p52^{PAI-1} gene expression in butyrate-induced flat revertants of v-*ras*-transformed rat kidney cells: mechanism of induction and involvement in the morphological response

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Sodium *n*-butyrate-induced flat reversion in v-*K-ras* oncogene-transformed rat kidney (KNRK) cells is associated with transcriptional activation of the p52^{PAI-1} gene (which encodes the type-1 inhibitor of plasminogen activator). Butyrate-initiated expression of p52^{PAI-1} mRNA and protein correlated with induced cell spreading and preceded development of cell-to-substrate focal adhesions. Such undersurface matrix contact structures, which are absent from parental KNRK cells, require proximal PAI-1 deposition for their stabilization. Stimulated p52^{PAI-1} expression by flat revertants (approximating 25-fold that of control cells) and the accompanying cytoarchitectural reorganization appeared to be programmed responses to butyrate as both events required *de novo* RNA and protein synthesis, metabolic characteristics consistent with a secondary pathway of gene regulation. To assess the relevance of p52^{PAI-1}

induction to the process of flat reversion more critically, a molecular genetic approach was designed to maintain high-level constitutive p52^{PAI-1} synthesis in the absence of butyrate. KNRK cells transfected with a Rc/CMVPAI plasmid construct, in which expression of a p52^{PAI-1} cDNA insert was driven by enhancer–promoter sequences from the immediate-early gene of human cytomegalovirus, formed colonies comprised of flat-revertant-like cells with a greater frequency than did cells transfected with the Rc/CMV vector alone (24.8% and 1.7% respectively). Comparative analysis of randomly selected Rc/CMVPAI clones indicated that a 10-fold increase in immunoreactive p52^{PAI-1} protein, relative to Rc/CMV isolates, correlated with generation of the flat phenotype. These data suggest that induced p52^{PAI-1} expression probably functions in the development of morphological revertants in the KNRK cell system.

INTRODUCTION

Malignant transformation of mammalian cells with *ras* family oncogenes results in dramatic changes in cytoarchitecture and growth behaviour [1]. Conversion of such transformants into a more normal phenotype, either spontaneously or by chemical induction, is often referred to as ‘flat reversion’ [2]. Flat revertants of *ras*-oncogene-transformed cells derive from the uncoupling of specific mediator genes from *ras*-dependent transduction pathways [2]. Since significant deviations from normal morphology and substrate adhesion typify the *ras*-transformed phenotype [1–5], genes that encode proteins that influence cytoskeletal organization and/or adhesive contact structures represent potential functional mediators [4]. Candidates include gelsolin [6,7], vinculin [8], tropomyosin [9], fibronectin and laminin [10], certain integrins [11], actin [12] and the 52 kDa type-1 inhibitor of plasminogen activator (p52^{PAI-1}) [4,13]; each appear to be transformation-sensitive in specific cell systems [4]. Indeed, expression of a specific 92 kDa/pI 5.7 gelsolin variant coincided with morphological reversion in human EJ*ras*^{val-12}-transformed NIH 3T3/R1 cells [6], and increases in gelsolin, vinculin and p52^{PAI-1} synthesis correlated with cytoarchitectural reorganization in sodium butyrate-induced flat revertants of v-*ras*-transformed rat kidney (KNRK) cells [4].

In normal renal cells, p52^{PAI-1} is deposited in close juxtaposition to, but not actually within, focal adhesion complexes [14] where it is presumed to stabilize cell-to-matrix contact structures [15]

via inhibition of associated serine proteases [e.g. urokinase plasminogen activator (uPA)] [16]. Deficiencies in either p52^{PAI-1} expression or its substrate deposition, both typical sequelae of rat mesenchymal cell transformation by the retroviral v-*K-ras* or activated human EJ*ras*^{val-12} oncogenes [4,17,18], would probably alter the pericellular proteolytic balance, thereby contributing to the marked pleiomorphism and increased motility characteristic of *ras* transformants [3]. Re-establishment of cell-to-substrate adhesive contacts in flat revertants appears to be a consequence of reduced pericellular proteolysis as the result of, at least in part, augmented p52^{PAI-1} gene transcription and matrix p52^{PAI-1} protein accumulation [4,19].

This paper examines morphological reversion in KNRK cells with specific regard to the mechanism of butyrate-induced p52^{PAI-1} expression. Induction required continuous RNA synthesis during the period of butyrate exposure and involved a secondary transduction pathway. Analysis of KNRK cells genetically engineered to express p52^{PAI-1} in the absence of butyrate provided evidence for participation of p52^{PAI-1} in phenotypic reversion.

MATERIALS AND METHODS

Cell culture, metabolic labelling and gel electrophoresis

KNRK cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) [18]

Abbreviations used: p52^{PAI-1}, plasminogen activator inhibitor type 1; KNRK, v-*ras*-transformed rat kidney; uPA, urokinase plasminogen activator; tPA, tissue-type plasminogen activator; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; SP, secreted proteins; CMF-PBS, Ca²⁺/Mg²⁺-free PBS.

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with or without butyrate (2 mM, final concentration). Cells were washed twice with Hanks balanced salt solution (HBSS; 1.3 mM CaCl_2 , 5 mM KCl, 0.3 mM KH_2PO_4 , 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 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) and labelled in serum-/methionine-free RPMI 1640 medium containing 50 μCi of [^{35}S]methionine/ml (specific radioactivity 1100 Ci/mmol) with or without butyrate [20]. Exposure to actinomycin D (5 $\mu\text{g}/\text{ml}$) and cycloheximide (50 μM) (optimal concentrations determined previously [20]) was for 1 h or 15 min respectively before butyrate addition. The conditioned labelling medium, containing secreted proteins (SP), was removed, centrifuged at 13000 g , diluted with an equal volume of electrophoresis sample buffer (50 mM Tris/HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol) and boiled. Cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (CMF-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 CMF-PBS to isolate focal contact structures and associated undersurface material [13]; such saponin fraction residues were scraped into sample buffer and boiled. Equivalent c.p.m. of trichloroacetic acid-insoluble SP or saponin fraction protein were separated on SDS/10% polyacrylamide slab gels [5]. Labelled proteins were visualized by fluorography and quantified by scanning densitometry. Cytoskeletal fractions were prepared by extraction of adherent cells at 4 °C for 5 min in TN/T buffer (140 mM NaCl, 10 mM Tris/HCl, pH 7.6, 1% Triton X-100). The extract buffer with solubilized proteins was aspirated, and the remaining cytoskeletal residue was scraped into TN/T buffer, vortex-mixed and collected at 13000 g for solubilization by boiling in electrophoresis sample buffer.

RNA analysis

Cytoplasmic fractions were diluted 1:1 in digest buffer (0.2 M Tris/HCl, pH 7.5, 25 mM EDTA, 0.3 M NaCl, 2% SDS, 200 μg of proteinase K/ml) and incubated at 37 °C for 30 min. Proteins were extracted with phenol/chloroform, and nucleic acids were ethanol-precipitated from the aqueous phase and digested with DNase I. RNA was separated by agarose-gel electrophoresis, blotted on to nitrocellulose in 20 $\times$ SSC (1.5 M NaCl, 0.15 M sodium citrate; 2 H_2O , pH 7.5) in sets of 20, 10, 5 and 2.5 μg of RNA/slot and the membranes vacuum-baked at 80 °C for 4 h. Plasmids for hybridization included pSS1-3, containing a p52^{PAI-1} cDNA insert in the pBSK(−) vector [21], pBSK(−) vector without insert, and a mouse β -actin cDNA [4]. *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 previously [21].

Immunoprecipitation

Immune precipitation of p52^{PAI-1} from metabolically labelled cells utilized procedures and antisera developed previously [13]. Immune complexes were collected with agarose-Protein G beads (Genex Corp., Gaithersburg, MD, U.S.A.) and washed three times by centrifugation in detergent buffer (10 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate); precipitated antigen was released by boiling in electrophoresis sample buffer before fractionation by SDS/PAGE.

Immunofluorescence microscopy

The focal-contact-associated protein vinculin was localized with

the monoclonal antibody VIN-11-5 in 10% neutral-buffered formalin-fixed 0.5% Triton X-100-extracted cell cultures as described [4]. All reagents were prepared in modified HBSS (i.e. without CaCl_2 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and containing 2 mM EGTA, 5.5 mM MgCl_2 and 5 mM Pipes) [18].

Construction and transfection of a sense p52^{PAI-1} expression vector

pBluescript containing a full-length rat PAI-1 cDNA was digested with *Eco*RI and *Hind*III to generate a 2.6 kb insert (representing nucleotides −118 to +2572). Agarose-gel-purified DNA was blunt-ended with Klenow fragment/dNTPs using a fill-in reaction. *Not*I linkers were ligated, the fragments digested with *Not*I and purified by agarose-gel electrophoresis. Ligation was confirmed by electrophoresis of the reaction products on a 6% acrylamide/TBE gel before and after *Not*I digestion to demonstrate the presence and absence of a linker ladder respectively. Flanked inserts were ligated to *Not*I-digested calf intestinal phosphatase-treated gel-purified Rc/CMV expression vector DNA and subsequently transformed into competent INV α F' *Escherichia coli*. Plasmid DNA was isolated from ampicillin-resistant colonies; restriction enzyme digestion and Southern-blot analysis confirmed sense (Rc/CMVPAI) and antisense (Rc/CMVIAP) insert orientation. For Southern-blot analysis, a 726 bp *Pst*I/*Apa*I-digested cDNA fragment was labelled with [^{32}P]dCTP and used to probe Oncor membrane blots of electrophoretically separated endonuclease-digested DNAs. For transfection, 5 μl of Lipofectamine reagent (Gibco, Gaithersburg, MD, U.S.A.) was added to 100 μl of serum-/antibiotic-free DMEM containing 1–2 μg of circular or linearized vector (either Rc/CMV or Rc/CMVPAI), incubated at room temperature for 30 min, diluted with 0.8 ml of DMEM and the mixture pipetted into 35 mm cultures of 10⁵ adherent freshly DMEM-washed KNRK cells. This DNA/Lipofectamine ratio produced maximal numbers of G418 (Geneticin)-resistant cells. After 5 h, 1 ml of DMEM/20% FBS was added and 18 h later the growth medium was aspirated and replaced with DMEM/10% FBS. The next day, cultures were treated with trypsin, and cells were seeded into 100 mm culture dishes in DMEM/10% FBS containing 200 $\mu\text{g}/\text{ml}$ G418 (concentration titration indicated that this level of G418 effectively killed 100% of control non-transfected cells); growing cells were subcultured after approx. 1 week and individual clones isolated 2–3 weeks later.

RESULTS

Newly synthesized p52^{PAI-1} protein accumulated in the saponin fraction of KNRK cells within 24 h of initial exposure to butyrate (Figure 1), correlating with the onset of induced cell spreading

Figure 1 Effect of butyrate on the saponin fraction of KNRK cells

Exposure of KNRK cells to butyrate effectively stimulated p52^{PAI-1} accumulation in the saponin fraction within 24 h; p52^{PAI-1} derived from constitutively expressing normal renal (NRK) cells [23] served as an internal standard. The positions of the mature 52 kDa p52^{PAI-1} protein, the intermediate-glycosylated 50 kDa variant of p52^{PAI-1} [4,13,18], and the major extracellular protein p60/62 [14,18] are indicated. NaB, sodium butyrate.

Table 1 Summary of phenotypic changes in KNRK cells over the time course of generation of flat revertants in response to butyrate

Units for the three phenotypic parameters measured are as follows: incidence of well-spread cells (% of the total population); p52^{PAI-1} content of saponin fraction (arbitrary densitometric units obtained by scanning SDS/PAGE separations of [³⁵S]methionine-labelled saponin fraction protein and representing both the 52 and 50 kDa species [23]); number of vinculin-containing focal contacts per cell (50 randomly selected cells assessed per culture condition). Data represent means $\pm$ S.D. for measurements made on at least triplicate cultures per time point.

Time in butyrate (h)	Spread of cells (%)	Saponin fraction p52 ^{PAI-1}	Focal contacts/cell
0	2.3 $\pm$ 2.1	3.5 $\pm$ 3.2	3.1 $\pm$ 3.0
24	22.4 $\pm$ 5.3	67.9 $\pm$ 4.8	5.0 $\pm$ 2.7
48	43.1 $\pm$ 4.4	60.6 $\pm$ 13.0	28.2 $\pm$ 12.9
72	66.7 $\pm$ 3.9	58.0 $\pm$ 7.1	37.8 $\pm$ 10.2

Table 2 Metabolic profiles of KNRK cells and butyrate-induced flat revertants

Cellular label incorporated is the fraction of total cellular [³⁵S]methionine converted into trichloroacetate-insoluble protein. Data represent means $\pm$ S.D. for triplicate assays. Total labelled protein was measured by liquid-scintillation spectroscopy in aliquots of total cellular extracts [23] precipitated with trichloroacetic acid. Data represent means $\pm$ S.D. for 11 determinations normalized to equivalent cell numbers. [³⁵S]Methionine-labelled saponin fraction protein was solubilized in electrophoresis sample buffer, boiled, and trichloroacetate-precipitable radioactivity determined. Data represent means $\pm$ S.D. for triplicate assays.

Cells	Cellular label incorporated (%)	10 ⁻⁷ $\times$ Total labelled protein (c.p.m.)	10 ⁻⁴ $\times$ Saponin protein (c.p.m.)
KNRK	85.0 $\pm$ 11.3	1.5 $\pm$ 0.5	10.7 $\pm$ 0.2
Revertants	81.0 $\pm$ 4.2	1.7 $\pm$ 0.8	9.9 $\pm$ 0.2

and preceding development of the first identifiable focal contacts (both cytoarchitectural correlates of the revertant phenotype; see e.g. ref. [4]) by at least 24 h (Table 1). Total *de novo*-synthesized as well as saponin fraction protein for parental KNRK cells and butyrate-induced revertants were similar (Table 2), suggesting that the approximately 25-fold increase in saponin fraction p52^{PAI-1} protein evident in butyrate-stimulated cells (Table 1) is relatively specific to p52^{PAI-1} and to development of the revertant phenotype. This co-ordinated display of early morphological (i.e. induced cell spreading) and metabolic (p52^{PAI-1} expression) events associated with flat reversion appeared to be a programmed response to butyrate, as both processes required *de novo* RNA and protein synthesis (Figure 2, Table 3). Indeed, actinomycin D (5 μ g/ml; which suppressed [³H]uridine incorporation by more than 90 %) completely blocked and cycloheximide (50 μ M; inhibited [³⁵S]methionine incorporation by 96 %) markedly reduced the extent of butyrate-induced p52^{PAI-1} synthesis, although cycloheximide itself stimulated low levels of *de novo* p52^{PAI-1} accumulation. (In expression analysis using cycloheximide, KNRK cells were exposed to the drug before and during butyrate treatment and then labelled with [³⁵S]methionine after removal of the inhibitor.) The reduced saponin fraction p52^{PAI-1} protein evident in cycloheximide/butyrate-treated cultures approximated that seen in populations treated with cycloheximide alone. Such expression, although low, was evident at the transcription level and, as for the morphological response, required continual RNA synthesis as it was effectively blocked by actinomycin D (Figure 2, Table 3). Direct comparison of the three major compartments of p52^{PAI-1} accumulation (i.e. the SP, saponin and cytoskeletal fractions [24,25]) moreover indicated that the low level of cycloheximide-inducible saponin fraction p52^{PAI-1} deposition in KNRK cells was not an artifact of altered subcellular protein targeting as a consequence of exposure to inhibitor (Table 4).

Figure 2 Metabolic requirements for butyrate-induced p52^{PAI-1} expression in KNRK cells

De novo RNA synthesis during the period of butyrate exposure was required to induce p52^{PAI-1} at both the mRNA (A) and protein (B,C) levels. Actinomycin D, when used, was given 1 h before the addition of butyrate. RNA was isolated after a 24 h exposure to butyrate-supplemented or control growth medium; actin mRNA abundance served as a monitor for input RNA (A). At the protein level, exposure to actinomycin D before addition of butyrate effectively blocked p52^{PAI-1} accumulation in both the SP and saponin (SAP) fraction compartments (C). Cycloheximide alone was a weak p52^{PAI-1} inducer (when metabolic labelling was carried out after removal of cycloheximide from the culture system) although it effectively reduced stimulated expression in response to butyrate; this slight induction by cycloheximide alone was inhibited by actinomycin D (B,C). Low-level cycloheximide-inducible actinomycin D-sensitive p52^{PAI-1} expression was also evident in KNRK cells (B) confirming that both protein and RNA synthesis was necessary for full induction in response to butyrate (C). As p52^{PAI-1} expression in cycloheximide-treated culture also required *de novo* RNA synthesis, induction was not the result of post-transcriptional effects exclusively (e.g. increased stability) on a low-abundance pool of p52^{PAI-1} mRNA. NaB, sodium butyrate; ACT, actinomycin D; CX, cycloheximide.

Table 3 Dependence of the development of the revertant phenotype on RNA synthesis during butyrate exposure

Quantitative measurements of cell spreading (% of the total population), densitometric assessments (arbitrary values) of saponin fraction p52^{PAI-1} protein content and p52^{PAI-1} mRNA levels indicated that reduced p52^{PAI-1} mRNA and protein synthesis as a consequence of actinomycin D (Act D) treatment 1 h before and during, a 24 h butyrate exposure correlated with inhibited development of the well-spread revertant phenotype.

Treatment	Spread of cells (%)	Saponin fraction p52 ^{PAI-1}	p52 ^{PAI-1} mRNA
None	2.1 ± 1.8	2.9 ± 1.3	0
Butyrate	26.4 ± 4.9	63.7 ± 5.2	49.8 ± 3.1
Butyrate + Act D	5.6 ± 2.5	2.0 ± 1.1	1.4 ± 1.0

Confirmation of the efficacy of cycloheximide to inhibit synthesis of specific high-abundance proteins was obtained by using the predominant p60/62 complex (Figure 1) as an indicator; cycloheximide (50 µM) effectively suppressed p60/62 synthesis by 95–97%. Recovery from cycloheximide blockade occurred rapidly (within 3 h), indicating that assessments of metabolic requirements for p52^{PAI-1} induction were not influenced by inhibitor toxicity (Table 5). Collectively these data indicate that full induction of p52^{PAI-1} expression in butyrate-treated cells occurs via a secondary-response (i.e. protein synthesis-dependent) pathway and requires continued transcription.

Only morphological revertants (i.e. well-spread cells) accumulate p52^{PAI-1} protein in the undersurface region [4]. To assess specifically the relevance of p52^{PAI-1} expression to the process of phenotypic reversion, an approach was devised to initiate high-level p52^{PAI-1} synthesis in KNRK cells in the absence of butyrate. The cloning strategy used to derive a sense p52^{PAI-1} expression vector (Rc/CMVPAI) is detailed in the Materials and methods section and summarized below (Figure 3). Southern blotting of DNA restriction fragments with a 726 bp PAI-1 cDNA-specific probe confirmed insert orientation (Figure 4). Analysis of uncloned G418-resistant cultures 2 weeks after initiation of antibiotic selection indicated a significant difference in the incidence of 'flat' cells (i.e. cytoplasmic/nuclear area ratios ≥ 2) between Rc/CMV (control vector) and Rc/CMVPAI transfectants. Rc/CMV derivatives (like parental KNRK cells) had a low fraction of such clones (1.7 ± 0.8 % of the total number of colonies examined) whereas Rc/CMVPAI cultures had a substantial number (24.8 ± 7.1 %) of flat-cell colonies. Flat-cell colonies were 4-fold more abundant in cultures transfected with linearized compared with circular sense vector plasmids. To evaluate potential differences in levels of p52^{PAI-1} expression in such morphologically distinct populations, seven clones were derived from each of the Rc/CMV and Rc/CMVPAI mass cultures. A major distinguishing feature between these two clonal groups was the almost 3-fold increase in total saponin fraction protein evident in Rc/CMVPAI isolates as compared with vector controls (i.e. 63 320 ± 26 920 c.p.m. and 21 960 ± 4680 c.p.m. respectively). In order to analyse transfectants more critically, four clones were arbitrarily selected from both the Rc/CMV (clones 1–4) and Rc/CMVPAI (clones 5–8) isolates. Cells were assessed with regard to morphology and level of p52^{PAI-1} expressed. Rc/CMV clones 1–4 consisted of spindle-shaped poorly spread cells identical in morphology with uncloned KNRK controls (Table 6); such cells, like the parental KNRK line, were devoid of vinculin-containing focal contact structures and lacked well-developed microfilament bundles (see e.g. ref. [5]). Rc/CMVPAI

Table 4 Accumulation of *de novo*-synthesized p52^{PAI-1} in specific subcellular compartments after release of KNRK cells from cycloheximide blockade

Cells were exposed to cycloheximide for the times indicated and then metabolically labelled for 3 h with [³⁵S]methionine in the absence of cycloheximide. p52^{PAI-1} content in the SP, saponin and cytoskeletal (CSK) fractions was assessed by densitometric analysis of SDS/PAGE separations and expressed relative to control (i.e. non-cycloheximide-treated) cells. p52^{PAI-1} levels in all three compartments increased (by approximately similar increments) as a function of time of cycloheximide treatment before labelling. p52^{PAI-1} accumulation in the saponin fraction of KNRK/cycloheximide cells (Figure 2) therefore was not an artifact of altered subcellular targeting of *de novo*-synthesized protein after exposure to cycloheximide.

Time in cycloheximide (h)	p52 ^{PAI-1} levels (cycloheximide/control)		
	SP fraction	Saponin fraction	CSK fraction
1	1.01	1.10	1.25
2	1.20	1.51	1.43
4	1.63	2.14	1.48

Table 5 Recovery of KNRK cells from cycloheximide blockade

To confirm the efficacy of cycloheximide (50 µM) to inhibit *de novo* protein synthesis, KNRK cells were pretreated with cycloheximide for 1 or 4 h before a 3 h pulse in labelling medium containing [³⁵S]methionine and cycloheximide. Compared with non-cycloheximide-blocked cells, exposure to inhibitor reduced conversion of isotope into trichloroacetic-insoluble product (specifically, the p60/62 protein complex as quantified by scanning densitometry of autoradiographs derived from SDS/PAGE separations) by 95–96%. Such inhibition was transient and quickly reversed on removal of cycloheximide. Pretreatment of KNRK cells with cycloheximide for 4 h followed by labelling in the absence of cycloheximide resulted in the rapid recovery of protein synthesis. The metabolic requirements accompanying p52^{PAI-1} induction during flat reversion (i.e. necessitating continual protein synthesis) therefore are not likely to be an artifact of inhibitor toxicity.

Treatment conditions	p60/62 levels (arbitrary densitometric units)
3 h label	62.5 ± 10.0
1 h cycloheximide pretreatment and 3 h cycloheximide + label	0.9 ± 1.1
4 h cycloheximide pretreatment and 3 h cycloheximide + label	1.3 ± 2.2
4 h cycloheximide pretreatment and 3 h no cycloheximide + label	57.6 ± 4.8

transfectants (clones 5–8), in contrast, had significantly flatter profiles (i.e. were comprised of cells with cytoplasmic-to-nuclear area ratios ≥ 2). Extremely low levels of the 52 and 58 kDa p52^{PAI-1} variants were evident in Rc/CMV isolates. Rc/CMVPAI clones, in contrast, expressed on average 18-fold more p52^{PAI-1}; this increase involved predominantly the 58 kDa species of immunoreactive p52^{PAI-1} (Figure 5).

DISCUSSION

Distinct ultrastructural localizations of plasminogen activators and PAI-1 are evident in cultured cells as well as *in vivo*. The extracellular face of the fibroblast focal contact, which represents the region of closest cell–substratum apposition [27], is a major site of uPA accumulation [28,29], as confirmed by its co-distribution with vinculin-containing adhesive structures [24]. Since uPA levels appear to correlate directly with the degree of cell growth and motility, even a small imbalance in the delicate uPA/PAI-1 ratio necessary to maintain normal cell adhesion might dramatically affect cell morphology and/or substrate

Figure 3 Construction of a p52^{PAI-1} expression vector

Cloning of 2.6 kb of the PAI-1 cDNA (nucleotides -118 to +2572) into the 5.4 kb Rc/CMV vector was as detailed in the Materials and methods section. (A) Topography of known restriction sites (P, *Pst*I; N, *Nco*I; A, *Apa*I) for both the sense (Rc/CMVPAI) and antisense (Rc/CMVPAIAP) orientations (A) was used to interpret restriction endonuclease digest patterns of plasmid DNA derived from ampicillin-resistant colonies (B). The size of DNA fragments predicted from single endonuclease digests for inserts of both orientations is indicated in (C). The cross-hatched region in the cloned cDNA insert represents the 726 bp *Pst*I-*Apa*I fragment used in Southern-blot analysis (see Figure 4).

Figure 4 Confirmation of sense and antisense p52^{PAI-1} cDNA insert orientations by Southern blotting

Plasmid DNA was separated either undigested (—) or digested with the indicated endonucleases, transferred to Oncor membranes, and blots were probed with the p52^{PAI-1} insert-specific ³²P-labelled 726 bp *Pst*I–*Apa*I fragment (as in Figure 3). Comparison of digest hybridization patterns with expected fragment length (Figures 3B and 3C) served to confirm insert orientation.

adhesion (reviewed in refs. [13,14,25,30,31]). Such events would probably have a significant impact on cellular growth characteristics. Indeed, gene-transfer experiments have conclusively shown that cells expressing high levels of recombinant plasminogen activator (either uPA or tPA) were significantly more invasive than untransfected controls [32,33]. Conversion of active plasmin from the proenzyme plasminogen is catalysed by either uPA or tPA [25,34], the activities of which, in turn, are regulated largely by the specific inhibitors PAI-1 and PAI-2 [35]. The intrinsic extracellular matrix-associated PAI-1 protects the matrix from plasminogen-dependent degradation [36] and, as a consequence, may efficiently stabilize cellular substrate contacts, in particular those mediated by vitronectin [15]. This delicate balance between plasminogen activators and their inhibitors is adversely affected by transformation in the NRK cell system [4,13,14,17,18] and may be mechanistically associated with generation of the pleomorphic shape, loss of matrix (fibronectin) structure and microfilament disorganization typical of transformed cells [4,5]. In accord with this suggestion are findings that inhibition of

Table 6 Comparative morphology of Rc/CMV and Rc/CMVPAI transfectants

Clones were isolated from G418-resistant mass cultures of KNRK cells transfected with the Rc/CMV or Rc/CMVPAI vectors. Like parental cells, Rc/CMV clones (1–4) consisted of poorly spread spindle-shaped cells. In contrast, a significant fraction (16–55%) of cells in Rc/CMVPAI clonal isolates (clones 5–8) had cytoplasmic-to-nuclear size ratios of ≥ 2 .

Clonal isolate	Well spread cells (%)
Rc/CMV clone 1	0.6 $\pm$ 0.5
Rc/CMV clone 2	1.4 $\pm$ 1.3
Rc/CMV clone 3	1.0 $\pm$ 0.8
Rc/CMV clone 4	0
Rc/CMV clone 5	46.2 $\pm$ 8.6
Rc/CMV clone 6	37.6 $\pm$ 10.1
Rc/CMV clone 7	40.3 $\pm$ 13.7
Rc/CMV clone 8	16.0 $\pm$ 6.2

Figure 5 Expression of p52^{PAI-1} by transfectant KNRK cells

Clones were isolated from G418-resistant mass cultures of KNRK cells transfected with the Rc/CMV (CI-1 to CI-4) or Rc/CMVPAI (CI-5 to CI-8) vectors. Immunoprecipitation of [³⁵S]methionine-labelled anti-p52^{PAI-1}-reactive protein from both Rc/CMV and Rc/CMVPAI clones confirmed that Rc/CMV cells expressed low levels of p52^{PAI-1} whereas Rc/CMV transfectants synthesized approximately 14- to 22-fold more immunoreactive p52^{PAI-1} [in particular, the 58 kDa species which appears to be a variant produced specifically by KNRK cells under conditions of induced expression, either in response to butyrate (see, for example, [26]) or vector-driven]. p52^{PAI-1} immunoprecipitated from normal rat kidney (NRK) and butyrate-treated KNRK (K/NaB) cells served as markers of the 50, 52 and 58 kDa p52^{PAI-1} protein species in SDS/PAGE separations. The 58 kDa K/NaB-expressed variant can be resolved by high-definition two-dimensional gel electrophoresis of substrate-associated protein [26] or by immunoprecipitation. Such approaches are necessary to discriminate the 58 kDa species from the p60/62 protein band, which predominates in standard one-dimensional separations of saponin fraction protein. The inability to subsequently process the 58 kDa p52^{PAI-1} protein to the 52 kDa mature form probably reflects the defect in glycoconjugate processing evident in parental KNRK cells and KNRK transfectants but which is partially reversed in butyrate-treated populations (P. J. Higgins, M. P. Ryan and D. M. Jelley, unpublished work).

plasminogen activator catalytic activity in Rous-sarcoma-virus-transformed fibroblasts prevents the development of growth and morphological anomalies associated with retroviral transformation [37,38], and increases in PAI-1 mRNA and protein expression in human tumour cells by stable transfection specifically inhibits aggressive growth behaviour [39]. The present findings are concordant with these observations and illustrate that vector-driven constitutive expression of p52^{PAI-1} in KNRK cells can specifically initiate (and apparently maintain) development of a revertant phenotype similar to that induced by butyrate. Maintenance of p52^{PAI-1} expression as well as the revertant phenotype, in addition, required the continued presence of butyrate since its removal resulted in conversion to the parental phenotype (i.e. spindle-shaped morphology, p52^{PAI-1}-negative status) within 24–36 h. Loss of saponin fraction p52^{PAI-1} preceded this conversion, consistent with the previously estimated half-life of matrix-associated p52^{PAI-1} of approx. 2 h [4]. Clearly, induced p52^{PAI-1} synthesis may be an important but perhaps not the only modulator of cell growth or cytoarchitecture expressed as a

consequence of exposure to butyrate. Exposure of Rc/CMVPAI clones 5 and 7 to butyrate, however, did not alter the incidence of cells displaying the revertant phenotype, and Rc/CMV clones 1–4 were each converted into the revertant morphology and concomitant p52^{PAI-1}-positive status on addition of butyrate (results not shown). Rc/CMVPAI clones 6 and 8, moreover, exhibited a greater frequency of percentage revertant cells and an increased level of p52^{PAI-1} expression when cultured in butyrate-containing medium. It appears therefore that cytoarchitectural restructuring mediated by butyrate in *ras* transformants is probably a function of this increased p52^{PAI-1} synthesis. Whether this reorganization specifically involves uPA-focalized contacts [28,29], as evident in cells cultured in serum-containing growth medium and thereby utilizing predominantly vitronectin-dependent adhesive complexes [15], is at present under evaluation.

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REFERENCES

- Sistonen, L., Keski-Oja, J., Ulmanen, I., Holttä, E., Wikgren, B. J. and Alitalo, K. (1987) *Exp. Cell Res.* **168**, 518–530
- Yamada, H., Omata-Yamada, T., Wakabayashi-Ito, N., Carter, S. G. and Lengyel, P. (1990) *Mol. Cell. Biol.* **10**, 1822–1827
- Egan, S., Wright, J. A., Jarolim, L., Yanagihara, K., Bassin, R. H. and Greenberg, A. H. (1987) *Science* **238**, 202–205
- Higgins, P. J. and Ryan, M. P. (1991) *Biochem. J.* **279**, 883–890
- Ryan, M. P. and Higgins, P. J. (1988) *J. Cell. Physiol.* **137**, 25–34
- Fujita, H., Suzuki, H., Kuzumaki, N., Mullauer, L., Ogiso, Y., Oda, A., Ebisawa, K., Sakurai, T., Nonomura, Y. and Kijimoto-Ochiai, S. (1990) *Exp. Cell Res.* **186**, 115–121
- Banyard, M. R. C., Medveczy, C. J. and Tellam, R. L. (1990) *Exp. Cell Res.* **187**, 180–183
- Burridge, K. (1986) *Cancer Rev.* **4**, 18–78
- Matsumura, F., Lin, J. J. C., Yamashiro-Matsumura, S., Thomas, G. P. and Topp, W. C. (1983) *J. Biol. Chem.* **258**, 13954–13964
- Hayman, E. G., Engvall, E. and Ruoslahti, E. (1981) *J. Cell Biol.* **88**, 352–357
- Plantefaber, L. C. and Hynes, R. O. (1990) *Cell* **56**, 281–290
- Leavitt, J. and Kakunaga, T. (1980) *J. Biol. Chem.* **255**, 1650–1661
- Higgins, P. J., Chaudhari, P. and Ryan, M. P. (1991) *Biochem. J.* **173**, 651–658
- Ryan, M. P. and Higgins, P. J. (1994) in *Actin: Biophysics, Biochemistry and Cell Biology* (Estes, J. E. and Higgins, P. J., eds.), pp. 215–230, Plenum Press, New York
- Ciambrone, G. J. and McKeown-Longo, P. J. (1990) *J. Cell Biol.* **111**, 2183–2195
- Pollanen, J., Ross, W. and Vahe, A. (1991) *Adv. Cancer Res.* **57**, 273–328
- Cohen, R. L., Niclas, J., Lee, W. M. F., Wun, T. C., Crowley, C. W., Levinson, A. D., Sadler, J. E. and Shuman, M. A. (1989) *J. Biol. Chem.* **264**, 8375–8383
- Higgins, P. J. and Ryan, M. P. (1989) *Biochem. J.* **257**, 173–182
- Higgins, P. J., Staiano-Coico, L. and Ryan, M. P. (1995) *Biochem. J.* **306**, 497–504
- Ryan, M. P. and Higgins, P. J. (1993) *J. Cell. Physiol.* **155**, 376–384
- Higgins, P. J., Ryan, M. P., Zeheb, R., Gelehrter, T. D. and Chaudhari, P. (1990) *J. Cell. Physiol.* **143**, 321–329
- Higgins, P. J. and Ryan, M. P. (1992) *Biochem. J.* **284**, 433–439
- Higgins, P. J., Ryan, M. P. and Providence, K. M. (1994) *J. Cell. Physiol.* **159**, 187–195
- Herbert, C. A. and Baker, J. B. (1988) *J. Cell Biol.* **106**, 185–193
- Dano, K., Andreassen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S. and Skriver, L. (1985) *Adv. Cancer Res.* **44**, 139–266
- Ryan, M. P. and Higgins, P. J. (1989) *Int. J. Biochem.* **21**, 31–37
- Izzard, C. S. and Lochner, L. R. (1976) *J. Cell Sci.* **21**, 129–159
- Pollanen, J., Hedman, K., Nielsen, L. S., Dano, K. and Vahe, A. (1988) *J. Cell Biol.* **106**, 87–95
- Pollanen, J., Saksela, O., Salonen, E. M., Andreassen, P., Nielsen, L., Dano, K. and Vahe, A. (1987) *J. Cell Biol.* **104**, 1085–1096
- Laiho, M., Saksela, O. and Keski-Oja, J. (1987) *J. Biol. Chem.* **262**, 17467–17474
- Laiho, M. and Keski-Oja, J. (1989) *Cancer Res.* **49**, 2533–2553
- Cajot, J.-F., Schleuning, W.-D., Medcalf, R. L., Bamat, J., Testuz, J., Liebermann, L. and Sordat, B. (1989) *J. Cell Biol.* **109**, 915–925
- Axelrod, J. H., Reich, R. and Miskin, R. (1989) *Mol. Cell. Biol.* **9**, 2133–2141
- Blasi, F., Vassalli, J. D. and Dano, K. (1987) *J. Cell Biol.* **104**, 801–804
- Andreassen, P. A., Pyke, G., Riccio, A., Nielsen, L. S., Blasi, F. and Dano, K. (1987) *Mol. Cell. Biol.* **7**, 3021–3025
- Knudsen, B. S. and Nachman, R. L. (1988) *J. Biol. Chem.* **263**, 9476–9481
- Quigley, J. P. (1979) *Cell* **17**, 131–141
- Sullivan, L. M. and Quigley, J. P. (1986) *Cell* **45**, 905–915
- Soff, G. A., Sanderowitz, J., Gately, S., Verrusio, E., Weiss, I., Brem, S. and Kwann, H. C. (1995) *J. Clin. Invest.* **96**, 2593–2600

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