Cell-shape-associated transcriptional activation of the p52(PAI-1) gene in rat kidney cells

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The microfilament-disrupting agent cytochalasin D (CD) increased (by 10–22-fold) the synthesis *de novo* and extracellular matrix deposition of plasminogen-activator inhibitor type-1 [p52(PAI-1)] in normal rat kidney (NRK) cells. Transition from a flat to a round phenotype occurred concomitantly with, and may actually precede, p52(PAI-1) induction; both the morphological and p52(PAI-1) responses were dose-dependent. Augmented synthesis became evident between 4 and 5 h of treatment of NRK cells with 100 μ M-CD, correlating with a transition from 25 to more than 60% rounded cells. CD-associated increases in p52(PAI-1) mRNA abundance and protein biosynthesis were maximal between 6 and 8 h of continuous CD exposure, declined by 50% thereafter, but remained elevated (by at least 6–21-fold respectively over control values) for 24 h. Changes in p52(PAI-1) mRNA abundance at this 24 h point reflected an approx. 5-fold increase in p52(PAI-1)-gene transcription. These data confirm previous suggestions, based on actinomycin D-sensitivity of the inductive response [Higgins & Ryan (1992) *Biochem. J.* 284, 433–439], that CD-mediated increases in p52(PAI-1) expression are at least partly due to transcription-level events. Since CD also augments specific cellular responses to growth factors or cytokines, the potential effectiveness of this inducer was evaluated both in the presence and absence of serum growth factors using quiescent NRK cells [a growth state in which p52(PAI-1) is not expressed] as a model system. Induction of p52(PAI-1) synthesis and matrix deposition in CD-stimulated quiescent NRK cells was as efficient under growth-factor-deficient conditions as when CD was added simultaneously with serum. CD alone is thus a complete inducer of p52(PAI-1) expression in NRK cells, an observation that supports the contention that cell shape is an important regulatory element in p52(PAI-1)-gene control.

INTRODUCTION

Cell shape, the extracellular matrix and the cytoskeleton play interacting, albeit complex, roles in tissue morphogenesis, cell differentiation and regulation of eukaryotic gene expression (Ben-Ze'ev, 1986; McDonald, 1989; Bissell *et al.*, 1982; DiPersio *et al.*, 1991; Hay & Svoboda, 1989). Reorganization of the internal cytoskeleton with specific drugs [e.g. the actin-microfilament-disrupting cytochalasins (Godman & Miranda, 1978; Cooper, 1987)] provides one approach to assess cell-shape-dependent metabolic pathways (reviewed by Hay & Svoboda, 1989). CD-initiated morphological changes parallel co-ordinate increases in the relative rates of synthesis of particular proteins in specific cell types (see, e.g., Higgins *et al.*, 1990; Unemori & Werb, 1986) including the 52 kDa type-1 inhibitor of plasminogen activator [p52(PAI-1)] and the microfilament-associated proteins actin, α -actinin and tropomyosin isoform-1 (Tannenbaum, 1986; Higgins & Ryan, 1992). CD-induced p52(PAI-1) and actin biosynthesis was accompanied by increases in mRNA abundance and inhibited by actinomycin D (Sympson & Geoghegan, 1990; Higgins *et al.*, 1989, 1990; Higgins & Ryan, 1992), suggesting involvement of transcriptional events. Explicating molecular mechanisms underlying such cell-shape-associated reprogramming of gene expression, however, is complicated by recent findings that CD modulates biological responses to specific cytokines or growth factors. Indeed, CD enhances interferon- γ -mediated expression of class II major-histocompatibility-complex genes (Ulevitch *et al.*, 1991), augments neutrophil/monocyte responses to formylated peptides (Honeycutt & Niedel, 1986; Jesaitis *et al.*, 1988) and stimulates epidermal-growth-factor-

induced transin gene expression (Matrisian *et al.*, 1986). Furthermore, PAI-1 synthesis is known to be modulated (often in a cell-type-dependent manner) by various serum-derived growth factors (Santaren & Bravo, 1987; Sporn *et al.*, 1987; Keski-Oja *et al.*, 1988; Westerhausen *et al.*, 1991). CD-hyperinduced p52(PAI-1) mRNA/protein accumulation, in fact, was originally defined in serum-supplemented cultures of normal rat kidney (NRK) cells (Higgins *et al.*, 1989, 1990). To clarify mechanisms which modulate p52(PAI-1)-gene expression, therefore, experiments were designed to evaluate the potential contribution of serum to the CD-associated p52(PAI-1)-inductive response in NRK cells.

MATERIALS AND METHODS

Cell culture, morphological assessments and immunofluorescence

NRK cells (epitheloid clone 52E) were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal-bovine serum (FBS) (GIBCO, Grand Island, NY, U.S.A.) (Ryan & Higgins, 1988). CD (from *Zygosporium mansonii*) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was prepared as a 10 mM stock in dimethyl sulphoxide (DMSO) and diluted in growth medium (final concns. 1–100 μ M). DMSO without added CD served as control. Cell-shape changes were assessed using the high-affinity F-actin probe rhodamine (Rh)-labelled phalloidin (Wulf *et al.*, 1979; Verderame *et al.*, 1980). Cells were fixed *in situ* for 20 min in 3.7% formaldehyde in phosphate-buffered saline (PBS; 0.14 M-NaCl/8.1 mM- $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ /2.7 mM-KCl/1.1 mM- KH_2PO_4 /0.9 mM- CaCl_2 /0.5 mM- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), rinsed, then extracted for 20 min with 1% Nonidet P40/PBS (Wahrman *et al.*, 1985). After

Abbreviations used: p52(PAI-1), plasminogen-activator inhibitor type-1; CD, cytochalasin D; NRK, normal rat kidney; FBS, fetal-bovine serum; DMSO, dimethyl sulphoxide; Rh, rhodamine; PBS, phosphate-buffered saline; TN, Tris/NaCl; CSK, cytoskeletal; HBSS, Hanks' balanced salt solution; SAP, saponin-resistant protein; i.e.f., isoelectric focusing; SSC, saline/sodium citrate; AUBF, adenyluridine-binding factor.

Fig. 1. Induced changes in cellular microfilament organization and progressive morphological rounding of NRK cells as a function of CD concentration

NRK cultures were treated with various concentrations of CD (1–100 μM ; control cells received DMSO vehicle alone) for 24 h. Actin microfilaments were revealed by epifluorescence microscopy of Rh-phalloidin-stained cells; magnification $\times 325$.

a final PBS rinse, cells were incubated in Rh-phalloidin (1 $\mu\text{g}/\text{ml}$ in PBS) for 2 h at 37 $^{\circ}\text{C}$, rinsed, and coverslips mounted in 50% (v/v) glycerol/PBS (Ryan & Higgins, 1988). Localization of p52(PAI-1) in PBS-rinsed, methanol-fixed, NRK and NRK/CD cells utilized p52(PAI-1)-specific antisera (Higgins *et al.*, 1990) and indirect immunocytochemical procedures described previously (Higgins & Ryan, 1991). In certain instances, washed cells were extracted *in situ* with Tris/NaCl (TN)/Triton buffer [140 mM-NaCl/10 mM-Tris/HCl (pH 7.6)/1% Triton X-100] (Higgins & Ryan, 1991) at 4 $^{\circ}\text{C}$ for 5 min; substrate-adherent cytoskeletal (CSK) fractions were fixed for immunocytochemistry with 3.7% formaldehyde in PBS.

Metabolic labelling and cell extraction

NRK cells were cultured in 35 mm-diameter dishes for treat-

Fig. 2. CD-concentration-dependence of induced p52(PAI-1) accumulation into the NRK cellular secreted protein and SAP fraction compartments

Equivalent numbers of NRK cells were treated with the indicated concentration of CD for 24 h, labelled for 6 h with [^{35}S]methionine, and aliquots of conditioned labelling medium (SP) and SAP residues fractionated by gel electrophoresis. Regions of the fluorographs representing p52(PAI-1) (above) were scanned and p52(PAI-1) content expressed as arbitrary densitometric units (DU). Results are means of two independent determinations; the bars indicate ranges of measurements.

ments indicated in the Results section. Media were removed, the monolayers washed twice with Hanks' balanced salt solution (HBSS; 0.14 M-NaCl/4.0 mM- NaHCO_3 /0.3 mM- Na_2HPO_4 , 7H $_2\text{O}$ /0.4 mM- MgSO_4 , 7H $_2\text{O}$ /0.3 mM- KH_2PO_4 /5.0 mM-KCl/0.5 mM- MgCl_2 , 6H $_2\text{O}$ /1.3 mM- CaCl_2 /5.6 mM-glucose) and 1 ml of labelling medium (FBS- and methionine-free RPMI 1640 medium containing 50 μCi of [^{35}S]methionine; sp. activity > 1100 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) added to each culture. After 3–6 h, conditioned media containing [^{35}S]methionine-labelled secreted proteins were harvested and clarified at 13000 g. Monolayers were washed twice with HBSS. Subcellular fractions enriched in cell-substratum contact regions and associated ventral carpet material [saponin-resistant protein (SAP) fractions] (Higgins & Ryan, 1989) were prepared using saponin (0.2%, w/v) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (Higgins *et al.*, 1989, 1991; Neyfakh & Svitkina, 1983). SAP residues were solubilized in one-dimensional-electrophoresis sample buffer [50 mM-Tris/HCl (pH 6.8)/10% (v/v) glycerol/1% SDS/1% (v/v) 2-mercaptoethanol] (Ryan & Higgins, 1988) and boiled. Aliquots (5 μl) of conditioned media or solubilized SAP residues were diluted (in triplicate assays) in 0.5 ml of BSA (0.1 mg/ml) and precipitated with 5 ml of ice-cold 10% (v/v) trichloroacetic acid. After vigorous mixing, solutions were placed on ice for 30 min then filtered through Millipore HA 0.45 μm -pore-size filters which were subsequently washed three times with cold 10% (w/v) trichloroacetic acid, dried, and counted for radioactivity.

Gel electrophoresis

One-dimensional slab gels (SDS/10% acrylamide) were loaded with 25000 c.p.m. of trichloroacetic acid-insoluble secreted or SAP fraction protein/lane in electrophoresis sample buffer. After fluorography, individual protein bands were quantified using the Molecular Dynamics Image Quant system. For two-dimensional electrophoresis, aliquots of conditioned media containing

Fig. 3. Immunocytochemical localization of p52(PAI-1) in NRK and NRK/CD cells

Panel A, p52(PAI-1) in methanol (MEOH)-fixed control NRK cells was non-homogeneously localized, by careful focusing, to the ventral undersurface region. Panel B, similar substratum concentration was evident in NRK/CD cells with additional immunoreactivity evident in the rounding cell body. Panel C, removal of the rounded cell body by forceable pipetting retained p52(PAI-1) in the cell-free substrate-attached matrix. Panel D, extraction of NRK/CD cells with TN/Triton buffer to generate CSK fractions before immunocytochemical manipulations successfully removed cell-body-associated immunoreactive p52(PAI-1), although cell-body structures proper were still evident in such CSK preparations, but had no effect on substrate-deposited p52(PAI-1). Two nuclei are evident in each rounded NRK cell as CD, in the concentration used, prevents cytokinesis but does not affect DNA synthesis and subsequent nuclear division. Panel E, high-power examination confirmed that TN/Triton extraction did, indeed, remove cell-body-associated p52(PAI-1) in rounded NRK/CD cells. In panel A the plane of focus is at the ventral undersurface; in panels B–E the focus is at the centre of the cell body. Treatment with CD (100 μ M) was for 24 h.

5×10^5 c.p.m. of trichloroacetic acid-insoluble secreted protein (diluted in first-dimension buffer; 9.8 M-urea/2 % Nonidet P40/2 % pH 7–9 Ampholytes/100 mM-dithiothreitol) (Ryan & Higgins, 1988; Ryan *et al.*, 1989) were separated on pre-run 1.5 mm-diameter tube gels (9.8 M-urea/5 % 2-mercaptoethanol/2 % Nonidet P40/6 % Ampholytes pH 5–7/1.2 % Ampholytes pH 3–10); isoelectric focusing (i.e.f.) was for 18 h before molecular-mass separation on SDS/10 % acrylamide slab gels (Ryan & Higgins, 1988).

mRNA and nuclear-run-off transcription analyses

Cytoplasmic extracts from control and CD-treated cells were diluted in an equal volume of buffer [0.2 M-Tris/HCl (pH 7.5)/25 mM-EDTA/0.3 M-NaCl/2 % SDS], containing proteinase K (200 μ g/ml) and incubated at 37 °C for 30 min. Proteins were extracted with phenol/chloroform, and nucleic acids were precipitated from the aqueous phase with ethanol for digestion with DNAase I (Favaloro *et al.*, 1980; Higgins *et al.*, 1990). RNA was collected by ethanol precipitation at –20 °C, ribosomal RNA in each sample quantified by agarose-gel electrophoresis followed by staining with ethidium bromide (White *et al.*, 1992), and 20 μ g of RNA slot-blotted to nitrocellulose in 20 $\times$ SSC (1.5 M-NaCl/0.15 M-sodium citrate, 2H₂O, pH 7.5) (Higgins *et al.*, 1990),

followed by vacuum baking at 80 °C for 4 h. The endonuclease-*Eco*RI-linearized phagemid pSS1-3, containing a 2.5 kb p52(PAI-1) cDNA insert in the pBSK(–) vector (Zeheb & Gelehrter, 1988; Higgins *et al.*, 1990) was labelled with [³²P]dCTP by random-primer extension and separated free from unincorporated label on nick columns (Pharmacia LKB, Piscataway, NJ, U.S.A.). Hybridization conditions and washing of blots were as described by Higgins *et al.* (1990) and Higgins & Ryan, (1992). For assessment of p52(PAI-1)-gene transcription, 10⁷ control or CD-treated cells were lysed by vortex-mixing in 10 mM-Tris/HCl buffer, pH 8.4, containing 10 mM-NaCl, 2 mM-MgCl₂, 0.5 % Nonidet P40 and 10 mM-vanadyl ribonucleoside complexes; nuclei were collected by centrifugation through 24 % (w/v) sucrose at 10000 *g* for 20 min (Higgins *et al.*, 1985). Isolated nuclei were washed in cold 20 mM-Tris buffer, pH 8.1, containing 20 % glycerol, 140 mM-KCl, 5 mM-MgCl₂, 1 mM-MnCl₂ and 14 mM-2-mercaptoethanol, then incubated at 25 °C for 15 min in 2 vol. of reaction buffer [10 mM-Tris/HCl (pH 8.0)/5 mM-MgCl₂/300 mM-KCl/0.5 mM each ATP, CTP and GTP/[³²P]UTP (100 μ Ci; 800 Ci/mmol)] (Greenberg & Ziff, 1984) to allow for nascent RNA chain elongation. Nuclei were lysed, RNAase-free DNAase (2 μ g/ml) was added, and RNA extracted with phenol/chloroform/3-methylbutan-1-ol (Clayton *et al.*,

1985; Higgins, 1991). pSS1-3 or pBSK(–) vector alone was blotted to nitrocellulose in $6 \times \text{SSC}$ ($10 \mu\text{g}$ of DNA/slot) and baked. Hybridization of labelled nuclear RNA (5×10^6 input c.p.m./ml) to membranes containing immobilized DNA and digestion with RNAase A, followed by proteinase K, was done as described by Clayton *et al.* (1985). Autoradiographs were scanned, and the p52(PAI-1) transcriptional signal was calculated by subtracting hybridization values due to pBSK(–) alone.

RESULTS

To evaluate the relationship between cell shape and p52(PAI-1) expression, NRK cultures were treated with various concentrations ($1\text{--}100 \mu\text{M}$) of CD for 24 h. One set of cultures was used for assessment of cell morphology, whereas the second group was labelled with [^{35}S]methionine. Both CD-mediated cell shape and p52(PAI-1)-inductive responses were dose-dependent (Figs. 1 and 2). Low levels ($1 \mu\text{M}$) of CD did not significantly alter cell morphology or accumulation of p52(PAI-1) in either the SAP or secreted protein compartments. Higher concentrations of CD ($2\text{--}100 \mu\text{M}$) were associated with progressively greater cell rounding (Fig. 1), paralleling increases in p52(PAI-1) deposition into both SAP and secreted protein fractions (Fig. 2). p52(PAI-1) specifically accumulated at the ventral undersurface region of NRK cells; in NRK/CD cultures this distribution *in situ* was maintained as p52(PAI-1) localized within the original cellular boundaries (Fig. 3). Whereas the rounding cell body did contain immunoreactive p52(PAI-1), such cellular p52(PAI-1) was soluble in TN/Triton buffer; matrix-associated p52(PAI-1) was not. These data directly confirm previous biochemical assessments of p52(PAI-1) cellular partitioning, which indicated that, once deposited into the saponin-resistant-matrix, p52(PAI-1) acquires the property of detergent-insolubility (Higgins & Ryan, 1992).

Long-duration (24 h) kinetic assessments (designed to avoid long periods of methionine deprivation) indicated that p52(PAI-1) SAP fraction levels increased within 4–5 h after CD addition (Fig. 4a). Maximal morphological change, assessed by phase-contrast microscopy, occurred even earlier (i.e. transition from 25% to > 60% round cells by 2 h of CD treatment). These data placed the peak window of newly synthesized p52(PAI-1) SAP fraction deposition (estimated by scanning densitometry to be 15–22-fold that of control) between 6 and 8 h after addition of CD. After this time, the p52(PAI-1) SAP fraction content declined slightly, but still remained elevated (7–10-fold) throughout the remaining 24 h exposure period relative to non-CD-treated cells at equivalent population densities. In contrast, the p52(PAI-1) content in non-CD-stimulated cultures never increased over basal levels (e.g., $0 \mu\text{M}$ -CD; Fig. 2). p52(PAI-1) levels in the culture medium increased to their highest accumulated values by 9 h of CD treatment and remained constant thereafter (Fig. 4a). Augmented medium levels thus had a lag of 1–3 h relative to SAP fraction p52(PAI-1) accumulation, consistent with previous assessments of p52(PAI-1) half-life in the NRK saponin-resistant matrix (Higgins & Ryan, 1989). This 6–8 h window of maximal CD-induced p52(PAI-1) protein deposition into the cellular SAP fraction coincided with a peak in cytoplasmic p52(PAI-1) mRNA abundance occurring 6–8 h after CD addition (Fig. 4b). Although p52(PAI-1) mRNA levels did decline subsequently, p52(PAI-1) mRNA abundance in 12–24 h-CD-treated NRK cells remained elevated (approx. 6-fold) relative to NRK controls. Enhanced p52(PAI-1) mRNA abundance at this time point paralleled a 5-fold increase in gene transcription (Fig. 5). The decline in SAP fraction p52(PAI-1) content after 9 h of CD treatment (Fig. 4a) would appear to be due to the coupled decrease in mRNA abundance (Fig. 4b) and short half-life of p52(PAI-1) in the

Fig. 4. Increased p52(PAI-1) protein deposition and mRNA abundance in NRK cells as a function of time of exposure to CD

(a) Kinetics of p52(PAI-1) accumulation in the secreted protein and SAP fractions of NRK/CD cells. Aliquots of conditioned labelling media (SP) and SAP fractions containing 25000 c.p.m. of trichloroacetic acid-insoluble protein, derived from CD ($100 \mu\text{M}$)-treated cultures at the indicated time points (followed by a 3 h labelling period), were fractionated by SDS/PAGE. p52(PAI-1) protein bands were revealed by fluorography (insets) and quantified by scanning densitometry. Results are means $\pm$ s.d. of triplicate scan values presented in arbitrary densitometric units. $\circ$, SP values; $\bullet$, SAP values. (b) Cytoplasmic p52(PAI-1) mRNA content in NRK cells also increased as a function of time of exposure to CD. Relative p52(PAI-1) mRNA abundance in NRK/CD cells was determined by slot-blot hybridization (inset). The hybridization signal was quantified by scanning densitometry and the fold increase in specific mRNA expressed relative to zero-time value. Results are means $\pm$ s.d. of triplicate determinations.

Fig. 5. Increased p52(PAI-1)-gene transcription and steady-state mRNA levels in 24 h-CD (100 μ M)-treated NRK cells

p52(PAI-1) cytoplasmic mRNA abundance and gene-transcriptional activity were assessed by slot-blot hybridization and run-off transcription assays respectively. Autoradiographs were densitometrically scanned and p52(PAI-1) mRNA cytoplasmic abundance and transcriptional activity expressed in relative absorbance units. Results are averages of duplicate determinations. □, Control untreated NRK cells; ▨, CD-treated cells.

matrix (Higgins & Ryan, 1989). It cannot be excluded, however, that CD-induced cell rounding and subsequent disruption of the relatively 'protected' cellular undersurface region may influence the stability of matrix-associated p52(PAI-1) protein.

Previous studies on p52(PAI-1) induction utilized treatment strategies involving exposure of NRK cells to CD for 24 h (before labelling) in the continued presence of serum growth factors [i.e. 10% (v/v) FBS]. Since cell shape and growth-factor-responsiveness may be interrelated (see, e.g., Ulevitch *et al.*, 1991), it was necessary to assess the potential contribution of serum-derived growth factors to the CD-mediated p52(PAI-1) inductive response. Initial experiments indicated that CD could induce p52(PAI-1) expression in post-confluent NRK cells, even

under serum-free (0% FBS) conditions. To further define conditions of p52(PAI-1) induction by CD, the quiescent (i.e., growth-factor-deprived) NRK-cell system, in which p52(PAI-1) synthesis is down-regulated to virtually undetectable levels (Ryan & Higgins, 1992), was used to assess whether CD stimulated p52(PAI-1) expression through a growth-factor-dependent or independent transduction pathway. Cells were cultured under standard conditions in medium supplemented with either 10% or 20% FBS and then changed to the treatment protocols indicated (Table 1). Cells cultured under standard growth conditions (10 or 20% FBS) had similar levels of SAP-fraction-associated p52(PAI-1). Growing cells (in either 10 or 20% FBS) or cells made quiescent (0.5% FBS for 48 h) were inducible for enhanced p52(PAI-1) expression upon exposure to CD under fully-growth-factor-supplemented culture conditions (10/20% FBS) as well as under partially (0.5% FBS) or completely (0% FBS) growth-factor-deficient conditions. Although there was some variation in the levels of response, induced SAP fraction p52(PAI-1) accumulation was as efficient when exposure to CD occurred under growth-factor-deficient conditions as when CD was added simultaneously with serum growth factors (Table 1). Two-dimensional electrophoretic analysis confirmed that the 52 kDa protein induced by CD alone, serum alone or CD + serum was, in fact, p52(PAI-1) (Fig. 6).

DISCUSSION

The present data illustrate several important points relative to the potential cell-shape-dependency of p52(PAI-1) gene control in NRK cells. Although augmented synthesis of this protein in response to CD has previously been shown to occur in the continued presence of serum growth factors (Higgins *et al.*, 1989, 1990), modulation of NRK cell shape (in response to CD) is clearly sufficient to induce p52(PAI-1) expression in quiescent cells without the concomitant addition of serum. Two-

Table 1. Relative efficiency of p52(PAI-1) induction by CD or serum in NRK cells

Group	Treatment*	Relative SAP fraction content†		
		Actin‡	p50	p52(PAI-1)
1	10% FBS	10.67 ± 0.08	0.25 ± 0.01	1.83 ± 0.03§
2	CD + 10% FBS (24 h)	10.55 ± 0.11	4.79 ± 0.06	30.84 ± 0.08
3	CD + 0% FBS (24 h)	10.99 ± 0.69	2.76 ± 0.08	19.30 ± 0.07
4	0% FBS (24 h)	3.66 ± 0.07	0.21 ± 0.02	0.23 ± 0.04
5	0.5% FBS (48 h)	2.98 ± 0.12	0.15 ± 0.03	0.19 ± 0.02
6	0.5% FBS (24 h)			
7	0.5% FBS (48 h)	3.21 ± 0.05	2.22 ± 0.07	16.26 ± 0.07
	CD + 0.5% FBS (24 h)			
8	0.5% FBS (48 h)	9.29 ± 0.18	6.43 ± 0.06	32.33 ± 0.16
	CD + 0% FBS (24 h)			
9	0.5% FBS (48 h)	13.78 ± 0.13	0.77 ± 0.04	13.28 ± 0.04
	20% FBS (24 h)			
10	0.5% FBS (48 h)	2.39 ± 0.05	2.37 ± 0.05	28.36 ± 0.14
	CD + 20% FBS (24 h)			
11	20% FBS	5.46 ± 0.08	1.29 ± 0.05	1.07 ± 0.06§
12	20% FBS (24 h)	5.13 ± 0.07	1.68 ± 0.04	1.15 ± 0.03§
	CD + 20% FBS (24 h)	8.05 ± 0.06	2.23 ± 0.05	20.43 ± 0.10

*All cultures were grown to 80% confluency in either 10% (groups 1–9) or 20% (groups 10–12) FBS-containing medium. Cultures were then switched to the treatment conditions indicated. Groups 5–9 were made quiescent by growth-factor-deprivation (0.5% FBS for 48 h) as a first treatment, followed by a subsequent treatment (for 24 h) as indicated.

†Gels were loaded with 25000 c.p.m. of trichloroacetic acid-insoluble SAP fraction protein. Actin, p50 and p52(PAI-1) bands were quantified by scanning densitometry (data are expressed in arbitrary densitometric units).

‡Residual actin associated with saponin-resistant focal-contact structures (Higgins & Ryan, 1989).

§Constitutive SAP fraction levels of p52(PAI-1) in either 10 or 20% FBS-containing control cultures never exceeded the arbitrary densitometric units indicated.

Fig. 6. Two-dimensional electrophoretic identification of the induced 52 kDa protein in growth-factor- and/or CD-treated NRK cells as p52(PAI-1)

As is evident from analysis of the secreted protein fraction of growing cultures (top four panels), quiescent cultures (middle five panels) or growing cultures maintained in 20% FBS (bottom three panels) (all culture conditions established before the indicated treatments, i.e., those described in Table 1), exposure to CD with or without concomitant addition of FBS was sufficient to induce the full complement of p52(PAI-1) isoforms. These include the unglycosylated 'core' protein (p43), several 50 kDa intermediate glycosylated derivatives (p50) and the various mature 52 kDa (p52) glyco-derivatives [see Higgins *et al.* (1990) for a full description of isoforms]. Abbreviation: M, molecular mass.

dimensional electrophoretic mapping not only confirmed the identity of the 52 kDa protein produced by NRK/CD cells under serum-free stimulation as, in fact, p52(PAI-1) but also

illustrated the competence of these cells to carry out normal post-translational processing of this protein in a growth-factor-deficient environment. Elevated p52(PAI-1) levels, moreover,

were not simply due to increased deposition (into the SAP compartment) or enhanced release (into the conditioned labelling medium) on the part of NRK/CD cells. Indeed, induction was both accompanied by transcriptional activation of the p52(PAI-1) gene (the present paper) and effectively blocked by inhibitors of RNA synthesis (see, e.g., Higgins *et al.*, 1989, 1990). The increased p52(PAI-1) mRNA abundance evident at 24 h of continuous CD exposure closely approximated the nuclear-run-off rate at the same time point. Enhanced p52(PAI-1) biosynthesis by NRK cells in response to CD, as is true for an increasing variety of growth factors active in other cell types (Loskutoff & Mimuro, 1989), thus is primarily a transcriptional event at least at 24 h of exposure. Assessment of p52(PAI-1) gene transcription at earlier times (i.e., 4–10 h) after addition of CD was not possible, owing to the extreme fragility of NRK nuclei isolated during these initial phases of treatment (CD may transiently destabilize the nuclear actin skeleton). It is possible, therefore, that increased mRNA stability may partly contribute to the elevated p52(PAI-1) mRNA abundance seen during the peak window of 6–8 h. In this regard, CD does stimulate *c-fos*-gene expression in HeLa cells through a complicated mechanism involving increases in both transcription and mRNA stability (Zambetti *et al.*, 1991). Analysis of the 3' untranslated region of the rat p52(PAI-1) cDNA (Zeheb & Gelehrter, 1988), using the IBI/Pustell subsequence similarity search program, has revealed three sites (positions 1935, 1999 and 2610 relative to the translation start codon) of sequence identity to the instability-associated pentanucleotide sequence AUUUA, as well as nine and six additional sites of 80% similarity corresponding to the sequences AUUU(N) and (N)UUUA respectively. Interaction of the AU (adenyluridine) binding factor (AUBF) with multiple reiterations of AUUUA motifs has been suggested as one possible mechanism to increase the half-life of intrinsically unstable mRNAs (Gillis & Malter, 1991). Indeed, phorbol esters stabilize certain labile mRNAs and also increase AUBF activity (Mitchell *et al.*, 1985; Malter & Yin, 1991). Whether CD increases p52(PAI-1) mRNA abundance within the peak period of 6–8 h solely through transcriptional mechanisms, as appears to be the case at later (24 h) time periods, or partly by induced changes in transcript stability (perhaps involving AUBF complexes) requires further study.

Increased *c-fos*-gene expression by CD suggests one molecular mechanism by which this cell-shape-modulating agent may affect p52(PAI-1) transcription. As is the case in HeLa cells, CD stimulates both FOS and JUN (AP-1) protein expression in NRK cells; induction closely paralleled the time course of augmented p52(PAI-1) expression and remained elevated for at least 24 h after initial addition of CD (P. J. Higgins & M. P. Ryan, unpublished work). Cell-shape change, therefore, may impart at least one transduction signal, perhaps through disruption of integrin-extracellular matrix complexes (Werb *et al.*, 1989; Kornberg *et al.*, 1991), resulting in activation of genes encoding specific transcriptional control factors (e.g., *fos* and *jun*) (Shaw *et al.*, 1990; Yamada *et al.*, 1991). Indeed, several known inducers of p52(PAI-1) synthesis (i.e., sodium butyrate, transforming growth factor β and platelet-derived growth factor), in addition to CD, activate (at least transiently) members of the *fos/jun*-gene families (reviewed by Higgins & Tanaka, 1991; Higgins, 1992). Such events may be particularly relevant to transcriptional control of p52(PAI-1)-gene expression. Sequence analysis of the 5' flanking region of the rat p52(PAI-1) gene has revealed various potential regulatory sequences, including a concentration of FOS/JUN (AP-1)-like binding sites (Bruzdzinski *et al.*, 1990). At least seven regions of approx. 85% sequence similarity to the AP-1 consensus sequence (TGACTCA) have been identified in the p52(PAI-1) promoter, in both (+) and

(–) position orientations, spanning the upstream region from –592 to –2112 nt. Experiments are required to determine whether occupancy of such AP-1-like sites by FOS/JUN heterodimers or JUN/JUN homodimers actually stimulates p52(PAI-1)-gene transcription in response to CD-initiated cell-shape change.

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