

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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Accepted 9 July 2002

Journal of Cell Science 115, 3767-3777 © 2002 The Company of Biologists Ltd

doi:10.1242/jcs.00051

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; Mazziari 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- β 1 (TGF- β 1)-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- β 1-initiated epithelial-to-mesenchymal transition in various cell systems (Santibanez et al., 1999; Akiyoshi et al., 2001; Zavadil et al., 2001). Variations 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- β 1-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) (Ricchio 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. Deoxyoligonucleotide 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 ³²P-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 μ l 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 μ l 10% Nonidet NP-40. Nuclei were collected by centrifugation for 30 seconds at 14,000 g, resuspended in 50 μ l 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 μ g/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 μ g 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/ μ l) in 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM MgCl₂, 5 mM DTT and [γ -³²P]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'-TACACACACGTTGCCAG-3';

PAI-1 mutant E-box #1, 5'-TACACACACGGATGCCAG-3';

PAI-1 mutant E-box #2, 5'-TACACATCCGGTTGCCAG-3';

standard consensus E-box, 5'-GGAAGCAGACCACGTTGCTCT-GTGCTTCC-3';

AP-1 consensus sequence, 5'-CGCTTGATGACTCAGCCGGAA-3'.

Nuclear extracts were incubated with 50,000–100,000 cpm ³²P end-labeled target deoxyoligonucleotides in 5 \times gel shift buffer (20% glycerol, 5 mM MgCl₂, 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 μ g per reaction) were added to the formed extract protein/³²P-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 ³²P 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 μ Joules/cm²) followed by DNase-I treatment (2 μ g/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 \times 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 isothiocyanate (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 *SacI/KpnI* 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 *KpnI/EcoRI* sites of pEGFP-N3. The same PAI-1 coding region was also transferred into the *BamHI/AgeI* 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 \times 10⁵ cells/cm² prior to transfection with 1–2 μ g DNA using Lipofectamine-Plus. Transfected cells were trypsinized 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 Ca²⁺/Mg²⁺-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

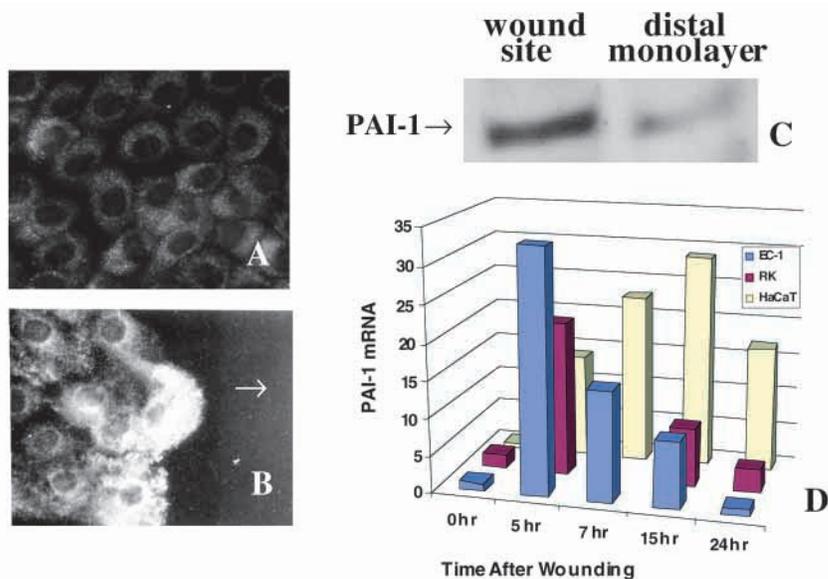
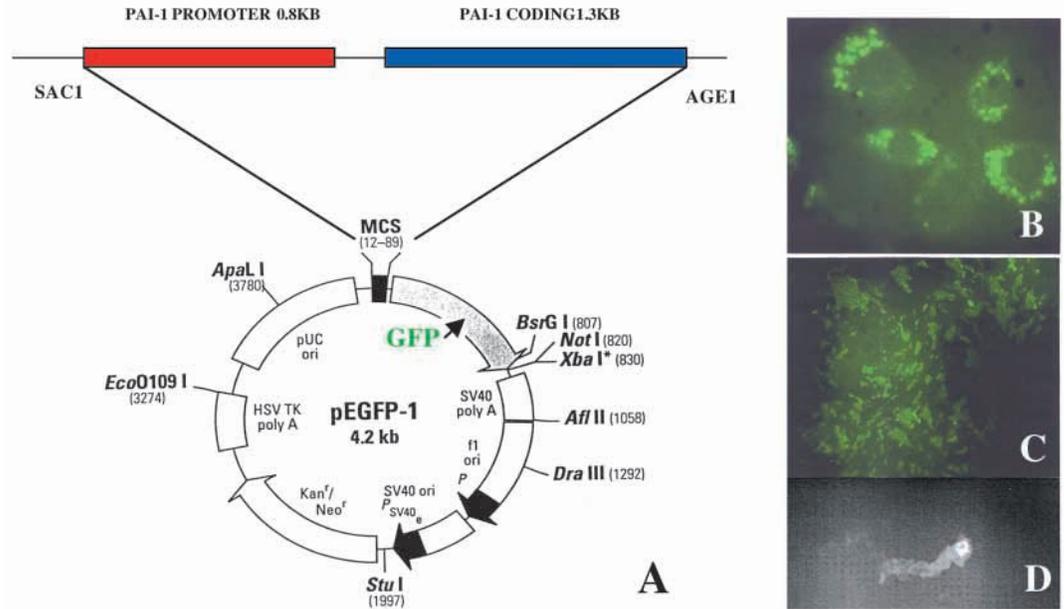


Fig. 1. Monolayer injury stimulates PAI-1 synthesis specifically in wound-edge cells. Quiescent contact-inhibited cultures of RK, HaCaT and EC-1 cells were maintained either intact or scrape-wounded with a pipette tip. RK cultures were fixed and PAI-1 protein visualized by immunocytochemistry (A,B). 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 hybridization 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 'dumbbell-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'-CGCTTGATGACTCAGCCGGAA-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)

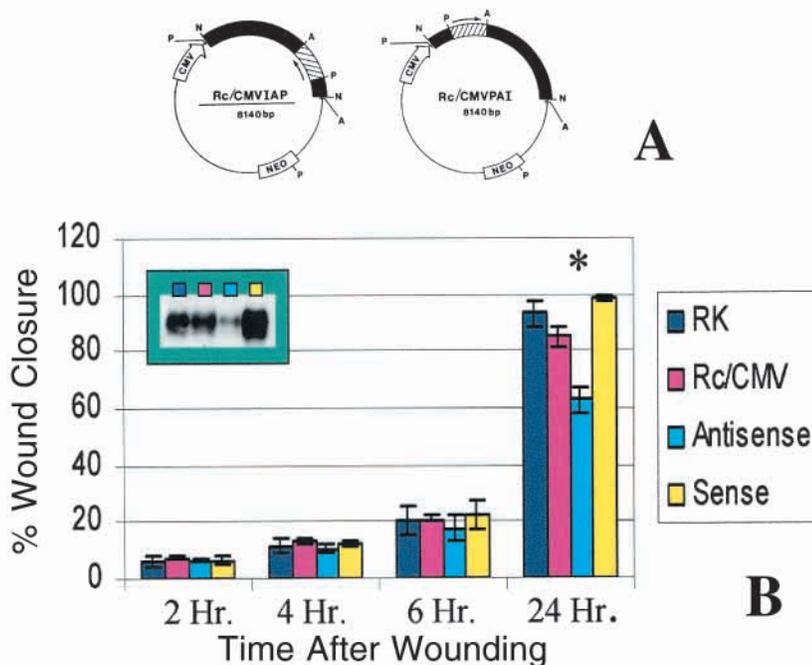


Fig. 3. Targeted PAI-1 downregulation inhibits wound-stimulated cell motility. RK cells were transfected with a non-insert-bearing vector (Rc/CMV) or with constructs in which full-length PAI-1 coding sequences were cloned in antisense (Rc/CMVIAP) or sense (Rc/CMVPAI) orientation (A). Cultures were grown 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 \pm 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.

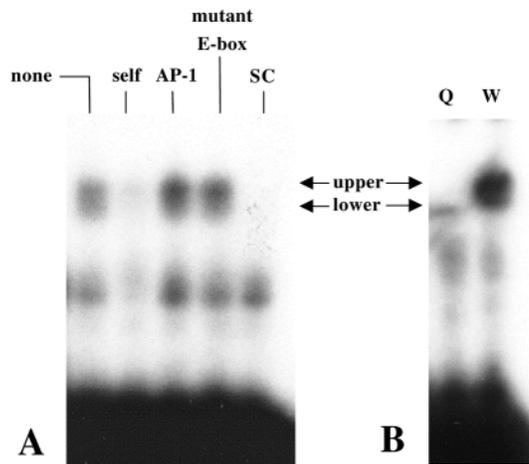


Fig. 4. Binding of a PAI-1 E-box probe by nuclear proteins from growing and wound-stimulated RK cells. The 18 bp ^{32}P -end labeled PAI-1 E-box probe (see Materials and Methods) was incubated with 10 μg of the nuclear protein fraction isolated from growing (A) or quiescent as well as wound-edge (B) RK cells and complexes resolved by gel electrophoresis. Two closely-spaced, 'dumbbell-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- β 1 (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

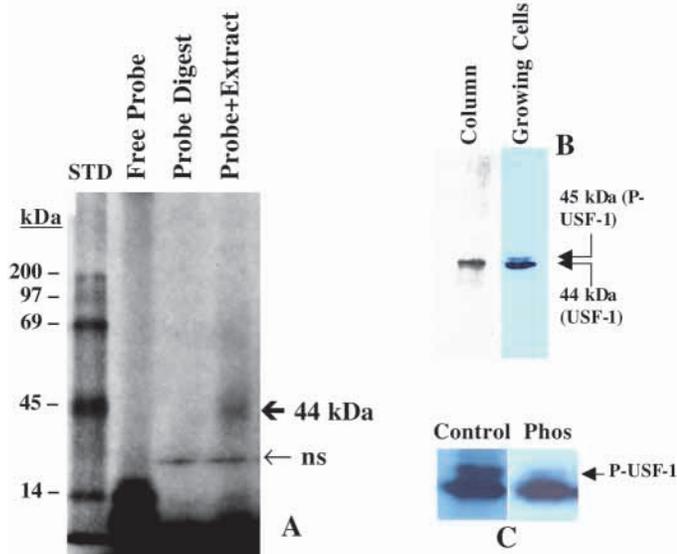


Fig. 5. Identification of USF-1 as PAI-1 E-box probe-binding factor. A 32 P-body labeled PAI-1 wild-type (WT) E-box deoxyoligonucleotide probe was generated by PCR. Gel-purified probe was incubated with nuclear extracts from serum-stimulated EC-1 cells prior to UV irradiation and treated with DNase-1; 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 systems, 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

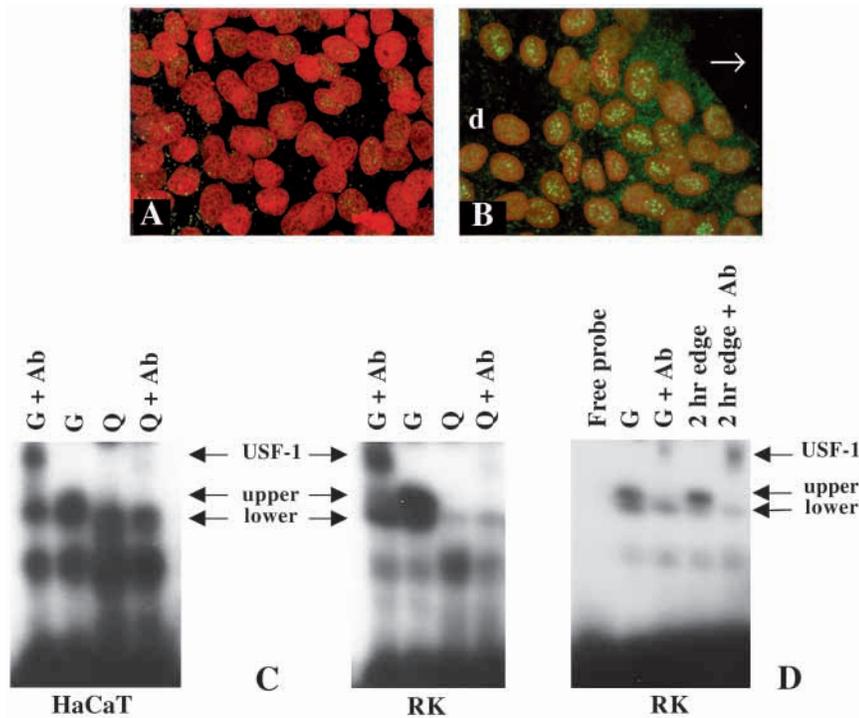


Fig. 6. USF-1 in situ localization and PAI-1 E-box-binding activity in wound-proximal keratinocytes. Compared with quiescent intact monolayer regions (A), HaCaT cells juxtaposed to the wound site (arrow in B indicates direction of migration into a denuded zone) exhibit significant cytoplasmic and nuclear 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 (preceding 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 dumbbell-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 α_v 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 (Ricchio 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 a motile phenotype.

This work was supported by grants from the NIH (GM57242, GM42461) and the US Army (DAMD17-98-1-8015 and DAMD17-00-1-0124).

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