Cell-shape-dependent modulation of p52(PAI-1) gene expression involves a secondary response pathway

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Expression of the rat p52(PAI-1) gene is positively regulated by agents that influence cellular microfilament organization and/or cell-to-substrate adhesion [e.g. cytochalasin D (CD) and sodium n-butyrate (NaB)] [Higgins, Chaudhari and Ryan (1991) *Biochem. J.* **273**, 651–658; Higgins, Ryan and Providence (1994) *J. Cell. Physiol.* **159**, 187–195]. As shape-responsive genes may be subject to inducer-specific controls, the biochemical mechanisms underlying the shape-dependent pathway of p52(PAI-1) gene regulation were examined in *v-ras*-transformed rat kidney (KNRK) cells. NaB and/or CD effectively stimulated p52(PAI-1) run-off transcription and augmented *de novo* p52(PAI-1) mRNA and protein synthesis in KNRK cells; induction at both

the mRNA and protein levels was inhibited by actinomycin D. Pretreatment with cycloheximide (CX) markedly attenuated NaB- and/or CD-stimulated p52(PAI-1) expression. CX alone, however, induced low levels of p52(PAI-1) mRNA; increased p52(PAI-1) protein synthesis was evident after release of KNRK cells from CX blockade. Such CX-mediated induction was also sensitive to actinomycin D. Full stimulation of p52(PAI-1) expression in KNRK cells in response to the shape modulators NaB and/or CD involves transcriptional activation of the p52(PAI-1) gene, requires *de novo* RNA synthesis and occurs through a secondary-response (i.e. protein-synthesis-dependent) pathway.

INTRODUCTION

Plasminogen activator inhibitor type 1 (PAI-1) regulates the activity of urokinase plasminogen activator, serving thereby as an effective modulator of the plasmin-based pericellular proteolytic cascade (Laiho et al., 1987; Laiho and Keski-Oja, 1989). Rat p52(PAI-1) is a complex glycoprotein which exists in at least 14 distinct isoforms (Higgins et al., 1994), probably as a result of differential utilization of four potential N-linked glycosylation sites (Zeheb and Gelehrter, 1988). The combined nomenclature 'p52(PAI-1)' therefore specifies the considerable microheterogeneity evident in the 'core' (43 kDa), intermediate glycosylated (50 kDa) and mature (52 kDa) variants of p52(PAI-1) (Higgins et al., 1994). Although the significance of such variation is unknown, potential cellular functions of p52(PAI-1) have been addressed by evaluating the consequences of expression perturbation. Indeed, rat cells transformed with the activated human *EJras*^{val-12} or retroviral *v-K-ras* oncogenes are deficient in p52(PAI-1) expression (Cohen et al. 1989; Higgins and Ryan, 1989; Newman et al., 1990; Higgins et al., 1991). This deficit might affect urokinase plasminogen activator-localized proteolytic activity at cell-to-substrate focal contacts (Pollanen et al., 1987, 1988), thus contributing to the reduced adhesivity and pleomorphism typical of *ras* transformants. The apparent requirement for p52(PAI-1) expression and its accumulation in extracellular matrix during generation of flat revertants of *v-ras*-transformed rat kidney (KNRK) cells in response to sodium butyrate (NaB) (Higgins and Ryan, 1989, 1991) is consistent with this hypothesis. The specific cytoarchitectural rearrangements

that accompany development of the revertant phenotype (i.e. elaboration of fine microfilament alignments, formation of vinculin-containing focal adhesions) (Altenburg et al., 1976; Ryan and Higgins, 1988; Higgins et al., 1991) may involve a PAI-1-dependent stabilization of induced focal contact sites (Ciambrone and McKeown-Longo, 1990).

p52(PAI-1) appears to function as a morphoregulator in NaB-stimulated KNRK cells (Higgins and Ryan, 1991). In this context, it is of interest that the expression of genes involved in the determination of cellular morphology are often regulated in a shape-dependent manner (Ben-Ze'ev, 1992). The p52(PAI-1) gene may also be subject to such control, as compounds that alter the morphology of normal rat kidney (NRK) cells and their transformed derivatives [e.g. the microfilament-disrupting fungal metabolite cytochalasin D (CD)] effectively induce, as does NaB, p52(PAI-1) expression in this cell system (Chaudhari et al., 1990; Higgins et al., 1990, 1994). The mechanisms underlying shape-regulated p52(PAI-1) expression are unknown; their clarification is important in the light of recent findings implicating cell-to-substrate adhesion and/or cytoskeletal organization in p52(PAI-1) gene control (Ryan and Higgins, 1994; Higgins and Ryan, 1994). The present paper focuses, therefore, on defining such pathways.

MATERIALS AND METHODS

Cell culture

NRK and KNRK cells were grown in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (Higgins and Ryan,

Abbreviations used: NaB, sodium butyrate; CD, cytochalasin D; KNRK cells, *v-ras*-transformed rat kidney cells; PAI-1, plasminogen activator inhibitor type 1; hPAI-1, human PAI-1; p52(PAI-1), p52 plasminogen activator inhibitor type 1; NRK cells, normal rat kidney cells; CX, cycloheximide; ACT D, actinomycin D; SP, secreted proteins; SAP fraction, saponin-resistant fraction; 1 × SSC, 1.5 M NaCl plus 0.15 M sodium citrate, 2H₂O, pH 7.5; TGF-β, transforming growth factor β.

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1989). Exposure to NaB (2 mM) and/or CD (100 μ M) was for 24 h unless indicated otherwise. Actinomycin D (ACT D) and cycloheximide (CX) were used at 5 μ g/ml and 50 μ M respectively (Ryan and Higgins, 1993). To inhibit N-linked glycosylation, tunicamycin was added to the culture medium (2 μ g/ml final concentration) 24 h before and during metabolic labelling (Higgins and Ryan, 1991). Primary cultures of normal human epidermal keratinocytes were prepared as described by Staiano-Coico et al. (1986).

Metabolic labelling, cell extraction and gel electrophoresis

Cells were washed twice with Hank's balanced salt solution (1.3 mM CaCl_2 , 5 mM KCl, 0.3 mM KH_2PO_4 , 0.5 mM MgCl_2 , $6\text{H}_2\text{O}$, 0.4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.14 M NaCl, 4 mM NaHCO_3 , 0.3 mM Na_2HPO_4 , 5.6 mM glucose) then labelled in serum-/methionine-free RPMI 1640 medium containing 50 μ Ci of [^{35}S]methionine/ml (specific radioactivity 1100 Ci/mmol) (Ryan and Higgins, 1993). Cells treated with NaB or CD were labelled in their presence unless indicated. Pre-exposure to ACT D or CX was for 1 h or 15 min respectively before addition of inducer. Cellular protein synthesized *de novo* was assessed as described by Higgins and Ryan (1991). The conditioned labelling medium, containing secreted proteins (SP), was removed, centrifuged at 13000 g, diluted with an equal volume of one-dimensional-electrophoresis sample buffer (50 mM Tris/HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol) and boiled. Monolayers were washed with Ca^{2+} / Mg^{2+} -free PBS (2.7 mM KCl, 1.2 mM KH_2PO_4 , 0.14 M NaCl, 8.1 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) and extracted with 0.2% (w/v) saponin in Ca^{2+} / Mg^{2+} -free PBS to isolate cell-substratum contact regions and associated ventral undersurface material (Higgins et al., 1990, 1991). These saponin-

resistant (SAP fraction) residues were scraped into one-dimensional-electrophoresis sample buffer and boiled or solubilized directly in two-dimensional-electrophoresis sample buffer (9.8 M urea, 2% Nonidet P-40, 2% pH 7-9 Ampholytes, 100 mM dithiothreitol). Equivalent c.p.m. of trichloroacetic acid-insoluble SP or SAP-fraction protein were separated by SDS/PAGE (10% acrylamide slab gels) (Ryan and Higgins, 1988). Labelled protein bands were visualized by fluorography and quantified by computerized densitometry (Smith et al., 1992). Identification of the rat p52(PAI-1) protein in one-dimensional-electrophoretic separations utilized criteria described previously (Higgins et al., 1989, 1990). The position of the 50 kDa human PAI-1 in one-dimensional separations of epidermal proteins was confirmed by mobility comparison with that of PAI-1 from human endothelial cells and fibroblasts (Higgins and Smith, 1993; Smith and Higgins, 1993; Higgins et al., 1991) as well by immunochemical reactivity with PAI-1-specific antibodies (Higgins et al., 1990). For two-dimensional analysis, 10^5 c.p.m. of trichloroacetic acid-insoluble SAP-fraction protein was solubilized in two-dimensional-electrophoresis sample buffer and separated first by isoelectric focusing on pre-run 1.5 mm-diameter tube gels (9.1 M urea, 2% Nonidet P-40, 6% pH 5-7 Ampholytes, 1.2% pH 3-10 Ampholytes) and then by SDS/PAGE (Ryan et al., 1989).

Analysis of mRNA and nuclear run-off transcription

Cytoplasmic fractions from control and treated cells were diluted in an equal volume of digest 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 $^{\circ}\text{C}$ for 30 min. Proteins were extracted with phenol/chloroform, nucleic acids were ethanol-

Figure 1 Differential levels of p52(PAI-1) in the SAP fractions of NRK and KNRK cells

Immunofluorescence examination of methanol-fixed SAP fractions clearly indicated that p52(PAI-1) was restricted to the undersurface periphery of individual NRK cells (a). p52(PAI-1) is a major component of the [^{35}S]methionine-labelled SAP-fraction protein complement of NRK cells; in contrast, only low levels of p52(PAI-1) are evident in the SAP fraction of KNRK cells (b). Metabolic labelling in the presence of the N-linked glycosylation inhibitor tunicamycin (t) reduces the molecular mass of the mature glycosylated 52 kDa p52(PAI-1) protein to 43 kDa (p43) [see Higgins et al. (1990, 1994) for two-dimensional map profiles of the mature, intermediate and core species of p52(PAI-1)]. The abundant and minor SAP-fraction p52(PAI-1) proteins of NRK and KNRK cells respectively were reduced to 43 kDa bands of corresponding relative intensity. The low level of p52(PAI-1) protein evident in the SAP fraction of KNRK cells thus represents decreased deposition and is not simply an artifact of misidentification due to aberrant N-linked glycosylation.

precipitated from the aqueous phase for digestion with DNAase I (Higgins et al., 1990) and RNAs were quantified by agarose-gel electrophoresis followed by staining with ethidium bromide (Higgins and Ryan, 1991). RNA was slot- or dot-blotted to nitrocellulose in $20 \times \text{SSC}$ ($1 \times \text{SSC}$ is $0.15 \text{ M NaCl}/0.015 \text{ M}$ sodium citrate, $2\text{H}_2\text{O}$, pH 7.5) in sets of 20, 10, 5 and $2.5 \mu\text{g}$ of RNA/slot, and the membranes were vacuum-baked at 80°C for 4 h. Plasmids for hybridization included pSS1-3, containing a 1.2 kb p52(PAI-1) cDNA insert in the pBSK(–) vector (Higgins et al., 1990; Zeheb and Gelehrter, 1988), pBSK(–) vector without insert and a mouse β -actin cDNA probe (Higgins and Ryan, 1991). Endonuclease *Eco*RI-linearized plasmids were labelled with [^{32}P]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, 1991). For assessment of p52(PAI-1) gene transcription, 10^7 cells were lysed by vortex mixing in 10 mM Tris/HCl buffer, pH 8.4, containing 10 mM NaCl, 2 mM MgCl_2 , 0.5% Nonidet P-40 and 10 mM vanadyl ribonucleoside complexes. Nuclei were collected by centrifugation through 24% (w/v) sucrose at $10000 g$ for 20 min, washed in cold 20 mM Tris/HCl buffer, pH 8.1, containing 20% glycerol, 140 mM KCl, 5 mM MgCl_2 , 1 mM MnCl_2 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 MgCl_2 , 300 mM KCl, 0.5 mM each ATP, CTP and GTP, and $100 \mu\text{Ci}$ of [^{32}P]UTP (800 Ci/mmol)] (Greenberg and Ziff, 1984) to allow for nascent RNA chain elongation. Nuclei were lysed, RNase-free DNase ($2 \mu\text{g}/\text{ml}$) was added, and RNA extracted with phenol/chloroform/3-methylbutan-1-ol (Clayton et al., 1985). pSS1-3 or pBSK(–) vector 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/denatured DNA and digestion with RNase A followed by proteinase K were as described by Clayton et al. (1985). Autoradiographs were scanned and p52(PAI-1) transcriptional signal was calculated by subtracting hybridization values due to pBSK(–) alone (Higgins et al., 1992).

Flow cytometry

Trypsin-treated cells were collected by centrifugation, washed once in PBS, resuspended in 1 ml of PBS and forcefully pipetted into 9 ml of ice-cold 80% (v/v) methanol at a concentration of 10^6 cells/ml. For assay, 10^6 fixed cells were centrifuged and resuspended in PBS containing 1% (w/v) BSA (PBS/BSA); rabbit anti-p52(PAI-1) serum (Higgins et al., 1990) or normal rabbit serum was added to a final dilution of 1:100 for a 1 h incubation on ice. After three PBS/BSA washes, cells were resuspended in 1:100 diluted fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for a subsequent 1 h incubation on ice followed by three PBS/BSA washes. Cells were resuspended in PBS containing $50 \mu\text{g}/\text{ml}$ propidium iodide and 500 units/ml RNase A, placed on ice for 1 h then analysed by flow cytometry.

RESULTS

p52(PAI-1) is a prominent SAP-fraction protein of NRK cells but is significantly reduced in their *ras* transformants (Figure 1). Although deficient in constitutive expression, KNRK cells were inducible for p52(PAI-1) accumulation in response to NaB and/or CD (Figure 2a). Induction, moreover, was not unique to transformed rat cells, as NaB markedly enhanced epidermal

Figure 2 Induction of p52(PAI-1) and hPAI-1 protein by NaB and/or CD

Exposure of KNRK cells to NaB (2 mM) (K/NaB) or CD ($100 \mu\text{M}$) (K/CD) for 24 h effectively stimulated p52(PAI-1) accumulation in the SAP fraction; treatment with both inducers (K/NaB+CD) yielded a significantly greater response than either independently (a). Electrophoresis of p52(PAI-1) derived from the constitutively producing NRK cell line provided an internal standard. The positions of the intermediate glycosylated 50 kDa variant of p52(PAI-1), designated p50 (Higgins et al., 1989, 1990), and the major extracellular protein p60/62 (Higgins and Ryan, 1989) are indicated. Lanes K/CD and K/NaB+CD were intentionally underexposed to illustrate the significant difference in p52(PAI-1) levels between CD and dual-inducer-treated cells. NaB similarly induced PAI-1 accumulation in normal human keratinocyte (NHK) cultures (b). hPAI-1 migrates as a 50 kDa protein as determined by immunoprecipitation (IP) of [^{35}S]methionine-labelled protein from cultured human abdominal and leg fibroblasts (AF, LF). Orbital fibroblasts (OF) produce low levels of hPAI-1 constitutively, thus serving as a negative control, but can be induced to synthesize hPAI-1 when treated with interferon- γ (OF/INF) (Smith et al., 1992; Smith and Higgins, 1993; Higgins and Smith, 1993). The 52 kDa (p52) and 50 kDa (p50) isoforms of rat p52(PAI-1) [expressed by CD-treated NRK cells (Higgins et al., 1990)] and hPAI-1 from human abdominal aortic endothelial (HAAE) cells (Higgins et al., 1991) provide internal standards. hPAI-1 levels in NHK/NaB cultures approximated to that of p52(PAI-1) in K/NaB cells.

Table 1 Protein-synthetic capabilities of control and inducer-treated cells

Portions of total cell extracts (prepared by direct lysis in two-dimensional-electrophoresis sample buffer) were precipitated with trichloroacetic acid for scintillation spectrophotometry. Exposure to NaB (2 mM final concentration) was for 2 days, and exposure to CD ($100 \mu\text{M}$ final concentration) was for 24 h. NHK, normal human epidermal keratinocytes. Data represent means $\pm$ S.D. of 11 determinations normalized to equivalent cell numbers.

Cell type	$10^{-7} \times$ Total cellular [^{35}S]methionine-labelled protein (c.p.m.)
KNRK	1.51 ± 0.50
K/NaB	1.70 ± 0.81
K/CD	1.46 ± 0.67
NHK	3.11 ± 0.28
NHK/NaB	2.93 ± 0.13

expression of the 50 kDa human PAI-1 (hPAI-1) protein (Figure 2b). It was important, however, to determine whether p52(PAI-1) or hPAI-1 substrate accumulation was simply a function of a

Table 2 Effect of inducer treatment on accumulation of protein synthesized *de novo* in the cellular SAP-fraction protein compartment

[³⁵S]Methionine-labelled substrate-associated protein was solubilized in 200 μ l of electrophoresis sample buffer, boiled, and trichloroacetic acid-precipitable c.p.m. determined in a 2 μ l sample. NHK, normal human epidermal keratinocytes. Data represent means $\pm$ S.D. of triplicate assays.

Test compound	10 ⁻² $\times$ SAP-fraction protein (c.p.m.)
KNRK cells	
None	1067 $\pm$ 214
NaB (2 mM final concn.)	987 $\pm$ 238
Dexamethasone (1 μ M final concn.)	1889 $\pm$ 182
TGF- β (10 ng/ml)	2102 $\pm$ 410
CD (100 μ M final concn.)	1293 $\pm$ 308
NaB (2 mM) + CD (100 μ M)	1751 $\pm$ 397
NHK cells	
None	2989 $\pm$ 661
NaB (2 mM final concn.)	2402 $\pm$ 715

generalized increase in SAP-fraction protein in response to NaB or CD. No relationship between SAP-fraction p52(PAI-1) protein content and total cellular (Table 1) or substrate-associated (Table 2) protein/culture was evident for either cell type. Comparison of the effects of NaB and CD with two other inducers of p52(PAI-1) synthesis in KNRK cells, dexamethasone and transforming growth factor β (TGF- β), clearly established that p52(PAI-1) expression stimulated by NaB or CD (unlike dexamethasone or TGF- β) does not reflect changes in levels of total SAP-fraction protein (Table 2). These and previous observations that neither methionine pool size nor the percentage label incorporated was different between control KNRK cells and flat revertants (Higgins and Ryan, 1991) strongly suggest that NaB (2 mM) and CD (100 μ M) do not significantly affect either overall or specifically SAP-fraction protein synthesis in this cell type. A similar conclusion was reached about the effects of NaB and CD in the epidermal and NRK cell systems respectively (Staiano-Coico and Higgins, 1992; Higgins et al., 1994).

p52(PAI-1) mRNA was virtually undetectable in control KNRK cells, consistent with the low levels of p52(PAI-1) protein expressed constitutively (Figure 1), whereas both NaB and CD effectively induced p52(PAI-1) transcripts within 24 h (Figure 3a). In contrast, and despite dramatic differences in cell shape evident among control and treated cultures (e.g. Higgins et al., 1992; Via et al., 1980), actin mRNA abundance was similar in cell types. p52(PAI-1) and hPAI-1 induction at the mRNA (Figure 3b) and protein (below) levels was blocked by pre-treatment with ACT D before addition of NaB or CD, indicating a requirement for *de novo* RNA synthesis during the exposure period. Indeed, transcriptional activation of the p52(PAI-1) gene in KNRK cells was evident in response to both agents (Figure 3c); the relative p52(PAI-1) mRNA abundance for both K/NaB and K/CD cultures approximated p52(PAI-1) protein levels in the SAP fraction (Figure 2a). Analysis of the time course of induction revealed a short delay (6–10 h) between addition of NaB to KNRK cells and stimulated accumulation of p52(PAI-1) in the SAP fraction (Figure 4a); once induced, however, expression was relatively stable (Figures 4a and 4b). This lag was also evident, with more protracted kinetics (24–36 h), for hPAI-1 induction in NaB-stimulated epidermal cells. The inductive response in keratinocytes, however, was transient with regard to

Figure 3 Induction of p52(PAI-1) and hPAI-1 mRNA

Cytoplasmic RNA was isolated from control and treated KNRK cells, blotted to nitrocellulose and probed with ³²P-labelled cDNAs specific to p52(PAI-1) and actin mRNAs (a). p52(PAI-1) mRNA was virtually undetectable in KNRK cells; NaB and CD, both alone and in combination, effectively induced p52(PAI-1) mRNA expression. In contrast, the relative abundance of actin transcripts was similar among the culture groups. The slight increase (approx. 1.5-fold) in actin mRNA abundance in NaB + CD-treated cells was a consistent finding. *De novo* RNA synthesis during the period of inducer exposure was required to effect p52(PAI-1) and hPAI-1 expression in both KNRK cells and human epidermal keratinocytes (NHK) at the mRNA (b) and protein (Figure 5) levels. ACT D, when used, was given 1 h before the addition of inducer. RNA was isolated from KNRK and epidermal cells at 24 and 48 h respectively after addition of NaB or CD. For epidermal cultures, exposure to NaB and ACT D was for 24 h after which the cells were changed over to NaB/ACT D-free medium for an additional 24 h culture period. Actin mRNA abundance served as a control for RNA input. Inducibility of mRNA that hybridized with the p52(PAI-1) cDNA probe was completely blocked by ACT D in both cell types. Actin mRNA was evident in KNRK cells 24 h after the addition of ACT D as a result of the relatively long half-life of actin transcripts. The abundant levels of actin mRNA in epidermal cell cultures was probably due to transcriptional recovery after removal of ACT D 24 h before isolation of RNA. The increase in steady-state p52(PAI-1) mRNA abundance evident in NaB- or CD-treated KNRK cells reflected a corresponding increase in p52(PAI-1) run-off transcriptional activity (c). Scanning densitometry was carried out within the linear range of autoradiographic sensitivity for each data set [mRNA (■) compared with run-off hybridization signal (▨)]; values represent means $\pm$ S.D. of triplicate experiments.

Figure 4 Time course of NaB-induced p52(PAI-1) and hPAI-1 expression

SAP-fraction proteins (25000 trichloroacetic acid-insoluble c.p.m. loaded/lane) were separated by SDS/PAGE; labelled bands representing the 52 kDa p52(PAI-1) protein (for KNRK cells) or the 50 kDa hPAI-1 protein [for epidermal cells (NHK)] were quantified by scanning densitometry (a). For the 0–24 h times, all cultures were incubated directly in labelling medium containing [³⁵S]methionine with or without NaB. □, KNRK; ■, K/NaB; ▨, NHK; ▩, NHK/NaB. KNRK cells were also exposed to NaB in serum-containing medium for 48–72 h, then labelled with [³⁵S]methionine for 6 h. In order to obtain the 48 and 72 h epidermal cell time points, cultures were initially exposed to NaB in serum-containing medium for the first 24 h or 48 h respectively, then changed over to NaB-free labelling medium for a subsequent 24 h period. Control cultures were treated identically except for omission of NaB. Data represent the mean ± S.D. of triplicate scans for duplicate experiments. In contrast with K/NaB cultures (■), which maintained a constant elevated fraction of anti-p52(PAI-1)-reactive cells (quantified by flow cytometry), NaB-stimulated epidermal populations (□) exhibited a bell-shaped profile of immunoreactive cells consistent with the transient nature of the hPAI-1-inductive response in this cell type (b).

SAP-fraction hPAI-1 deposition (Figure 4a) and percentage of expressing cells (Figure 4b).

To define metabolic requirements involved in NaB- and CD-induced p52(PAI-1) synthesis, KNRK cells were exposed to CX before and during treatment with NaB, CD or NaB plus CD, then labelled with [³⁵S]methionine after removal of the inhibitor. Although CX markedly reduced the extent of NaB- and/or CD-induced p52(PAI-1) expression (Figures 5a and 5b), this protein synthesis inhibitor alone stimulated low levels of p52(PAI-1) accumulation. The reduced SAP-fraction p52(PAI-1) abundance in CX/NaB-, CX/CD- and CX/NaB plus CD-treated cultures approximated that seen in populations treated with CX alone and was not simply a function of inhibitor-associated decline in *de novo*-synthesized SAP-fraction protein (Table 3). Such expression, although low, was evident at the transcript level (Figure 6) and required continuing RNA synthesis as it was effectively blocked by ACT D (Figure 5). Two-dimensional electrophoresis confirmed that the 52 kDa SAP-fraction protein synthesized by KNRK cells after release from CX blockade was indeed p52(PAI-1); CX-induced p52(PAI-1) protein had an isoform complexity at least equivalent to p52(PAI-1) expressed in K/NaB cultures (Figure 5c). In epidermal cells exposed to CX before NaB addition, a similar inhibition of induced hPAI-1 expression was evident (Figure 5d). Owing to the 24 h offset in induced hPAI-1 accumulation as compared with KNRK cells, however, no stimulation was detected in keratinocyte cultures exposed to CX alone and was not expected because of the relatively short duration of the CX effect.

DISCUSSION

Although the constitutive pathway of p52(PAI-1) expression is defective in KNRK cells (Higgins et al., 1991), the p52(PAI-1) gene remains responsive to certain stimuli. For NaB and CD, the induction is largely transcriptional and involves a secondary (i.e. protein-synthesis-dependent) transduction pathway. CD also induces synthesis of p52(PAI-1) and actin in NRK cells (Higgins and Ryan, 1992); this response similarly reflects transcriptional controls, as augmented p52(PAI-1) and actin protein levels parallel the increase in mRNA abundance for both proteins and is effectively blocked by ACT D (Higgins and Ryan, 1992; Higgins et al., 1992). Such inducibility, moreover, is not restricted by cell-type as NaB effectively stimulated accumulation of hPAI-1 mRNA and protein in normal human keratinocytes. Epidermal hPAI-1 expression was transient, however, with kinetics virtually identical with those seen in NaB-treated human colon carcinoma cells (Reeder et al., 1993). Although the actual mechanism of induction is unclear, previous and present data shed light on this issue. Both NaB and CD are effective inducers of *c-fos* expression in several cell types (Tichonicky et al., 1990; Tang et al., 1990; Naranjo et al., 1990; Rius et al., 1991; Zambetti et al., 1992) including NRK and epidermal cells (Higgins, 1992). CD stimulates *c-fos* gene expression in HeLa cells via increases in both translation and mRNA stability (Zambetti et al., 1992); a similar complexity in *c-fos*-gene control appears to operate in NaB-treated cells (Souleimani and Asselin, 1993). Our analysis of the 3' untranslated region of rat p52(PAI-1) cDNA has revealed three sites (positions 1935, 1999 and 2610 relative to the translation-start codon) of identity with the instability-associated pentanucleotide sequence AUUUA, as well as nine and six additional sites of 80% homology corresponding to the sequences AUUU(N) and (N)UUUA respectively. Interaction of the AU-binding factor with multiple AUUUA motifs may function to increase the half-lives of intrinsically unstable mRNAs (Gillis

Figure 5 Effects of metabolic inhibitors on expression of p52(PAI-1) and hPAI-1 induced by NaB, CD or NaB plus CD

In K/NaB cells, exposure to ACT D before addition of NaB blocked p52(PAI-1) accumulation in the SP and SAP fractions (a). CX alone was a weak inducer although it effectively reduced stimulated expression in response to NaB. This slight induction by CX alone was inhibited by ACT D. An identical trend was seen in KNRK cells treated with NaB plus CD (a). Although the combination of the two inducers greatly enhanced p52(PAI-1) expression, this effect was completely blocked by ACT D and the weak stimulation observed in NaB + CD/CX cultures approximated to levels seen in NaB/CX cells and was inhibited by ACT D. Such low-level CX-inducible ACT D-sensitive p52(PAI-1) expression was similarly evident in control (KNRK) and CD-treated (K/CD) populations (b), confirming that full induction in response to NaB, CD or NaB plus CD required both protein and RNA synthesis. As p52(PAI-1) expression in CX-treated cultures also necessitated *de novo* RNA synthesis, induction was not the result of post-transcriptional effects (e.g. increased stability) on a low-abundance pool of p52(PAI-1) mRNA. Two-dimensional electrophoresis confirmed the identity of the induced 52 kDa SAP-fraction protein in K/CX cultures as p52(PAI-1) (c). Computer-generated spot profiles (top left) illustrate the microheterogeneity evident in electrophoretic separations of rat p52(PAI-1) derived from NRK/CD cells. Large arrowheads indicate the map positions of the known 52, 50 and 43 kDa variants of p52(PAI-1). Curved arrows point to four previously unidentified but potential minor p52(PAI-1) species. Distribution and relative abundance of individual p52(PAI-1) isoforms in the SAP fraction of K/NaB cells (bottom left) is identical with that expressed by K/CX populations (bottom right). The positions of actin and vimentin are indicated by small arrowheads. Two-dimensional gel-loading titrations indicated that the level of p52(PAI-1) protein in the SAP fraction of K/CX cells was 25–30% of that of K/NaB populations necessitating a 4-fold increase in K/CX cellular SAP-fraction protein loading to attain reasonable separations. Similarly to KNRK cells, hPAI-1 induction in NaB-treated epidermal cells was blocked by pretreatment with either ACT D or CX (d). No induction of hPAI-1 was evident in cells exposed to CX alone since culture conditions similar to those detailed in the legend to Figure 4 for the 48 h time point were used and metabolic labelling was performed 24 h after removal of CX.

and Malter, 1991; Malter and Huong, 1991). As there is a strong transcriptional component to the p52(PAI-1)-inductive response, however, steady-state p52(PAI-1) mRNA levels in NaB- and/or CD-treated cells are probably not regulated exclusively by stability mechanisms.

In KNRK cells, CD- or NaB-induced Fos and Jun (AP-1) protein expression paralleled the time course of p52(PAI-1) induction and remained elevated for at least 24 h (E. Dickerson and P. J. Higgins, unpublished work). NaB-induced Fos and Jun

function as biologically active AP-1 DNA-binding complexes in mobility-shift assays using consensus oligonucleotide target sequences (Mollinedo et al., 1993). Both NaB and CD therefore impart at least one transduction signal (Higgins et al., 1994; Kruh et al., 1992), perhaps via restructuring of integrin–matrix complexes (Werb et al., 1989; Kornberg et al., 1991), resulting in the activation of genes encoding specific transcriptional regulators (Shaw et al., 1990; Yamada et al., 1991). Indeed, several stimulators of p52(PAI-1) synthesis in cultured cells (e.g.

Table 3 Effect of metabolic inhibitors on accumulation of protein synthesized *de novo* in the SAP fraction of KNRK, K/CD and K/NaB cells

KNRK, K/CD and K/NaB cells were exposed to ACT D, CX or both and then labelled with [³⁵S]methionine before SAP-fraction preparation as described in the Materials and methods section. [³⁵S]Methionine-labelled SAP-fraction protein was solubilized in 200 μ l of electrophoresis sample buffer, boiled, and trichloroacetic acid-precipitable radioactivity was determined for a 2 μ l portion. Data represent means $\pm$ S.D. of triplicate assays.

Cell type	$10^{-2} \times$ SAP-fraction protein (c.p.m.)
KNRK	1187 $\pm$ 129
+ ACT D	1164 $\pm$ 293
+ CX	2105 $\pm$ 639
+ ACT D + CX	1771 $\pm$ 440
K/CD	1309 $\pm$ 303
+ ACT D	1448 $\pm$ 481
+ CX	1321 $\pm$ 219
+ ACT D + CX	1857 $\pm$ 502
K/NaB	1211 $\pm$ 58
+ ACT D	1317 $\pm$ 222
+ CX	2168 $\pm$ 934
+ ACT D + CX	1859 $\pm$ 70

Figure 6 Induction of p52(PAI-1) mRNA in CX-treated KNRK cells

Cytoplasmic RNA was isolated from control and CX-treated (24 h) KNRK cells; nitrocellulose blots were probed with ³²P-labelled p52(PAI-1) cDNA and signal intensity expressed relative to 18S RNA to control for RNA input. Scanning densitometry was carried out within the linear range of autoradiographic sensitivity. Data represent means $\pm$ S.D. of triplicate assessments. A 7-fold increase in KNRK p52(PAI-1) mRNA abundance was evident as a function of CX exposure.

TGF- β), in addition to CD or NaB, activate (at least transiently) members of the *fos/jun* gene families (Higgins and Tanaka, 1991; Higgins, 1992). Such events may be relevant to transcriptional control of p52(PAI-1) gene expression. The 5'-flanking region of the rat p52(PAI-1) gene has various potential regulatory sequences, including a concentration of Fos/Jun (AP-1)-like binding sites (Bruzdzinski et al., 1990). Seven regions of approx. 85% sequence homology to the AP-1 consensus sequence (TGACTCA) have been identified, in both (+) and (−) orientations, in the 5'-upstream region from −592 to −2112 nt. It

remains to be determined whether occupancy of such AP-1 sites by specific Fos/Jun heterodimers or Jun/Jun homodimers actually stimulates p52(PAI-1) transcription in response to CD. In addition, NaB-response elements exist in the mouse calbindin-D28K, CCP1 and CCP2 protease genes (Gill and Christakos, 1993; Fregeau et al., 1992) which confer NaB responsiveness when ligated to a heterologous *fos* promoter (Fregeau et al., 1992). The calbindin-D28K gene also possesses a vitamin D-response element immediately upstream of a proximally situated NaB-response element (Gill and Christakos, 1993). We have identified 11 NaB-response-element-like sites in the 5'-flanking region of the p52(PAI-1) gene; perhaps not coincidentally, one such NaB-response element at −154 nt is contiguous with a vitamin D-response element-like sequence located at −143 nt in the proximal promoter.

PAI-1-inductive characteristics are quite variable with respect to transcriptional and/or post-transcriptional requirements and are probably inducer- and cell-type-specific. The hPAI-1 gene in HepG2 cells, for example, is transcriptionally activated by treatment with phorbol ester or epidermal growth factor; induction, however, occurs in a protein-synthesis-independent and -dependent manner in response to phorbol ester and epidermal growth factor respectively, suggesting two regulatory pathways (Bosma and Kooistra, 1991; Hopkins et al., 1991). By comparison, transcriptional induction of p52(PAI-1) mRNA in quiescent NRK cells after serum stimulation occurs rapidly (within 1 h), is independent of on-going protein synthesis and approximates to an immediate-early response (Ryan and Higgins, 1993). The pathway of stimulated p52(PAI-1) expression in NaB-and/or CD-treated KNRK and epidermal cells therefore more closely resembles that utilized by epidermal-growth-factor-stimulated HepG2 cells.

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