to do in other cell types (Stefansson and Lawrence, 1996; Waltz et al., 1997; Brooks et al., 2001). To evaluate this possibility, RK cells were transfected with the PAI-1 antisense expression vector Rc/CMVIAP, cultured under quiescence conditions, then scrape injured. Two criteria were specifically evaluated including effects on PAI-1 synthesis and on 2D planar motility. The resultant attenuation of wound-induced PAI-1 protein synthesis in Rc/CMVIAP transfectants (confirmed by western blotting) reflected a significant inhibition of injury site closure (Fig. 3).

Monolayer wounding stimulates PAI-1 E-box-binding activity

Since PAI-1 expression was clearly a critical modulator of epithelial cell migration, it was important to clarify molecular events involved in PAI-1 expression control in response to

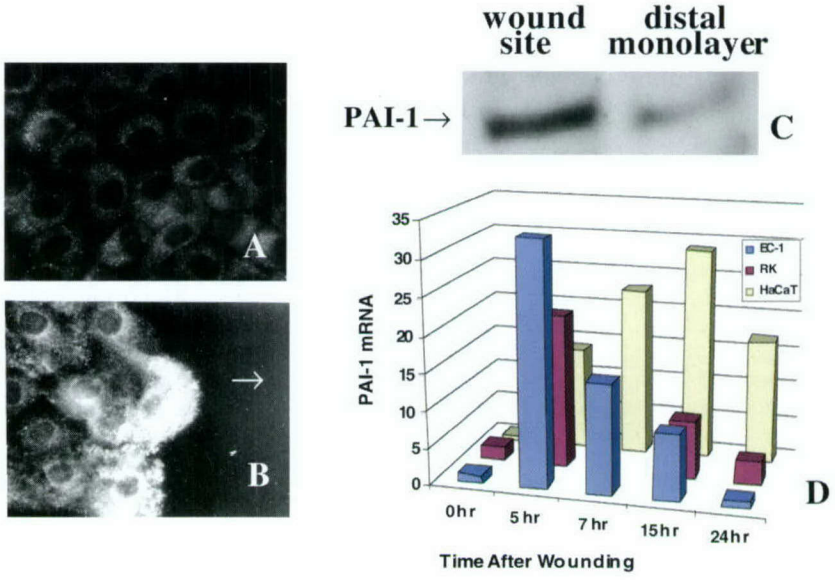

Fig. 1. Monolayer injury stimulates PAI-1 synthesis specifically in wound-edge cells. Quiescent contact-inhibited cultures of RK, HaCaT and EC-1 cells were maintained either intact or scrape-wounded with a pipette tip. RK cultures were fixed and PAI-1 protein visualized by immunocytochemistry (A,B). Control quiescent RK cells expressed relatively low levels of PAI-1 (A). Within 6 hours after scraping, PAI-1 was readily detected in motile RK cells immediately juxtaposed to the injury site (B; arrow indicates direction of migration into the denuded area). Western analysis of lysates of HaCaT cells differentially harvested 24 hours after scrape-trauma confirmed a significant increase in PAI-1 expression by epithelial cells bordering the injury site compared with cells in the distal uninvolved monolayer (C). PAI-1 mRNA transcripts were upregulated in cells harvested from the wound edge within 5 hours after scrape-injury. PAI-1 mRNA abundance (normalized to A-50 and GAPD hydridization signal for EC-1/RK and HaCaT cells, respectively) remained elevated over the time course of wound repair (D). RK total RNA was not isolated at the 7 hour post-wounding time point in the analysis series summarized in D. The difference in PAI-1 mRNA kinetic profiles for HaCaT versus EC-1/RK cells reflects the relatively protracted time frame for HaCaT monolayer injury site closure compared with RK/EC-1 populations (i.e. 48-72 hours versus 24-36 hours).

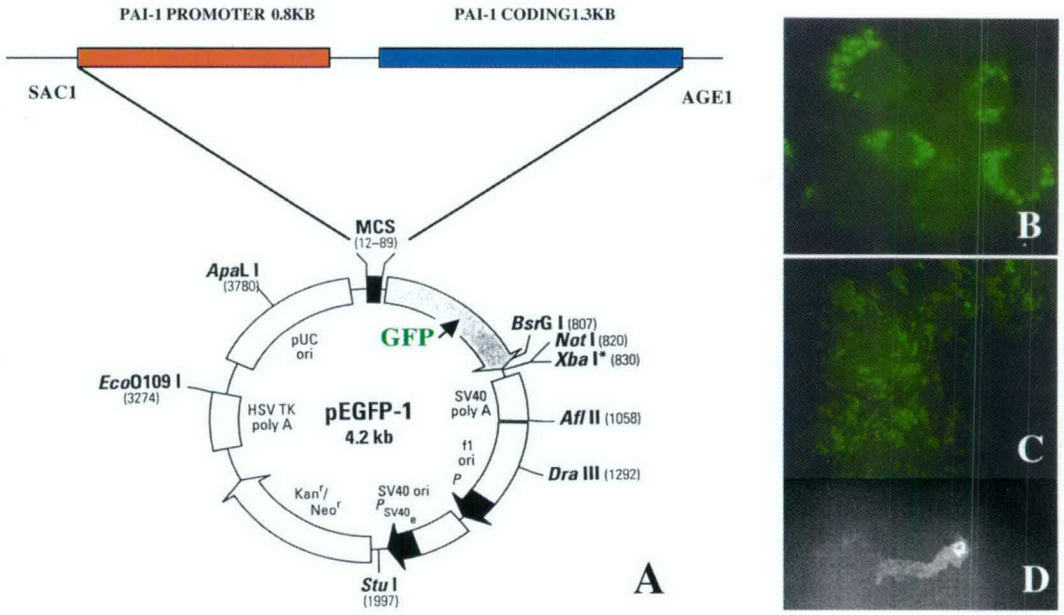

Fig. 2. Visualization of PAI-1-GFP in cellular migration tracks. Schematic of a pEGFP-1-based vector in which a chimeric transcript consisting of 1.3 kb of PAI-1 coding sequences and GFP is expressed under the control of a 0.8 kb PAI-1 'promoter' (A). Transfection of RK cells and re-seeding in serum-containing medium resulted in synthesis of PAI-1-GFP detected initially in perinuclear Golgi-like structures (B). Approximately 6-12 hours later, PAI-1-GFP can be found in the matrix (probably vitronectin)-rich undersurface region upon removal of cells with saponin (C). The green 'footprint' of a single cell is shown in C. Seeding of PAI-1 promoter-PAI-1 coding-GFP transfectants at low density in EGF-containing medium provides for the clear visualization of the chimeric PAI-1-GFP protein in cellular migration trails (D). The small bright image at the extreme right of the trail is the cell body. Transfection of RK cells with the PAI-1 promoter-PAI-1 coding-GFP vector followed by growth to confluency and subsequent scrape injury indicated that the resulting motile population deposited GFP-'tagged' PAI-1 into the cellular migration trails similar to that illustrated in D.

monolayer wounding. A search of the 5′ flanking region of the PAI-1 gene, originally to identify potential cis-acting elements involved in growth state-dependent gene expression (Ryan et al., 1996; Boehm et al., 1999), identified a consensus E-box motif (CACGTG) at nucleotides –165 to –160 upstream of the transcriptional start site (White et al., 2000). This region is protected from DNase I digestion in growing epithelial cells (Johnson et al., 1992). E-box-binding activity consisting of two closely-spaced 'dumbell-shaped' upper and lower bands, assessed using an 18 bp PAI-1 sequence as a target in which the CACGTG motif was flanked both 5′ and 3′ by PAI-1-specific sequences, was evident in RK (Fig. 4), EC-1 and HaCaT (see below) cells. This probe shift pattern was maintained when an unlabeled AP-1 deoxyoligonucleotide (5′-CGCTTGA*TGACTCA*GCCGGAA-3′), in 100-fold molar excess, was included in the reaction mixture. Band shifts were successfully competed, however, upon simultaneous addition of a 100-fold molar excess of an unlabeled wild-type (self)

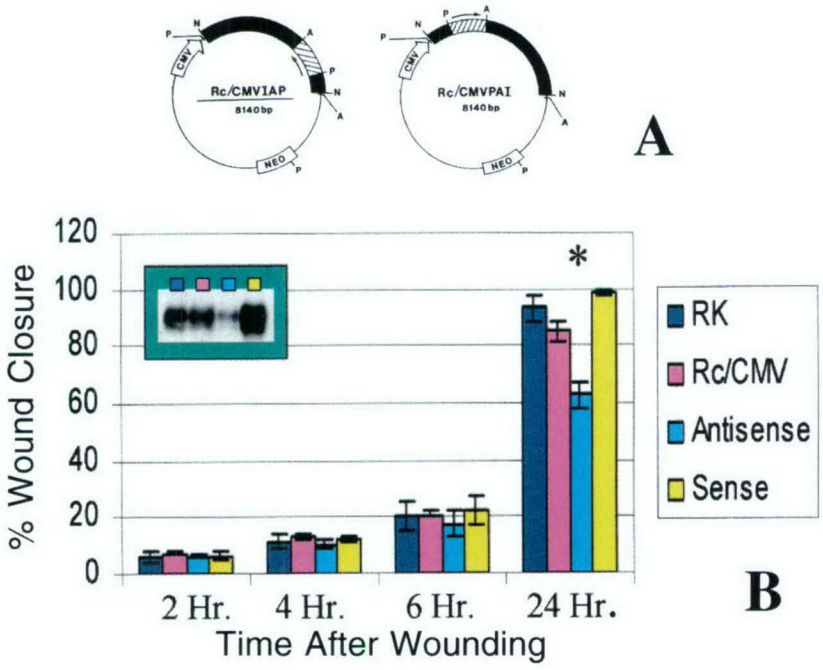

Fig. 3. Targeted PAI-1 downregulation inhibits wound-stimulated cell motility. RK cells were transfected with a non-insert-bearing vector (Rc/CMV) or with constructs in which full-length PAI-1 coding sequences were cloned in antisense (Rc/CMVIAP) or sense (Rc/CMVPAI) orientation (A). Cultures were grown to confluency and scrape-wounded. Extent of repair-associated migration (% wound closure) was measured over a 24 hour period (B). There was no difference in stimulated motility among Rc/CMV- or Rc/CMVPAI-transfectants compared with non-transfected controls (RK). The rate of monolayer scrape repair by Rc/CMVIAP (antisense PAI-1)-transfected cells, in contrast, was significantly impaired (asterisk) relative to control RK cultures or to sense (Rc/CMVPAI) or empty vector (Rc/CMV) transfectants. Data plotted is mean±standard deviation of three independent wound repair determinations. Inset in B is a western blot of PAI-1 levels in the various cell types at the 24 hour time point illustrating downregulation of PAI-1 expression in the Rc/CMVIAP transfectants.

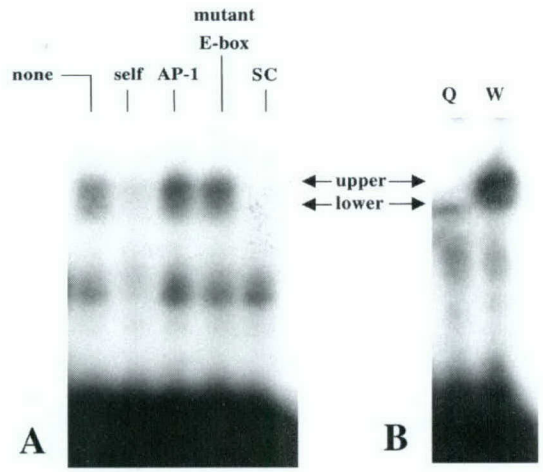

Fig. 4. Binding of a PAI-1 E-box probe by nuclear proteins from growing and wound-stimulated RK cells. The 18 bp ^{32}P-end labeled PAI-1 E-box probe (see Materials and Methods) was incubated with 10 μg of the nuclear protein fraction isolated from growing (A) or quiescent as well as wound-edge (B) RK cells and complexes resolved by gel electrophoresis. Two closely-spaced, 'dumbell-shaped', bands (arrows) were evident in shifts produced by nuclear extracts derived from growing cells in the absence (none) of competing sequences (A). Incubation with the unlabeled WT PAI-1 18 bp E-box deoxyoligonucleotide (self) or the unlabeled standard consensus (SC) E-box construct (i.e. a CACGTG motif flanked by non-PAI-1 sequences) (both at a 50- to 100-fold molar excess) effectively blocked complex formation with the labeled probe. The AP-1 deoxyoligonucleotide and a mutant E-box construct (5′-CACGGA-3′), the latter in the context of PAI-1 flanking sequences, each failed to compete for probe binding (A). In contrast to PAI-1 probe patterns developed with nuclear extracts from quiescent (Q) RK cultures and which failed to form complexes that co-migrated with the slower mobile (i.e. upper) band, extracts prepared from wound-edge keratinocytes 2 hours post-scrape injury (W) produced the characteristic two-band complex (B).

competitor or a standard consensus (SC) E-box construct (i.e. an E-box hexanucleotide motif with non-PAI-1 flanking sequences). A mutant E-box motif (either CACGGA or TCCGTG) flanked by PAI-1-specific sequences failed to compete for probe binding (Fig. 4). These same mutant constructs were also incapable of forming shifted complexes when ^{32}P end-labeled and used as targets in gel retardation assays (see below). Collectively, these findings indicate a requirement, and specificity, for an intact consensus hexanucleotide E box for protein binding to the homologous site in the PAI-1 gene. Once probe E-box site occupancy by nuclear factors isolated from constitutively-growing, PAI-1-expressing, RK cells was established, it was necessary to determine whether a similar binding activity could be detected in wound-stimulated cultures and, if so, the kinetics of site occupancy relative to wound-induced expression of the endogenous PAI-1 gene. Nuclear extracts from differentially harvested wound-edge and distal quiescent monolayer cells were incubated with the ^{32}P-labeled 18 bp PAI-1 E-box probe and the formed complexes resolved by electrophoresis. Relative to cells that are in immediate proximity to the denudation injury and that have the demonstrable characteristic 2-band pattern (upper and lower) probe-binding activity, nuclear extracts of distal monolayer isolates generally formed only a single complex corresponding in mobility to the lower band (Fig. 4). E-box-binding activity was evident soon after scrape injury in wound-edge cells consistent with the subsequent increase in PAI-1 transcripts in the migrating cohort (Fig. 1).

USF-1 is a PAI-1 E-box binding protein

An intact E-box site at nucleotides –160 to –165 in the proximal promoter of the rat PAI-1 gene is an important platform for protein binding in response to proliferative stimuli, mild hypoxia as well as to individual growth factors including TGF-β1 (Kietzmann et al., 1999; White et al., 2000) (L.A.W. and P.J.H., unpublished). In order to identify specific transcriptional effectors capable of binding to the PAI-1 E-box site (based on data summarized in Fig. 4), a ^{32}P body-labeled, PCR-amplified, fragment of the PAI-1 promoter containing the CACGTG motif was UV crosslinked to nuclear proteins isolated from growing EC-1 cells. A major complex of approximately 44-45 kDa was resolved after DNase-1 digestion of the probe-extract reaction products and electrophoresis on SDS-acrylamide gels (Fig. 5). Addition of proteinase K to the UV-irradiated nuclear extract/deoxyoligonucleotide binding reaction for a 5 minute incubation before gel electrophoresis eliminated the 44-45 kDa band, suggesting involvement of a crosslinked nuclear protein in the formed complex (not shown). Prominent among E-box-binding proteins in this mass range are several helix-loop-helix transcription factors most notably members of the USF1/2 family (Littlewood and Evan, 1995). Tethered deoxyoligonucleotide affinity chromatography was used, therefore, to isolate PAI-1 E-box-binding proteins from the nuclear fraction of growing EC-1 cells. Bound proteins were eluted and western blotting, in fact, confirmed USF-1 as one PAI-1 E-box target sequence binding element (Fig. 5). Two immunoreactive USF-1 species, corresponding in mobility to USF-1 and phospho-USF-1 (Galibert et al., 2001), were resolved in extracts of growing EC-1 cells (Fig. 5). Blot analysis suggested an approximately threefold increase in USF-1 levels in growing cells compared with quiescent cells (L.A.W. and P.J.H., unpublished). Phosphorylation of USF-1 is necessary for DNA binding (Cheung et al., 1999) and, consistent with this requirement, the predominant form of USF-1 eluted from PAI-1 deoxyoligonucleotide affinity columns co-migrated with the 45 kDa (phospho-USF-1) species (Fig. 5). That the slower migrating 45 kDa species was phospho-USF-1 was confirmed by potato acid phosphatase treatment of nuclear extracts from serum-stimulated cells (which have abundant levels of the 45 kDa USF-1 immunoreactive protein) prior to western analysis. Once identified, it was important to assess whether E-box-dependent USF-1 binding could be resolved in nuclear extracts of scrape injury-stimulated cells (since PAI-1 transcripts were upregulated specifically in wound-edge keratinocytes; Fig. 1). Initial analysis of wounded monolayers indicated that increased levels of immunoreactive USF-1 were evident in cells immediately adjacent, and in close proximity, to the denuded site. Compared with intact cultures, scrape injury-juxtaposed cells had significantly greater cytoplasmic and nuclear USF-1 immunoreactivity (Fig. 6) correlating with the

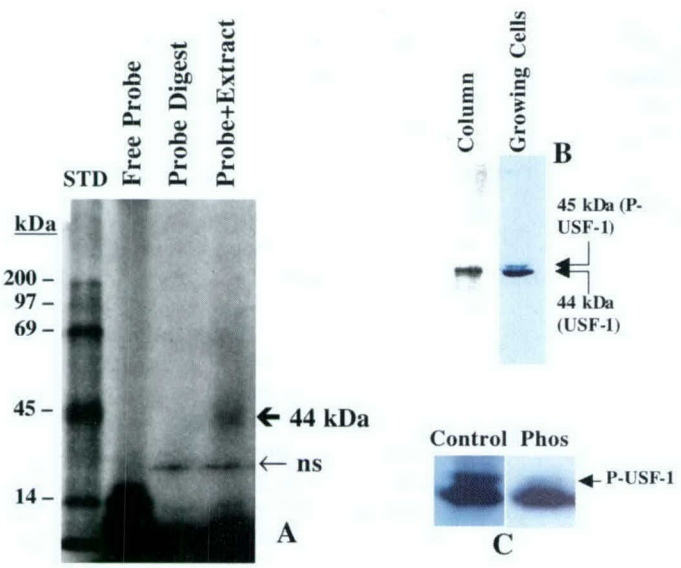

Fig. 5. Identification of USF-1 as PAI-1 E-box probe-binding factor. A ^{32}P-body labeled PAI-1 wild-type (WT) E-box deoxyoligonucleotide probe was generated by PCR. Gel-purified probe was incubated with nuclear extracts from serum-stimulated EC-1 cells prior to UV irradiation and treated with DNase-1; the complexes were then boiled in sample buffer and resolved on SDS/9% polyacrylamide slab gels (A). A single major band at 44-45 kDa was resolved in the lane containing probe crosslinked to EC-1 nuclear extract (Probe+Extract). This band was not detected upon electrophoresis of probe alone (Free Probe) or in reactions where the probe was DNase-digested prior to addition of nuclear extract and UV crosslinking (Probe Digest). One low molecular weight nonspecific (ns) band was evident with the latter control. PAI-1 E-box-binding proteins were isolated from the nuclear fraction of growing EC-1 cells by tethered deoxyoligonucleotide affinity chromatography (B). Bound proteins were eluted and separated by gel electrophoresis. Western blotting confirmed USF-1 as one PAI-1 E-box target sequence binding element (B, Column). Two USF-1 species, corresponding in mobility to USF-1 (44 kDa) and phospho-USF-1 (P-USF-1, 45 kDa), were resolved by western analysis of extracts derived from growing EC-1 cells (B, Growing Cells). The predominant form of USF-1 eluted from PAI-1 deoxyoligonucleotide affinity columns co-migrated with the slower mobility (i.e. phosphorylated) USF-1 species. Acid phosphatase treatment (Phos) of nuclear extracts from serum-stimulated cells prior to gel electrophoresis and western blotting significantly decreased the abundance of the anti-USF-1 immunoreactive 45 kDa (P-USF-1) band compared with non-phosphatase-treated (Control) extracts (C).

specific in situ-restricted expression of PAI-1 transcripts/protein in the wound-edge cohort (Fig. 1). Mobility shift studies were designed, therefore, to evaluate whether this augmented USF-1 nuclear accumulation, at least following wound stimulation, correlated with an increase in USF-1 PAI-1 E-box construct binding activity. Nuclear extracts from constitutively growing HaCaT and RK cells produced the typical upper and lower doublet band shift pattern with the target 18 bp PAI-1 E-box probe. The upper band was specifically supershifted by antibodies to USF-1 indicating that at least one contributing factor in this slower migrating complex was USF-1 (Fig. 6). Extracts from quiescent cells (i.e. contact-inhibited cultures maintained in serum-free medium for 3 days) did not form the upper banding component with the target PAI-1 probe and the complexes that were resolved were generally unreactive with USF-1 antibodies. Comparison of the probe gel retardation patterns obtained with extracts from growing RK cells to those developed with nuclear extracts isolated from wound-edge harvested cells (2 hours post-scrape injury), in contrast, confirmed that the upper component in the doublet complex resolved with extracts from injury site cells, like that in proliferating keratinocytes, could also be supershifted by USF-1 antibodies (Fig. 6).

Discussion

Recapitulation of events associated with injury repair in vivo (i.e. regional uPA/PAI-1 expression, spatial/temporal distinctions among the differentiated, motile and proliferative compartments) (Romer et al., 1991; Romer et al., 1994; Reidy et al., 1995) are modeled, in certain sytems, during cell migration into the denuded areas of a scrape-injured monolayer (Pepper et al., 1987; Pepper et al., 1992; Garlick and Taichman, 1994; Pawar et al., 1995; Coulombe, 1997; Zahm et al., 1997; Providence et al., 2000). PAI-1 mRNA/protein are rapidly synthesized by keratinocytes immediately adjacent to experimentally-created wounds and remain elevated over the course of monolayer 'healing', similar to findings in the wounded epidermis (Romer et al., 1991; Jensen and Lavker, 1996; Providence et al., 2000). PAI-1 deposition into migration tracks, moreover, is a characteristic of a mobile cohort (Seebacher et al., 1992; Pepper et al., 1992), although whether such accumulation is due to encoded 'trafficking' information, resulting in specific targeting to the cellular undersurface region, remains to be determined. Use of the serum/EGF-responsive PAI-1 promoter to drive expression of a chimeric PAI-1-GFP transcript, nevertheless, is the first demonstration of PAI-1 localization in migratory trails during the real time of stimulated expression. PAI-1 synthesis, moreover, is an essential component of the motile program in cultured basal keratinocytes since PAI-1 downregulation with the Rc/CMVIAP vector effectively attenuated scrape injury closure. The present observations in established keratinocytes confirm wound repair anomalies reported for the PAI-1-deficient 4HH cell line, in which PAI-1 synthesis is specifically ablated by stable antisense targeting (Higgins et al., 1997; Providence et al., 2000). The kinetics of induction and in situ distribution of this protein are, in fact, consistent with a function in cell locomotion. Indeed, the approximately 10 hour offset in maximal PAI-1 transcript expression in wounded HaCaT versus RK/EC-1 monolayers actually reflects cell type differences in injury closure rates and supports the concept that this gene is regulated as a function of cellular motile status. The PAI-1 insert, as used in the Rc/CMV expression system, when ligated in the antisense configuration in a T7/T3 selectable promoter vector (Higgins et al., 1997) yielded a transcript that hybridized to both the 2.2- and 3.0-kb species of human PAI-1 mRNA (Li et al., 2000). Planar migratory defects observed in Rc/CMVIAP transfectants in both the RK and EC-1 cell types, moreover, correlated with significant reductions in de novo PAI-1 synthesis. Similar findings in HaCaT keratinocytes expressing inducible PAI-1 antisense transcripts (Li et al., 2000) strongly suggest that the motile deficit in each case was a direct result of an antisense effect on

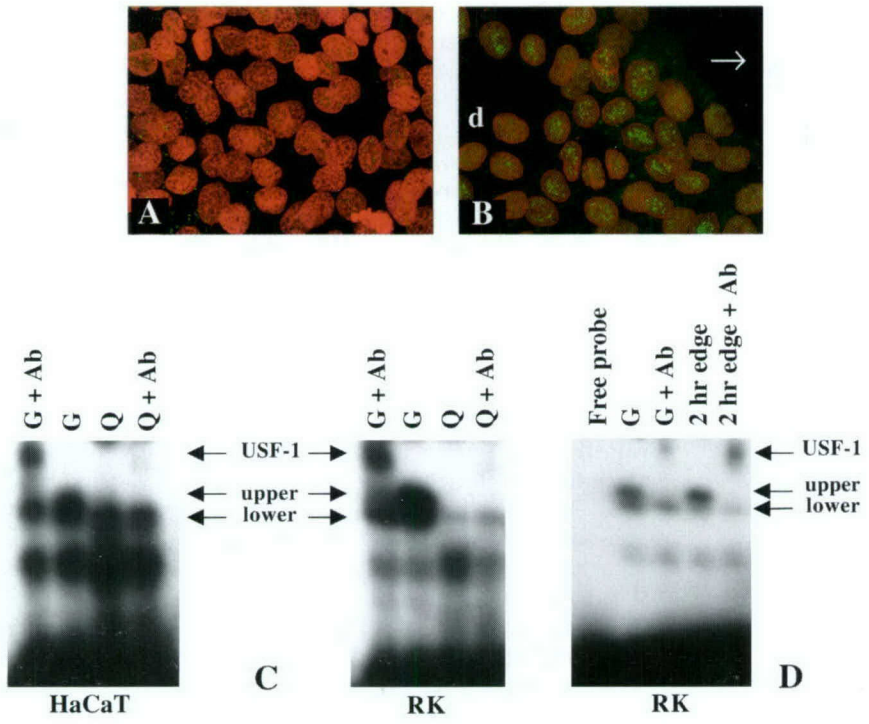

Fig. 6. USF-1 in situ localization and PAI-1 E-box-binding activity in wound-proximal keratinocytes. Compared with quiescent intact monolayer regions (A), HaCaT cells juxtaposed to the wound site (arrow in B indicates direction of migration into a denuded zone) exhibit significant cytoplasmic and nuclear immunocytochemical reactivity for USF-1 (B). In both panels A and B, nuclei are stained (red) with propidium iodide while green speckles indicate immunoreactive USF-1. Cells situated more distal (d) from the wound edge had considerably lower cytoplasmic and nuclear USF-1 (B). Nuclear USF-1 accumulation could be detected as early as 2 hours post-monolayer scraping (preceeding the increase in PAI-1 transcripts) and remained evident throughout the period of wound repair. The protracted (48-72 hour) time course of injury resolution in HaCaT cultures reflected (even at 24 hours after wounding) continued PAI-1 expression (Fig. 1) and nuclear USF-1 localization (B) by the migrating epithelium. The typical upper and lower dumbell-shaped gel retardation pattern was resolved upon incubation of nuclear extracts from growing (G), but not quiescent (Q), HaCaT and RK cultures with the ^{32}P-labeled 18 bp PAI-1 E-box probe (C). The upper band was specifically supershifted upon addition of antibodies to USF-1 after formation of the protein-probe complex. Similarly, the USF-1-containing upper complex was also resolved upon incubation of nuclear extracts from RK cells harvested from the wound site (D). The upper-lower doublet retardation pattern was evident as early as 2 hours after scrape injury (2 hr edge) and, like growing RK cultures (G), addition of USF-1 antibodies specifically supershifted this upper complex.

PAI-1 synthesis with concomitant modulation of cellular adhesive traits.

De novo synthesized PAI-1 protein accumulates in the cellular undersurface region likely in a complex with matrix vitronectin (Higgins and Ryan, 1989; Seiffert et al., 1994; Lawrence et al., 1997), although PAI-1 has been suggested to also associate with fibronectin and/or laminin deposits in migration tracks (Seebacher et al., 1992). This SERPIN is well-positioned, therefore, to modulate integrin-ECM or uPA/uPAR-ECM interactions as well as ECM barrier proteolysis. PAI-1 may dissociate bound vitronectin from the uPAR, detaching cells that use this receptor as a vitronectin anchor (Deng et al., 1996; Deng et al., 2001; Kjoller et al., 1997; Loskutoff et al., 1999). Alternatively, PAI-1 may directly inhibit αv integrin-mediated attachment to vitronectin by blocking accessibility to the RGD sequence located proximal to the uPAR binding site (Stefansson and Lawrence, 1996; Loskutoff et al., 1999). uPAR-associated uPA/PAI-1 complexes, furthermore, are internalized by endocytosis promoting uPA receptor recycling (Andreasen et al., 1997) and, thereby, vitronectin-dependent cell movement. Transgenic approaches suggest, however, that PAI-1 promotes vitronectin-independent angiogenesis specifically by inhibiting plasmin proteolysis, and thus preserves an appropriate matrix 'scaffold' to support cell migration or provide required neovessel stability (Bajou et al., 2001). These findings highlight the complexity of cellular motile controls that collectively reflect the level of expression of participating elements, the nature of the 'matrix' encountered, the system context (i.e., 2D vs 3D migration) and the growth factor environment. The rapid kinetics of wound-stimulated PAI-1 induction and relatively short matrix-associated half-life (Higgins and Ryan, 1989) suggests that this protein may influence cellular adhesive events for a specified duration during injury repair.

Similar to PAI-1 induction under conditions of mitogenic stimulation (White et al., 2000) is the rapid wound-related recruitment of USF-1 to the same defined E-box site in the PAI-1 promoter. Site occupancy, moreover, likely requires conservation of the CANNTG motif as mutations outside of the two central nucleotides resulted in loss of competitive binding activity. USF dimers as well as TFE3, HIF and MYC/MAX family member homo- or heterodimers recognize E-box motifs within certain genes including p53 and PAI-1 (e.g. Riccio et al., 1992; Reisman and Rotter, 1993; Hua et al., 1998; Hua et al., 1999; Dennler et al., 1998; Kietzmann et al., 1999; White et al., 2000) and present data are consistent with the preference of USF proteins for CACGTG or CACATG sequences (Littlewood and Evan, 1995; Ismail et al., 1999). Successful PAI-1 probe competition by a CACGTG 'core' flanked by non-PAI-1 sequences and failure of specific E-box mutants with PAI-1 homologous flanking DNA to similarly compete (or to produce band shifts when used as targets) indicate, furthermore, that an intact hexanucleotide E-box motif is necessary and sufficient for USF-1 binding in both serum- and wound-simulated cells. The enrichment for phospho-USF-1 by DNA affinity chromatography of extracts from growing cells compared with the relative abundance of phospho- and non-phosphorylated species resolved by western blotting of cell extracts indicated that USF-1 that bound to DNA was almost exclusively phosphorylated, whereas only a fraction of the total cellular USF-1 in proliferating cultures was phosphorylated at any given time. These data are consistent with the known

phosphorylation requirement of certain HLH factors for E-box motif recognition (Nozaki et al., 1997; Cheung et al., 1999).

The mechanism of USF-1 functional mobilization (i.e. DNA-binding) in response to wounding is speculative. Monolayer injury is associated with the induced expression of several growth factors (e.g. FGF, HB-EGF, TGF-β) and with MAP kinase activation in cells bordering the denudation site (Sato and Rifkin, 1988; Dieckgraefe et al., 1997; Song et al., 2000; Ellis et al., 2001). Certain growth factors, particularly those of the TGF-β family, stimulate occupancy of E-box sequences in several genes including PAI-1 (Riccio et al., 1992; Hua et al., 1998; Hua et al., 1999) as well as activate MAP kinases (Kutz et al., 2001; Yue and Mulder, 2001). Specific E-box-binding factors, including USF-1 and TFE3, are phosphorylated at consensus MAP kinase target residues (Galibert et al., 2001; Weilbaecher et al., 2001) facilitating DNA site interactions. At least one member of the stress family of MAP kinases (p38) does, in fact, phosphorylate USF-1 (Galibert et al., 2001), although other growth-related kinases may also target USF-1. In synchronized cells, for example, the DNA-binding activity of USF-1 is regulated by cyclin A-$p34^{cdc2}$- or cyclin B1-$p34^{cdc2}$-dependent phosphorylation within the USF-specific region (USR), the likely target site (Cheung et al., 1999). Phosphorylation of residues within the USR appears to initiate a conformational switch that exposes the DNA-binding domain (Cheung et al., 1999). Similar to the requirements for interaction of MAX with its target E-box sequence, USF-1 DNA-binding activity may be regulated, therefore, in a growth state- or wound-responsive manner apart from direct controls on USF-1 or MAX synthesis (Miltenberger et al., 1995; Lun et al., 1997). One possibility is that MAP kinase activation in the injured epithelium, dependent or independent of an autocrine growth factor-initiated loop, results in USF-1 phosphorylation and subsequent trans-activation of specific USF-1 target genes (e.g. PAI-1) as part of the switch from a sessile to motile phenotype.

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APPENDIX 3

Research Signpost
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1 Use of chromatin immunoprecipitation to identify E box-binding transcription factors in the promoter of the growth state-regulated human PAI-1 gene

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Abstract

Genes encoding proteins required for G_1 transit and entry into DNA synthetic phase are activated within a defined time frame and for a specified duration following mitogen-induced recruitment of quiescent cells into the proliferative cycle. Cooperation between integrins and growth factor receptors is required to optimally relay mitogenic signals likely reflecting specific adhesive or "shape" controls on cell cycle progression. The induced expression of plasminogen activator inhibitor type-1 (PAI-1) occurs as part of the immediate-early response to serum. PAI-1 transcription is maximal within 4 hours of growth factor addition to quiescent cells with a rapid decline to basal levels prior to S phase entry. The PAI-1 gene encodes a serine

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protease inhibitor (SERPIN) that regulates cell-to-substrate attachment and generation of the trypsin-like protease plasmin. By virtue of its ability to modulate cell-to-matrix interactions, PAI-1 may be a critical element in the growth program affecting both adhesion-dependent G_o/G_1 and G_1/S transitions as well as cell survival. E box motifs (5'-CACGTG-3'), important in the transcription of several growth state-dependent genes, have been mapped to growth factor-response sites in the PAI-1 promoter. Nuclear extract immunodepletion and supershift/complex-blocking approaches identified one PAI-1 E box-binding protein to be upstream stimulatory factor-1 (USF-1). Dinucleotide substitutions (CACGTG→TCCGTG) that ablate USF-1 occupancy of the E box site also attenuate PAI-1 promoter activity in growing cells. Changes in USF-1/E box interactions as a function of cell cycle progression may well influence PAI-1 transcriptional kinetics during G_1 transit. This review describes basic mechanisms operative in growth cycle-dependent PAI-1 gene expression and the use of the relatively new technique of chromatin immunoprecipitation to provide insights to in vivo promoter site occupancy. The available data support the hypothesis that specific E box motifs in the PAI-1 gene, previously implicated as growth factor-response elements, function as platforms for recruitment of transcriptional repressor/activator complexes during the $G_o \rightarrow G_1$ transition.

Growth state-dependent PAI-1 gene expression

Mitogenic stimulation of quiescent (G_o) cells initiates a cascade of gene activity required for G_0/G_1 transit and subsequent progression through the cell cycle [1-4]. Such growth-associated genes are generally activated within temporally-defined "windows" and segregate among the immediate-early (IER), delayed-early (or mid) and late response sets [1,2,5-10]. While a significant fraction of IER genes encode transcription factors (which subsequently target the delayed-early and late genes that sustain the proliferative response) **(Fig. 1)**, many structural proteins that comprise the cytoskeletal network, extracellular matrix (ECM), and cell-to-matrix adhesive complexes including certain matrix-active proteases and their inhibitors are also encoded by IER genes [7,11]. Recruitment of quiescent fibroblasts into the growth cycle, for example, is accompanied by the increased synthesis of actin, tropomyosin, alpha-actinin, vinculin, fibronectin, the β1 integrin, and ECM-remodeling proteases (e.g., urokinase plasminogen activator; uPA) [3,6,7,12-14]. This suggests a role for cytoarchitectural and ECM-modulating proteins in proliferative activation [10,15] and is consistent with earlier work implicating cell shape/spreading in cell growth control [16-18]. Indeed, cooperation between integrins and growth factor receptors is essential for transduction of mitogenic signals [19-22]. Cytostructural "reformatting" as part of the cellular response to serum growth factors is likely necessary, therefore, for transition through the adhesion-dependent restriction point in late G_1 **(Fig. 2)**.

Prominent among the repertoire of genes activated in response to serum is that encoding the type 1 inhibitor of plasminogen activator (PAI-1) [15,23,24]. Synthesis of PAI-1 mRNA occurs rapidly and transiently upon addition of serum to quiescent cells [23,25,26] **(Fig. 3)** as well as during compensatory regeneration following tissue injury [27,28]. PAI-1 complexes with urokinase and tissue-type plasminogen activators limiting, thereby, the generation of plasmin, a broad spectrum protease involved in fibrin surveillance and extracellular matrix turnover [29]. PAI-1 may function, therefore, as

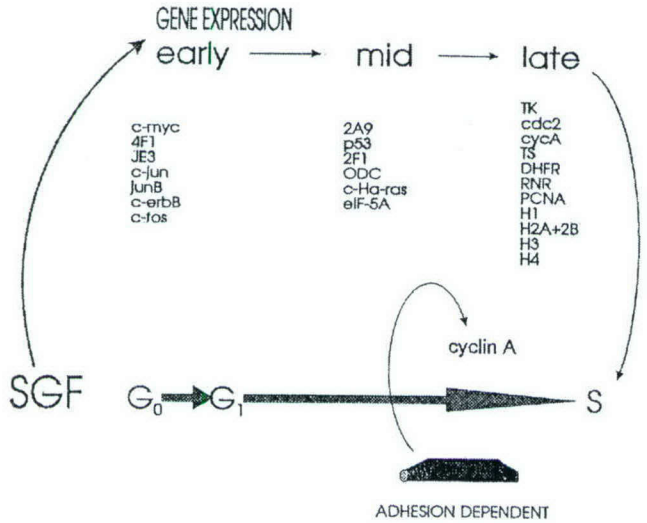

Figure 1. Relative time course for transcription of immediate-early (early), delayed-early (mid) and late genes following stimulation of quiescent cells with serum growth factors (SGF) superimposed on a topographic map of G_1 progression. Re-entry into a cycling G_1 state from a condition of G_0 arrest involves the rapid induction of early gene transcripts, many of which encode transcription factors, coincident with the $G_0 \rightarrow G_1$ transition. Metabolic genes and those encoding proteins essential for DNA synthesis, are highly represented among the mid and late gene sets. Expression of cyclin A, a late gene product, is adhesion-dependent as is S phase entry (at least for normal cells).

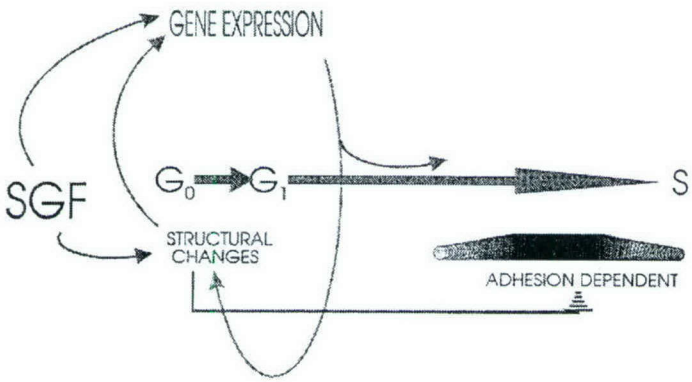

Figure 2. Stimulation of quiescent cells with serum growth factors (SGF) initiates transcriptional reprogramming required for proliferative commitment as well as for cellular structural reorganization [15,24]. Specific growth "activated" changes in cytoarchitecture (i.e., increased cell spreading, formation of new matrix contact sites) [e.g., 15] modulates growth factor signaling events to maintain expression of gene products involved in cell cycle progression. Such growth-associated alterations in cell structure are necessary for exit from G_0 into G_1 and for transition through the adhesion- dependent restriction point.

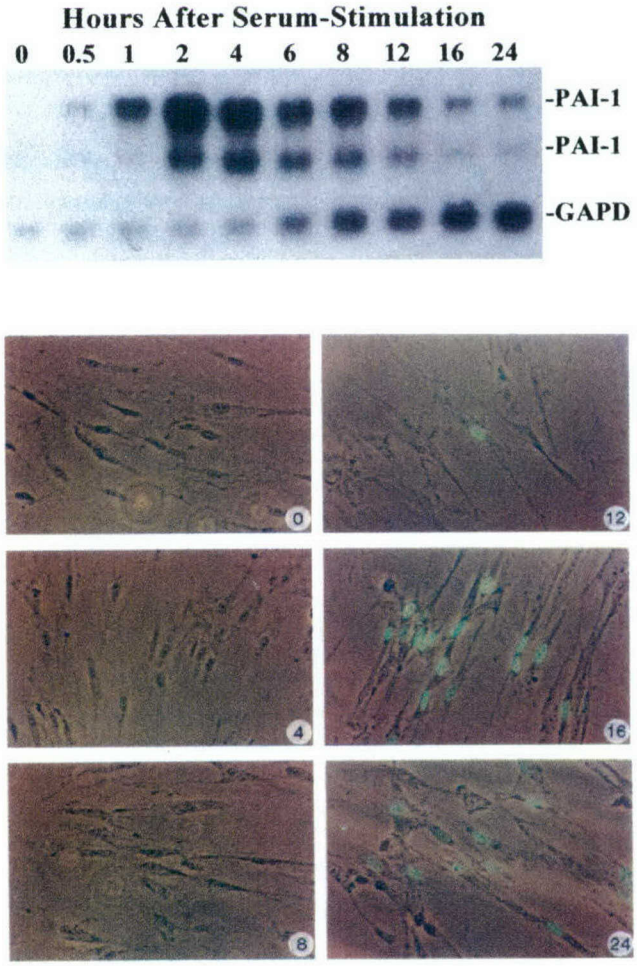

Figure 3. Kinetics of PAI-1 mRNA accumulation in response to serum (top panel). Subconfluent IMR-90 human diploid fibroblasts were serum-deprived for 3 days then stimulated with medium containing FBS (20% final concentration). At the indicated times post-stimulation, RNA was isolated for Northern blotting. Induced PAI-1 transcripts (3.0 and 2.2 kb species) were maximal 2 hours after serum addition. GAPD mRNA levels gradually increase as a function of time after serum addition. BrdU was added at the time of stimulation for immunocytochemical visualization of S phase cells (bottom panels). DNA synthesis was synchronously detected between 12 and 16 hours following release from G_0. Collectively, these data indicate that there is a rapid decline in PAI-1 transcripts prior to entry into S phase.

part of a global program of tissue remodeling/wound repair [24] or, more specifically, within the context of the growth-activated phenotype [15], to create an adhesive environment permissive for anchorage-dependent cell proliferation and/or migration (**Fig. 2**). PAI-1 protein does, in fact, accumulate in the cellular undersurface likely in a complex with matrix vitronectin [30-33]. This serine protease inhibitor is well-

positioned, therefore, to modulate integrin-ECM or uPA/uPAR (uPAR)-ECM interactions as well as pericellular proteolytic activity [29,34]. Since PAI-1 protein deposition occurs quickly after serum-stimulation [15,26], changes in PAI-1 expression may subsequently impact on the stability of newly formed focal contacts [35] affecting, thereby, maintenance of the growth-activated phenotype. The rapid kinetics of PAI-1 undersurface deposition following mitogenic stimulation and short matrix-associated half-life are consistent with recent suggestions that this protein may influence cellular adhesive characteristics for a specified duration within the context of "activated" cell growth [15,26,36,37]. Indeed, PAI-1 has dramatic effects on cell-to-ECM attachment [34,38]. It appears that PAI-1, α_v integrins, and uPA likely function coordinately to regulate cell-to-substrate adhesion (a necessary prerequisite for G_1/S transition [39,40]), cell shape (and shape-dependent metabolic pathways [41-43]), and adhesive events important in the control of cell motility [44-49]. The actual mechanism(s) by which PAI-1 influences cellular adhesive state is the subject of considerable speculation. Recent findings suggest that PAI-1 may dissociate vitronectin from the uPAR, detaching cells that utilize this receptor as a vitronectin anchor [33,50-52]. Alternatively, PAI-1 may directly inhibit α_v integrin-mediated attachment to vitronectin by blocking accessibility to the RGD sequence located proximal to the uPAR binding site [33,46]. uPAR-associated uPA/PAI-1 complexes, moreover, are internalized by endocytosis promoting uPA receptor recycling and, thereby, vitronectin-dependent cell movement [53]. PAI-1 may also influence cell adhesion directly, functioning as one component in the molecular complex that bridges the cell and the ECM [34,38,54]. Clearly, the association between the activated phenotype and targeted accumulation of PAI-1 in close proximity to newly formed focal adhesions [26] is consistent with this function. Most recently, PAI-1 has been found to inhibit insulin-induced signaling by competing with the $\alpha_v\beta_3$ integrin for vitronectin binding in a uPA receptor-independent manner reinforcing the pivotal role of PAI-1 in cellular transduction pathways [55]. PAI-1 has emerged, therefore, as a key element in a global program of tissue remodeling where it likely regulates the adhesive environment permissive for anchorage-dependent cell proliferation and/or migration [15,24,35,39].

Cell cycle controls on PAI-1 transcription

Assessment of G_1 transit (using BrdU incorporation to delineate onset of S phase) indicated that maximal PAI-1 transcript levels are restricted to approximately early-to-mid-G_1 and decline prior to entrance into the DNA synthetic phase (**Fig. 3**). Cell cycle-associated transcriptional controls, thus, are super-imposed on this growth state-dependent program of PAI-1 gene regulation following exit from G_0 [25,26]. The PAI-1 gene, in fact, exhibits a dual mode of regulation upon entry of G_0-arrested cells into a cycling G_1 condition [23] with the amplitude of induction as well as the maintenance of expression through the G_1 phase being anchorage responsive [23,25]. Late in G_1 phase, however, secondary events predominate and likely mediate a rapid decline in mRNA abundance [57]. Such adhesion-dependent effects, unlike the initial inductive event, require on-going protein synthesis [23] and highlight the complexity of PAI-1 gene control during G_1 progression [25].

While the specific signals for PAI-1 promoter activation during the G_0-G_1 transition are unknown, the E box at nucleotides -165 to -160 in the rat PAI-1 5' flanking genomic

region is footprinted in growing cells [58]. This site is critical for PAI-1 expression since incorporation of the dinucleotide substitution CACGTG→TCCGTG in a CAT reporter construct driven by 764 bp of PAI-1 promoter sequences reduced serum-stimulated CAT expression by 74% [57]. The helix-loop-helix/leucine zipper (HLH-Zip) protein upstream stimulatory factor-1 (USF-1) binds to an E box probe target with PAI-1-specific flanking sequences in a growth state-dependent manner [49,57]. First described as HLH-Zip *trans*-activators of adenovirus major-late promoter expression via an upstream E box motif (CACGTG), USF-1 and USF-2 influence expression of several cellular genes (e.g., fibrinogen, class 1 alcohol dehydrogenase, cyclin D1, p53, and human PAI-1) [59-61]. These factors likely facilitate TFII-D binding to the TATA box [59,62]. While USF may also stimulate Inr-dependent transcription by stabilizing the TFII-1 preinitiation complex [63,64], the MYC protein negatively influences Inr-mediated transcriptional responses by sequestering TFII-1 [65]. Depending upon the promoter configuration (TATA box, Inr, and/or an E box motif), the availability of specific HLH-Zip transcription factors with E box recognition domains (e.g., USF and MYC), and elements composing the preinitiation complex, therefore, the effects on transcription may be quite different [66-68]. While USF can be a stimulatory factor, in several systems USF-1 and 2 have repressive or negative regulatory influences [69-70]. Transcriptional inhibition appears due to direct interference with the *trans*-activator TFE3. Indeed, it has been suggested that USF is not an efficient *trans*-activator and that displacement of a strong activator by USF down-regulates gene expression [70]. Since USF-1 effectively converts VP16 into a *trans*-activator, E box occupancy by USF may recruit other regulatory proteins to the site in addition to functioning independently. Clearly, such effects may be inhibitory as well as stimulatory [71] and likely involve direct protein-protein interactions (e.g., sequestration of Fra1 by USF resulting in transcriptional repression; formation of USF-2/Fos dimers [71,72]). Simple identification of USF-1 as an E box-bound species does not resolve this issue as overexpression of USF-1 or USF-2 as homodimers effectively repressed rRNA transcription in CHO cells whereas USF-1/USF-2 heterodimeric complexes *trans*-activated gene expression [73]. Indeed, repression is a critical aspect of cell cycle-dependent PAI-1 gene control. Decreased PAI-1 mRNA abundance prior to S phase entry of serum-stimulated cells correlated with increases in E2F1 protein levels while pharmacologic inhibition of pRB phosphorylation prevented the late G_1 decline in PAI-1 transcripts [37]. pRB phosphorylation blockade prevents E2F-pRB dissociation and E2F1-dependent PAI-1 suppression. These data strongly implicate E2F1 as a negative regulator of cell cycle-dependent PAI-1 gene transcription, although no actual E2F1-DNA complexes were resolved [37]. Overexpression of E2F1-3 family members each inhibited cell cycle-dependent expression of the PAI-1 gene whereas E2F4 and 5 were without effect; both the DNA-binding and *trans*-activation domains of E2F1 are required for this suppressive effect [36].

E box elements in growth state-responsive regions of the PAI-1 promoter are occupied by USF-1

The footprinted E box motif located at nucleotides -160 to -165 (upstream of the transcription start site) in the promoter of the rat PAI-1 gene [58] binds USF-1 [57]. Site occupancy, moreover, or at least an intact consensus CACGTG hexanucleotide

sequence, is required for PAI-1 promoter-driven CAT reporter activity in growing cells [57]. Electrophoretic mobility shift analysis of E box target probe/nuclear factor formed complexes, moreover, indicate that the gel retardation patterns clearly distinguish quiescent from cycling cells (**Fig. 4**) and, thereby, PAI-1 non-expressing from expressing cells, respectively (**Fig. 3**). While USF-1/E box binding activity was evident in both G_0 and proliferating cells, the complexes formed between the E box probe and nuclear extract protein had different mobilities with USF-1 present in both (**Fig. 4**). It is tempting to speculate, based on these findings, that changing dynamics between USF-1 and recruited (repressing vs. activating) regulatory proteins at the bound E box site determines the transcriptional status of the PAI-1 gene in quiescent and cycling cells.

5'-GGAAGCAGACCACGTGGTCTGTGCTTCC-3'

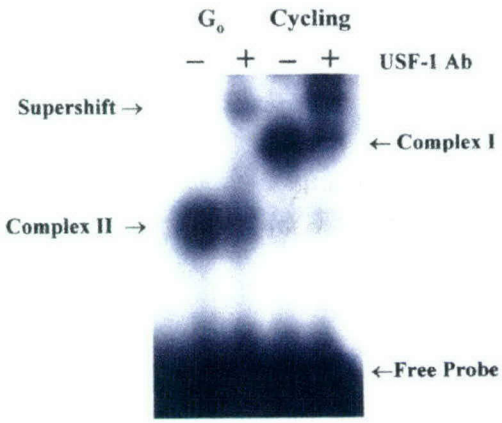

Figure 4. A double-stranded 28-mer E box (CACGTG) target probe (only 5'→3' sequence indicated) was end-labeled and incubated with nuclear extracts from G_0 or cycling cells for electrophoretic mobility shift analysis. Two major complexes with different relative migration patterns were resolved (complex I and complex II) that served to distinguish quiescent from proliferating cells. Addition of antibodies to USF-1 after incubation of nuclear factors with the target DNA produced a marked supershift for complex I and II indicating that USF-1 was present in both.

Similar to the TATA-proximal E box at nucleotides –160 to –165 in the rat PAI-1 gene [57], the CACGTG sequence within the growth-responsive –550 to –596 region of the human PAI-1 gene was originally identified as a USF-1 binding site [67]. This E box is 3' of three adjacent SMAD recognition motifs (AGAC); collectively, these sequences map to the PE2.1 segment of the PAI-1 gene (5'-CCTAGACAGACAAAA CCTAGACAATCACGTGGCTGG-3') and have been implicated in transforming growth factor-β (TGF-β)-inducible expression [74]. Recent *in vitro* findings suggested that the CACGTG hexanucleotide and its adjoining AGAC sites are occupied by the HLH-Zip factor TFE3 and SMAD-3, respectively, under conditions of TGF-β stimulation [75]. USF-1 may also bind to this same E box [67] but, in this circumstance, the PAI-1 gene appears to be unregulated by TGF-β [74,75]. Such approaches to

promoter function are, at best, inferential. The use of deoxyoligonucleotide probes and isolated transcription factors or nuclear extracts to identify binding elements, while clearly advantageous for determining the sequence requirements for DNA recognition, can be influenced by the relative concentration of the individual proteins presented to the deoxyoligonucleotide and the binding/electrophoretic conditions used. Indeed, using such procedures, the human PAI-1 gene has been variously implicated as a target for several E box-resident HLH-Zip transcription factors including MYC/MAX, USF-1/2, HIF-1, and TFE3 [e.g., 49,66,74,75].

One technique that avoids some of the ambiguities associated with purely *in vitro* methods to the study of DNA-protein interactions is chromatin immunoprecipitation (ChIP). ChIP is a relatively new approach to define *in vivo* relationships between transcriptionally critical *cis* elements and specific *trans*-acting control factors. Living cells, cultured under particular growth conditions, are fixed with formaldehyde, a tight dipolar crosslinking agent that effectively produces both protein-protein and protein-nucleic acid crosslinks [76-78]. Isolated chromatin is sonicated to an appropriate size and fragments immunoprecipitated with antisera to specific chromatin-associated proteins. After reversal of the crosslinks, regions of DNA involved in protein binding are identified by PCR. The details of this procedure **(Fig. 5)** were the subject of a recent comprehensive review [79].

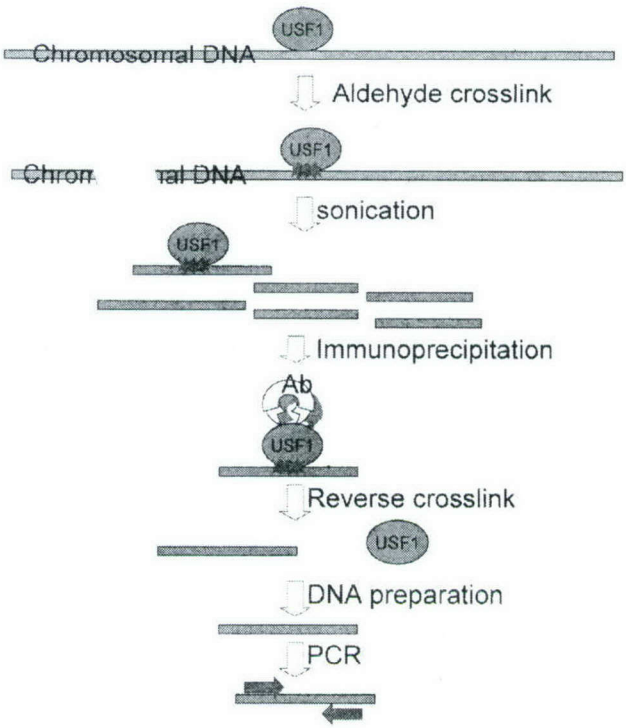

Figure 5. A summary flow chart describing the basic outline of a chromatin immunoprecipitation analysis of DNA-protein interactions.

ChIP assessment of the E box site in the PE2.1 region of the human PAI-1 gene suggested an unexpected dynamic occupancy by USF-1 as a function of growth state **(Fig. 6)**. This motif was clearly a platform for USF-1 binding in quiescent cells **(Fig. 6)** consistent with E box target probe analysis by electrophoretic mobility shift assay **(Fig. 4)**. In cells that were stimulated back into the cell cycle, however, USF-1 antibodies precipitated significantly less DNA (as indicated by a greatly reduced PCR signal) while analysis of DNA fragments obtained with antisera to acetylated histone 4 confirmed that the PAI-1 promoter underwent chromatin remodeling events associated with transcriptional activation. Such obvious cell cycle-dependent changes in the ChIP profile and the requirement for intact E box sites in PAI-1 expression [57,75] suggest that USF-1 may participate in multiple functions at the PAI-1 E box depending on particular protein partners and cellular growth state. Some anti-USF-1-precipitable DNA was, in fact, evident in G_1 chromatin raising the possibility that the critical epitopes may be masked by recruited co-activators during cell cycle progression. The potential existence of such higher order USF-1-containing chromatin complexes in proliferating cells is actually supported by resolution of the slowly migrating complex I in probe targeting studies **(Fig. 4)**. While it may be tempting to speculate that USF-1 complexes are simply replaced on the PAI-1 E box during growth activation, the available data provides for alternative explanations. One relevant model proposes that USF-1 or the closely-related family member USF-2, when present as homodimers, may actually repress PAI-1 expression in G_o cells **(Fig. 3)** and complex II **(Fig. 4)** could mediate this effect. Subsequent conversion of USF-1 to a *trans*-activator may occur alone or in cooperation with other regulatory elements (e.g., USF-2). Indeed, support for this hypothesis derives from the finding that both USF-1 and USF-2 homodimers repress rRNA transcription while USF-1/USF-2 heterodimeric complexes are effective *trans*-activators [73]. Dimer replacement at the critical E box motif likely occurs soon after, or coincident with, the $G_o \rightarrow G_1$ transition, may require USF-1 phosphorylation by mitogen-

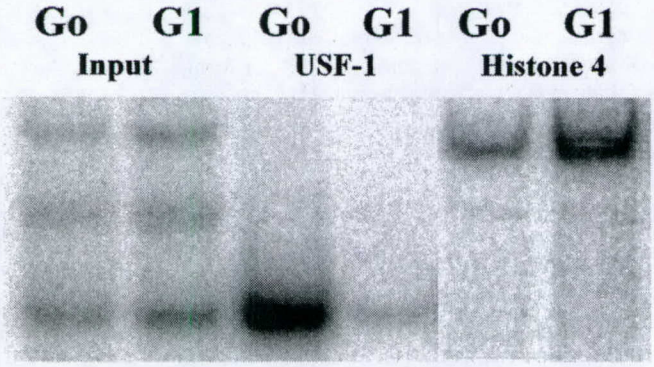

Figure 6. ChIP analysis of chromatin from G_o and growth-activated (G_1) cells using antibodies to USF-1 and acetylated histone 4. Primer sets were designed to amplify PAI-1 promoter segments corresponding to the E box region in the PE2.1 site and a TATA-proximal (histone 4) sequence. USF-1 was a major PE2.1 E box-binding protein in G_o but not G_1 cells (see text). The near-transcriptional start site of the PAI-1 gene bound significantly more acetylated histone 4 in G_1 as compared to G_o cells consistent with chromatin remodeling associated with stimulated expression.

activated protein kinases during growth stimulation [49] and may be followed (in mid-to-late G_1) by binding of the unique PAI-1 repressor E2F1 to the adjoining 3' GC-rich region of the PAI-1 promoter [36,37].

The continuing analysis of cell culture systems particularly amenable to molecular dissection of growth state-dependent PAI-1 expression controls [15,23-26,49] will provide fresh insights into the transcriptional constraints on this important gene. Most notably, these approaches can potentially define critical *in vivo* regulatory factors that may ultimately prove to be novel "therapeutic targets" for the treatment of various diseases associated with PAI-1 dysregulation including cancer, vascular disorders and progressive fibrosis. Molecular targeting of PAI-1 transcripts has already shown promise as one means to regulate cellular invasive/motile traits [49]. Future studies are likely to extend the available targets to include elements of the PAI-1 transcriptional control network.

Acknowledgments

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APPENDIX 4

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Upstream Stimulatory Factor Regulates E Box-Dependent PAI-1 Transcription in Human Epidermal Keratinocytes

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Certain growth factors (e.g., TGF-β1) initiate a "plastic" response in human keratinocytes (HaCaT cells) characterized by changes in gene expression and increased cell motility. While microarray analyses identified a number of involved genes, plasminogen activator inhibitor type 1 (*PAI-1*) is among the subset most highly responsive to TGF-β1. Previous antisense attenuation of PAI-1 synthesis confirmed an essential role for this protease inhibitor in cell motility (Providence et al., 2002, J Cell Sci 115:3767–3777; Providence and Higgins, 2004, J Cell Physiol 200:297–308). It was important, therefore, to clarify molecular mechanisms underlying PAI-1 expression control in human keratinocytes. A consensus E box motif (5′-CACGTG-3′) at nucleotides −566 to −561 in the PE2 region of the *PAI-1* gene was required for TGF-β1-induced transcription of a PAI-1 promoter-driven luceriferase reporter. Truncation of the PE2 E box or mutation of the CACGTG hexanucleotide to CAATTG inhibited growth factor-stimulated promoter function confirming the importance of this site in inducible expression. A similar mutation at the PE1 E box (nucleotides −682 to −677), in contrast, did not result in reduced luciferase activity. Competing CACGTG-containing DNAs, regardless of the presence or absence of PAI-1-specific flanking sequences or lacking accessory sequences (i.e., Smad-binding sites, AAT trinucleotide spacer), inhibited complex formation between HaCaT cell nuclear factors and a 45-mer PE2 region probe. A deoxyoligonucleotide that differed from the consensus E box by a CG → AT substitution (the same base change incorporated into the PAI-1p806-lucerifase reporter by site-directed mutagenesis) but with random (i.e., non-PAI-1) flanking sequences also failed to compete with the PE2 region probe for protein binding whereas the same construct with an intact CACGTG motif was an effective competitor. The major protein/DNA interactions in the PE2 segment, therefore, are E box-dependent. USF-1, a member of the upstream stimulatory factor family, bound the PE2 construct suggesting a role for USF proteins in E box residence and *PAI-1* gene expression. Chromatin immunoprecipitation, using primers designed to amplify a 300-bp PE2-associated promoter fragment and containing no other E box motifs except the target CACGTG at nucleotides −566 to −561, confirmed that this site was occupied by USF-1 or a USF-1-containing complex in both quiescent and TGF-β1-stimulated cells. Transfection of a dominant-negative USF construct effectively attenuated serum- and TGF-β1-induced PAI-1 synthesis as well as TGF-β1-stimulated Matrigel barrier invasion. Dominant-negative USF-expressing keratinocytes, moreover, had a significantly reduced capacity for Matrigel barrier invasion. USF elements, therefore, are important regulators of growth factor-initiated PAI-1 transcription (as predicted from the identification of *PAI-1* as a direct USF target gene) and the associated epithelial migratory response. J. Cell. Physiol. 9999: 1–10, 2004. © 2004 Wiley-Liss, Inc.

Cells utilize several multicomponent proteolytic systems to migrate through stromal barriers with invasive ability frequently dependent on conversion of plasminogen to the broad-spectrum protease plasmin by urokinase plasminogen activator (uPA) (e.g., Lund et al., 1999). The catalytic properties of uPA are regulated, in turn, by the fast-acting type-1 serine protease inhibitor (SERPIN) plasminogen activator inhibitor type 1 (PAI-1) (Andreasen et al., 2000). This cascade is a major determinant of the overall pericellular proteolytic balance, effectively modulating the highly inter-related processes of extracellular matrix (ECM) remodeling and cell migration (Okedon et al., 1992; Jeffers et al., 1996; Mazzieri et al., 1997; Bajou et al., 1998; Farina et al., 1998; Lund et al., 1999; Providence et al., 2000; Reijerkerk et al., 2000; Zhou et al., 2000; Brooks et al., 2001; Legrand et al., 2001).

PAI-1 is most prominent among the subset of TGF-β1-induced genes that accompany development of epithelial "plasticity" (Boehm et al., 1999; Akiyoshi et al., 2001; Zavadil et al., 2001), a phenotypic transition associated with enhanced expression of matrix-degrading proteases, cytoarchitectural restructuring, and increased cell motility (Gilles et al., 1997; Santibanez et al., 1999; Arias, 2001; Kutz et al., 2001; Thiery, 2002; Giusti et al., 2003). This protease inhibitor, moreover, is a major promigratory factor in invasive breast carcinoma cells (e.g., Chazaud et al., 2002; Palmieri et al., 2002) and elevated tumor levels of PAI-1 correlate with a poor patient prognosis and reduced disease-free survival time (Dublin et al., 2000; Chappuis et al., 2001; Chazaud et al., 2002). PAI-1 appears to be a particularly critical element in the program of tissue invasion acting to limit plasmin generation, thus preserving an ECM "scaffold" permissive for stromal migration (Bajou et al., 1998, 2001). Although TGF-β1-stimulated PAI-1 synthesis may regulate cell motility via fine control of uPA-dependent matrix barrier proteolysis (Mignatti and Rifkin, 2000; Providence et al., 2003 for reviews), this SERPIN also functions in a more general context to modulate integrin–ECM or uPA/uPA receptor (uPAR)–ECM

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interactions affecting, thereby, cell-to-substrate adhesion (Deng et al., 1996, 2001; Kjoller et al., 1997; Loskutoff et al., 1999; Andreasen et al., 2000; Gutierrez et al., 2000; Koziczak et al., 2000; Mignatti and Rifkin, 2000; McMahon et al., 2001; Stefansson et al., 2001; Palmieri et al., 2002; Providence et al., 2002; Al-Fakhri et al., 2003; Czekay et al., 2003; Stefansson and Lawrence, 2003). Even minor changes in PAI-1 expression, therefore, would be expected to influence motile behavior by either or both mechanisms (e.g., Liu et al., 1995; Chapman, 1997; Loskutoff et al., 1999; Mignatti and Rifkin, 2000; Czekay et al., 2003; Providence and Higgins, 2004). Indeed, recent findings confirmed that antisense down-regulation of PAI-1 synthesis, or use of PAI-1 function-blocking antibodies, effectively inhibits basal as well as growth factor-stimulated cell migration (Brooks et al., 2001; Chazaud et al., 2002; Providence et al., 2002, 2003).

TGF-β1 controls on specific "invasion-modulating" target genes (i.e., *PAI-1*) is largely transcriptional (e.g., Riccio et al., 1992; Petzelbauer et al., 1996; Kutz et al., 2001). Prominent TGF-β1-activated sites include the hexanucleotide E box motif (5′-CACGTG-3′) in the PE2 region of the PAI-1 promoter and related sequences that are recognized by a subgroup of basic helix-loop-helix/leucine zipper (bHLH-LZ) transcription factors (e.g., MYC, MAX, TFE3, USF-1, USF-2, and HIF-1α) (Riccio et al., 1992; Dennler et al., 1998; Hua et al., 1998, 1999; White et al., 2000; Fink et al., 2002; Grinberg and Kerppola, 2003). The most TATAA-proximal consensus E box, situated at nucleotides −160 to −165 upstream of the transcription start site in the rat *PAI-1* gene, for example, binds USF-1 and is required for PAI-1 promoter-driven reporter expression in growing, TGF-β1-stimulated and hypoxia-stressed cells (Kietzmann et al., 1999; White et al., 2000). This motif likely functions, therefore, as a "platform" for recruitment of positive and negative regulators of PAI-1 transcription (e.g., Samoylenko et al., 2001) which may vary depending on the stimulus and/or growth state (White et al., 2000; Qi and Higgins, 2003).

Recent chromatin immunoprecipitation analysis identified USF family members as in vivo DNA-binding proteins at the PE2 site of the *PAI-1* gene in proliferating human keratinocytes (Qi and Higgins, 2003). This study details the critical involvement of the PE2 region E box in TGF-β1-stimulated PAI-1 transcription as well as the sequence requirements for USF-1/PAI-1 E box interactions. Data are also presented indicating that expression of a dominant-negative USF construct (USF-1A) decreased both PAI-1 levels and the invasive ability of immortalized keratinocytes, implicating the importance of USF target genes in migration through stromal barriers.

MATERIALS AND METHODS

Cell culture

HaCaT and RK epidermal keratinocytes were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Boehm et al., 1999; Providence et al., 2002). Confluent cultures were washed with HBSS and maintained in serum-free medium for 3 days to initiate a state of growth arrest before stimulation with serum (20%) or recombinant TGF-β1 (1 ng/ml; R&D Systems, Minneapolis, MN). Pre-exposure to the p38 inhibitor III (380 nM; Calbiochem, San Diego, CA) or the MEK inhibitors PD98059 (50 μM; BioSource, Camarillo, CA) and U0126 (10 μM; Promega, Madison, WI) was for 30 min prior to growth factor addition. Inhibitor III (a potent, selective, ATP site-directed p38 MAPK inhibitor) was selected to target p38 since the more commonly used compound SB203580 also attenuates TGF-β receptor signaling.

Northern blots

Cellular RNA was isolated with Purescript (Gentra, Minneapolis, MN), denatured at 55°C for 15 min in 1× MOPS, 50% formamide, and 6.5% formaldehyde prior to separation in agarose/formaldehyde gels (1.2% agarose, 1.1% formaldehyde, 1 × MOPS), transferred to Nytran membranes in 10 × SSC (3M NaCl, 0.3 M Na citrate, pH 7.0) and UV-crosslinked. Membranes were incubated for 2 h at 42°C in 50% formamide, 5 × Denhardt's solution, 5 × SSC, 1% SDS, 100 μg/ml sheared/heat denatured salmon sperm DNA (ssDNA) then hybridized overnight with ^{32}P-labeled cDNA probes for human PAI-1 and GAPD at 42°C in 50% formamide, 2.5 × Denhardt's solution, 1% SDS, 5 × SSC, 100 μg/ml ssDNA, and 10% dextran sulfate. Blots were washed three-times (for 15 min each) at 42°C followed by three additional washes at 55°C in 0.1 × SSC/0.1% SDS before exposure to film.

Chromatin immunoprecipitation (ChIP)

Quiescent and TGF-β1-treated HaCaT cells were fixed with 1% formaldehyde, chromatin isolated, sonicated (fragment range = 300–1,000 bp), precleared with protein A agarose beads coated with ssDNA, and incubated overnight at 4°C with 2 μg IgG (see text[Q2]). Immune complexes were collected with protein A agarose/ssDNA beads, washed, eluted, and cross-links reversed at 65°C for 5 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. A 300 bp region encompassing the PE2 E box of the human PAI-1 promoter was amplified by PCR.

Nuclear extracts

Cells were rinsed in ice-cold PBS, scrape-harvested, washed twice in Tris-buffered saline (0.14 M NaCl, 0.5 mM KCl, 1.4 mM Na_2HPO_4, 25 mM Tris, 1.4 mM $CaCl_2$, 0.5 mM $MgCl_2$), resuspended in 800 μl 10 mM HEPES buffer, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail (PIC; leupeptin, aprotinin, pepstatin A [each at 10 μg/ml], 1 mM PMSF), incubated on ice for 15 min, and 50 μl 10% NP-40 added prior to a 10 sec votex lysis. Nuclei were collected by centrifugation, resuspended in 100 μl cold extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, PIC), rocked on ice for 15 min, and the soluble fraction recovered by centrifugation at 14,000 rpm for 5 min. Where indicated, incubation of nuclear extracts with potato acid phosphatase (PAP, 1 U/5 μg nuclear protein; Sigma Chemical Co., St Louis, MO) was for 15 min at room temperature.

Electrophoretic mobility shift assay (EMSA)

Double-stranded deoxyoligonucleotides (5 pM) (Table 1) were end-labeled by incubation for 10 min at room temperature in 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM $MgCl_2$, 5 mM DTT, γ-^{32}P-dATP (3,000 Ci/mmol) and T4 polynucleotide kinase then purified by elution from 5 kDa cellulose spin columns (Millipore, Bedford, MA). Target probes (25–50,000 cpm) were incubated for 20 min at room temperature in a final reaction volume of 20 μl with 2–10 μg nuclear extract protein/ml in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DDT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1 μg/μl poly dI:dC) in the absence or presence of the indicated antibody (2 μg) or a 100-fold molar excess of unlabeled competing DNA (White et al., 2000). Gel loading buffer (25 mM Tris-HCl, pH 7.5, 0.02% bromophenol blue, 0.02% xylene cyanol, 4% glycerol) was added and reaction mixtures separated on 4% acrylamide Tris/Borate/EDTA gels. Gels were pre-run at 100 V for 30 min prior to sample separation at 125 V at room temperature for 3 h.

Oligonucleotide pulldown assay

Nuclear extract protein (50–100 μg) was incubated with 100 pM biotinylated DNA (Table 1) for 18 h at 4°C in binding buffer (as above) but containing 0.5 μg/μl poly dI:dC and PIC. Complexes were harvested with magnetic streptavidin-coated beads (Pierce, Madison, WI) for 30 min on ice prior to three

TABLE 1. Double-stranded deoxyoligonucleotides used for EMSA

Deoxyoligonucleotide description	Sequence
Human PAI-1 PE2 construct	5′-CAAGTCCTAGACAGACAAAACCTAGACAATCACGTGGCTGGCTGC-3′
Human PAI-1 PE2 CG → AT mutant	5′-CAAGTCCTAGACAGACAAAACCTAGACAATCAATTGGCTGGCTGC-3′
Human PAI-1 PE2 CA → TC mutant	5′-CAAGTCCTAGACAGACAAAACCTAGACAATTCCGTGGCTGGCTGC-3′
Human PAI-1 PE1 construct	5′-GTCTGGACACGTGGGGGGAGTCAGCCGTGTATCATCGGAG-3′
PE2 Smad mutant (AGAC → CTTG)(SBE mutation)	5′-CAAGTCCTCTTGCTTGAAAACCTCTTGAATCACGTGGCTGGCTGC-3′
PE2 distal SBE construct (SIS2)	5′-CAAGTCCTAGACAGACAAAAC-3′
PE2 AAT deletion (−AAT)	5′-CAAGTCCTAGACAGACAAAACCTAGACCACGTGGCTGGCTGC-3′
PE2 18-mer fragment with the AAT spacer and E box (PE2S)	5′-AATCACGTGGCTGGCTGC-3′
PE2 proximal SBE, AAT spacer and E box (PE2SBE)	5′-CTAGACAATCACGTGGCT-3′
Rat PAI-1 promoter proximal E box construct	5′-TACACACACGTGTCCCAG-3′
Consensus E box (no PAI-1-flanking sequences)	5′-CACCCGGTCACGTGGCCTACACC-3′
Mutant E box (no PAI-1-flanking sequences)	5′-CACCCGGTCAATTGGCCTACACC-3′

washes with 2 M NaCl, boiled for 10 min in 2 × Laemmli sample buffer, separated on 9% SDS/PAGE gels and proteins transferred to nitrocellulose membranes for immunoblotting with USF-1 antibodies.

Luciferase reporter assay

Regions of the human PAI-1 promoter (nucleotides −806 to +72, −606 to +72, −506 to +72) were PCR-amplified using primers designed to add 5′ SacI and 3′ XhoI restrictions sites. PCR products were gel-purified, ligated into TOPO TA (Invitrogen, Carlsbad, CA), and cloned into the SacI/XhoI site of the pGL3 luciferase vector (Promega) to create the reporter PAI-1p806-Luc. The dinucleotide substitution (CACGTG → CAATTG) in the PE1 and PE2 region E boxes was created using the Quick-Change site-directed mutagenesis kit (Stratagene, San Diego, CA). HaCaT cells were transfected with the full-length lucerifase reporter or derivatives thereof (see text[Q3]) using Lipofectamine, serum-starved for 1 day, then stimulated with TGF-β1 for 8 h prior to extraction. Luciferase activity was normalized by co-transfection with an SV40 driven β-galactosidase-expressing plasmid and development of the respective signals using a luciferase/β-galactosidase assay kit (Promega).

USF-1 expression construct transfection and PAI-1 Western blotting

RK cells were transfected with a dominant-negative USF-1 construct (USF-1A) or wild-type (WT) USF-1 expression plasmid using Lipofectamine Plus (Invitrogen). Serum-starved (3 days) cells were stimulated with serum (20%) or TGF-β1 (1 ng/ml), extracted in 50 mM HEPES buffer (pH 7.0, containing 0.5% deoxycholate and 0.1% SDS), lysates clarified by centrifugation at 14,000 × g, 10–20 μg extract protein diluted in 2 × Laemmli sample buffer, separated on SDS/9% acrylamide gels and transferred to nitrocellulose. Membranes were blocked in 3% milk (in 0.05% Triton-X 100/PBS) prior to sequential incubations with rabbit antibodies to rat PAI-1 (#1062; American Diagnostica, Stamford, CT) and horseradish peroxidase-conjugated goat anti-rabbit IgG and reactive bands visualized using ECL/autoradiography. All blots were subsequently stripped and reprobed with antibodies to ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA) to assess protein loading levels.

Matrigel invasion assay

For assessment of invasive traits, quiescent (5×10^4) cells were added to Matrigel-coated chambers (8 μm pore size; BD Clontech, Palo Alto, CA). TGF-β1 (1 ng/ml) or solvent was added to the upper chamber while the lower chamber contained DMEM/10% FBS. After 24 h, membranes were fixed and cells stained with Hoechst dye. The number of keratinocytes that migrated to the bottom of the filter was determined by microscopy. To determine the fraction of HA-tagged construct-expressing keratinocytes, cells were fixed, permeabilized, and stained sequentially with anti-HA antibodies (Santa Cruz BioTechnology) and Alexa-Fluor 568-labeled secondary antibodies.

RESULTS

TGF-β1 activation of PAI-1 transcription requires an intact E Box within the PE2 region of the *PAI-1* gene

Several TGF-β1 response elements, including three SMAD-binding sites (AGAC) (SBEs) and two E box motifs (CACGTG), map within the PE1 and PE2 regions of the human PAI-1 promoter (Fig. 1A, Table 1). While nucleotides −598 to −532 (upstream of the transcription start site) specifically appear to be involved in the response to TGF-β1, only the CACGTG motif (and a 5′ 5 nucleotide flank as well as a 3′ segment consisting of approximately 20 nucleotides) yielded a DNase I-protected footprint (Riccio et al., 1992). To topographically position TGF-β1 regulatory elements within the 5′-PE1-PE2-3′ domains of the *PAI-1* gene in the HaCaT cell system, luciferase reporter assays were designed to assess the consequences of specific truncations and nucleotide substitutions on PAI-1 promoter activity. Compared to the full-length PAI-1p806-Luc construct, PAI-1p606-Luc (in which 0.2 kb of 5′ sequence was removed including the PE1 region E box) retained approximately 50% of the TGF-β1-inducible signal indicating that major (albeit not the only) TGF-β1-response sites reside within the proximal 606 bp of the PAI-1 promoter (Fig. 1B). Further elimination of nucleotides −606 to −506, removing the three SBEs and the PE2 region E box, reduced reporter expression by >7-fold. The dinucleotide substitution CACGTG to CAATTG in the PE2 region E box (−806 E2 mut), moreover, resulted in a significant attenuation in full-length PAI-1p806-driven transcriptional activity (Fig. 1B), highlighting the functional significance of this site in the reporter response to TGF-β1. The identical mutation at the PE1 E box (−806 E1 MUT) was without effect. Decreased expression as a consequence of the −806 to −606 truncation, therefore, likely eliminated sequences essential for full reporter activation other than just the PE1 region hexanucleotide E box.

Since an intact consensus PE2 region E box motif is required for the maximal transcriptional response of the PAI-1p806-Luc reporter to TGF-β1 (Fig. 1B), it was important to identify specific E box-binding nuclear proteins in human keratinocytes that may mediate gene control. A double-stranded 45-mer PE2 region deoxyoligonucleotide was designed (Fig. 2A; Table 1) (with eight and nine base pair extensions 5′ of the last SBE and 3′ of the E box, respectively), ^{32}P end-labeled and used in mobility shift assays. Factors capable of binding this DNA target were evident in nuclear extracts from quiescent as well as constitutively-growing and TGF-

COLOR

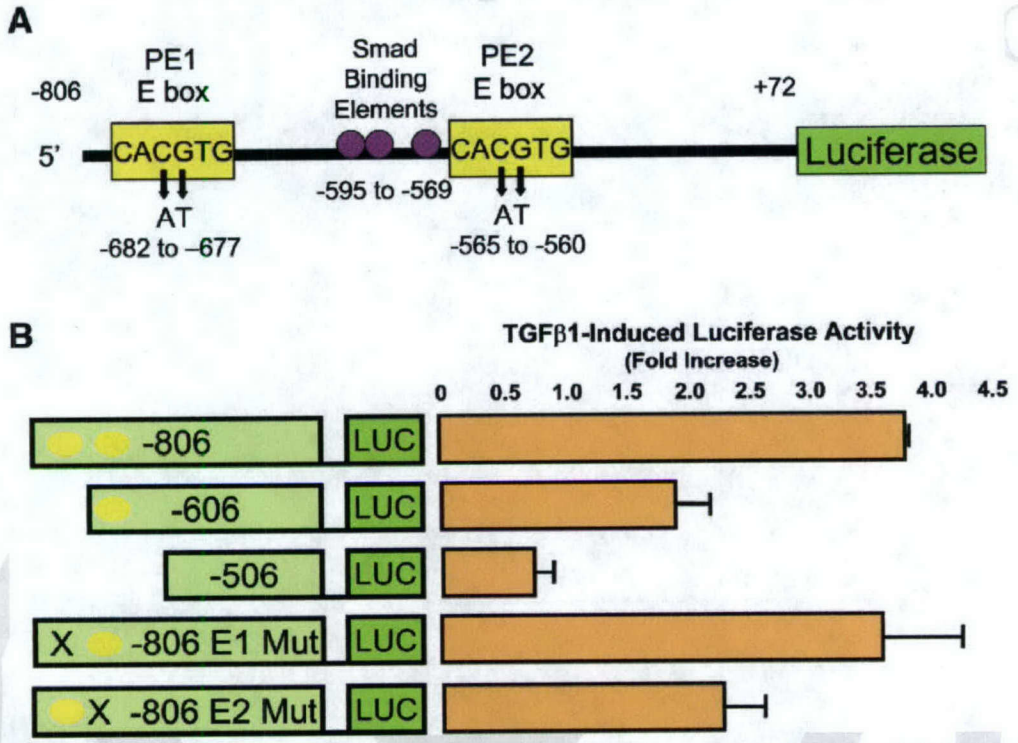

Fig. 1. TGF-β1-induced PAI-1 transcription is E box-dependent. Topography of the PAI-1p806-Luc reporter construct illustrating the relative positions of the two (PE1 and PE2 region) E box sequences, the three Smad binding elements and the two central E box nucleotides (CG) selected for mutation (**A**). Luciferase assays in HaCaT transfectants used the indicated promoter constructs and a relatively short (i.e., 8 h) exposure to TGF-β1 (**B**); ovals depict the wild-type (WT) E box motifs while X indicates the mutated (CG → AT) PE1 and PE2 region E boxes. Luciferase signal was normalized by co-transfection with a SV40-driven β-galactosidase expression vector and expressed as fold-increase relative to non-TGF-β1-stimulated cells. Data plotted is the mean ± standard error of four independent experiments.

β1-stimulated cells (Fig. 2B). This finding provided an opportunity to utilize the 45-mer PE2 construct to assess the relative contributions of the SBEs, E box flanking sequences, and the E box motif to nuclear protein binding. All unlabeled CACGTG hexanucleotide-containing DNAs, regardless of the presence or absence of PAI-1-specific 5′ and 3′ flanking sequences or SBEs (Table 1), significantly decreased probe complex formation (Fig. 2C). Most importantly, a deoxyoligonucleotide that differed from the consensus E box by a CG → AT substitution (identical to the base change incorporated into the PAI-1p806-Luc by site-directed mutagenesis) and containing no PAI-1 flanking sequences failed to compete with the PE2 region probe for factor binding, whereas the same construct with an intact CACGTG motif was an effective competitor (Fig. 2C; Table 1). The major protein/DNA interactions in the PE2 segment, therefore, appear to be E box-dependent. Double-stranded E box containing deoxyoligonucleotides with all three SBEs mutated (AGAC → CTTG) or lacking the AAT trinucleotide spacer sequence (Fig. 3A; Table 1) also successfully competed for protein binding with the labeled 45-mer PE2 DNA target (Fig. 3B), and formed nuclear protein/DNA complexes when presented as labeled probes (not shown[Q4]), further minimizing the potential contribution of the PE2 region AAT spacer or SBEs to site occupancy. These data are consistent with the absence of DNAse I protection at the PE2 segment SBEs (Riccio et al., 1992). Fragments containing the E box sequence and either three 5′ or three 3′ flanking base pairs, however, competed less effectively (Fig. 3B) or failed to bind protein to the same extent as the labeled PE2 target (Fig. 3C), suggesting the necessity for more than three flanking nucleotides for efficient protein/DNA interactions.

The PE2 region E box binds bHLH-LZ proteins of the USF family

A proximal promoter E box in the rat *PAI-1* gene is an important platform for protein binding in response to proliferative stimuli and mild hypoxia as well as to individual growth factors including TGF-β1 (Kietzmann et al., 1999; White et al., 2000). Previous nuclear extract immunodepletion analysis and tethered deoxyoligonucleotide affinity chromatography identified USF-1 as one PAI-1 E box binding element (White et al., 2000; Providence et al., 2002). Inhibition of complex formation by USF-1 antibodies (Fig. 3B) and supershift approaches (Fig. 4A) confirmed that USF-1 similarly recognized the 45-mer PE2 construct. In accord with the sequence constraints for probe binding defined by EMSA (Figs. 2C, 3B), deoxyoligonucleotide pull-down assays additionally substantiated that an intact consensus CACGTG motif was required for optimal site recognition by USF-1 (Fig. 4B). It appears that USF proteins are primarily responsible for binding the PE2 PAI-1 promoter region construct as addition of antibodies capable of recognizing the bHLH-LZ factor TFE3 were unable to either supershift, or disrupt formation of, protein/DNA complexes (Fig. 4A). Phosphorylation of USF-1, moreover, is necessary for DNA binding (Cheung et al., 1999; Galibert et al., 2001) and, consistent with this requirement, the predominant form of USF-1 that eluted from PAI-1 deoxyoligonucleotide affinity columns co-migrat-

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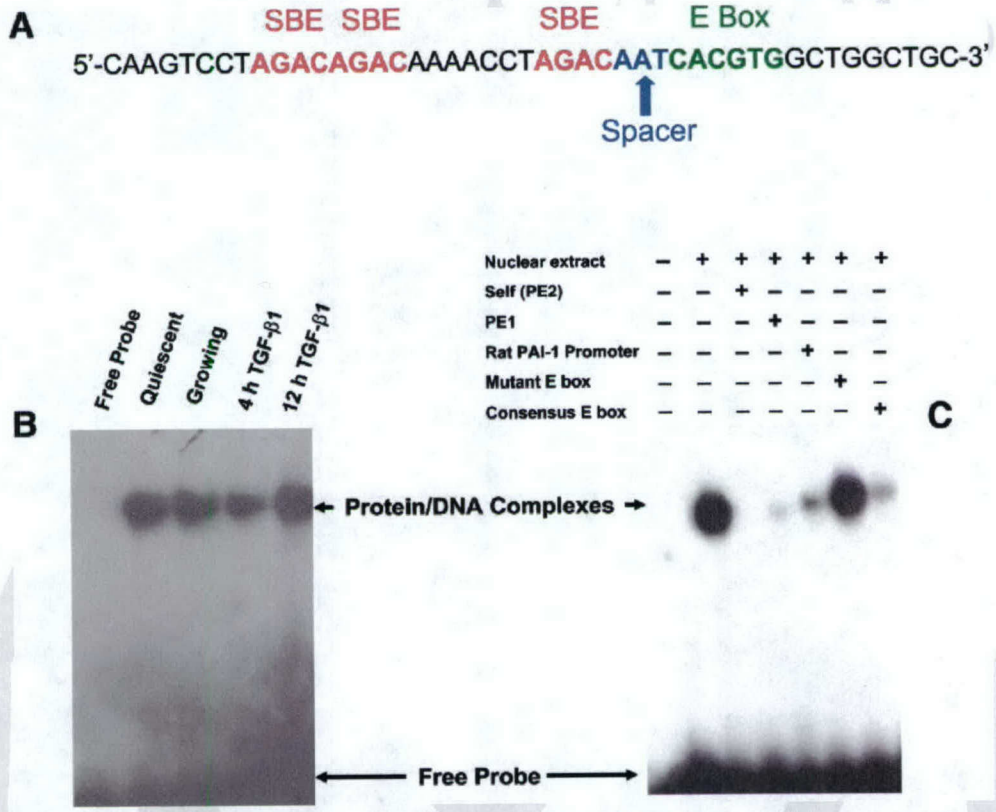

Fig. 2. PE2 probe-binding activity by HaCaT cell nuclear factors is growth state-independent and E box-specific. Nuclear extracts from quiescent, constitutively-growing and TGF-β1-stimulated HaCaT cells were incubated with the double-stranded 45-mer ^{32}P-labeled PE2 region deoxyoligonucleotide (**A**; Table 1) (only one strand is shown) and the reaction products separated on non-denaturing 4% acrylamide gels (**B**). For competition assays, extracts were incubated with a 100-fold molar excess of the indicated unlabeled double-stranded DNA constructs (as described in Table 1) prior to addition of ^{32}P-labeled PE2 target probe and electrophoretic separation of complexes (**C**). The apparent differences in band migration upon addition of cold competing sequences (C) is an electrophoretic artifact and not an actual change in complex mobility.

ed with the 45 kDa (phospho-USF-1) species (Providence et al., 2002). PE2 binding ability was TGF-β1-independent but did, indeed, require phosphorylation as PAP treatment of nuclear extracts from quiescent or TGF-β1-stimulated cells prior to addition of the 45-mer target sequence eliminated the characteristic band shift resolved by EMSA (Fig. 4A). Control experiments confirmed that USF-1 protein levels were not affected by PAP treatment (Fig. 4C). While the activity of USF proteins and other E box-binding transcription factors is modulated by phosphorylation, the involved enzymes (i.e., the mitogen-activated protein [MAP] kinases) may differ (e.g., Cheung et al., 1999; Galibert et al., 2001). A pharmacologic approach was designed to address this issue specifically with regard to *PAI-1* gene control. The MEK inhibitor PD98059 effectively reduced TGF-β1-stimulated PAI-1 transcripts by >75% (Fig. 5A). The p38 inhibitor effectively attenuated growth factor-induced HSP27 phosphorylation (not shown[Q5]), attesting its functionality in the present experimental system, but failed to affect either PAI-1 mRNA induction or protein/DNA complex formation (Fig. 5B,C).

USF regulates induced PAI-1 protein expression and barrier invasion

Although both EMSA and pull-down approaches can yield important information regarding specific sequence requirements for probe recognition, it was necessary to establish that the PE2 region was, in fact, a platform for USF/DNA interactions in vivo. Chromatin immunoprecipitation, using primers designed to amplify a 300-bp PE2-associated promoter fragment and containing no other E box motifs except the target CACGTG at nucleotides −566 to −561 (Fig. 1), confirmed that this site was occupied by USF-1 or a USF-1-containing complex in both quiescent and TGF-β1-stimulated cells (Fig. 6A). This finding, moreover, provided the rationale to directly evaluate the role of USF proteins in the regulation of PAI-1 transcription. RK cells were transiently transfected, therefore, with a dominant-negative USF-1 construct (USF-1A) or wild-type (WT) USF-1 expression vector. Substitution of the WT USF-1 basic DNA-binding region with an acidic domain confers increased homo- or hetero-dimer stability between USF-1A and endogenous USF-1 or USF-2, respectively (Sirito et al., 1998); such dimers, however, are unable to interact with their target DNA motif (Galibert et al., 2001). Thus, the overall effect of USF-1A introduction is to disrupt the formation of functional USF homo- or hetero-dimers. Cells were cultured under quiescent conditions, stimulated with serum or TGF-β1 and PAI-1 protein levels assessed by Western blotting. A significant decrease in PAI-1 protein was evident in dominant-negative USF-1A transfectants upon stimulation with TGF-β1 or FBS when compared with untransfected or WT USF-1 controls (Fig. 6B). Introduction of the WT USF-1 construct into RK cells did not alter levels of induced PAI-1 expression relative to untransfected cultures, confirm-

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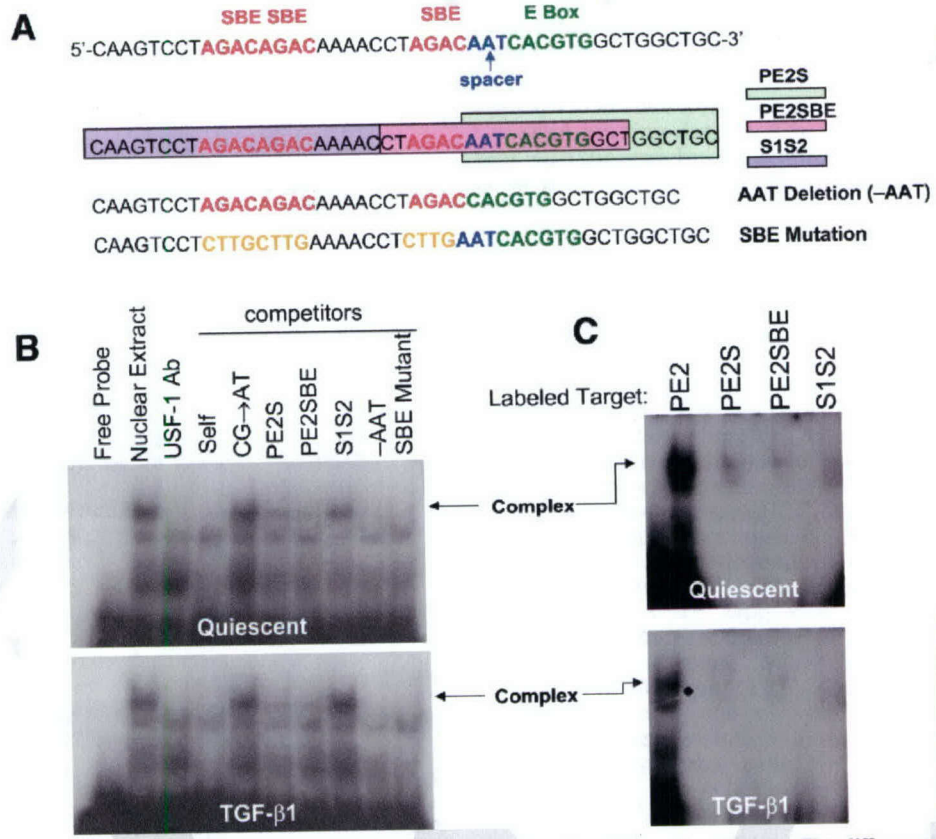

Fig. 3. Sequence requirements for probe binding. Schematic of the PE2 PAI-1 promoter sequence illustrating the position of the SMAD binding elements (SBE), the trinucleotide (AAT) spacer, and the E box motif; truncated and mutated sequences are highlighted (**A**). Nuclear extracts from quiescent and TGF-β1-treated cells were incubated with ^{32}P-labeled PE2 DNA (A) in the presence or absence of a 100-fold molar excess of the indicated unlabeled competing DNA (see also Table 1; **B**) or incubated with ^{32}P-labeled WT 45-mer PE2 DNA or truncated probe targets (**C**) and reaction products separated on non-denaturing 4% acrylamide gels. The difference in the low-affinity complexes resolved with the S1S2 probe, as compared with the PE2S and PE2SBE fragments, may indicate SMAD recognition of the AGAC repeat element in the S1S2 construct (C). An antibody specific for USF-1 (USF-1 Ab) was used (B) in complex-blocking mode (White et al., 2000). This approach avoids resolution of supershifted complexes that might complicate the banding profile and identifies the topmost band as the major USF-1-binding species. Arrow indicates position of specific formed complexes.

ing its suitability as a plasmid control (see "Discussion"). Attenuation of the TGF-β1-associated increase in PAI-1 synthesis, moreover, correlated with a complete inhibition in growth factor-stimulated keratinocyte invasion through basement membrane (Matrigel) barriers (Fig. 6C).

DISCUSSION

An E box motif in the PE2 region of the human *PAI-1* gene is required for the maximal response of the PAI-1p806-Luc reporter to TGF-β1. Site occupancy and transcriptional activity, furthermore, require conservation of the PE2 core E box structure as the CACGTG → CACGGA and TCCGTG dinucleotide substitutions (in the rat gene) (White et al., 2000) and a CACGTG → CAATTG or TCCGTG replacement in the human gene, with retention of PAI-1 flanking sequences, resulted in loss of both competitive binding and TGF-β1-dependent reporter activity. The dinucleotide PE2 domain CACGTG → CAATTG mutation (within the context of the full-length PAI-1p806-Luc plasmid), moreover, significantly attenuates TGF-β1-stimulated transcriptional signal, confirming the importance of this site in inducible expression. The present data are also consistent with the known hexanucleotide preference (CACGTG or CACATG) of USF proteins (Littlewood and Evan, 1995; Ismail et al., 1999; Samoylenko et al., 2001) and strongly suggest that USF family members with PAI-1 PE2 site E box-occupancy potential are constitutively present (and active) regardless of cellular growth state. Successful PAI-1 probe competition by a CACGTG "core" flanked by non-PAI-1 sequences and the failure of specific E box mutants to similarly compete (or to produce band shifts when used as targets) further indicate that an intact hexanucleotide E box motif is necessary and sufficient for USF-1 binding. This appears to contrast with the highly cooperative constraints for E box recognition by other bHLH-LZ proteins (e.g., TFE3) that utilize accessory factors (Grinberg and Kerppola, 2003). Depending on the relative abundance of E box-binding factors in individual cell types, the promoter context and specific flanking nucleotides (e.g., Szentirmay et al., 2003), proteins that dock at adjoining sites may also be required (e.g., Hua et al., 1998). The relative abundance of USF-1 in HaCaT cells (Providence et al., 2002) and low, virtually undetectable, levels of TFE3, moreover, are likely significant determinants in the final composition of specific E box-binding complexes.

ChIP analysis confirmed that USF-1 is a PE2 region E box resident factor in vivo and preliminary EMSA data additionally indicate that USF-2 can potentially recognize the same site (not shown[Q6]). Considering the

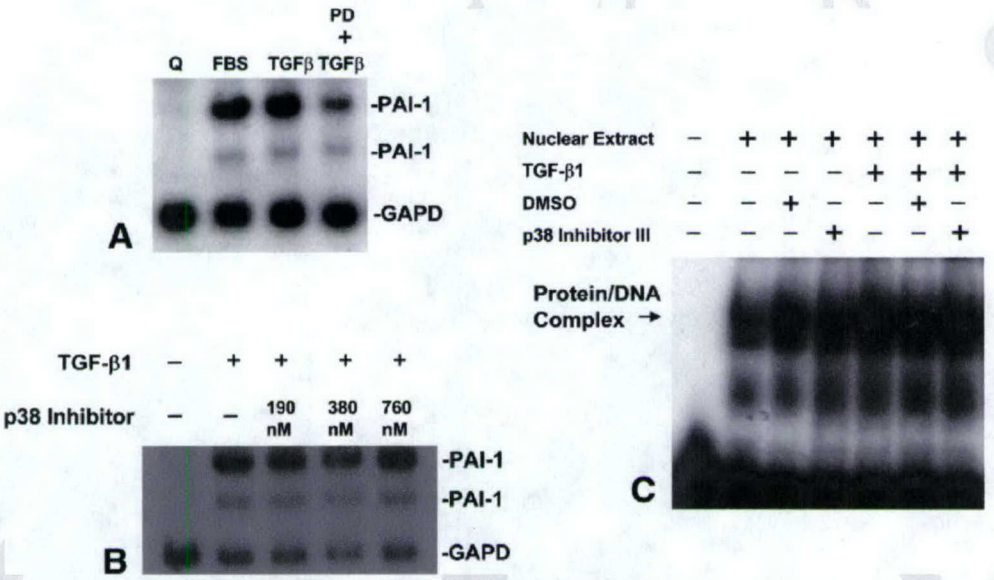

Fig. 5. Signaling requirements for TGF-β1-induced PAI-1 expression. The typical response of the endogenous *PAI-1* gene to a 4-h exposure to serum (FBS) or TGF-β1 is illustrated in the Northern blot in (**A**). The absence of PAI-1 mRNA in serum-deprived HaCaT cells (Q) is consistent with quiescence-associated PAI-1 repression (e.g., Ryan et al., 1996; Qi and Higgins, 2003). Treatment with the MEK inhibitor PD98058 (50 μM) for 30 min prior to addition of TGF-β1 effectively attenuates (by >75%) induced-expression. The p38 inhibitor III, in contrast, did not affect either TGF-β1-induced PAI-1 transcript levels (**B**) or nuclear protein/PE2 region DNA complex formation (**C**). Hybridization signals that represent the two PAI-1 transcripts (3.0- and 2.2-kb) and the normalization control GAPD are indicated in (A, B).

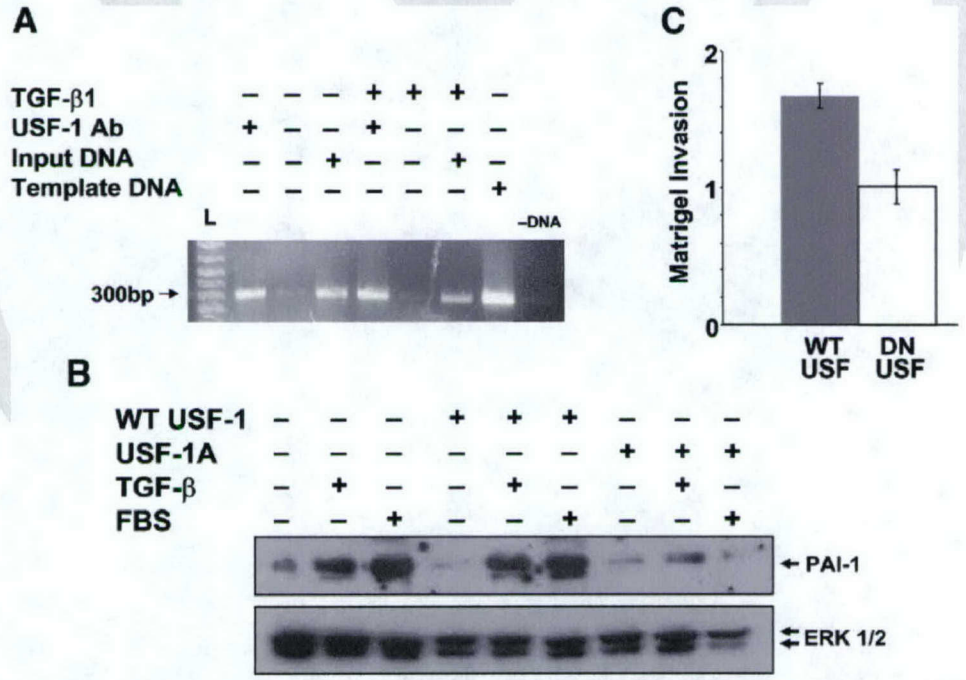

Fig. 6. Dominant-negative USF-1 (USF-1A) attenuates TGF-β1-induced PAI-1 protein expression and barrier invasion. USF-1 was confirmed to be an endogenous PAI-1 promoter PE2 region DNA-binding factor by chromatin immunoprecipitation using antibodies to USF-1 (**A**). Controls included addition of sonicated cellular DNA but without immunoprecipitation (input DNA), addition of PAI-1p806-Luc DNA as a control template for PCR (template DNA) and PCR reaction mixtures with H_2O in place of DNA (−DNA). L = sizing ladder. To evaluate the effects of molecular genetic interference with USF function, RK cells were untransfected or transfected with CMV-driven WT USF-1 or CMV-driven dominant-negative USF-1A. Whole cell lysates from quiescent cultures or cells stimulated with 20% FBS or 1 ng/ml TGF-β1 were collected, separated on 9% SDS–PAGE, proteins transferred, and blots probed with anti-rat PAI-1 antibody (**B**). Transfers were reprobed with antibodies to ERK1/2 to assess protein loading. Invasion of RK cells expressing either WT (USF-1) or dominant-negative (USF-1A) expression constructs was compared ± TGF-β1 (**C**). Data in (C) specifically represent TGF-β1-induced fold-invasion of Matrigel-coated barriers (i.e., TGF-β1-associated invasion/untreated controls); histogram represents mean ± standard error from eight migration evaluations. Approximately 60% of the RK cells used in invasion assays were anti-HA positive (i.e., 60% transfection efficiency; Providence et al., 2002). Only 5% of cells that invaded the Matrigel barrier, in contrast, stained positively with the HA antibody, indicating that the majority of USF-1A-expressing cells were non-invasive.

TGF-β1. USF-1A transfection and subsequent PAI-1 down-regulation, in fact, completely ablated the TGF-β1-associated increase in invasive potential of RK cells compared to WT USF-1-expressing controls. USF elements are clearly important regulators of both *PAI-1* gene control (as predicted from the identification of *PAI-1* as a direct USF target gene) and cellular migratory processes. Although it remains to be determined if USF regulates the expression of "migration modifer" genes other than *PAI-1*, these approaches may ultimately lead to the identification of novel molecular targets and subsequent design of transcription-focused "therapies" to manage specific in vivo consequences of PAI-1 overexpression (tumor progression, tissue fibrosis, vascular disease).

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APPENDIX 5

Transworld Research Network
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1 Plasminogen activator inhibitor type-1 expression targeting: New therapeutic approaches to regulate tumor growth and angiogenesis

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Abstract

Angiogenesis is a complex physiological and, in certain settings, pathophysiologic process involving the formation of new blood vessels from the existent vasculature or by recruited precursor cells. An efficient angiogenic response is highly dependent on focalized stromal remodeling by cooperating, often interdependent, proteolytic cascades and involves generation of the broad-spectrum protease plasmin from plasminogen through the catalytic action of urokinase plasminogen activator (uPA). uPA localizes to the sprouting endothelium while its major, fast-acting, type-1 inhibitor (PAI-1) is expressed either by the uPA-synthesizing endothelial cohort or by stromal cells (i.e., fibroblasts) closely juxtaposed to the developing neovessels. The spatial restriction of critical elements in the plasmin cascade likely facilitates vessel outgrowth by maintaining a matrix

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"scaffold" permissive for cell migration. This review discusses the potential role of PAI-1 in tumor angiogenesis and the complex, concentration-dependent, effects of this protease inhibitor on cell migration and the angiogenic process. Evidence is presented that genetic ablation of PAI-1 expression in intact animals as well as in cultured cells has defined phenotypic outcomes. Collectively, these findings support development of PAI-1 antagonists, mutant PAI-1 molecules or reagents that target the PAI-1 expression network as potential anti-angiogenic chemotherapeutics.

Introduction

Angiogenesis is essential for embryonic development, menstrual cycling and wound healing as well as a major underlying contributor to the pathogenesis of psoriasis, rheumatoid arthritis, diabetic retinopathy and tumor growth. Endothelial cell "activation" by angiogenic factors is closely followed by degradation of the parent vessel basement membrane, stromal invasion and matrix remodeling as part of the outgrowth process. Following loss of basement membrane integrity, endothelial cells extend processes in the direction of the angiogenic stimulus and begin to migrate into the surrounding stroma forming a capillary sprout. A lumen gradually develops and reconstitution of the basement membrane signals sprout maturation [1,2]. Proliferation occurs in a cohort of cells just behind the migrating front and is likely required for continued maintenance of vessel growth. Stimulated endothelial cell locomotion within the angiogenic "field", furthermore, requires cycles of extracellular matrix (ECM) adhesion/detachment and precise control of the pericellular proteolytic environment [2-4]. These events are regulated (temporally and spatially) by members of the matrix metalloproteinase (MMP) family and by uPA-mediated conversion of plasminogen to plasmin [5-11]. The plasmin-generating cascade directly influences the overall pericellular proteolytic balance and is a critical determinant in directed cell movement, matrix remodeling and tissue invasion [12-24]. PAI-1 functions in this process to limit plasmin generation by inhibiting the catalytic activity of uPA [5,6], restricting downstream MMP activation and affecting, thereby, uPA-dependent ECM degradation and *in vivo* cell motility [6,25,26]. Targeted knockout/reconstitution studies, moreover, identified PAI-1 as essential for, and an efficient modulator of, the neovascularization process. Recent findings, in fact, support a multifunctional role for PAI-1 in angiogenesis (e.g., this uPA inhibitor regulates stromal barrier proteolysis, facilitates endothelial cell migration by focalized exposure of cryptic matrix-binding sites, stabilizes nascent vessel structure and controls cell-to-matrix adhesion/de-adhesion). Indeed, analyses of systems that model specific stages in angiogenesis have disclosed that PAI-1 can exert potent pro- and anti-angiogenic effects depending on the context and the concentration (as well as specific activity) of this SERPIN (serine protease inhibitor) in the tissue microenvironment.

PAI-1 in the angiogenic response

Endothelial cell migration and capillary sprouting requires proteolysis [2,27]. Studies in mice deficient in elements in the plasmin activation cascade confirmed the importance of uPA, PAI-1 and plasmin in cell migration [28]. Excessive protease activity as typically evident in chronic wounds, however, prevents the coordinated assembly of endothelial cells into capillary structures highlighting the requirement for an appropriate proteolytic "balance" in tubular network formation [29,30]. Genetic studies *in vivo*,

moreover, have implicated PAI-1 as an important regulator of this balance [31,32]. Indeed, PAI-1 is expressed specifically in angiogenic "cords" and migrating endothelial cells (**Figure 1**) as well as in stromal cells in direct contact with the sprouting neovessels but not in the quiescent endothelium [33-38]. PAI-1$^{-/-}$ mice, moreover, are incapable of mounting an efficient angiogenic response to transplanted tumors or implants of potent angiogenic growth factors (e.g., bFGF) [33,35,37] although this deficiency could be corrected by injection of PAI-1-expressing adenovirus. PAI-1 appears to promote angiogenesis by inhibition of plasmin activity, thus preserving an appropriate matrix scaffold for capillary network formation or providing required neovessel stability [32]. Exogenous PAI-1, under specific circumstances, can also significantly limit vessel construction (at least in the chick chorioallantoic membrane model; CAM) [38]. These findings [i.e., 32,38] in all probability reflect the context and complexity of different angiogenic processes which, in turn, depend on the level of expression of participating elements, the nature of the ECM involved, the system studied and the growth factor environment. PAI-1 deficit (as in the PAI-1$^{-/-}$ mouse) would likely result in loss of ECM integrity (due to excessive protease activity) and compromise endothelial cell-ECM adhesion, each required for a successful angiogenic response (**Figure 2**). Relatively high PAI-1 concentrations in the local environment, on the other hand, may also be detrimental to angiogenesis by inhibiting the required proteolytic activity necessary for focalized dissection of the ECM by activated endothelial cells. Indeed, expression of a long half-life PAI-1 mutant protein inhibited sprout formation by human endothelial

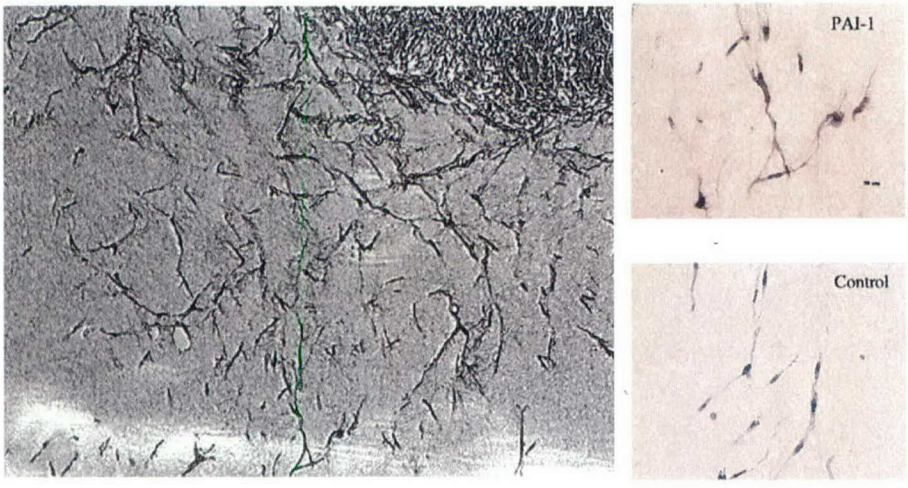

Figure 1. Identification of PAI-1-expressing endothelial cells in outgrowth culture. Human saphenous vein endothelial cells migrate into a fibrin gel within 2 weeks after placement of vein "rings" into a fibrin matrix (left panel). The original ring is at the upper right. Cells that migrate out of the vein segment express PAI-1 (upper right panel). Substitution of pre-immune rabbit serum in place of PAI-1 antibodies resulted in loss of immunoreactivity (Control, lower right panel). Unlike the obvious expression of PAI-1 by endothelial elements locomoting through the fibrin gel, the residual endothelium in the explant was PAI-1-negative.

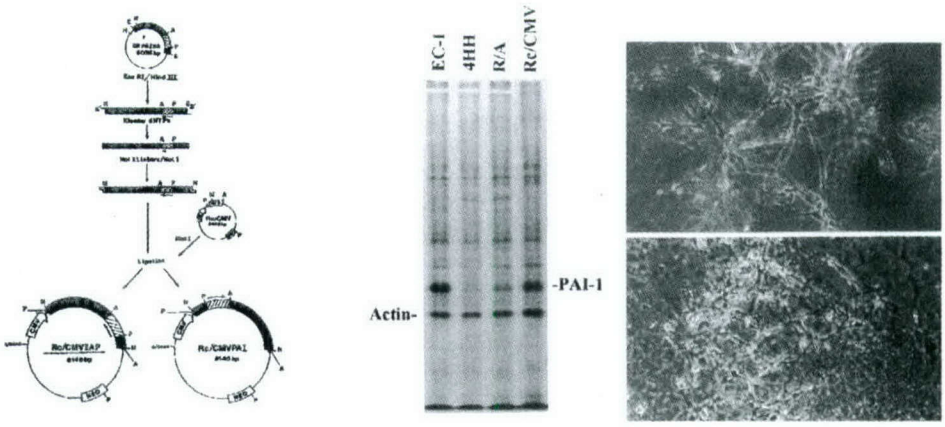

Figure 2. Targeted down-regulation of PAI-1 synthesis inhibits *in vitro* tubulogenesis. Construction of Rc/CMV plasmid vectors that drive expression of a full-length PAI-1 cDNA insert, cloned in sense (PAI) and antisense (IAP) orientations, under control of a CMV promoter (left panel). The black fill-in region corresponds to the PAI-1 cDNA inset. EC-1 parental cells were transfected with the Rc/CMVIAP vector and stable clones derived. To assess the success of Rc/CMVIAP driven down-regulation of PAI-1 synthesis and matrix accumulation, saponin-extracts of ^{35}S-methionine-labeled cells were separated by gel electrophoresis and proteins visualized by fluorography (middle panel). One derivative (4HH) did not express detectable PAI-1 protein nor accumulate PAI-1 in the matrix (middle panel). Cells transfected with Rc/CMV vector without insert expressed levels of PAI-1 similar to that of EC-1 controls. Wild-type T2 cells (top right panel) formed highly-branched and anastomizing capillary networks when suspended in a complex support matrix consisting of a 3:1 mixture of Vitrogen-Matrigel. Many of these tubular processes had clearly evident lumens. Extensive sprout formation was evident at the tips of T2 branches, moreover, indicative of both invasive and differentiated compartments. PAI-1$^{-/-}$ 4HH cells (bottom right panel), in contrast, failed to construct stable tubular structures and extensively degraded the gel matrix.

cells in fibrin gels, blocked angiogenesis in the CAM assay and inhibited prostate tumor growth in SCID mice [39]. Recent findings [40], using the Matrigel implant model of *in vivo* angiogenesis, have confirmed that PAI-1 is essential for efficient neovessel formation and implant invasion but that the PAI-1 "tissue" content critically influences the angiogenic outcome. This SERPIN is a potent stimulator of angiogenesis at low or physiologic levels but significantly impairs or completely inhibits vessel formation, lumen development and implant invasion at high concentrations [40]. This last point is highly relevant therapeutically and supported by recent findings highlighting the role of PAI-1 as an inhibitor of capillary dissolution. Indeed, uPA-mediated plasmin generation activates MMP 1 and 9 causing endothelial cell collagen gel contraction and capillary tube regression (with associated apoptosis); both events are inhibited by PAI-1 [41]. Moreover, attenuation of PAI-1 activity with neutralizing antibodies accelerates tube regression supporting the contention that endogenous PAI-1 is the major negative regulator of this process [42]. These results suggest that continued PAI-1 expression by formed capillary structures is required to maintain their stability and, in fact, to prevent vessel loss.

PAI-1 and cell motility

Cell migration within a complex tissue structure involves several interactive protease systems [e.g., 10,18,20,43-46] and is often dependent on plasmin generation by uPA [47-50]. PAI-1 synthesis and matrix accumulation closely accompany acquisition of the motile phenotype [21,51,52]. Recently, a unique vector that encodes a PAI-1-GFP chimeric protein driven by PAI-1 promoter sequences was developed in this laboratory (**Figure 3**) [22]. This construct provides for the direct visualization of induced PAI-1 deposition into cellular migration trails during the real time of stimulated motility. Secreted PAI-1 most likely accumulates in the cellular undersurface region in a complex with the matrix protein vitronectin [53,54]. *In vitro* studies suggest that PAI-1 occupancy of its docking site on vitronectin may dissociate uPA receptor (uPAR)-vitronectin interactions, resulting in the detachment of cells that utilize uPAR as a vitronectin anchor [55-57]. Alternatively, PAI-1 may directly inhibit αv integrin-mediated attachment to vitronectin by blocking accessibility to the RGD sequence located proximal to the uPAR binding site [57-59] (**Figure 4**). uPAR-associated uPA/PAI-1 complexes, furthermore, are internalized by endocytosis promoting uPA receptor recycling [8] and, thereby,

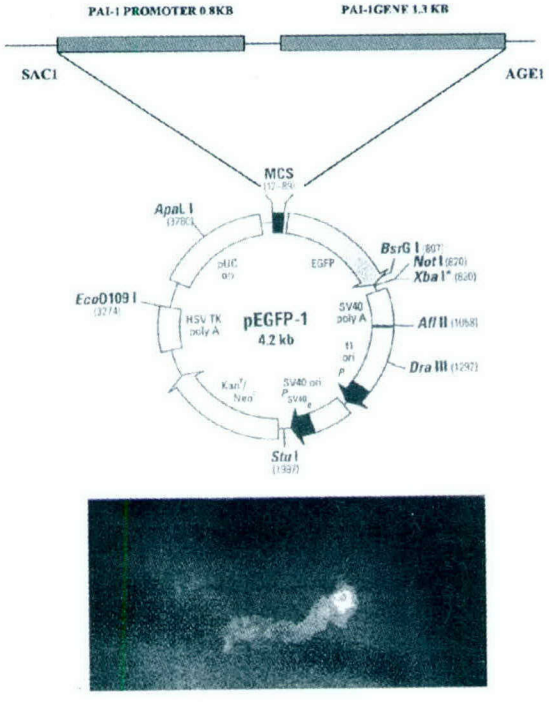

Figure 3. Visualization of PAI-1-GFP chimera deposition into cellular migration tracks. Schematic of a pEGFP-1-based vector in which a fusion transcript consisting of 1.3 kb of PAI-1 coding sequences and GFP is expressed under the control of a 0.8 kb PAI-1 "promoter" (top panel). Seeding of PAI-1 promoter-PAI-1 coding-GFP transfectant RK cells at low density provides for the clear visualization of the PAI-1-GFP fusion protein in cellular migration trails (bottom panel). The small bright image at the extreme right end of the trail is the cell body.

transfectants) was associated with a significant impairment in cellular migratory ability [20,22]. Most importantly, targeted down-regulation of PAI-1 transcripts impairs cell motility *in vitro* [21] and inhibits endothelial capillary formation on basement membrane (Matrigel) coated culture surfaces (**Figure 5**), a rapid process dependent on cell migration.

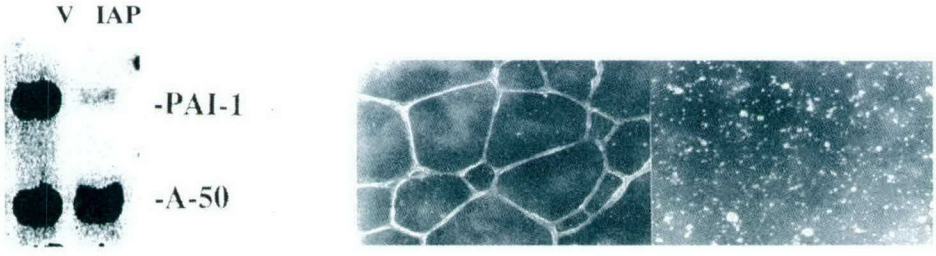

Figure 5. Capillary formation on Matrigel requires migration. T2 cells were stably transfected with the "empty" Rc/CMV vector (V) or vector bearing a full-length PAI-1 cDNA insert in antisense configuration (IAP) (as in Figure 2). Northern analysis indicated that control T2 cells or T2/V cells expressed abundant PAI-1 transcripts (left panel) and formed well-developed highly-branched tubular structures with luminal spaces within 48 hours after plating onto hydrated Matrigel-coated surfaces (middle panel). T2/IAP transfectants, in contrast, had significantly reduced PAI-1 mRNA levels (left panel) and failed migrate and coalesce into a defined capillary network. T2/IAP cells remained either as single cells (without any evidence of an associated migratory track) or formed small multicellular aggregates (right panel).

Several recent studies, using dominant-negative constructs and pharmacologic inhibitors, confirmed that cellular motile responses, *in vitro* angiogenesis and associated PAI-1 induction are dependent on ERK signaling [20,68]. These findings, coupled with the success of the genetic-based approach to modulate PAI-1 expression directly at the mRNA transcript level [20,22] or indirectly, by disruption of the involved transduction pathway [69], support the contention that PAI-1 expression modulation can be utilized to manipulate specific aspects of the angiogenic process. PAI-1-specific antagonists, mutant PAI-1 molecules or reagents to target the PAI-1 signaling or transcriptional networks are currently under development [38]. The potential of this approach was highlighted by the ability of a non-uPA-inhibitory PAI-1 mutant protein to attenuate angiogenesis *in vivo* most likely by interference with cellular attachment to vitronectin in the provisional ECM [38]. Although in many cases the mechanism of action of such PAI-1-focused therapeutics remains to be determined, initial results indicate this strategy may lead to novel, clinically-relevant, options for cancer treatment.

Acknowledgements

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