MEK/ERK pathway mediates cell-shape-dependent plasminogen activator inhibitor type 1 gene expression upon drug-induced disruption of the microfilament and microtubule networks

Rohan Samarakoon and Paul J. Higgins*

Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York 12208, USA
*Author for correspondence (e-mail: higginp@mail.amc.edu)

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Summary

Changes in cellular morphology induced as a consequence of direct perturbation of cytoskeletal structure with network-specific targeting agents (i.e. microfilament- or microtubule-disrupting drugs) results in the stimulated expression of a specific subset of genes. Transcription of c-*fos*, collagenase, transforming growth factor-β, actin, urokinase plasminogen activator and its type-1 inhibitor (PAI-1) appears to be particularly responsive to shape-activated signaling pathways. Cytochalasin D (CD) or colchicine treatment of contact-inhibited and serum-deprived vascular smooth muscle (R22) cells was used, therefore, as a model system to evaluate morphology-associated controls on PAI-1 gene regulation in the absence of added growth factors. PAI-1 transcript levels in quiescent R22 cells increased rapidly and in a CD-concentration-dependent fashion, with kinetics of expression paralleling the morphological changes. Colchicine concentrations that effectively disrupted microtubule structure and reduced the cellular 'footprint' area (to approximately that of CD treatment) also stimulated PAI-1 synthesis. Shape-related increases in PAI-1 mRNA synthesis were ablated by prior exposure to actinomycin D. Unlike the mechanism of induction in growth-factor-stimulated cells, CD- and colchicine-induced PAI-1 expression required on-going protein synthesis (i.e. it was a secondary response). Although PAI-1 is a TGF-β-regulated gene and TGF-β expression is also shape dependent, an autocrine TGF-β loop was not a factor in CD-initiated PAI-1 transcription. Since CD exposure resulted in actin microfilament disruption and subsequent morphological changes, with uncertain effects on interactions between signaling intermediates or 'scaffold' structures, a pharmacological approach was selected to probe the pathways involved. Signaling events leading to PAI-1 induction were compared with colchicine-treated cells. CD- as well as colchicine-stimulated PAI-1 expression was effectively and dose dependently attenuated by the MEK inhibitor PD98059 (in the 10 to 25 μM concentration range), consistent with the known MAP kinase dependency of PAI-1 synthesis in growth-factor-stimulated cells. Reduced PAI-1 mRNA levels upon exposure to genistein prior to CD addition correlated with inhibition of ERK1/2 activity, implicating a tyrosine kinase in shape-dependent MEK activation. S*rc*-family kinases, moreover, appeared to be specific upstream elements in the CD- and colchicine-dependent pathways of PAI-1 transcription since both agents effectively activated pp60$^{c\text{-}src}$ kinase activity in quiescent R22 cells. The restrictive (*src*-family) kinase inhibitor PP1 completely inhibited induced, as well as basal, ERK activity in a coupled immunoprecipitation myelin-basic-protein-phosphorylation assay and ablated shape-initiated PAI-1 mRNA expression. These data suggest that PP1-sensitive tyrosine kinases are upstream intermediates in cell-shape-associated signaling pathways resulting in ERK1/2 activation and subsequent PAI-1 transcription. In contrast to the rapid and transient kinetics of ERK activity typical of serum-stimulated cells, the ERK1/2 response to CD and colchicine is both delayed and relatively sustained. Collectively, these data support a model in which MEK is a focal point for the convergence of shape-initiated signaling events leading to induced PAI-1 transcription.

Key words: PAI-1 transcription, Cytoskeleton, Signal transduction, Cell shape

Introduction

Eukaryotic cell morphology is regulated by the internal organization of the actin-, tubulin- and intermediate filament-based cytoskeletal networks as well as by constraints imposed by cell-to-cell and cell-to-matrix contacts (Stein and Bronner, 1989; Fuchs and Karakesisoglou, 2001). Elements that comprise these distinct, but highly interactive, architectural domains are critical determinants of cell form and function. Indeed, cell growth and related basic metabolic processes (e.g., cell cycle transit, DNA synthesis) are profoundly influenced by cell shape and substrate adhesion/cell spreading (Folkman and Moscona, 1978; Ben-Ze'ev et al., 1980; Crossin and Carney, 1981; Chou et al., 1982; Maness and Walsh, 1982; Assoian, 1997; Ruoslahti,

1997; Aplin and Juliano, 1999; Roovers et al., 1999; Aplin et al., 2001).

Perturbation of cell morphology with cytoskeleton-targeting drugs provides one important approach to the identification of shape-responsive genes and, in some cases, the involved signaling pathways (Aggeler et al., 1984; Zambetti et al., 1991; Higgins et al., 1992; Bershadsky et al., 1996; Irigoyen et al., 1997; Varedi et al., 1997; Schmid-Alliana, 1998; Wang et al., 1998). The transcription of genes that encode proteins involved in tissue remodeling processes, such as urokinase plasminogen activator (uPA) and its type-1 inhibitor (PAI-1), is particularly closely associated with dynamic changes in cellular morphology and shape-altering physiologic processes (Higgins et al., 1992; Ryan and Higgins, 1993; Bayraktutan and Jones, 1995; Ailenberg and Silverman, 1996; Irigoyen et al., 1997; Providence et al., 2000; Yan et al., 2000). Growth-factor-initiated epithelial-to-mesenchymal transition or disruption of E-cadherin-dependent cell-to-cell contacts and cadherin-associated actin structures, for example, stimulates PAI-1 expression and uPA secretion (Frixen and Nagamine, 1993; Zavadil et al., 2001). Cellular migration, both over planar surfaces and through complex 'tissue' barriers, moreover, also involves extensive morphological restructuring and is similarly accompanied by induced PAI-1 and uPA transcription (Pepper et al., 1987; Pepper et al., 1992; Lauffenburger and Horwitz, 1996; Friedl and Brocker, 2000; Providence et al., 2000; Ridley, 2001). Such findings consistently link expression controls on this protease-protease inhibitor pair to specific cytoarchitectural changes, probably as part of the motile program (Providence et al., 2000), and suggest involvement of the cytoskeleton in the signaling apparatus. Indeed, targeted reorganization of cell morphology with the cytoskeleton-active agent cytochalasin D (CD) does, in fact, transcriptionally activate both the uPA and PAI-1 genes (Higgins et al., 1992; Lee et al., 1993). Microfilament-disrupting agents similarly increase transforming growth factor-β1 (TGF-β1), c-*fos*, collagenase and fibronectin transcription (Zambetti et al., 1991; Varedi et al., 1997), suggesting a specific genetic response to cytoskeletal remodeling. Cell-shape-related induction of TGF-β is particularly relevant since PAI-1 transcription in CD-stimulated cells is a secondary (i.e., protein-synthesis-dependent) event (Higgins et al., 1995), and PAI-1 is a major TGF-β1-inducible gene (Boehm et al., 1999). It was important, therefore, to assess the potential involvement of an autocrine TGF-β1 loop in shape-initiated PAI-1 expression. Drug-initiated alterations in both the microfilament and microtubule networks, moreover, also mobilize intracellular signaling elements activating the ERK, JNK and p38 mitogen-activated protein kinases (MAPKs) (Irigoyen et al., 1997; Rijken et al., 1998; Schmid-Alliana, 1998; Sotiropoulos et al., 1999; Ren et al., 1999; Irigoyen and Nagamine, 1999; Yujiri et al., 1999; Subbaramhiah et al., 2000). The relationship between kinase stimulation in response to cytoskeletal disruption and the associated reprogramming of gene expression, however, remains to be defined. The present study was designed, therefore, to identify signaling intermediates involved in this unique pathway of PAI-1 gene regulation. Strategies were utilized to manipulate both the actin- and tubulin-based cytoskeletons in order to distinguish potential network-specific controls on intracellular signaling cascades/PAI-1 expression from cell-shape-dependent events.

Materials and Methods

Cell culture

R22 smooth muscle cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and standard concentrations of penicillin/streptomycin (Ryan and Higgins, 1993). The medium in near-confluent (80-90%) cultures was aspirated, the cells washed three times with phosphate-buffered saline (PBS) then maintained in serum-free DMEM for 3 days to initiate a state of quiescence (Ryan et al., 1996). Acridine-orange-based 2-parameter (DNA versus RNA content) flow cytometry confirmed that such serum-deficient culture conditions resulted in withdrawal of R22 cells from the proliferative cycle and subsequent entry into G0. [The criteria used for this were detailed previously (Higgins et al., 1984)]. CD, colchicine, pharmacologic inhibitors (at final concentrations indicated in the text) or DMSO solvent vehicle alone were added directly to the conditioned serum-free DMEM to avoid potential changes in either the quiescent state or level of PAI-1 expression as a consequence of medium replacement.

Northern blotting

Total cellular RNA was isolated and resuspended in TE buffer; 10-15 μg RNA was separated in 1.2% agarose/formaldehyde gels, transferred to Nytran membranes and immobilized by UV crosslinking. Blots were incubated at 42°C for 2 hours in 50% formamide, 5× Denhardt's reagent, 1% SDS, 200 μg/ml heat-denatured salmon sperm DNA (ssDNA), 5× SSC then hybridized overnight at 42°C in 50% formamide, 1% SDS, 2.5% SSC, 5× Denhardt's reagent, 100 μg/ml ssDNA, 20% dextran sulfate to ^{32}P-labeled PAI-1 and A50 cDNA probes. A50 was selected as a normalizing transcript since expression of A50 is unaffected by serum, growth factors or cell-shape perturbation (Ryan et al., 1996; Providence et al., 1999; Kutz et al., 2001). Membranes were washed at 55°C in 0.1×SSC/0.1%SDS and hybridization signals quantified with a Storm phosphorimager (Molecular Dynamics, CA).

MAP kinase assay

Cells were extracted for 30 minutes in ice-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 vanadate, 0.01% aprotinin, 4 μg/ul pepstatin A, 10 μg/ul leupeptin, 1mM phenylmethanesulfonyl fluoride; 1 ml/100 mm dish) and lysates clarified at 14,000 ***g*** for 15 minutes. Extract protein concentration was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL), and 500 μg aliquots from control and CD-treated cells were incubated with ERK1 and/or ERK2 antibodies (2 μg each; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours (or overnight) in a reaction volume of 500 μl at 4°C while rocking. Protein A/G Plus-agarose (30 μl) was added and immune complexes collected 2 hours later by centrifugation, washed twice with lysis buffer and twice with 100 mM NaCl in 50 mM HEPES buffer (pH 8.0). Complexes were 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 $MgCl_2$, 1mM DTT, 1mM benzamidine, 0.3 mg/ml myelin basic protein [MBP]), diluted in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol), boiled and 15 μl aliquots separated on SDS/15% acrylamide slab gels. Proteins were transferred to nitrocellulose, and radiolabeled MBP was quantified by phosphorimager analysis and total MBP assessed by Ponceau S staining. Nitrocellulose membranes were blocked in 3% milk, incubated overnight with ERK1 and ERK2 antibodies (0.2 μg/ml), washed, incubated with horseradish-peroxidase-conjugated secondary antibodies (0.13 μg/ml), and the proteins were visualized by enhanced chemiluminescence.

pp60$^{c\text{-}src}$ kinase assay

Cells were extracted for 30 minutes in ice-cold lysis buffer as used for MAP kinase activity assessments (above) but containing 0.1% SDS and lysates clarified at 14,000 ***g*** for 15 minutes. Protein aliquots (300 μg) from control and CD-treated cells were incubated with monoclonal antibodies to pp60$^{c\text{-}src}$ (clone GD11; Upstate Biotechnology, Lake Placid, NY) (2 μg) for 2 hours in a reaction volume of 300 μl. Immune complexes were collected on Protein A/G Plus-agarose (as above) and washed twice with lysis bufer without SDS and twice with wash buffer (20 mM HEPES pH 7.4, 10 mM MgCl2, 10 mM $MnCl_2$, 150 mM NaCl). Complexes were incubated at 30°C for 20 minutes in SRC kinase reaction buffer (10 μCi ^{32}P-ATP, 5 μM ATP, 20 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 10 mM MnCl2, 150 mM Nacl) followed by addition of 10 μl electrophoresis sample buffer. After boiling, 15 μl aliquots were separated on SDS/15% acrylamide slab gels, and proteins were transferred to nitrocellulose. Radiolabeled pp60$^{c\text{-}src}$ and IgG heavy chain (a substrate for immunoprecipitated SRC kinase) were quantified by phosphoimager analysis. pp60$^{c\text{-}src}$ and IgG heavy chain levels in each lane were assessed by western blotting. Briefly, nitrocellulose membranes were blocked in 3% milk, incubated overnight with pp60$^{c\text{-}src}$ antibody (GD11); the immunoblots were then washed, incubated with the appropriate secondary antibodies and proteins visualized by enhanced chemiluminescence.

Microscopy

Quiescent cells were treated with CD or colchicine (for the times and at the concentrations indicated) or DMSO and fixed in 10% formalin for phase-contrast microscopy. For each culture condtion, 50 random cells were selected for area measurements using Image Pro-Plus analytical software; cellular perimeters were outlined, the spread cell area ('footprint') calculated (mean±standard deviation) and plotted as a function of treatment. Cytoskeletal structures and intracellular PAI-1 protein were visualized by two-color fluorescence microscopy. Formalin (10%)-fixed cells were permeabilized in 1% NP-40/PBS for 20 minutes, incubated in 1% BSA for 20 minutes then with rabbit anti-rat PAI-1 IgG (10 μg/ml) for 1 hour and washed three times. Cells were incubated simultaneously with Alexa-488-labeled goat anti-rabbit IgG and rhodamine-conjugated phalloidin (1 μg/ml) to visualize PAI-1 and microfilament organization, respectively. Coverslips were mounted with anti-fade reagent (Molecular Probes, Eugene, OR). For analysis of cellular microtubules, formalin-fixed cells were washed three times with PBS (without Ca^{+2}/Mg^{+2}) then permeabilized in 0.5% Triton X-100/PBS for 10 minutes followed by three PBS washes. After a BSA block (as above), cells were incubated with a monoclonal antibody to β-tubulin (1-2 μg/ml) and rabbit anti-PAI-1 (10 μg/ml) for 1 hour at room temperature, washed three times and incubated in Alexa-568-labeled goat anti-mouse IgG and Alexa-488-labeled goat anti-rabbit IgG for 45 minutes to visualize tubulin and PAI-1, respectively. Cells were rinsed for 15 minutes in PBS followed by two additional washes for 5 minutes each, incubated in DAPI (to stain the nuclei), washed twice and mounted with anti-fade reagent.

PAI-1 western blotting

Cells were extracted for 30 minutes in ice-cold lysis buffer (as used in MAP kinase assays but containing 0.1% SDS) and lysates clarified at 14,000 ***g*** for 15 minutes. PAI-1 protein was detected on nylon membrane transfers of electrophoretic separations of 20 μg of total cellular lysate (quantified with the BCA Protein Assay Kit) by chemiluminescence using the IgG fraction of rabbit anti-rat PAI-1 (3-5 μg/ml) and horseradish-peroxidase-conjugated secondary antibodies (0.13 μg/ml).

Results

Kinetics and concentration dependency of CD-induced PAI-1 gene expression in R22 cells

To assess the mechanisms associated with cell shape controls on PAI-1 expression, we took advantage of the finding that quiescent cells significantly downregulate PAI-1 mRNA synthesis (i.e. growth-state-dependent PAI-1 transcription is largely restricted to the G0→G1 transition period and early G1 phase) (Ryan et al., 1996; Mu et al., 1998; White et al., 2000). Near-confluent R22 cells were incubated in FBS-free DMEM for 3 days, therefore, to initiate a state of growth-arrest as well as to eliminate any influence of concomitant exposure to serum on CD-mediated PAI-1 transcription (Higgins et al., 1992). Induced PAI-1 mRNA expression increased as a function of CD concentration (from 2-10 μM) (Fig. 1). Relative PAI-1 transcript abundance was minimally elevated (compared with quiescent control cultures) at 2 μM CD (1.5- to 2.0-fold) but increased significantly at CD concentrations of 5 μM (3.2- to 4.0-fold) and 10 μM (5.7- to 9.0-fold). Cellular PAI-1 protein levels also increased as early as 1-2 hours of CD treatment and were near maximal by 4 hours of continuous CD exposure **(**Fig. 1**)**. A greater range of CD concentrations was tested, therefore, to determine if induction reflected the extent of morphological

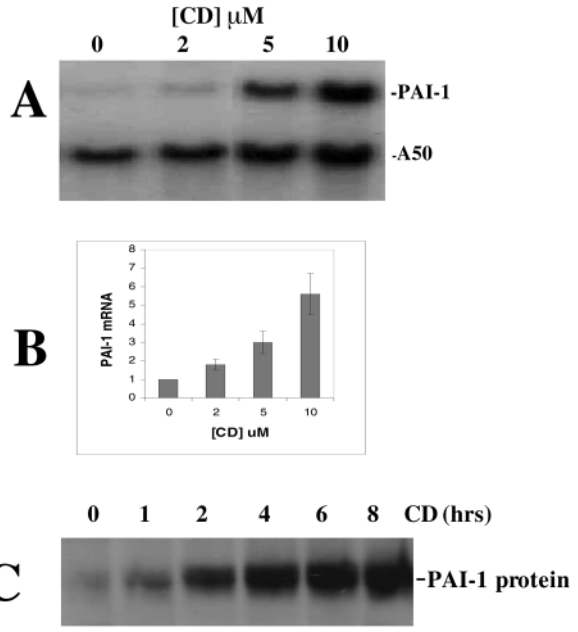


Fig. 1. CD induces PAI-1 expression in growth-arrested R22 cells as a function of concentration and time of exposure. CD (in final concentrations of 2, 5 or 10 μM) or DMSO vehicle alone (0 μM) was added to serum-deprived R22 cell cultures. Total RNA was isolated 4 hours later and northern blots hybridized with 32P-labeled PAI-1 and A50 cDNAs (A). PAI-1 mRNA levels increased as a function of CD concentration; maximal expression was attained with 10 μM CD (Hawks and Higgins, 1998). The histogram in (B) represents a quantitative analysis of PAI-1 mRNA abundance (mean±s.d.) from three different experiments normalized to the A50 signal. Immunoblot detection of PAI-1 protein on transfers of electrophoretic separations of R22 cell extracts (20 μg protein/lane) at various times after CD treatment (C). PAI-1 increased as early as 1 hour post-stimulation and reached maximal levels by approximately 4 hours.

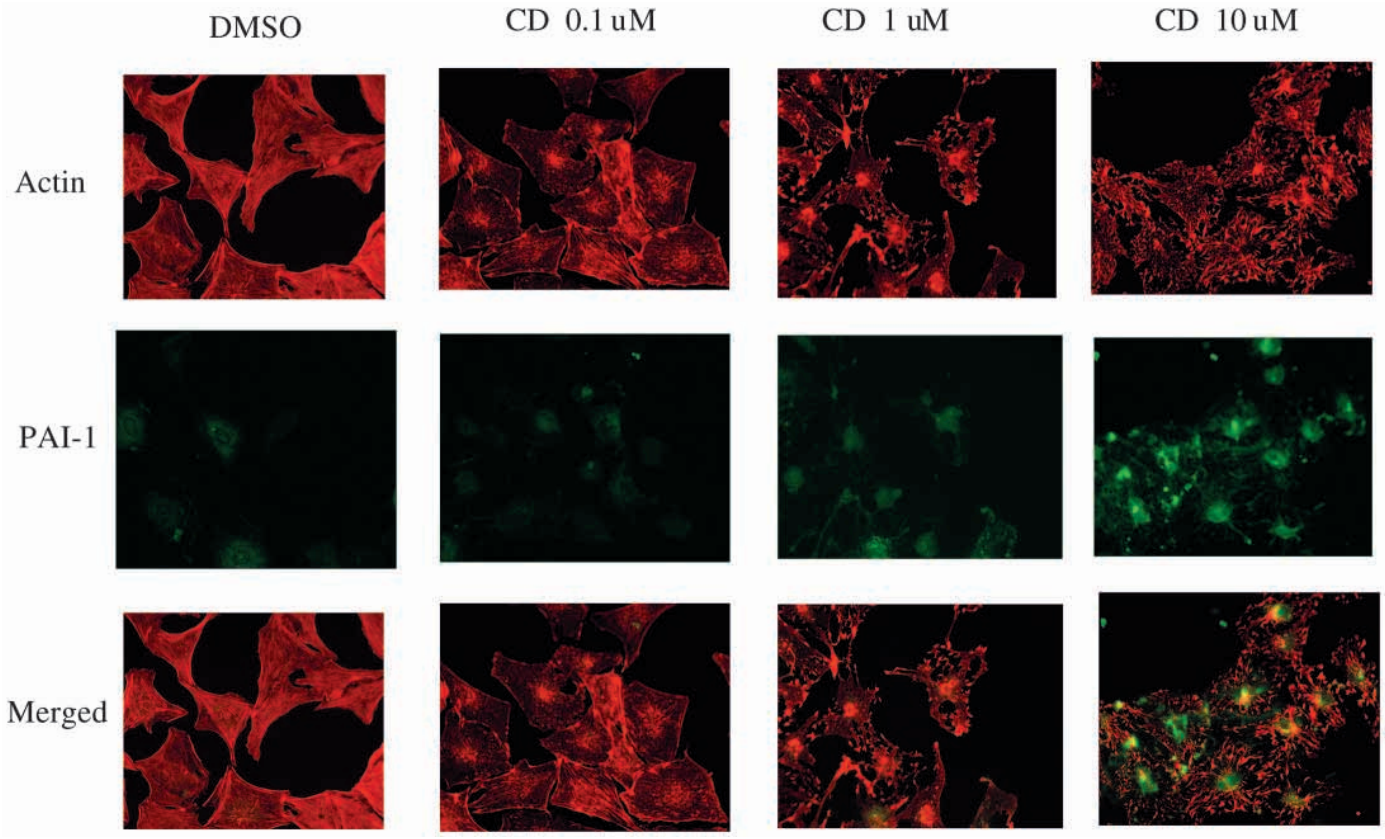
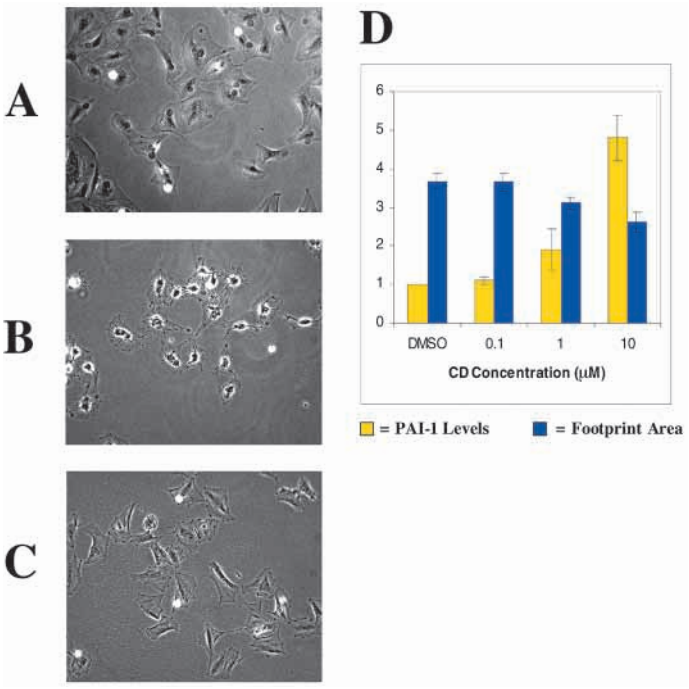


Fig. 2. In situ distribution of CD-induced PAI-1 protein in quiescent R22 cells. The fractions of PAI-1 protein-expressing cells and the relative intensity of immunoreactive PAI-1 staining were assessed by fluorescence microscopy; both increased as a function of the time of CD exposure. Nuclei were visualized by DAPI staining. Although microfilament structure was progressively disrupted in 0.1 and 1.0 μM CD-treated cells, PAI-1 expression was only evident in 10 μM CD-stimulated cultures. Induction was more closely associated with changes in cell shape (Fig. 3) than actin skeleton disorganization. The apparent nuclear region accumulation of PAI-1 is probably the collective result of CD-induced cellular arborization and Golgi collapse around the nucleus.



change. Parameters assessed included stress fiber loss, appearance of dense actin bodies, progressively aborized cell shape and reduced spread cell area (i.e., cytoarchitectural correlates of the CD-initiated PAI-1$^+$ phenotype) (Cooper, 1987; Schliwa, 1982; Hawks and Higgins, 1998). Similar to findings in the NRK cell system (Higgins et al., 1989), changes in actin filament organization sufficient to generate the

Fig. 3. Inverse correlation between extent of morphological response of quiescent R22 cells to cytoskeletal disruption and PAI-1 transcript abundance. Cells maintained under serum-free culture conditions for 3 days were generally well spread with little membrane ruffling (A). A 4 hour exposure to 10 μM CD resulted in an arborized phenotype (B), although the cellular periphery remained well demarcated and easily visible for footprint imaging. Colchicine exposure (10 μM, 4 hours) **(**C**)** did not produce the same arborized effect as CD, although the cellular footprint area was similarly reduced (by 35%) in CD- and colchicine-treated cultures. Computerized imaging was done on 75 randomly selected individual cells per culture condition in three sets of 25 cells each. The footprint area was significantly reduced by treatment with either drug at the maximal PAI-1 transcript-inducing concentration of 10 μM (Fig. 4). Comparison between DMSO and CD-treated cells indicated an approximate reciprocal relationship between cellular footprint area and PAI-1 expression (D). The histogram in (D) illustrates the mean±s.d. (in arbitrary units) for triplicate determinations of footprint area and PAI-1 levels.

aborized morphology with a significant reduction in cellular footprint area appear to be general cytoarchitectural concomitants of PAI-1 induction (Figs 2 and 3). It was important, however, to assess whether PAI-1 expression was a specific attribute of actin cytoskeleton disruption or a more general response to cell-shape perturbation. Quiescent R22 cells were exposed to the tubulin-binding drug colchicine using a concentration (10 μM; optimal level as determined by titer analysis) that not only effectively disrupted the microtubule network but also reduced the cellular 'footprint' area to an extent equivalent to that of 10 μM CD-treated cells (Fig. 3). Cell-shape change initiated by colchicine markedly induced PAI-1 expression in a time frame (4 hours) similar to that of CD-stimulated cells (Figs 3 and 4).

Metabolic requirements for induced PAI-1 expression

Assessments of signaling pathways involved in PAI-1 induction were standardized to a 4 hour exposure to 10 μM CD or colchicine (Fig. 1). R22 cells were incubated with metabolic inhibitors for 30 minutes prior to treatment. CD-stimulated PAI-1 expression was actinomycin D sensitive and largely inhibited by puromycin (Fig. 5). The increase in PAI-1 transcripts in CD-treated cultures was a secondary (i.e., protein-synthesis-dependent) response differing from the primary mode of PAI-1 gene activation by serum growth factors (Ryan et al., 1996; Boehm et al., 1999). Neither puromycin nor actinomycin D when used alone, moreover, affected PAI-1 mRNA levels relative to quiescent controls (Fig. 5), supporting their suitability to assess the mechanism (i.e., primary versus secondary) of CD-dependent PAI-1 induction. PAI-1 expression in response to colchicine treatment was similarly transcriptionally dependent and sensitive to puromycin (data not shown).

Cytochalasin also induces TGF-β1 transcription (Varedi et al., 1997), and TGF-β1 is a potent stimulator of PAI-1 synthesis (e.g. Boehm et al., 1999). Since PAI-1 expression in CD-treated R22 cells has a significant secondary component (Fig. 5), it was important to evaluate the potential contribution of an autocrine TGF-β1 loop to the inductive process. Addition of broad-spectrum TGF-β-neutralizing antibodies effectively blocked PAI-1 induction by exogenously added TGF-β1 but failed to inhibit CD-stimulated PAI-1 expression (Fig. 5). PAI-1 transcript abundance in CD-treated cultures, moreover, was consistently lower than the level of PAI-1 expression evident in 1 ng/ml TGF-β1-stimulated cells. Thus, the range of neutralizing antibodies used (2-20 mg/ml) is probably more than sufficient to block any PAI-1 induction

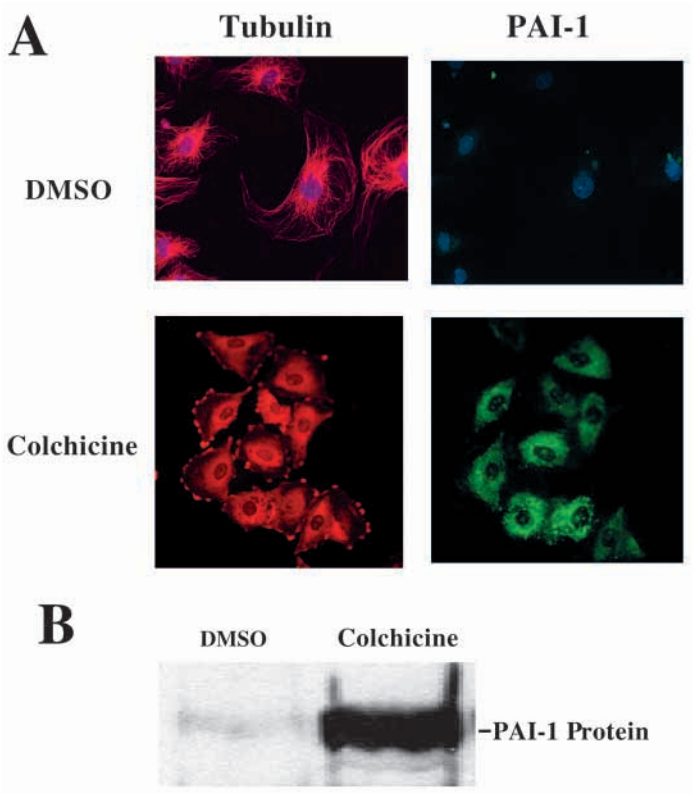


Fig. 4. Colchicine stimulates PAI-1 synthesis in quiescent R22 cells. Growth-arrested cells were treated with DMSO (solvent control) or colchicine (10 μM, final concentration) for 4 hours. The cellular microtubule network and PAI-1 protein were visualized in fixed and permeabilized cells by indirect immunocytochemistry with antibodies to tubulin and PAI-1, respectively (A). Cells exposed to DMSO were well spread with a highly branched microtubule network. Colchicine treatment disrupted the microtubule skeleton and significantly reduced the cell-spread area (Fig. 3, legend). PAI-1 protein was virtually undetectable by immunocytochemistry (A) and resolved at only extremely low levels by western blotting of extracts of control cells (B). Abundant PAI-1 protein was easily identified, by contrast, by both techniques in 4 hour colchicine-stimulated cells (A,B). Nuclei were stained with DAPI.

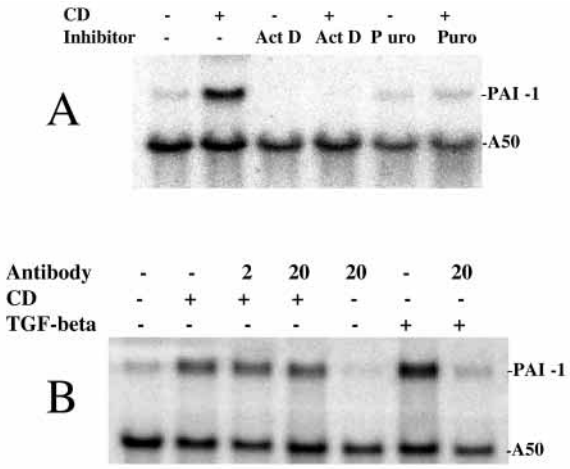


Fig. 5. Metabolic requirements for CD-induced PAI-1 mRNA expression. Serum-deprived cells were pretreated for 30 minutes with actinomycin D (Act D; 5 μg/ml) or puromycin (Puro; 100 μg/ml) prior to a 4 hour exposure to CD (final concentration 10 μM). Optimal concentrations of inhibitors were determined previously (e.g. Ryan et al., 1996; Hawks and Higgins, 1998). Northern blots were probed with ^{32}P-labeled cDNAs to PAI-1 and A50 (A). CD-induced PAI-1 transcription appeared to utilize a secondary (i.e. protein-synthesis-dependent) response mechanism. To evaluate the potential role of autocrine TGF-β as a secondary response intermediate, quiescent R22 cell cultures were pretreated for 30 minutes with TGF-β neutralizing antibodies prior to addition of either CD or TGF-β1. RNA was isolated, and blots were probed for PAI-1 and A50 transcripts (B). Representative blots are shown.

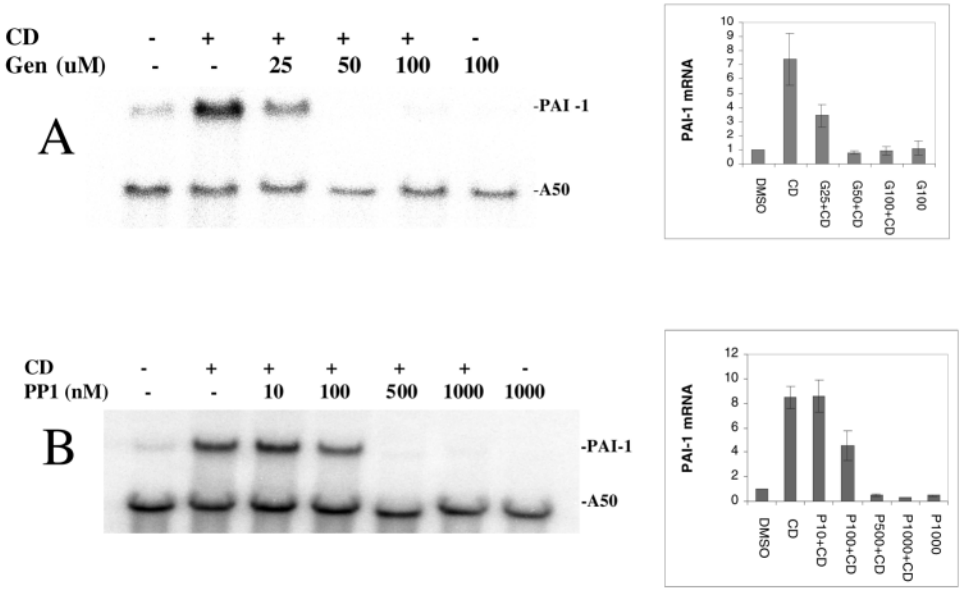


Fig. 6. CD-induced PAI-1 expression is sensitive to tyrosine kinase inhibitors. Quiescent R22 cultures were pretreated for 30 minutes with progressively increasing concentrations of genistein (A) or PPI (B) prior to CD treatment. RNA was isolated, and northern blots were probed for PAI-1 and A50 transcripts. Representative blots are shown in A and B. –/– refers to DMSO vehicle only. The corresponding histograms illustrate PAI-1 mRNA abundance normalized to A50 signal for replicate experiments (mean±s.d.). Concentrations of genistine and PP1 (>25 μM and 10 nM, respectively) effectively ablated PAI-1 transcript expression.

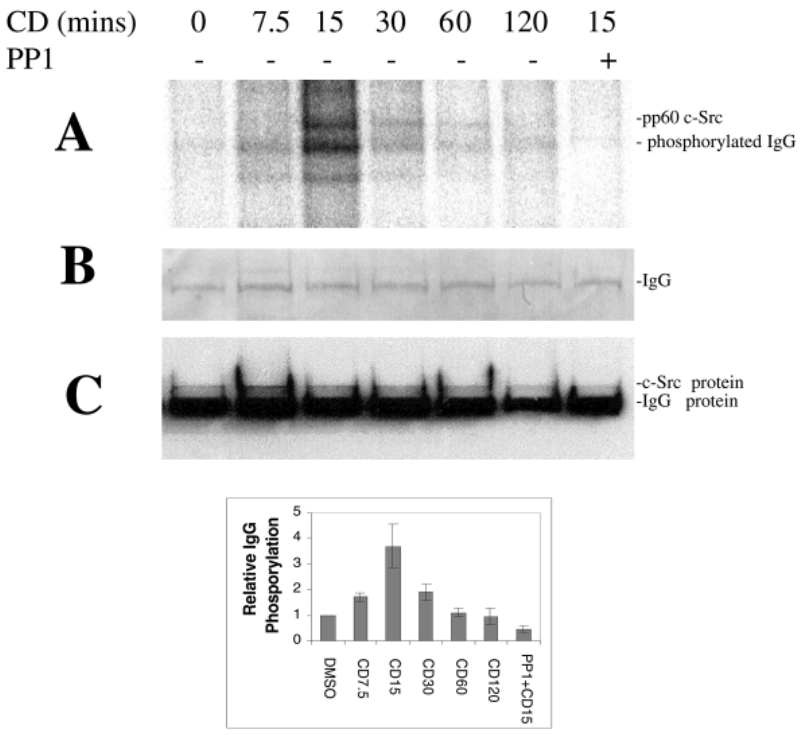


Fig. 7. CD activates pp60$^{c\text{-}src}$ kinase activity. Cells were incubated in 10 μM CD and extracts prepared at the times indicated for the immune complex assay of pp60$^{c\text{-}src}$ activity. Autophosphorylated pp60$^{c\text{-}src}$ kinase and phosphorylated IgG heavy chain were evident at 7.5 minutes after CD addition and maximal at 15 minutes post-stimulation (A). Kinase activity rapidly declined thereafter. Addition of PP1 to cultures prior to CD treatment effectively inhibited pp60$^{c\text{-}src}$ kinase activity at the optimal 15 minute time point. Equal gel loading was confirmed by visualization of the IgG heavy chain by staining with Ponceau S (B) and by western blotting for pp60$^{c\text{-}src}$ and IgG (C). The histogram indicates quantitative data (mean±s.d.) for three independent experiments.

that may have been mediated by an autocrine TGF-β-dependent pathway.

Cell-shape perturbation, particularly that induced by cytoskeleton-disrupting drugs, alters (in certain circumstances) the activity of specific signaling intermediates (Schmid-Alliana et al., 1998; Ren et al., 1999; Yujiri et al., 1999; Subbaramaiah et al., 2000). A pharmacological approach was selected to probe the involved pathways since changes in cell structure may disrupt signaling ‘scaffolds’ or interfere with intracellular proteins, making data obtained with dominant-negative constructs difficult to interpret. Several non-receptor tyrosine kinases, moreover, have been implicated as upstream elements in signaling events that involve cytoskeletal reorganization (Abram and Courtneidge, 2000; Schmitz et al., 2000). Consistent with these findings, the broad-spectrum tyrosine kinase inhibitor genistein effectively blocked CD-induced PAI-1 mRNA expression (>50% and 100% inhibition at 25 and 50 μM, respectively) (Fig. 6). pp60$^{c\text{-}src}$, in particular, can be specifically activated by actin network-modulating compounds (i.e. CD) in concentrations identical to those required to initiate PAI-1 transcription (Higgins et al., 1992; Lock et al., 1998), suggesting that *src*-family kinases may function in shape-dependent PAI-1 gene regulation. Indeed, CD stimulated pp60$^{c\text{-}src}$ kinase activity at least four-fold within 15 minutes of addition to quiescent R22 cultures (Fig. 7). Preincubation of R22 cells with the more restrictive (*src*-family) tyrosine kinase inhibitor PP1 markedly decreased CD-induced PAI-1 mRNA levels (Fig. 6) and pp60$^{c\text{-}src}$ activation (Fig. 7). Herbimycin A produced similar results in R22 (data not shown) as well as in NRK cells (Hawks and Higgins, 1998). PP1 was particularly effective (i.e. 50% inhibition

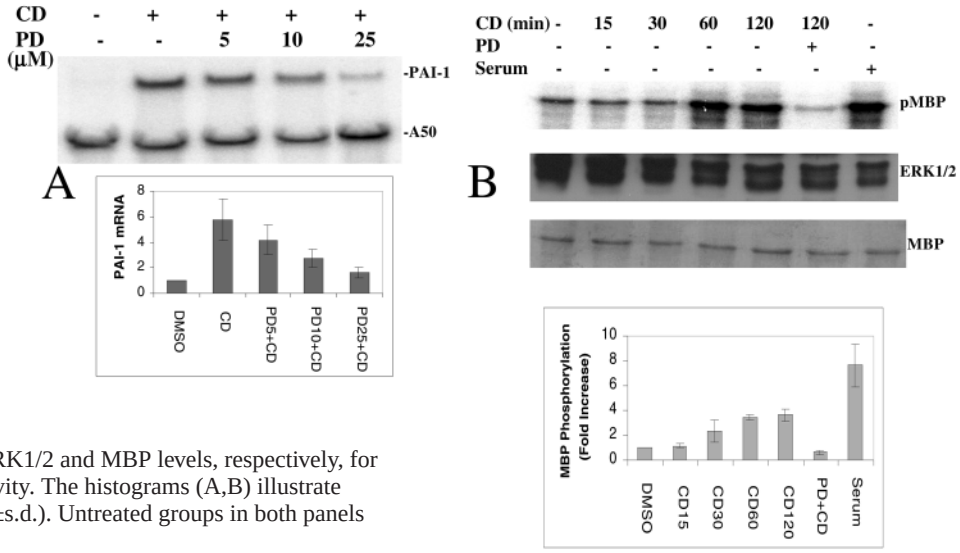


Fig. 8. CD-mediated PAI-1 expression and ERK1/2 activation is MEK dependent. Quiescent R22 cells were pretreated with PD98059 (at the indicated concentrations) prior to addition of CD. Northern blots of total cellular RNA were probed with ^{32}P-labeled cDNA probes to PAI-1 and A50; PAI-1 mRNA abundance was normalized to A50 signal (A). CD-induced ERK1/2 activity was assessed by a coupled immunoprecipitation–in-vitro-kinase assay using myelin basic protein (MBP) as a phosphorylation substrate with or without PD98059 pretreatment (B). Western blotting and Ponceau S staining (B) served to confirm ERK1/2 and MBP levels, respectively, for normalization of MBP phosphorylation activity. The histograms (A,B) illustrate results of three separate experiments (mean±s.d.). Untreated groups in both panels refer to DMSO vehicle only.

of PAI-1 expression at 100 nM and complete inhibition at 500 nM), strongly suggesting the participation of *src* kinases in the inductive process (Hanke et al., 1996).

Involvement of ERK1/2 in CD-mediated *PAI-1* gene expression

Elements of the *ras*, *raf*, MAPK cascade associate with a microfilament-linked signaling 'particle' (Carothers-Carraway et al., 1999; Li et al., 1999), suggesting a cell structural basis for MAPK activation similar to the regulation of *Rho* GTPase by the cytoskeleton (Ren et al., 1999). The potential involvement of specific MAPKs as downstream mediators of CD-initiated signaling was initially evaluated using PD98059 to inhibit MEK-dependent signaling. Pretreatment of R22 cells with PD98059 significantly reduced (at 10 μM) and virtually eliminated (at 25 μM) CD-stimulated PAI-1 expression (Fig. 8). CD-dependent ERK1/2 activation was confirmed using a coupled immunoprecipitation–in-vitro-kinase-assay in the presence of the exogenous substrate, MBP. While there was some variation in CD-stimulated ERK activity at early time points (i.e. 30 minutes) in replicate experiments, total ERK-targeted MBP phosphorylation consistently increased four-fold by 1 hour and remained elevated for at least 2 hours (Fig. 8). CD-responsive ERK activation was approximately 50% that of serum-stimulated cells (standardized to 10 minutes after FBS addition) and could be effectively suppressed (to levels even below that of quiescent control cells) by PD98059 (Fig. 8). CD treatment activated both ERK1 and ERK2 with only slight differences in kinetics (Fig. 9). The level of ERK1-directed

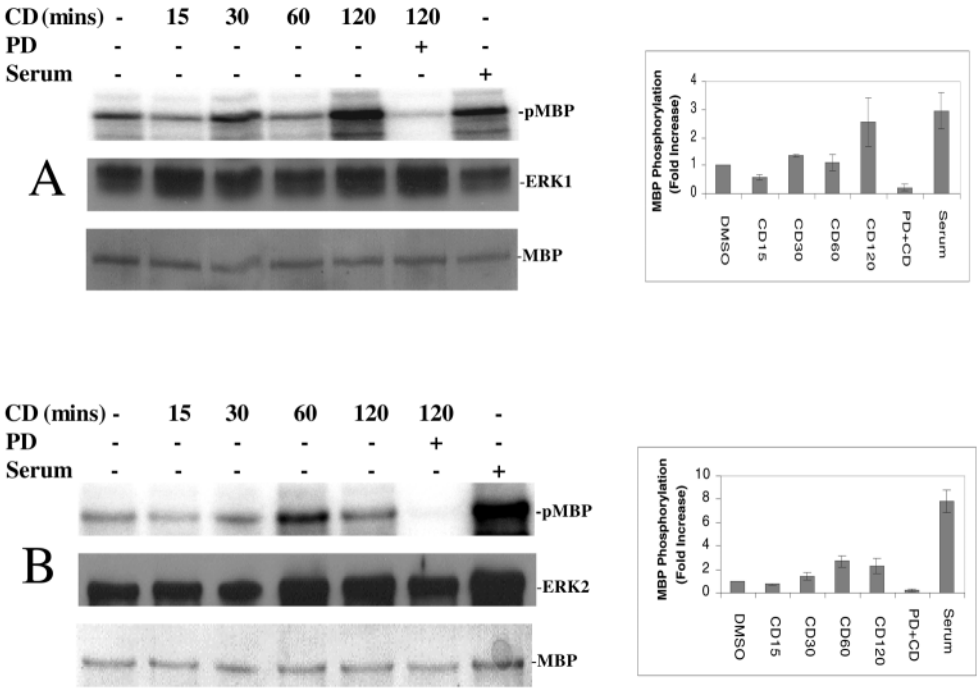


Fig. 9. ERK1 and ERK2 are activated by CD treatment. The upper panels in (A,B) illustrate MBP phosphorylation by activated ERK1 and ERK2, respectively, as determined by the coupled immunoprecipitation–in-vitro-kinase assay at various times (mins) following CD addition. PD98059 (20 μM) or serum (final concentration of 20%) was added where indicated. Western blotting and Ponceau S staining served to confirm ERK and MBP levels, respectively (middle and bottom panels in A,B), used for normalization of MBP phosphorylation activity. Data plotted are the means±s.d. for at least three different experiments.

MBP phosphorylation (at 2 hours) in response to CD approached that of serum-stimulation; ERK2, by contrast, was more responsive to serum than ERK1 in R22 cells (by at least two-fold) although the relative ability of ERK1 and ERK2 to phosphorylate MBP in response to CD was similar (Fig. 9). Total ERK1/2 protein levels remained unchanged regardless of treatment conditions or relative ERK1/2 activity. CD-mediated ERK1/2 activation coincided with the initial increase in PAI-1 transcripts; both events, moreover, occurred in a delayed and sustained fashion compared with the rapid and transient ERK1/2 phosphorylation response to serum.

To assess the relationships among signaling elements, pharmacological inhibitors that impacted on CD-mediated PAI-1 gene expression were evaluated for their ability to modulate ERK activity. Consistent with the requirements for PAI-1 induction (Figs 6 and 8), genistein and PP1 (and PD98059) completely inhibited CD-stimulated as well as basal ERK activity in the immunoprecipitation-in-vitro-kinase MBP assay, placing the PP1-sensitive kinases upstream of ERK (Fig. 10). The effect of these agents on PAI-1 expression/ERK activation profiles, moreover, was not simply caused by generalized toxicity or inhibition of CD-mediated microfilament disruption/cell-shape change. Neither CD or colchicine nor any of the pharmacological inhibitors used adversely affected cell viability over the time course used; PD98059 and PP1 also did not interfere with CD-initiated cytoskeletal rearrangements in R22 cells (data not shown). These data clearly position the PP1-sensitive kinase upstream of MEK and, more importantly, illustrate that cell-shape-dependent PAI-1 transcription is not merely a consequence of MEK-ERK autoactivation. Similar to the requirements for CD-dependent PAI-1 expression, colchicine-induced PAI-1 synthesis was significantly attenuated by pretreatment with PD98059, PP1 and puromycin. Inhibitor levels that ablated CD-mediated PAI-1 transcription (i.e., 25 μM PD98059, 170 nM PP1) were also sufficient to block both the colchicine-initiated PAI-1 response and ERK1/2 activation **(**Fig. 11**)**.

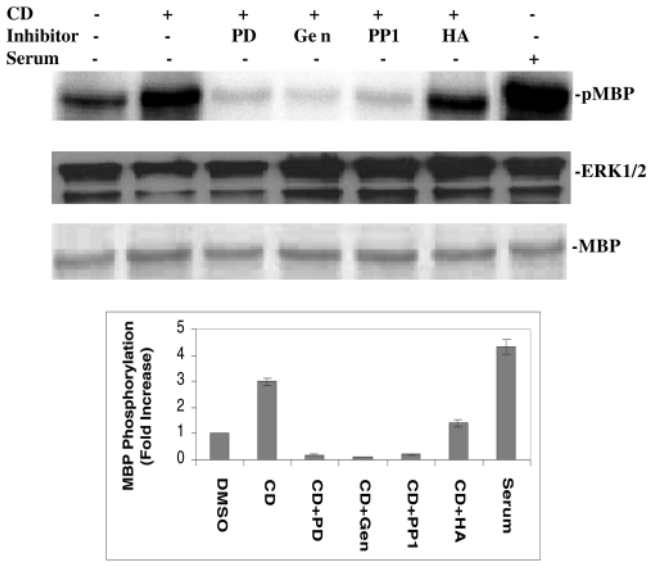


Fig. 10. CD-induced ERK1/2 activity is dependent on a *src*-like tyrosine kinase. Quiescent R22 cells were preincubated with the indicated inhibitors prior to a 2 hour treatment with CD (10 μM final concentration); control cultures were maintained without additives. Serum-stimulation (FBS to a final concentration of 20%) provided a positive control for ERK activation. The MAPK assay used myelin basic protein (MBP) as the phosphorylation substrate (pMBP; upper panel). Western blot probed with pan-ERK antibodies (middle panel) and Ponceau S staining of MBP (bottom panel) served to confirm equivalent protein loading as well as to confirm normalization of MBP phosphorylation (mean±s.d.) in replicate experiments (histogram).

Discussion

Transition from a proliferating to a quiescent substate, owing to growth factor deprivation or loss of substrate anchorage, is accompanied by reduced ERK1/2 activity (Aplin and Juliano, 1999; Rosenfeldt and Grinnell, 2000; Kutz et al., 2001) and attenuated PAI-1 transcription (Ryan et al., 1996; Boehm et al., 1999). Such growth-state-dependent constraints on particular signaling events (i.e. *src*- and/or MAPK-mediated) that impact on downstream transcription-level controls are relieved, at least in some cell types (including R22), by cytoskeleton/cell-shape perturbation (Higgins et al., 1992; Banes et al., 1995; Ailenberg and Silverman, 1996; Ren et al., 1999). The pathways involved, however, appear to be relatively restrictive, converging on specific target genes (e.g. Unemori and Werb, 1986; Higgins et al., 1992; Higgins et al., 1994; Lee et al., 1993; Bayraktutan and Jones, 1995; Ailenberg and Silverman, 1996; Hawks and Higgins, 1998; Castilla et al., 1999; Providence et al., 1999; Yan et al., 2000), unlike the global reprogramming of gene transcription typical of serum-stimulated cells (Iyer et al., 1999).

src-family/MAP kinase signaling to PAI-1 transcription is not unique to structural disruption in a specific cytoskeletal compartment, as CD and colchicine were each efficient activators. It is quite possible that altered dynamics in both the actin- and tubulin-based systems have common downstream effectors. The similarity in signaling requirements between the two stimuli (CD versus colchicine) used in the present paper to effect PAI-1 transcription, however, suggests that the associated changes in cell shape, while the result of cytoskeletal network perturbation, may be a more likely causative element in PAI-1 induction. CD- and colchicine-dependent PAI-1 expression, moreover, utilizes a tyrosine kinase/MEK-activated secondary response pathway that is distinct from the primary mode of growth-factor-induced PAI-1 transcription (Ryan et al., 1996; Kutz at al., 2001). TGF-β1 transcription is similarly stimulated by CD (Varedi et al., 1997) and TGF-β1-induced PAI-1 expression is, at least partly, MEK dependent (Kutz et al., 2001). Although this growth factor represents a likely secondary response intermediary in the shape-initiated pathway of PAI-1 gene control (Boehm et al., 1999), the present findings, clearly rule out an autocrine TGF-β loop as a PAI-1 inductive mechanism.

Induced *src*-like kinase and MAPK activity as well as crosstalk among the *ras*, *rho*, *rac*, cdc42 and related GTPase cascades may each relate to cytoskeletal/cell-shape controls on PAI-1 gene regulation (this paper) (Mucsi et al., 1996; Afti et al., 1997; Schmid-Alliana et al., 1998; Yujiri et al., 1999; Abram and Courtneidge, 2000). CD stimulates *rho* GTPase function (Ren et al., 1999) which, in turn, increases serum response factor (SRF) activity (Hill et al., 1995; Alberts et al.,

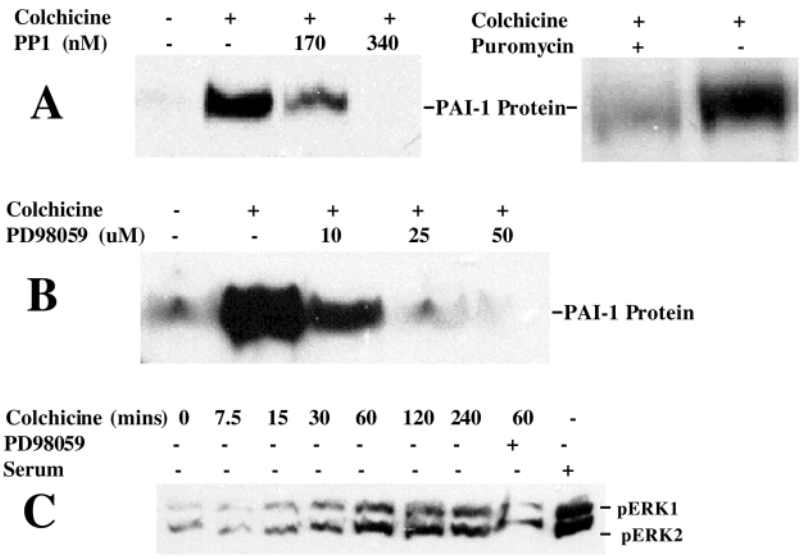


Fig. 11. Colchicine-induction of PAI-1 expression is sensitive to PP1, puromycin and PD98059 and associated with ERK activation. Pretreatment of quiescent R22 cultures with PP1 significantly attenuated and completely ablated colchicine-mediated PAI-1 expression at 170 and 340 nM, respectively (A, left panel). PAI-1 induction was also effectively inhibited by exposure to puromycin (100 μg/ml) (A, right panel). Similarly, PD98059 at a final concentration of 10 μM reduced cellular PAI-1 levels by 65% whereas use of the inhibitor at ≥25 μM effectively blocked CD-induced PAI-1 synthesis (B). Exposure of quiescent R22 cells to colchicine-stimulated ERK1/2 phosphorylation by 30 minutes and ERK remained activated for at least 4 hours after addition of the drug (C). ERK phosphorylation, like PAI-1 induction, was PD98059 sensitive. In all cases, colchicine was used at 10 μM.

1998) and (potentially) *rho*-mediated PAI-1 transcription (Afti et al., 1997; Park and Galper, 1999; Takeda et al., 2001). Indeed, the *rho*-GTPase-binding proteins mDia 1 and mDia 2 couple *rho* GTPase and pp60$^{c\text{-}src}$, with subsequent effects on cell signaling, SRF activation and actin organization (Tominaga et al., 2000). Microfilament disruption in response to CD, moreover, stimulates pp60$^{c\text{-}src}$ kinase activity (this paper) (Lock et al., 1998), amplifies tyrosine phosphorylation of Shc and promotes association of Shc with Grb2 as well as adhesion-dependent ERK activation (Barberis et al., 2000). These pathways may be relevant not only to shape-dependent regulation of the PAI-1 gene but also to its protease target uPA since a dominant-negative *src* expression construct effectively attenuated luciferase reporter expression driven by a uPA promoter sequences (Irigoyen et al., 1997; Irigoyen and Nagamine, 1999). Previous findings suggest, moreover, that cytoskeletal controls on ERK activity may proceed through two parallel but distinct (FAK- and Shc-dependent) pathways, with *src*-family intermediates common to both (Irigoyen and Nagamine, 1999; Barberis et al., 2000). Cytoskeletal disruption induces Shc phosphorylation and the formation of Shc/Grb2 and Shc/FAK complexes; both FAK and pp60$^{c\text{-}src}$ are required for optimal CD-initiated *ras*/ERK signaling. PAI-1 gene induction is, indeed, sensitive to the tyrosine kinase inhibitor PP1, implying that *src* kinases serve as upstream regulators of this pathway. CD-stimulated matrix metalloproteinase expression in human dermal fibroblasts is similarly a *src*-family kinase-dependent secondary-type response (Lambert et al., 2001). Recent data, furthermore, indicate that p130^{cas} enhances epidermal growth-factor-dependent signaling events by acting as a substrate for pp60$^{c\text{-}src}$. pp60$^{c\text{-}src}$ phosphorylates p130^{cas}, promoting interactions with Grb2 or Shc/Shp2, activating the *ras*/MEK/ERK cascade and inducing SRE-dependent gene transcription (Hakak and Martin, 1999). CD does, in fact, stimulate a genistein-sensitive phosphorylation of p130^{cas} as well as formation of p130^{cas}-FAK complexes (R.S. and P.J.H., unpublished). These and previous findings (Lock et al., 1998; Hakek and Martin, 1999; Barberis et al., 2000) suggest a model whereby cell shape changes initiate a pp60^{src}/p130^{cas}-dependent signaling cascade that results in MEK-ERK activation and PAI-1 expression.

The potential importance of morphology-linked controls on PAI-1 transcription is underscored by the marked increase in PAI-1 synthesis in transformed, migrating, mechanical- and hypoxia-stressed as well as growth-factor-stimulated cells (e.g. Seebacher et al., 1992; Eckstein and Bade, 1996; Ryan et al., 1996; Feng et al., 1999; Coats et al., 2000; Providence et al., 2000; Kutz et al., 2001) and the obvious changes in cytoskeletal dynamics associated with these processes. Recent data suggest that sudden loss of cell-cell contact in cultured epithelial cells by denudation injury stimulates PAI-1 mRNA/protein synthesis specifically in the wound edge cohort but not in the distal, still contact-inhibited, monolayer regions (Providence et al., 2000). Since cadherin-dependent cell-cell contacts are sites of *src* kinase localization (Calautti et al., 1998; Owens et al., 2000), one intriguing possibility is that loss of cell-to-cell junction integrity, a common aspect of CD- and colchicine-induced cell body retraction, may activate *src* kinases and downstream (MAPK) effectors resulting in PAI-1 transcription.

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