

Identification of the 52 kDa cytoskeletal-like protein of cytochalasin D-stimulated normal rat kidney (NRK/CD) cells as substrate-associated glycoprotein p52 [plasminogen-activator inhibitor type-1 (PAI-1)]

Expression of p52 (PAI-1) in NRK/CD cells is regulated at the level of mRNA abundance

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Cell shape profoundly affects cellular metabolic activity, protein and nucleic acid synthesis, and cytoskeletal organization. To examine the influence of cell shape on protein expression, normal rat kidney (NRK) cells were exposed to the microfilament-disrupting drug cytochalasin D (CD), labelled with [³⁵S]methionine, and newly synthesized cellular and cytoskeletal proteins examined by two-dimensional gel electrophoresis. CD produced dramatic changes in cell shape (from a flat to round phenotype) with concomitant 3–7-fold increases in the cellular content and cytoskeletal deposition of the microfilament-associated proteins actin, α -actinin, and tropomyosin isoform 1. Augmented actin protein content in NRK/CD cells was paralleled by a corresponding increase in actin mRNA abundance and was inhibited by prior addition of actinomycin D. A detergent-insoluble protein of 52 kDa was also detected at high levels in the cytoskeletal fraction of NRK/CD cells. Two-dimensional electrophoretic mapping of total cellular and cytoskeletal proteins revealed this 52 kDa protein to be the previously described glycoprotein p52 [Higgins & Ryan (1989) *Biochem. J.* 257, 173–182]. By using electrophoretic and immunochemical criteria, p52 was identified as plasminogen-activator inhibitor type-1 (PAI-1). Like actin, CD-induced p52(PAI-1) synthesis, cellular content, and partitioning to the detergent-insoluble cytoskeletal compartment reflected a corresponding increase in p52(PAI-1) mRNA. Such induction was similarly inhibited by actinomycin D. p52(PAI-1) expression in the NRK-cell system is thus responsive to CD-mediated shape changes and requires ongoing RNA synthesis for its induction. Differential extraction of detached cell bodies and the substrate-adherent 'remnant' fraction of NRK/CD cultures, furthermore, indicated that p52(PAI-1) was not an intrinsic internal cytoskeletal element but, rather, selectively localized to the extracellular residue. p52(PAI-1) retained its detergent-insoluble characteristics even in this isolated 'remnant' fraction, where it was also the predominant protein species resolved.

INTRODUCTION

Substantial evidence suggests that cell shape and the cytoskeleton play integral, albeit complex, roles in the regulation of gene expression [see Zambetti *et al.* (1990) for a review]. Control of cell shape itself is closely linked to the synthesis and organization of particular cytoskeletal elements (Ben-Ze'ev, 1989; Farmer & Dike, 1989; Hay & Svoboda, 1989). Disruption of the internal cytoskeleton, with specific cytoskeletal-active drugs, can be used as one approach to assess cell-shape-dependent changes in gene expression. Cytochalasins, for example, represent a complex group of fungal metabolites with marked effects on cytoarchitecture and cell motility [reviewed by Cooper (1987) and Godman & Miranda (1978)] and are frequently used as convenient modulators of cell shape. The cytostructural reorganization which typifies cytochalasin D (CD) treatment of cultured cells progresses through a sequence of three, temporally-related, stages involving (a) energy-independent 'disruption' of actin microfilaments and inhibition of spontaneous filament re-elongation, (b) accumulation of actin-containing 'dense foci' with initiation of cell retraction/arborization and (c) dis-

integration of cellular stress fibres (Schliwa, 1982). These induced morphological changes are accompanied by co-ordinate increases in the relative rates of synthesis of the microfilament-associated proteins actin, α -actinin and high-molecular-mass tropomyosin (Tannenbaum, 1986). In CD-treated murine erythroleukaemia cells, augmented actin biosynthesis reflected increases in both actin mRNA abundance and actin-gene transcriptional activity and was inhibited by actinomycin D (Simpson & Geoghegan, 1990). CD likely regulates actin synthesis (at least in erythroleukaemia cells), therefore, primarily by a transcriptional mechanism.

During a preliminary study of CD-modulated protein synthesis in normal rat kidney (NRK) cells, increased cellular actin content was found to be accompanied by a corresponding increase in the synthesis of a 52 kDa protein which possessed detergent-insolubility properties characteristic of cytoskeletal-like elements. The present study revealed this 52 kDa protein to be, in fact, the substrate-associated glycoprotein p52 and confirms identification of p52 as plasminogen-activator inhibitor type-1 (PAI-1) (Higgins *et al.*, 1990). Expression of p52 (PAI), like that of actin, is also regulated in NRK/CD cells at the level of mRNA abundance.

Abbreviations used: NRK, normal rat kidney; CD, cytochalasin D; i.e.f., isoelectric focusing; p52, glycoprotein p52 [plasminogen-activator inhibitor type-1 (PAI-1)]; TM1, tropomyosin isoform 1; SRE, serum response element; PBS (phosphate-buffered saline) and other buffers are defined in the text.

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MATERIALS AND METHODS

Cell culture, metabolic labelling and extract preparation

Culture of NRK (clone 52E) cells, labelling with [³⁵S]-methionine and exposure to CD (0.1 mM final concentration in the medium for 24 h) were as described by Higgins *et al.* (1989, 1990, 1991). After aspiration of the labelling medium, attached cells in control and CD-treated cultures were washed with phosphate-buffered saline (138 mM-NaCl/1.2 mM-KH₂PO₄/8.1 mM-Na₂HPO₄, 7H₂O/2.7 mM-KCl/0.5 mM-MgCl₂, 6H₂O/0.9 mM-CaCl₂), pH 7.4, containing Mg²⁺ and Ca²⁺ (PBS). Cytoskeletal fractions were prepared at 4 °C using four different extraction protocols *in situ*: (1) cells were extracted for 5 min in TN/T buffer [140 mM-NaCl/10 mM-Tris/HCl (pH 7.6) 1 % Triton X-100], extract buffer removed, and the cytoskeletal residue scraped into TN/T buffer, vortex-mixed and collected at 13000 g; (2) cells were sequentially extracted first in TN/T buffer (as described above) then in TN/T-KCl buffer [140 mM-NaCl/10 mM-Tris/HCl (pH 7.6) 1.5 M-KCl/0.5 % Triton X-100] (Ryan & Higgins, 1987), the cytoskeletal residue scraped into TN/T-KCl buffer, vortex-mixed and collected at 13000 g; (3) cells were extracted directly in TN/T-KCl buffer for 5 min at 4 °C, the cytoskeletal residue scraped into TN/T-KCl buffer and collected as above; or (4) cells were extracted for 5 min in THS buffer [20 mM-Tris/HCl (pH 7.3)/1 % Triton X-100/0.6 M-KCl/5 mM-EDTA] (Rheinwald *et al.*, 1987), the cytoskeletal residue scraped into THS buffer, vortex-mixed and collected at 13000 g. Total cellular lysates were prepared directly in first-dimension lysis buffer [9.8 M-urea/2 % (v/v) Nonidet P40/2 % (v/v) pH 7–9 Ampholytes/100 mM-dithiothreitol] (Ryan & Higgins, 1988).

One- and two-dimensional gel electrophoresis

[³⁵S]Methionine-labelled (5 × 10⁵–2 × 10⁶ c.p.m.) trichloroacetic acid-insoluble total cellular or cytoskeletal proteins in first-dimension lysis buffer were separated by isoelectric focusing on pre-run 1.5 mm-diameter tube gels (pH 5–7/8–10 Ampholytes, 5:1, v/v before molecular-mass separation by SDS/10 %-PAGE (Ryan & Higgins, 1988; Ryan *et al.*, 1989). One-dimensional gel electrophoresis of 12500–50000 c.p.m. cytoskeletal fraction protein was as described by Ryan & Higgins (1988, 1989). Labelled proteins were revealed by fluorography and quantified (as a function of c.p.m. loaded) with a Zeiss MOPS III digital image analyser (Higgins & Ryan, 1989). Proteins were identified by pI/molecular mass, by computer-based spot-set matching according to the REF52 database (Garrels, 1989; Garrels & Franza, 1989) as well as by immunoprecipitation with specific antisera (Higgins *et al.*, 1990).

RNA extraction, plasmid preparation and slot-blot hybridization

Total cytoplasmic RNA was isolated as described by Favalora *et al.* (1980) and ribosomal RNA was quantified by agarose-gel electrophoresis followed by staining with ethidium bromide. RNAs were slot-blotted to nitrocellulose in 20 × SSC (1.5 M-NaCl, 0.15 M-sodium citrate, 2H₂O, pH 7.5) (Davis *et al.*, 1986) in duplicate sets of 10 and 5 μg of RNA/slot and the filters vacuum-baked at 80 °C for 4 h. Plasmids for hybridization included pSS1-3, containing a 2.5 kb p52(PAI-1) cDNA insert in the pBSK(–) vector (Higgins *et al.*, 1990; Zeheb & Gelerhter, 1988), pBSK(–) vector without insert, and the mouse β-actin cDNA probe pActin (Higgins & Ryan, 1991) or restriction-endonuclease-released inserts (see the Results section). Endonuclease-*Eco*RI-linearized plasmids were 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). For Northern analysis of RNA species, 10 μg of cytoplasmic RNA was separated on 1.2 %-agarose/formaldehyde denaturing gels in 10 × SSC, transferred to nitrocellulose by capillary action in 20 × SSC and filters vacuum-baked at 80 °C for 4 h. Filters were prehybridized at 42 °C for 4 h in a solution consisting of (in 11.5 ml) 50 × Denhardt's solution (1 ml), formamide (5 ml), 1 M-Tris/HCl, pH 7.4 (0.5 ml), 5 % (w/v) sodium phosphate, pH 7.4 (0.2 ml), salmon sperm DNA (10 μg/ml; 50 μl), 10 % (w/v) SDS (1 ml) and heparin (20 mg/ml; 0.1 ml). ³²P-labelled probes (5 × 10⁶ c.p.m.) were added to the hybridization buffer and allowed to incubate at 42 °C for 24 h. Blots were washed twice for 15 min each in 1 × SSC containing 0.1 % SDS then twice for 15 min each in 0.25 × SSC containing 0.1 % SDS and exposed to Kodak X-Omat AR5 film using intensifying screens.

Concentration of NRK/CD secreted proteins

NRK cells were seeded to 100 mm-diameter culture dishes containing 10 ml of RPMI 1640 + 10 % (v/v) fetal-bovine-serum medium. At 80 % confluent growth, the medium was aspirated, monolayers washed twice with Hanks balanced-salts solution and 5 ml of serum-free RPMI 1640 medium containing CD added to each culture. After 24 h, media (containing secreted proteins) were harvested from 165 cultures, clarified at 1000 g, and concentrated 800-fold by using Millipore centrifugal ultra-free-60 HMLW low-binding cellulose filter units (no. UFC6LG02). The medium concentrate was diluted with an equal volume of labelling medium from an NRK/CD culture incubated for 6 h with [³⁵S]methionine to provide 'tracer' p52 (see, e.g., Higgins *et al.*, 1990).

One-dimensional Western blotting

The mixture of concentrate and tracer medium was clarified at 13000 g, diluted with an equal volume of electrophoresis sample buffer [50 mM-Tris/HCl (pH 6.8)/10 % (v/v) glycerol/1 % SDS/1 % 2-mercaptoethanol], boiled, and proteins were separated on SDS/10 %-acrylamide slab gels (Higgins *et al.*, 1990, 1991). Conditioned medium from dexamethasone-stimulated HTC-rat hepatoma cells provided a position PAI-1 marker for one-dimensional electrophoresis and blot detection (Zeheb *et al.*, 1987). Gels were equilibrated in transfer buffer (25 mM-Tris/192 mM-glycine) and proteins electrotransferred to nitrocellulose (70 V, 3 h). Membrane binding sites were blocked using 3 % BSA in TBS [20 mM-Tris (pH 7.5)/500 mM-NaCl], followed by incubation for 18 h in primary antibody [rabbit anti-(rat PAI-1)] (diluted 1:400 in BSA/TBS) (Zeheb *et al.*, 1987). After one rinse in water and two rinses (10 min each) in TBS/0.05 % Tween 20, secondary antibody (alkaline phosphatase-conjugated IgG fraction of goat anti-(rabbit IgG) serum, diluted 1:3000) was added for a 3 h incubation. Colour development of rinsed blots was as described for two-dimensional blots (see below) autoradiography of En³Hance-sprayed blots (for detection of tracer p52) was at –70 °C for 3 days.

Two-dimensional Western blotting

First-dimension i.e.f. gels were loaded with 40 μl of concentrate and tracer media for protein separation over the pH range 8–4 (Ryan *et al.*, 1989); second-dimension SDS/PAGE utilized 7.5 %-acrylamide slab gels. Proteins were transferred to Immobilon-P (100 V, 1 h) and the membranes dried at room temperature. Antibody incubations and membrane rinses were as for one-dimensional blotting, with the exceptions that Tween 20 was excluded from rinse buffers; secondary antibody was alkaline phosphatase-conjugated. Colour development utilized Nitro Blue Tetrazolium (0.02 %, w/v) and bovine calf intestinal phosphatase (0.01 %, w/v) in carbonate buffer [0.1 M-NaHCO₃

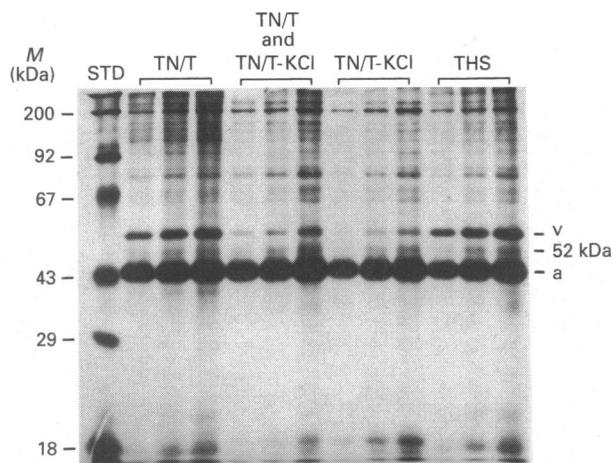


Fig. 1. Analysis of the detergent-insoluble cytoskeletal proteins of NRK/CD cells generated using the four different extraction buffer systems described in the Materials and methods section

For each extraction protocol, lanes were loaded with 1.25×10^4 , 2.5×10^4 and 5.0×10^4 c.p.m. of trichloroacetic acid-insoluble [^{35}S]-methionine-labelled cytoskeletal-fraction protein. The general composition of extracts so generated was similar for all buffer systems, except for greater quantitative recoveries of vimentin (v) obtained with buffers lacking very high (i.e., 1.5 M) KCl concentrations. Actin (a) recovery was approximately the same for each extraction method, as was the content of the 52 kDa cytoskeletal-associated protein. All gels were internally calibrated with protein standards (STD) of known molecular mass, *M* (in kDa).

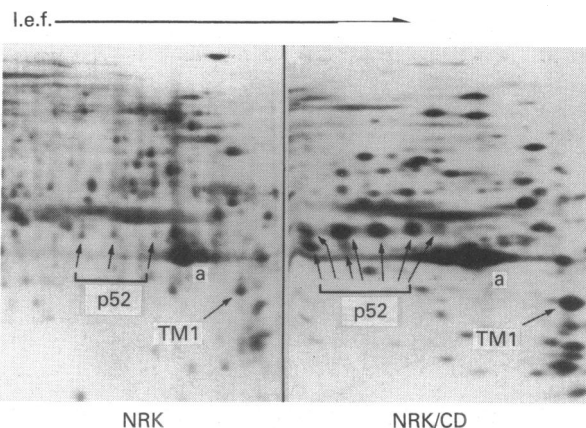


Fig. 3. Two-dimensional electrophoresis of total cellular proteins of NRK and NRK/CD cells

Increases in the deposition of actin (a), TM1 and p52(PAI-1) into the cytoskeletal fraction of NRK/CD cells (Fig. 2) are also evident at the level of total cellular protein.

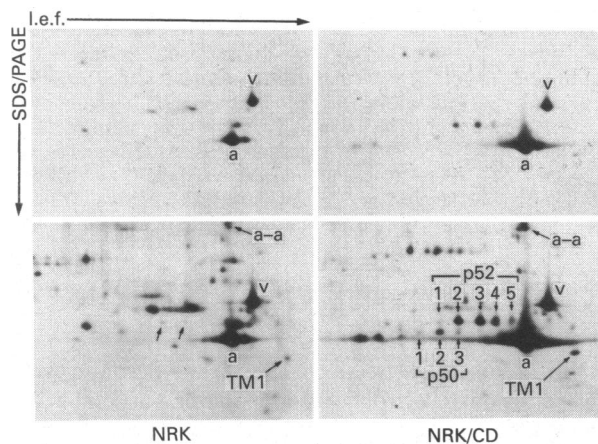


Fig. 2. Two-dimensional gel electrophoresis of the TN/T-insoluble cytoskeletal protein fractions of NRK and NRK/CD cells

First-dimension i.e.f. gels were loaded with equivalent-c.p.m. trichloroacetic acid-insoluble [^{35}S]-methionine-labelled cytoskeletal proteins from both cell types. The obvious increase in cytoskeletal actin in NRK/CD cells (see the text for details of quantification) is evident in light autoradiographic exposures (3 days; top panels). Significant quantitative changes in less-abundant cytoskeletal proteins required longer autoradiographic exposure periods (2 weeks; bottom panels). The only 52 kDa protein evident in the cytoskeletal residue of NRK/CD cells was found to possess a pI range and isoform distribution identical with those of the previously defined substrate-associated glycoprotein p52 (Higgins *et al.*, 1989, 1990, 1991). Only faint levels of the most abundant p52 isoforms (i.e. p52-2 and p52-3) were evident in the cytoskeletal fraction of NRK cells (unmarked arrows, bottom left panel). Obvious also in the NRK/CD cytoskeletal protein separations are increases in α -actinin (a-a) and TM1; similarly, longer exposures revealed the three known isoforms of the less-glycosylated 50 kDa variant (p50) of p52. In contrast, the concentration of the intermediate-filament protein vimentin (v) in the cytoskeletal fraction was similar for both cell types.

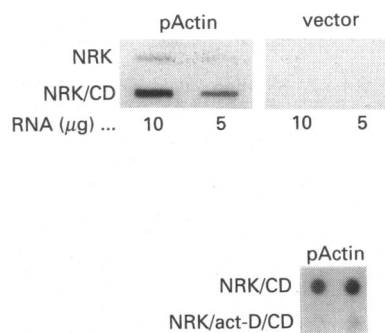


Fig. 4. Increased abundance of actin mRNA in NRK/CD cells as compared with NRK controls

Total cytoplasmic RNA was blotted on to nitrocellulose at 10 and 5 μg /slot before hybridization with ^{32}P -labelled DNA (either pActin or vector alone). A light autoradiographic exposure is shown which, when quantified by scanning densitometry, revealed an approx. 6–8-fold CD-associated increase in actin mRNA abundance. Input RNA content was confirmed by spectroscopy as well as by quantification of ribosomal RNA in each sample by electrophoretic fractionation in agarose gels followed by staining with ethidium bromide. Augmented actin mRNA levels as a consequence of CD treatment required ongoing RNA synthesis during the period of CD exposure, since actinomycin D (5 μM) added before the change-over to CD-containing medium effectively inhibited the increase in actin mRNA abundance (determined by hybridization of the pActin probe to duplicate dot-blot containing 10 μg of RNA from control and CD-treated cells).

(pH 9.8)/1 mM-MgCl₂]. Autoradiography of dried blots was as indicated above.

RESULTS

Retraction of normally well-spread NRK cells is evident within 15 min of exposure to CD and starts at the peripheral regions. Cellular margins are almost completely retracted by 30 min, although considerable substrate-attached residual material remained behind. Refractility becomes evident at 45 min and is most pronounced by 2 h, correlating with virtual complete

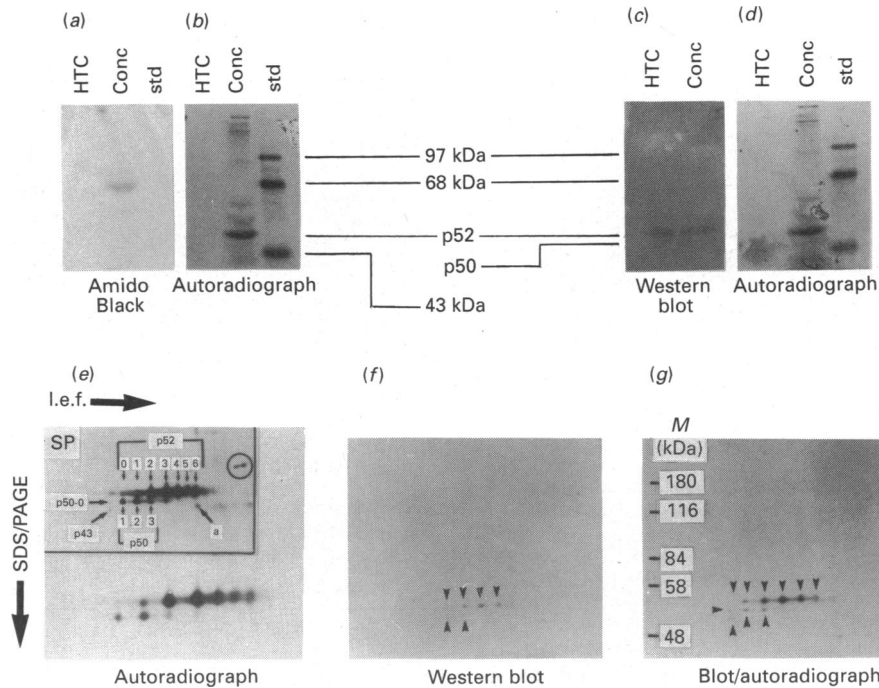


Fig. 5. Immunoechemical criteria for identification of p52 as rat PAI-1

(a–d) One-dimensional electrophoretic analysis. Conditioned medium from PAI-1-secreting HTC-rat hepatoma cells and the medium concentrate from NRK/CD cells + ‘tracer’ (‘Conc’) were fractionated on SDS/10% acrylamide gels and the proteins transferred to nitrocellulose, where they were revealed by Amido Black staining (a) or by reaction with anti-(rat PAI-1) antibodies (c). The protein content of HTC medium was too low for detection with Amido Black, and only residual serum albumin (derived from fetal-bovine serum used for cell growth) was evident in the Conc. Tracer [35 S]methionine-labelled proteins in the ‘Conc’ and 14 C-labelled standards (‘std’) provided markers for identification of p52 by autoradiography (b). Only p52 and the 50 kDa (p50) isoform of rat PAI-1 were reactive with antibodies to rat PAI-1 (c). Autoradiography of the one-dimensional Western blot indicated that the immunoreactive bands co-migrated with p52 and p50 (d). 14 C-labelled standards for one-dimensional electrophoresis included phosphorylase b (97 kDa), BSA (68 kDa) and ovalbumin (43 kDa). Two-dimensional electrophoretic analysis (e–g) confirmed that individual pI variants of p52 were reactive with antibodies to p52. [35 S]Methionine-labelled NRK/CD secreted proteins (SP) were fractionated in two-dimensions (e), as were Conc + tracer fractions (f, g). The inset (in e) indicates designation of the known secreted isoforms of p52 (from Higgins *et al.*, 1989, 1990, 1991; Higgins & Ryan, 1989); identity of p50 as a less-glycosylated variant of p52 has been established by one- and two-dimensional immunoechemical criteria, as well as by analysis of proteolytic digestion products (Higgins *et al.*, 1989, 1990). Transfer of proteins to Immobilon-P and reaction with antibodies to rat PAI-1 revealed six immunoreactive spots (f) which co-migrated [according to molecular mass (M)/pI] with six abundant isoforms of [35 S]methionine-labelled p52 (g). 14 C-labelled standards for two-dimensional gel calibration included α_2 -macroglobulin (180 kDa), β -galactosidase (116 kDa), fructose-6-phosphokinase (84 kDa), pyruvate kinase (58 kDa) and fumarase (48 kDa).

retraction. By 24 h, individual cell bodies are rounded and delicately attached to the plastic substrate [see Schliwa (1982) and Higgins *et al.* (1990) for complete morphological description]. Electrophoretic comparison of detergent-insoluble proteins obtained by the four different extraction protocols indicated that all procedures were approximately equivalent in their ability to generate actin-enriched cytoskeletal fractions (Fig. 1). The only obvious quantitative difference in the major cytoskeletal proteins resolved was an apparent loss in the intermediate-filament protein vimentin in protocols utilizing 1.5 M-KCl in either single- or two-stage extraction procedures. These orienting experiments indicated that the simple one-step TN/T extraction procedure yielded NRK cytoskeletons which were qualitatively and quantitatively at least as good as, if not better than, extraction procedures using high KCl concentrations. Scanning densitometry of two-dimensional gel separations of NRK and NRK/CD cytoskeletal fractions revealed CD-associated increases in actin (5–7-fold), tropomyosin isoform 1 (TM1) (4–6-fold), and α -actinin (3–5-fold) (Fig. 2). These quantitative changes in cytoskeletal deposition of newly synthesized microfilament-associated proteins were in sharp contrast with the intermediate-filament protein vimentin, which did not change in

content as a result of CD treatment (Fig. 2) and were also evident in two-dimensional electrophoretic separations of total cellular protein (Fig. 3). This latter point indicated that it was not merely the cytoskeletal deposition of actin and TM1 which was altered in CD-treated cells, but that the cellular content of these two microfilament-associated proteins was increased as well. Augmented total cellular and cytoskeletal-associated actin content seen in NRK/CD cells reflected a corresponding increase in actin mRNA abundance (Fig. 4). This induction required ongoing RNA synthesis during the period of CD exposure, since the increase in actin mRNA was prevented by addition of actinomycin D before exposure to CD (Fig. 4). In addition to actin and vimentin, a third major cytoskeletal element found in NRK/CD cells was a detergent-resistant protein of 52 kDa (Fig. 1). Only a single 52 kDa protein, possessing extensive pI microheterogeneity, was evident upon two-dimensional electrophoresis of the cytoskeletal fraction of CD-stimulated cells. This 52 kDa protein was also present in significantly increased abundance (as were actin and TM1) in total extracts of NRK/CD cells and was resolved, albeit as a relatively minor component, in unstimulated cells (Fig. 3). Electrophoresis of the cytoskeletal fraction of NRK/CD cells, however, clearly indicated that this

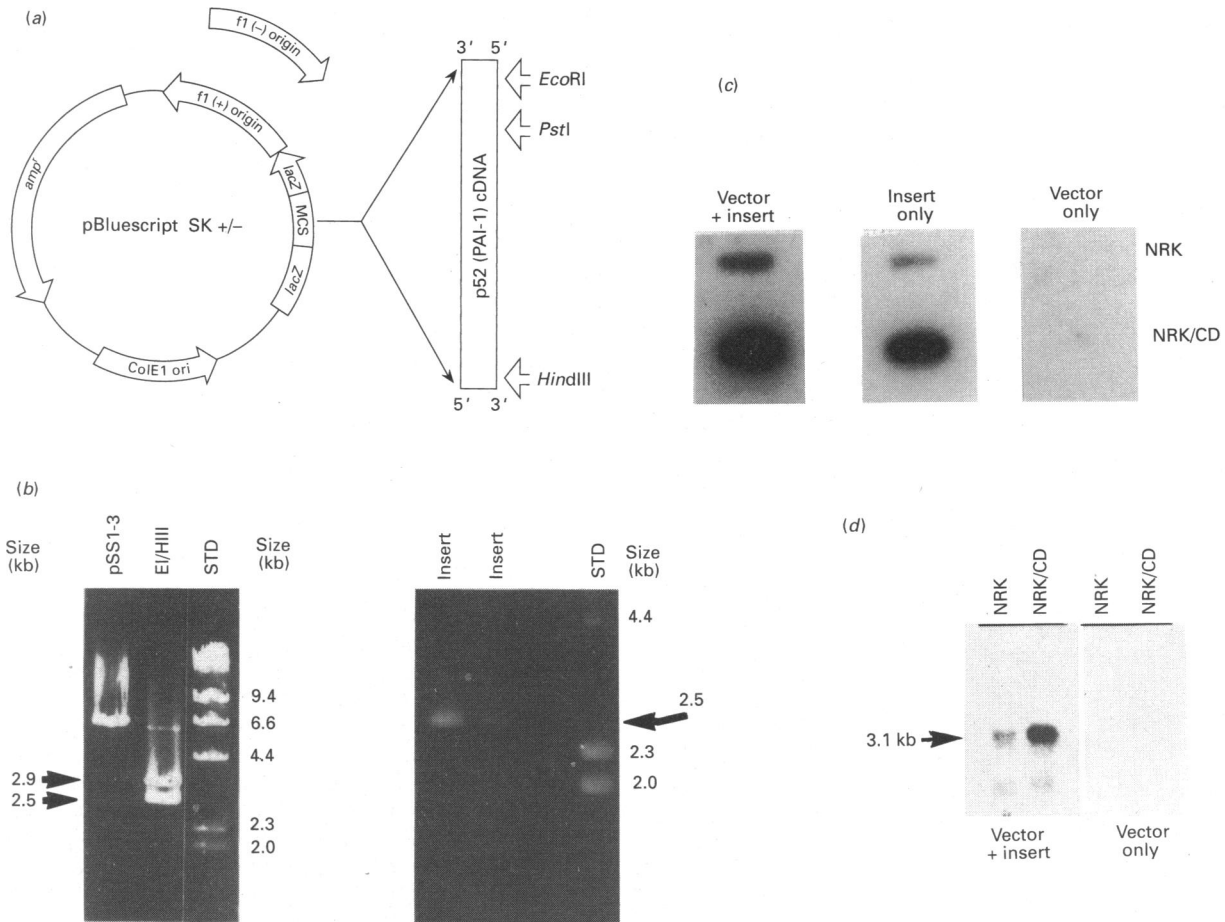


Fig. 6. Increased p52 (PAI-1) mRNA abundance in NRK/CD cells

A 2.5 kb cDNA containing the entire coding region of rat p52 (PAI-1) (Higgins *et al.*, 1991; Zeheb & Gelehrter, 1988) was inserted into the 2.9 kb phagemid pBluescript SK(-) and propagated in *Escherichia coli* strain XL1-Blue using ampicillin, isopropyl thiogalactoside and 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside to select for white recombinants (a). [Abbreviations used in the phagemid: fl(+/-) origin, (-) orientation; lacZ, β -galactosidase; MCS, multiple cloning site; ColE1 ori, replication origin; amp^r, ampicillin-resistance gene.] pSS1-3, containing vector (pBluescript) and insert [p52 (PAI-1) cDNA] sequences, was prepared in quantity, digested with *EcoRI*/*HindIII* restriction endonucleases (E1/HIII), and fragments were separated on 1% agarose gels, using *HindIII*-digested λ -phage DNA (STD) as internal markers (b). The 2.5 kb p52 (PAI-1) cDNA insert was cut out of the gel and re-run to ascertain the purity of the insert preparation. DNA fragments in agarose gels were revealed by staining with ethidium bromide (b). Equivalent amounts of RNA from NRK and NRK/CD cells were slot-blotted to nitrocellulose and probed with ³²P-labelled DNAs representing vector+insert, insert only or vector only. Identical results were obtained, showing a marked increase in p52 (PAI-1) mRNA abundance as a function of CD exposure, using either vector+insert or insert alone as probes; pBluescript vector alone did not hybridize to NRK mRNA and thus served as a negative control (c). Northern-blot analysis indicated that the CD-associated increase in p52 (PAI-1) mRNA abundance was due to increases in the 3.1 kb species of p52(PAI-1) mRNA (d).

52 kDa protein had a molecular mass and pI isoform diversity virtually identical with that of the substrate-associated protein p52(PAI-1) (Higgins *et al.*, 1990, 1991) (Fig. 2). Identity of the 52 kDa protein as p52(PAI-1) was confirmed by combined Western blotting (using antibodies specific for rat PAI-1; Zeheb *et al.*, 1987) and blot autoradiography at both the one- and two-dimensional separation levels (Fig. 5).

As was the case for actin, augmented p52(PAI-1) biosynthesis, cellular content and cytoskeletal deposition reflected a corresponding increase in p52(PAI-1) mRNA abundance (Fig. 6). p52(PAI-1) mRNA from unstimulated cells as well as that induced in response to CD migrated as a single 3.1 kb species (Fig. 6). This is the size predicted from sequence analysis of a full-length p52(PAI-1) cDNA clone (Zeheb & Gelehrter, 1988) and suggests that no unique p52(PAI-1) transcripts are produced as a consequence of CD treatment. [It was previously established that CD-stimulated p52 synthesis and mRNA accumulation, like that of actin mRNA (Fig. 4), was actinomycin D-sensitive (Higgins *et al.*, 1990).]

p52(PAI-1) was a prominent contributor to the cytoskeletal-protein fraction of NRK/CD cells (Fig. 2). It remained to be established, however, whether p52 (PAI-1) was an intrinsic cytoskeletal element of NRK/CD cells or if it co-isolated with the bulk of the cytoskeletal fraction proteins, owing to similarities in detergent phase partitioning. To distinguish between these possibilities, NRK/CD cells were labelled with [³⁵S]methionine, the cell bodies detached from the culture surface with a stream of buffer and collected by centrifugation. Compared with electrophoretic profiles of total culture protein (Fig. 3) or to the TN/T-insoluble fraction obtained from cells extracted *in situ* (Fig. 2), detached NRK/CD cells possessed relatively little p52 (PAI-1). The low level of p52 (PAI-1) that was present in the detached cell bodies did selectively partition to the TN/T-soluble phase (Fig. 7). By far the most preponderant fraction of p52 (PAI-1) recovered [$> 95\%$ of total culture p52 (PAI-1) content], however, was in the residue that remained adherent to the culture dish (remnant fraction) after detachment of the cell bodies. Extraction of this remnant fraction with TN/T

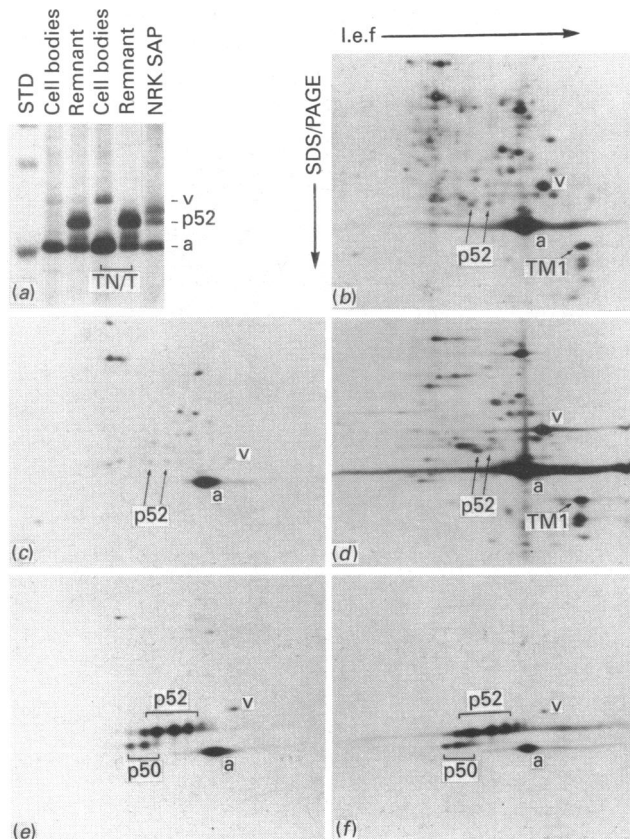


Fig. 7. Selective localization of p52(PAI-1) to the substrate-adherent residue of NRK/CD cells

Cell bodies or the substrate-attached 'remnant' protein fraction were obtained as described in the text and solubilized in electrophoresis sample buffer directly or extracted in TN/T buffer before solubilization. One-dimensional electrophoresis revealed selective enrichment of p52(PAI-1) in the remnant protein fraction and the insolubility of remnant-associated p52(PAI-1) in TN/T buffer (a) [STD, protein standards of 97, 68, and 43 kDa (top to bottom)]. Two-dimensional electrophoresis of lysates of detached NRK/CD cell bodies (b) revealed little cell-associated p52(PAI-1) compared with the abundant p52(PAI-1) levels observed in total culture lysates (e.g. Fig. 3). Comparison of the TN/T-soluble proteins of detached NRK/CD cells (c) with the TN/T-insoluble fraction (d) indicated that most of the cell-body-associated p52(PAI-1) partitioned to the TN/T-soluble phase. The p52(PAI-1) region (arrows) in (d) was occupied by non p52(PAI-1)-like protein species. By far the greatest fraction (> 95%) of total culture p52(PAI-1) localized to the substrate-attached remnant protein compartment, where it was also the predominant protein species (e). Virtually all of this remnant fraction-associated p52(PAI-1) was TN/T-insoluble (f). Other abbreviations: v, vimentin; a, actin; SAP, saponin-resistant matrix (Higgins *et al.*, 1990, 1991; provided p52 marker).

buffer before harvesting of the substrate-attached material indicated that virtually all of this remnant-associated p52 (PAI-1) was TN/T-insoluble.

DISCUSSION

Differential-fractionation data clearly indicate that the 52 kDa detergent-insoluble protein which co-fractionates with the cytoskeletal fraction from total NRK/CD cultures is, in fact, the matrix-associated protein p52 (PAI-1). Virtually all (> 95%) the p52 (PAI-1) produced in response to CD thus resides in the extracellular-matrix compartment, where (even *in situ*) it exhibits both sensitivity to exogenous proteinase (Higgins, 1992) and

detergent-insoluble traits (Fig. 7). Examination of the electrophoretic microheterogeneity of remnant-associated p52(PAI-1) both before and after extraction with TN/T buffer, furthermore, revealed no obvious quantitative difference in the species of p52 (PAI-1) retained on the matrix. This suggests that none of the three subtypes of p50 or the six subtypes of mature p52(PAI-1) resolved here exhibit a preferential association for the matrix nor differentially compartmentalize to the detergent-insoluble matrix fraction. These data, therefore, clarify previous observations made on human (Rheinwald *et al.*, 1987), murine (Santaren & Bravo, 1987) and porcine (White *et al.*, 1990) PAI-1-like proteins regarding their cytoskeletal-like solubility characteristics.

CD induces p52(PAI-1) and actin biosynthesis in NRK cells. This likely reflects largely transcriptional-level controls, since the increased p52(PAI-1)/actin protein content closely parallels the increase in mRNA abundance for the two proteins and is effectively blocked by actinomycin D. All of the induced p52 (PAI-1) mRNA corresponded to a single size species of 3.1 kb which is, in fact, the known length of rat PAI-1 mRNA (Zeheb & Gelehrter, 1988). It is apparent, therefore, that CD induces accumulation of fully mature rat p52(PAI-1) mRNA at an abundance level which closely corresponds to the increase in p52 (PAI-1) protein synthesis.

A major issue to be resolved concerns the molecular mechanisms by which CD induces p52 (PAI-1)-gene expression. Recent data indicate that CD is a strong stimulator of p52(PAI-1)-gene transcription (Chaudhari *et al.*, 1990) and that the nuclear run-on rate closely approximates the increase in mRNA abundance reported here. How might CD induce changes in p52(PAI-1) gene transcription? Disruption of HeLa-cell microfilaments with cytochalasin D was accompanied by a rapid increase in oncogene-*c-fos* steady-state mRNA levels due to, at least partially, increased *c-fos* transcription (Zambetti *et al.*, 1991). Augmented levels of FOS protein (product of *fos* gene) occurs in CD-treated NRK cells and was maintained for at least 24 h after initial addition of CD (P. J. Higgins & M. P. Ryan, unpublished work). The mechanism of *c-fos* induction by CD is not known, although speculation ranges from indirect effects on protein kinase C to perturbation of the nuclear matrix (which in turn affects transcription of certain genes), to signal transduction due to either microfilament disruption (Zambetti *et al.*, 1991) or cell-shape change. Drug-induced microtubule collapse will induce transcription of chloramphenicol acetyltransferase genes linked to *c-fos* or actin promoters (Ng, 1989). Both the *c-fos* and actin promoters share a specific transcription consensus sequence known as the serum response element (SRE) (Mohun & Garret, 1987). Positive transcriptional activation of the SRE might lead to increased actin mRNA accumulation in response to CD. A similar SRE in the p52 (PAI-1) promoter, however, has yet to be defined, suggesting that p52 (PAI-1) expression in CD-treated cells may be regulated by another mechanism. Analysis of the 5' flanking region of the rat p52 (PAI-1) gene has identified several potential regulatory sequences, most notably a closely grouped array of *fos/jun* (AP-1)-like binding sites (Bruzdinski *et al.*, 1990). AP-1 elements, either independently or in tandem arrangement, can mediate elevated transcription of specific genes via binding of FOS/JUN complexes (Curran & Franza, 1988). Since CD is an effective inducer of *c-fos*-gene transcription (Zambetti *et al.*, 1991), it is possible that the expression of genes containing *fos*/AP-1 regulatory sequences in their 5' flanking regions [such as p52 (PAI-1)] may be positively influenced by a CD-stimulated *fos*-dependent mechanism.

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