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Cell-shape regulation and matrix protein p52 content in phenotypic variants of *ras*-transformed rat kidney fibroblasts

Functional analysis and biochemical comparison of p52 with proteins implicated in cell-shape determination

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The 52 kDa transformation-sensitive protein p52 was previously identified as a major substrate-associated component of normal rat kidney (NRK) fibroblasts [Higgins & Ryan (1989) Biochem. J. **257**, 173–182]. p52 selectively localized to cellular fractions enriched in substrate focal-contact sites and associated ventral undersurface elements. Rapid attachment/spreading of NRK cells on to prepared p52 matrices and inhibition of fibroblast spreading by antibodies to p52 indicated that this protein participates in shape determination or cell-to-substrate adhesion. NRK cells transformed with Kirsten murine sarcoma virus (KiMSV), with a temperature-sensitive mutant (ts-371 KiMSV) and maintained at the permissive temperature, or with the cloned EJ*ras*$^{val\text{-}12}$ oncogene, exhibited down-regulated accumulation of p52 in the ventral undersurface region. Immunochemical, lectin-affinity and electrophoretic analyses indicated that p52 shares considerable sequence similarity with plasminogen-activator inhibitor type-1, which is consistent with its subcellular localization and likely morphoregulatory activity. The marked down-regulation of p52 expression seen in four different *ras*-mediated transformation systems, its induction prior to butyrate-induced morphological reorganization in KiMSV-transformed cells, and the morphological consequences of exogenously added p52 or p52 antibodies on NRK fibroblasts suggest that this protein probably functions in cell-shape regulation. Abrogation of p52 matrix accumulation typically seen in *ras* transformants may contribute, therefore, to the aberrant cytoarchitecture characteristic of malignant fibroblasts.

INTRODUCTION

Cell-to-substrate adhesion is usually altered in fibroblasts expressing mutant *ras* oncogenes. v-K-*ras*-transformed rat kidney (KNRK) cells, for example, exhibit several anomalies in substratum-attachment elements. These include loss of cell-associated laminin, failure to assemble a fibronectin (FN)-rich extracellular matrix (ECM) (Hayman *et al.*, 1981), reduced integrin content (including one FN receptor) (Plantefaber & Hynes, 1989), disruption of actin microfilaments (MFs) and associated focal-contact (FC) structures (Altenburg *et al.*, 1976; Ryan & Higgins, 1988), decreased ventral undersurface deposition of the 52 kDa glycoprotein p52 (Ryan & Higgins, 1989) and reduced ECM adherence (Hynes, 1976; Yamada, 1983; Sistonen *et al.*, 1987). Integrins link ECM components to the cytoskeleton, probably through the MF-associated proteins vinculin and talin (Damsky *et al.*, 1985; Chen *et al.*, 1986). Deficient integrin content, distribution or receptor activity might therefore contribute to the altered morphology, reduced adhesion and cytoskeletal (CSK) anomalies typical of *ras*-transformants (Plantefaber & Hynes, 1989). Cell adhesion, however, is a complex process involving at least 30 distinct protein species (Edelman, 1988). Understanding cell-shape regulation requires further study of the activities and interactions within this diverse protein group.

Normal rat kidney (NRK) fibroblast p52 localizes to cellular fractions enriched in substrate-anchoring elements (Ryan & Higgins, 1989; Higgins & Ryan, 1989). Morphologically abnormal KNRK cells fail to express p52 constitutively, but are inducible for p52 synthesis and substrate deposition upon exposure to sodium *N*-butyrate (NaB) (Ryan & Higgins, 1989). Pulse–chase studies indicated that the saponin-resistant ECM (consisting of FCs and the ventral undersurface 'carpet') is the initial site of p52 targeting (Higgins & Ryan, 1989). ECM deposition of p52 occurred before NaB-induced increases in KNRK-cell flattening (Altenburg *et al.*, 1976; Ryan & Higgins, 1989). Plating of KNRK fibroblasts on to prepared p52-enriched matrices, moreover, resulted in rapid spreading (Ryan & Higgins, 1989) similar to the temporary morphological reversions induced by FN (Ali *et al.*, 1977; Wagner *et al.*, 1981). These data stimulated additional analysis of p52 expression in normal and *ras*-transformed fibroblasts, including a comparison with proteins implicated in cell-shape regulation.

MATERIALS AND METHODS

Cell culture

NRK, KNRK (Ryan & Higgins, 1988, 1989) and ts371-NRK fibroblasts (which possess a temperature-sensitive lesion within

Abbreviations used: NRK, normal rat kidney; KiMSV, Kirsten murine sarcoma virus; KNRK, v-K-*ras*-transformed rat kidney; FN, fibronectin; ECM, extracellular matrix; MF, microfilaments; FC, focal contact; CSK, cytoskeletal; PE, porcine aorta endothelial; CPAE, bovine pulmonary-artery endothelial cells; FBS, foetal-bovine serum; DMEM, Dulbecco's modified Eagle's medium; ECGF, endothelial-cell growth factor; HAAE, human abdominal aorta endothelial; HUVE, umbilical-vein endothelial; CD, cytochalasin D; NaB, sodium *N*-butyrate; SP, secreted proteins; SAP fraction, substrate-attached residue; ESB, electrophoresis sample buffer; NP40, Nonidet P40; Con A, concanavalin A; IP, immunoprecipitation; MLV, Moloney leukaemia virus; PAI-1 plasminogen-activator inhibitor type-1; PA, plasminogen activator; AP-1, activator protein 1.

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the transforming $p21^{v\text{-}K\text{-}ras}$ gene) (Shih *et al.*, 1979) were grown in RPMI 1640 medium with 10% (v/v) fetal-bovine serum (FBS). Porcine aorta endothelial (PE) and bovine pulmonary-artery endothelial (CPAE) cells (A.T.C.C., CCL-209) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, non-essential amino acids, heparin (0.1 mg/ml; 166 units/mg), endothelial-cell growth factor (ECGF; 0.1 mg/ml; Meloy Laboratories, Springfield, VA, U.S.A.) (Phillips *et al.*, 1988; White *et al.*, 1990). Human abdominal aorta endothelial (HAAE) cells (AG09799; Institute for Medical Research, Camden, NJ, U.S.A.) and umbilical-vein endothelial (HUVE) cells (a gift from Dr. P. Del Vecchio, Albany Medical College) were grown on gelatin-coated dishes in M199 medium containing 20% (v/v) FBS, heparin and ECGF. Human mesothelial cells (AG07086; Institute for Medical Research) were cultured in DMEM containing 15% (v/v) FBS, 0.4 μg of cortisol (Sigma)/ml and 10 ng of epidermal growth factor (Collaborative Research, Bedford, MA)/ml. Cytochalasin D (CD; Sigma) or tunicamycin (Boehringer–Mannheim) were added at final concentrations of 0.1 mM or 2 μg/ml respectively to cultures 24 h before metabolic labelling (Higgins *et al.*, 1989). Treatment of KNRK-cell cultures with NaB (2 mM final concn.) was as detailed by Ryan & Higgins (1989) and Higgins & Ryan (1989).

Transfection

NRK cells were seeded into DMEM + 10%-FBS medium. DNA plasmids pWLneo and pEJ6.6, encoding the neomycin/G418-resistance and activated human $EJras^{val\text{-}12}$ genes, were combined (total DNA 20–30 μg) in 500 μl of 250 mM-$CaCl_2$, diluted with 500 μl of 2 × BBS [50 mM-Bes (pH 6.95)/280 mM-NaCl/1.5 mM-Na_2HPO_4], left for 20 min, then added to cell cultures (Chen & Okayama, 1987). Media were changed after 24 h and cells passaged on day 7 to 100 mm-diameter dishes containing RPMI 1640 + 10% FBS medium. G418 (300 μg/ml) was added 6 days later, and colonies of morphologically distinct cell types were isolated with cloning cylinders after 2 weeks of G418 selection.

Metabolic labelling, cell extraction and gel electrophoresis

Cells were labelled with [^{35}S]methionine as described by Ryan & Higgins (1988). Labelling media containing secreted proteins (SP) were aspirated and clarified at 13000 *g*. Cells were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (0.01 M-sodium phosphate/0.14 M-NaCl) (CMF-PBS), pH 7.1, then incubated at 25 °C in 0.2% (w/v) saponin/CMF-PBS for 20 min (Higgins *et al.*, 1989) and dislodged with a stream of CMF-PBS (Neyfakh & Svitkina, 1983). The substrate-attached residue (SAP fraction) was scraped into one-dimensional-electrophoresis sample buffer (ESB) [50 mM-Tris/HCl (pH 6.8)/10% glycerol/1% SDS/1% 2-mercaptoethanol] and boiled or solubilized in two-dimensional buffer [9.8 M urea/2% Nonidet P40 (NP40)/2% (v/v) pH 7–9 ampholytes/100 mM-dithiothreitol]. Electrophoresis of 2.4×10^4–5.0×10^5 c.p.m. trichloroacetic acid-insoluble SAP residue protein was as described by Ryan & Higgins (1989) and Ryan *et al.* (1989). Fluorographs of En^{3}Hance (New England Nuclear)-treated gels were densitometrically scanned and the SAP-fraction protein-p52 content quantified with a Zeiss MOP III digital image analyser (Higgins & Ryan, 1989).

Cell-spreading assays

NRK/CD SAP residues were scraped into PBS, pH 7.4, concentrated with ultrafilters and dialysed against PBS. Culture dishes were coated with 10 μg of SAP-fraction protein (containing 8.5 μg of p52) [calculated from the data of Higgins *et al.* (1989)] or with 10 μg of FBS protein (each in 1 ml of PBS). After 18 h, excess fluid was aspirated, matrices washed three times with growth medium, 2 ml of RPMI 1640 medium + 10% FBS were added to each 35 mm-diameter dish, and 5×10^4 NRK cells seeded/culture. Alternatively, cells were added to dishes containing 1 ml of growth medium supplemented with 10 μg of SAP-fraction or FBS protein. After 30 min at 37 °C, the medium was removed, the cells fixed in methanol and stained with haematoxylin/eosin, and the percentages of 'flat' cells (i.e. cytoplasmic diameters $\geqslant$ to 2 two nuclear diameters) determined microscopically. To assess effects of p52 antibodies on cell spreading, 2×10^4 NRK cells were added to Nunc plate wells containing 0.5 ml of RPMI 1640 + 10% FBS medium and 200–600 μg of the IgG fraction of pre-immunization or anti-p52 sera (Higgins *et al.*, 1990). After 1 h at 37 °C the medium was aspirated and the cells then fixed in methanol and stained. The percentage of well-spread fibroblasts was quantified as described above.

Characterization of the SP fraction

[^{35}S]methionine-labelled SP were fractionated according to differential solubility in $(NH_4)_2SO_4$ and affinity for concanavalin A (Con A)–Sepharose 4B beads (Sigma) (Canfield *et al.*, 1987). $(NH_4)_2SO_4$-insoluble precipitates were collected at 25000 *g*, resuspended in 0.05 M-Tris/HCl, pH 7.4, and dialysed against this buffer at 4 °C, as were the soluble fractions; further dilution was in one-dimensional ESB. For Con A-affinity assays, 50 μl of a 50% suspension of Con A–Sepharose beads in 0.1 M-sodium acetate buffer, pH 6.0, containing 1 M-NaCl, 1 mM-$MnCl_2$, 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$, was added to 150 μl of labelling medium containing 50000 c.p.m. of trichloroacetic acid-insoluble SP and the mixture placed on ice (with intermittent shaking) for 2 h. Con A–Sepharose beads were collected at 13000 *g* for 2 min, washed three times in PBS/0.01% Tween 80 at 13000 *g*, and bound proteins were released by boiling in one-dimensional ESB. Immunoprecipitation (IP) of individual SP utilized procedures and antisera developed previously (Higgins *et al.*, 1990). Immune complexes were collected with Protein A–Sepharose (Sigma) or Protein G–Sepharose (Genex Corp., Gaithersburg, MD, U.S.A.) beads, washed three times in IP buffer [10 mM-Tris/HCl (pH 8.0)/0.15 M-NaCl/1% Triton X-100/0.1% SDS/0.5% deoxycholate], and precipitated antigen was released by boiling in one-dimensional ESB. Evaluation of protein–protein complexes utilized strong nucleophilic agents (NH_4OH + SDS) and electrophoretic criteria as described by Booyse *et al.* (1988). All biochemical profiles were evaluated on SDS/10% (w/v)-acrylamide slab gels using, as input, 25000–50000 TCA-insoluble c.p.m. of [^{35}S]methionine-labelled protein/lane.

RESULTS

Altered p52 expression in *ras*-transformed fibroblasts

p52 comprises 29–37% of the SAP-residue protein of NRK fibroblasts, but was virtually undetectable in the morphologically aberrant KNRK-cell line (Fig. 1). Use of tunicamycin (2 μg/ml; added to medium 24 h before labelling), to generate the M_r 43000 (p43) 'core' peptide of p52, similarly failed to resolve significant SAP-fraction-associated p43 in KNRK fibroblasts (< 5% of the NRK level). Down-regulated SAP-fraction p52 content also correlated with morphological aberrancy in ts371-NRK cells. At 39 °C, ts371-NRK fibroblasts have a flat morphology and approximately normal SAP-fraction p52 content (25–32% of total SAP-fraction protein); these characteristics are maintained for the first 24 h after transfer to the permissive temperature of 31 °C. Transition (over the subsequent 3 days) to a transformed phenotype correlated with marked decreases in SAP-compart-

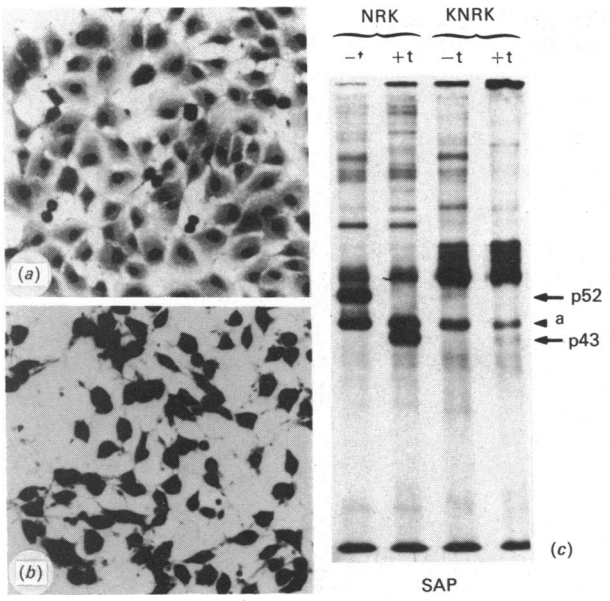


Fig. 1. Differences in SAP fraction p52 levels between normal and v-*ras*-transformed fibroblasts

NRK (*a*) and KNRK (*b*) cells were labelled with [^{35}S]methionine in the presence (+) or absence (−) of a 24 h pretreatment with tunicamycin (t) and SAP-fraction proteins separated on SDS/10%-acrylamide slab gels (*c*). The p52 SAP-residue protein of NRK fibroblasts is reduced to a molecular mass of 43 kDa (p43) as a consequence of exposure to tunicamycin. The identity of p43 as the unglycosylated 'core' of p52 was confirmed previously by immunoprecipitation and proteolytic fragment mapping (Higgins *et al.*, 1989, 1990). The faint bands corresponding to p52 and p43 in KNRK(−t) and KNRK(+t) SAP preparations respectively represent < 5% of the level of these proteins in the SAP residues of NRK fibroblasts. Further abbreviation: a, actin.

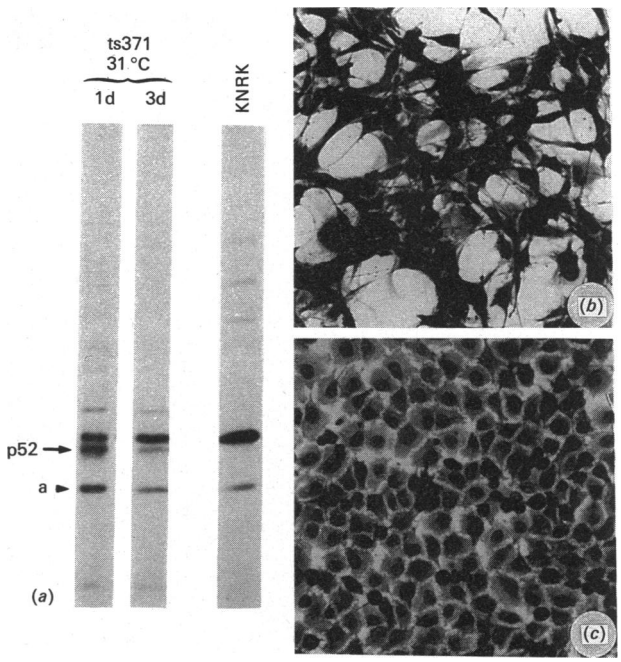


Fig. 2. Changes in cell morphology and SAP-fraction p52 content as a function of temperature in ts371-NRK fibroblasts

ts371-NRK cells cultured at 39 °C then switched to 31 °C for 1 day (1 d) maintained a relatively normal SAP residue p52 content (75% of NRK level) (*a*). After 3 days (3 d) at 31 °C, SAP-fraction p52 content declined significantly corresponding with a marked change in cell morphology. ts371-NRK cells cultured at 39 °C then at 31 °C for 3 days developed a typical transformed phenotype (*b*) compared with ts371-NRK fibroblasts before temperature down-shift (*c*).

ment p52 deposition (to < 10% of the NRK level) and collateral changes in SAP-fraction actin content (Fig. 2). Control experiments indicated that culture of NRK and KNRK cells at 39 or 31 °C did not alter the pattern of SAP-fraction p52 accumulation characteristic of either cell line at 37 °C. p52 down-modulation also typified morphological variants of NRK cells derived by acute infection with wild-type KiMSV–Moloney-leukaemia-virus complex (> 1×10^5 focus-forming units/ml; titred on C127 indicator fibroblasts). After 2 weeks, colonies of morphologically abnormal (KSV/MLV-NRK) cells were selected with cloning cylinders. These cells exhibited the same down-modulated SAP-fraction p52 content characteristic of chronic transformants (i.e. KNRK cells) (Table 1). Finally, decreased p52 accumulation was similarly associated with progressive morphological aberrancy in EJ*ras*-transformed fibroblasts.

Table 1. Characteristics of NRK fibroblasts and derived cell lines

For (*a*), parental NRK cells transfected with the cloned human EJ*ras*$^{val\text{-}12}$ oncogene or infected with either temperature-sensitive (ts) or wild-type (wt) KiMSV. For (*b*), 50–80% confluent cell cultures were fixed in 100% methanol and stained with haematoxylin/eosin for morphological assessments. (*c*) Shows the amount of [^{35}S]methionine-labelled p52 resolved upon one-dimensional electrophoresis of 25000 c.p.m. of trichloroacetic acid-insoluble SAP-fraction protein. Quantification was done by scanning densitometry and digital image analysis (Higgins & Ryan, 1989) and expressed as a percentage relative to NRK fibroblast SAP-fraction p52 content. Data are means ± S.D. for triplicate determinations.

Cells	(*a*) Transforming agent	(*b*) Phenotype	(*c*) Relative SAP-fraction p52 content (%)
NRK	None	Flat	100
cl-13	None	Very flat	1051 ± 273
cl-2A	None	Very flat	638 ± 104
cl-R3	EJ*ras*$^{val\text{-}12}$	Cuboidal, compact	26 ± 3
cl-R2	EJ*ras*$^{val\text{-}12}$	Spindle, piled	11 ± 2
ts371-NRK	tsKiMSV		
31 °C		Spindle, piled	9 ± 3
39 °C		Flat	91 ± 8
KSV/MLV-NRK	wt KiMSV	Spindle, piled	< 5
KNRK	wt KiMSV	Spindle, piled	< 5

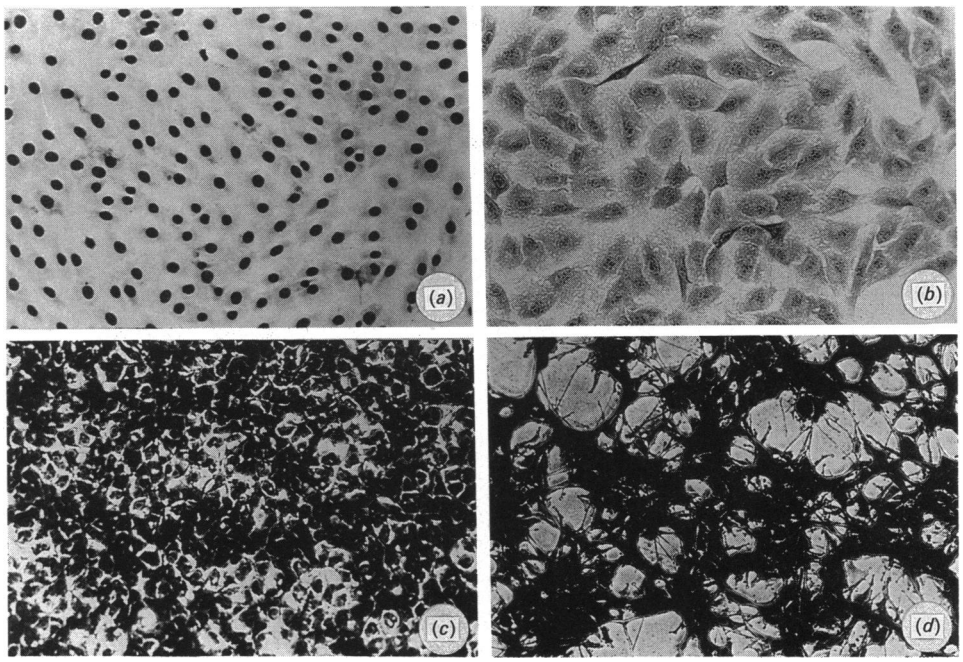
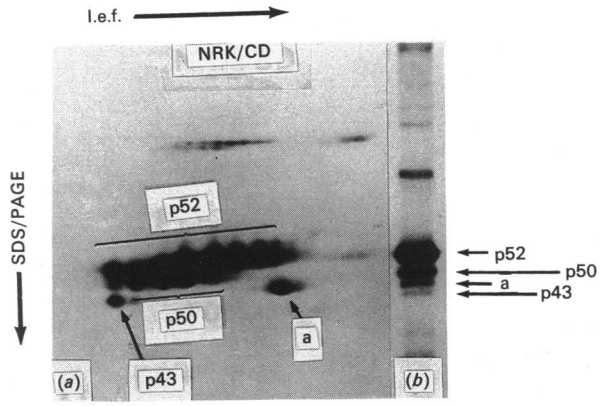
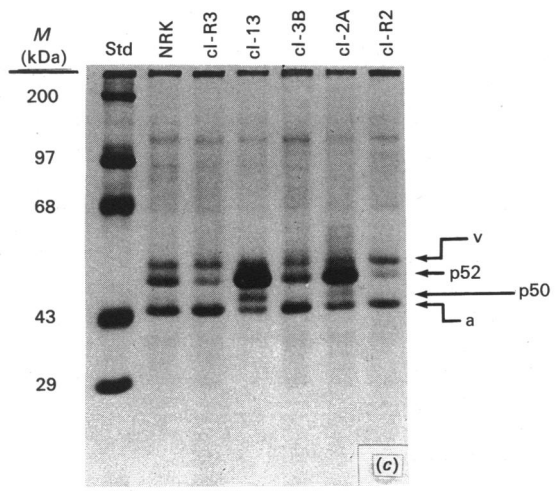


Fig. 3. Morphology of clonal isolates

NRK cells were co-transfected with the *neo*^{R} gene and the cloned EJ6.6*ras*$^{val\text{-}12}$ oncogene (Shih & Weinberg, 1982). Control cells (*a*) possessed typical contact-inhibited fibroblast morphology and grew to densities of 1.3×10^6 cells/50 mm-diameter dish. NRK isolates cl-13 (*b*) and cl-2A (not shown) consisted of extremely flat fibroblasts and attained maximum densities of just 0.7×10^6 cells/50 mm-diameter dish. NRK-EJ*ras* cl-R3 cells (*c*), in contrast, were cuboidal-shaped, tightly juxtaposed, and reached high densities (2.4×10^6 cells/dish), but did not form foci of piled cells. NRK-EJ*ras* cl-R2 fibroblasts (*d*) were spindle-shaped, grew to high density (2.7×10^6 cells/dish) and formed dense foci.





Characteristics of derived clones are summarized in Table 1 and Fig. 3. Very flat cl-13 and 2A cells accumulated significantly more SAP-fraction p52 than parental NRK fibroblasts. By comparison, the progressively abnormal EJ*ras* isolates cl-R3 and R2 were deficient in SAP-fraction p52 content (Fig. 4).

Characterization/properties of p52 and p52-like proteins

NRK cell spreading reflected SAP-fraction p52 content (Figs. 1–4), suggesting that p52 might function in cell-to-substrate adhesion. Indeed, plating of NRK cells directly on to p52-enriched matrices resulted in enhanced cell flattening compared with matrices prepared with an equivalent amount of FBS protein (in both cases, assays were carried out in a > 250-fold excess of FBS protein). SAP-fraction protein promoted cell spreading even more effectively when added directly to the medium immediately before seeding of cells (Table 2). Although SAP fractions consist predominantly of p52 (see the Materials and methods section), there was the possibility that a minor component of such preparations was the active mediator of cell

Fig. 4. Comparative SAP fraction p52 contents of phenotypic variants

Two-dimensional electrophoresis of NRK/CD cellular SAP-fraction proteins (*a*) was used to identify p50, p52, p43 and actin in corresponding one-dimensional separations (*b*) as detailed by Higgins *et al.* (1989, 1990). Relative to NRK fibroblasts, clones composed of well-spread very-substrate-adherent cells (NRK-cl-13 and cl-2A) deposited 6–10-fold more p52 into the SAP compartment, whereas more-aberrant-shaped less-substrate-adherent clones (NRK-EJ*ras* cl-R3 and cl-R2) accumulated significantly less SAP-fraction-associated p52 (*c*). Cl-3B is a *neo*^{R}-derived control cell line similar to NRK cells in morphology and p52 deposition. Abbreviations: a, actin; v, vimentin; Std, molecular-mass (*M*) standards; I.e.f., isoelectric focusing.

Table 2. Extent of NRK-cell spreading attained upon plating cells in the presence of p52-enriched SAP-fraction protein

For (*a*), NRK cells were seeded into RPMI 1640 growth medium + 10 % FBS on to matrices prepared with 10 μg of FBS protein or p52-enriched SAP-fraction protein. For (*b*) NRK cells were seeded into RPMI 1640 growth medium + 10 % FBS supplemented with 10 μg of p52-enriched SAP-fraction protein/ml or 10 μg of FBS protein/ml. For (*c*) the cells, 30 min after seeding, were fixed with methanol and stained with haematoxylin and eosin. Well-spread cells (defined as cells with a cytoplasmic diameter equal to, or exceeding, two nuclear diameters) were quantified microscopically. Results are means ± S.D. for counts made on four sets of 100 cells/set for each culture condition.

Treatment	(*c*) Well-spread NRK cells (%)
(*a*) FBS coated	12.6 ± 5.6
p52 SAP coating	38.2 ± 5.9
(*b*) FBS added	14.6 ± 4.7
p52 SAP added	66.0 ± 11.9

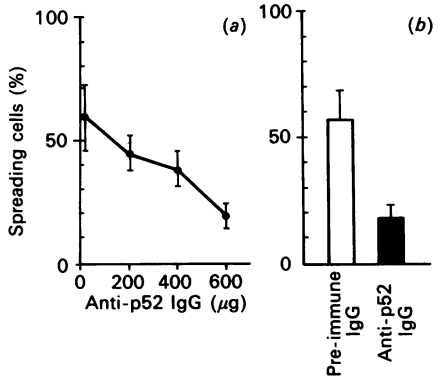


Fig. 5. Inhibition of NRK fibroblast spreading by the IgG fraction of a rabbit antiserum to p52

(*a*) Cells were seeded into Nunc wells containing RPMI 1640 + 10 %-FBS medium and the indicated concentration of anti-p52 IgG. The percentage of spreading cells was determined microscopically 1 h later. (*b*) Cells were seeded into Nunc wells in media containing 600 μg of either pre-immune IgG or anti-p52 IgG; the percentage of spreading cells was determined after 1 h at 37 °C. Comparable concentrations of pre-immune IgG did not influence NRK cell spreading (*b*) and yielded values similar to wells containing no IgG (*a*). In contrast, the IgG fraction of anti-p52 serum dramatically inhibited spreading of attached NRK fibroblasts. Results are means ± S.D. ($n = 9$).

spreading. Rabbit antibodies of p52 (previously shown to immunoprecipitate p52 specifically from NRK cells; Higgins *et al.*, 1990) were tested, therefore, as to their effect on spreading of substrate-attached NRK cells. The IgG fraction of anti-p52 serum, but not pre-immune serum IgG, effectively blocked NRK cell spreading in a concentration-dependent manner (Fig. 5).

On the basis of relative abundance (approx. 20 % of total SP), core size of 43000 M_r, two-dimensional-electrophoretic microheterogeneity and ECM association, p52 appeared similar to Gp47 (Canfield *et al.*, 1987, 1989) and p45 (White *et al.*, 1990) of bovine retinal and porcine/bovine arterial endothelial cells respectively (Fig. 6). $p45_{CPAE}$/p52 proteins possessed overlapping pI distributions (pI 5.6–6.2) compared with the more basic isoforms (pI 5.8–7.2) of $p45_{PE}$. SAP-fraction residues of NRK and PE cells contained similar levels of $p52_{NRK}$ and $p45_{PE}$

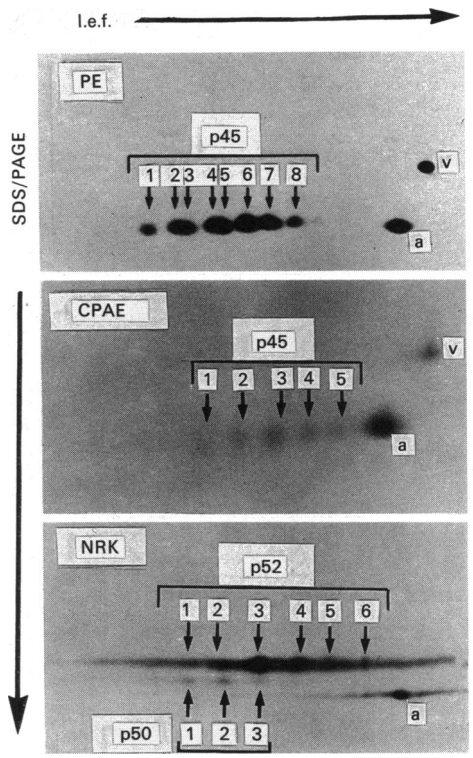


Fig. 6. Two-dimensional electrophoresis of [^{35}S]methionine-labelled SAP fraction proteins from PE, CPAE, and NRK cell cultures

The major SAP-residue proteins are actin (a), vimentin (v) and $p45_{PE/CPAE}$ or $p52_{NRK}$. $p45_{PE}$ resolves as eight distinct isoforms (shorter exposures resolved spots 2–3 and 4–5 as dumb-bell-shaped, each consisting of two closely spaced proteins) (White *et al.*, 1990). $p45_{CPAE}$ exhibits approximately 5, relatively low-abundance, pI variants. p52 maps as 6 isoforms with its less-glycosylated form (p50) resolving as 3 distinct species. p45/p52 proteins possess similar pI ranges while $p45_{PE}$ isotypes appear more basic. Further abbreviation: I.e.f., isoelectric focusing.

respectively, relative to actin (Fig. 6). CPAE SAP fractions, in contrast, had a relatively low $p45_{CPAE}$/actin ratio. Since Gp47 is identical with plasminogen-activator inhibitor type-1 (PAI-1) (Canfield *et al.*, 1989), the biochemical/immunochemical properties of p52 were compared with those of Gp47/PAI-1 and related proteins, including human PAI-1 and the 52 kDa protein induced in KNRK cells by NaB. Like Gp47, the majority (> 90 %) of p52 was insoluble in 30–70 % satd. $(NH_4)_2SO_4$ and could be bound by Con A–Sepharose [as was the 50 kDa human mesothelial PAI-1 protein ('mesosecrin')] (Rheinwald *et al.*, 1987; Cicila *et al.*, 1989) (Figs. 7*a* and 7*b*). p52 (and human mesothelial PAI-1) also reacted with the same antibody to bovine PAI-1 which immunoprecipitated Gp47 (Canfield *et al.*, 1989), with antibodies to purified human PAI-1, as well as with antibodies to rat PAI-1 [Figs. 7*b* and 7*c*; see also Higgins *et al.*, (1990)]. Consistent with previous two-dimensional electrophoretic data (Ryan & Higgins, 1989), p52 was undetectable in the SP of KNRK cells by metabolic labelling, IP or Con A binding (Figs. 7*c* and 7*d*). Both p52 and its less-glycosylated isoform, p50, however, were readily detected by all three methods in the SP of KNRK/NaB cells (Figs. 7*c* and 7*d*). By using these criteria, therefore, NaB-induced p52 in KNRK cells was indistinguishable from p52 constitutively expressed by NRK fibroblasts.

NRK p52, the PAI-1-like p45 protein of PE cells (Fig. 8) and the 50 kDa PAI-1 from human mesothelial (Fig. 7) and en-

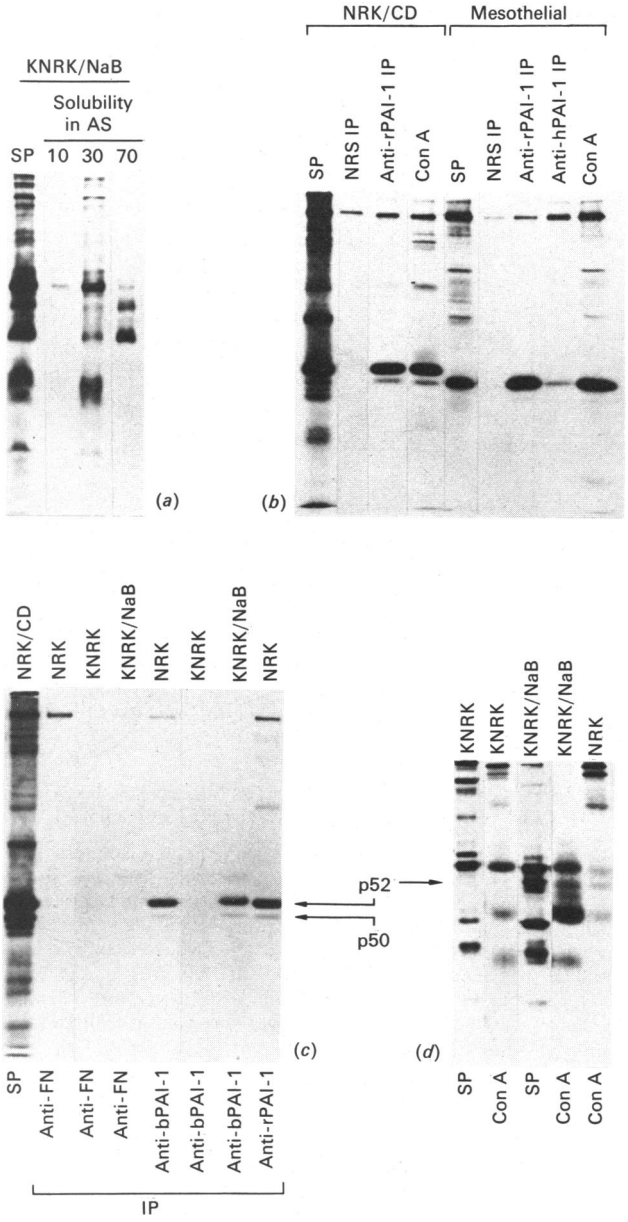


Fig. 7. Comparative solubility, Con A-affinity and immunological properties of p52 with other p52-like proteins

(*a*) Analysis of the fraction of KNRK/NaB secreted proteins (SP) insoluble in 10%-satd. $(NH_4)_2SO_4$ (AS) (10), soluble in 10%; but insoluble in 30%; $(NH_4)_2SO_4$ (30), and soluble in 30%; but insoluble in 70%; satd. $(NH_4)_2SO_4$ (70). Most of the p52 is precipitated between 30 and 70% saturation. (*b*) Using NRK/CD SP as source of p52, both p52 and its less-glycosylated isoform p50 were immunoprecipitated (IP) by antibodies to rat PAI-1 (rPAI-1) (Higgins *et al.*, 1990) and bound by Con A; substituting normal rabbit serum (NRS) for anti-rPAI-1 antibodies failed to precipitate p52. Similarly, the 50 kDa secreted PAI-1 of human mesothelial cells was also precipitated by antibodies to rPAI-1, by antibodies to human PAI-1 (hPAI-1) (American Diagnostica, Greenwich, CT, U.S.A.), and bound to Con A–Sepharose beads. (*c*) Immunoreactive p50/52 was absent from the SP fraction of KNRK cells, but evident in KNRK/NaB fibroblasts. Anti-FN serum was utilized as an unrelated antibody control. p50/p52 were precipitated by both anti-(bovine PAI-1) (bPAI-1) (a gift from Dr. D. Loskutoff) and anti-rPAI-1 antibodies. IP data in (*c*) were confirmed by Con A–Sepharose-binding studies (*d*). The absence of immunoreactive p52 in the KNRK SP fraction was reflected in the lack of a 52 kDa Con A-binding protein. Induction of p52 in KNRK/NaB cells correlated with the appearance of p52 in the Con A-bound fraction, which co-migrated with NRK SP Con A-bound p52.

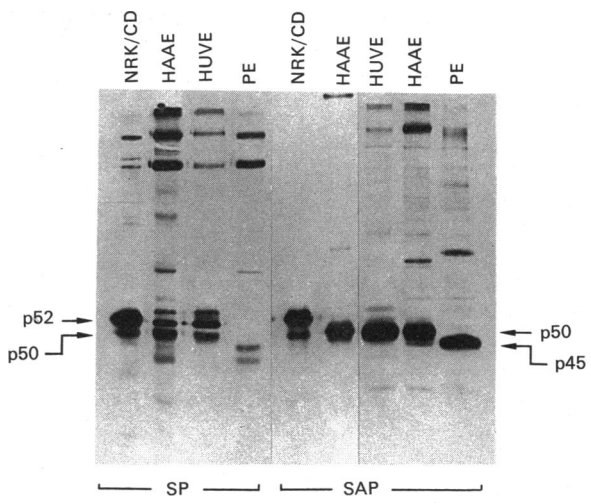


Fig. 8. Electrophoretic identification of the p52-like proteins p50 and p45 of human (HAAE, HUVE) and porcine (PE) endothelial cells as the major SAP-fraction elements of their respective cell types

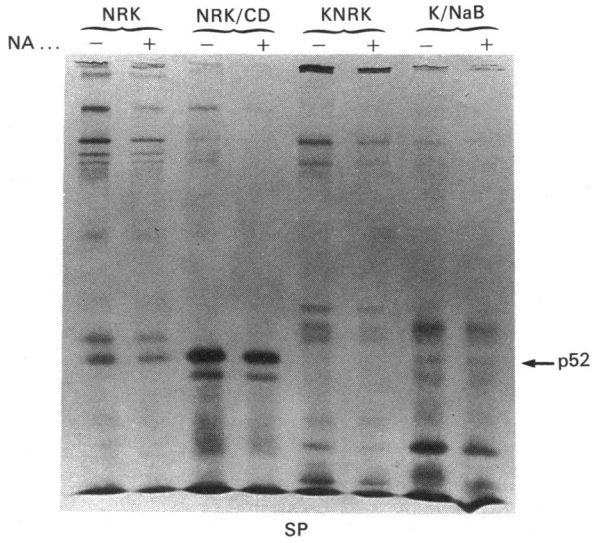


Fig. 9. Resolution of p52 in the SP fraction before and after treatment with nucleophilic agents (NA) (NH_4OH + SDS)

[^{35}S]methionine-labelled SP were treated with NH_4OH + SDS (see the Materials and methods section) to dissociate potential protein–protein complexes before electrophoresis. Treatment did not increase p52 band density in any sample, as would be expected if protein complexes involving p52 were dissociated, allowing p52 to migrate to its normal molecular-mass position of 52 kDa (the slight decrease in overall intensity of the lane with treated SP was expected, owing to sample dilution with nucleophilic agents. p52 was not resolved in KNRK SP exposed to nucleophilic treatment. Absence of this protein in KNRK SP, therefore, did not simply reflect altered p52 mobility as a consequence of protein–protein complexing.

dothelial cells (Fig. 8) each localized to their respective SAP fractions as the predominant protein species. Considering the collective similarities in biochemical and immunochemical properties, subcellular distribution and lectin affinity, p52 is classified as a 'PAI-1-like element'. Since PAIs form detergent-resistant high-molecular-mass complexes with specific plasminogen activators (PAs), failure to detect an M_r 52000 protein in *ras*-transformed NRK cells might simply reflect an altered

electrophoretic mobility (Laiho *et al.*, 1987). p52 was undetectable, however, in the SP or ECM of KNRK cells by electrophoretic mapping (Ryan & Higgins, 1989) by IP (Fig. 7) or by treatment with agents known to dissociate protein–protein complexes involving PA–PAI-1 (Fig. 9).

DISCUSSION

Down-modulation of p52 occurred in four different *ras*-mediated transformation systems. Reduced SAP fraction p52 accumulation is thus neither unique to retrovirus-transformed cells nor an artifact of long-term culture. p52 down-regulation was linked to morphological transformation and, for the most aberrant NRK EJ*ras*$^{val\text{-}12}$-transfectants, approached the low level characteristic of KNRK cells. The present data extend previous observations as to the potential role of p52 in cell spreading (Ryan & Higgins, 1989) and confirm, on the basis of immunochemical and lectin-affinity characteristics, the identity of the NaB-induced 52 kDa protein in KNRK cells as p52. The marked cell spreading associated with NaB exposure, therefore, may be mediated (in large part) by induced p52.

Rat p52, the 47 kDa protein of BHK (Aplin *et al.*, 1981) and mouse-embryo (Neyfakh & Svitkina, 1983) fibroblasts, the 43 kDa (Norris, 1989), FC-1 (Oesch & Birchmeier, 1982), and p51 proteins (Neyfakh & Svitkina, 1983) of chick-embryo fibroblasts (CEF) cells and human PAI-1 (Pollanen *et al.*, 1987; Rheinwald *et al.*, 1987) all localize to the cellular undersurface or ECM. Antibodies to each of these proteins inhibit cell-to-substrate adhesion, subsequent cell spreading or promote detachment of adherent cells (Oesch & Birchmeier, 1982; Rheinwald *et al.*, 1987; Norris, 1989). PAI-1 binds to vitronectin at the ECM (Declerck *et al.*, 1988). This interaction may regulate the stability and/or presentation of PAI-1 to target pericellular proteolytic elements (Levin & Santell, 1987). Adhesive inhibition by immune IgG may result from blocking ECM-binding sites on the p52 (PAI-1) molecule or by direct interference with PAI activity. Alternatively, since p52, p45 and mesosecrin have CSK-protein-like solubility properties (Rheinwald *et al.*, 1987; Santaren & Bravo, 1987; Higgins & Ryan, 1989), they may function as structural elements in cell-to-substrate adhesion.

Factors contributing to control of local proteolysis relate to the target cell, its stage of differentiation/transformation, the combination of growth factors used (Dano *et al.*, 1985; Laiho *et al.*, 1987; Thalacker & Nilsen-Hamilton, 1987; Laiho & Keski-Oja, 1989), and the spatial distribution and time course of change of each component involved in the response (e.g., ECM constituents, proteinases and their inhibitors) (Mayer *et al.*, 1988). Distinct ultrastructural localizations of PA and PAI-1 occur in cultured cells. Urokinase PA is abundant at FCs, whereas PAI-1 and PAI-1-like proteins, including p52, distribute throughout the ventral undersurface (Pollanen *et al.*, 1987, 1988). Given the relatively high concentration of PAs at FCs, even a small imbalance in the delicate PA/PAI-1 ratio necessary to effect normal cell adhesion [as could be achieved in *ras*-transformed cells by interference with PAI-1 synthesis (e.g. Cohen *et al.*, 1989) or overproduction of PA] might influence cell morphology/substrate adhesion.

Regulation of the PAI-1 gene in a given cell type is a complex event involving processing of multiple signals. Indeed, decreased PAI-1 secretion in rat cells occurs upon transformation with oncogenes which possess different mechanisms of action; these include v-*src* (membrane tyrosine kinase), v-*myc* (nuclear factor), and *ras* (GTP binding) (Cohen *et al.*, 1989; Higgins & Ryan, 1989). The rat PAI-1 gene contains multiple 5′ flanking region *fos–jun*/AP-1 binding sites (from −592 to −2112 bases upstream of the coding start site) (Bruzdzinski *et al.*, 1990). Tandem arrays of AP-1 (activator protein 1) sites initiate both positive and negative transcriptional signals (Curran & Franza, 1988). The sequence recognized by nuclear factor PEBP1, which is in consensus with AP-1 (Satake *et al.*, 1989), is stimulated by a transiently expressed *ras* oncogene (Imler *et al.*, 1988). In cells constitutively expressing high levels of *ras*, e.g. KNRK, however, PEBP1 responsiveness is lost (probably due to altered PEBP1 phosphorylation), leading to an inability to interact with its recognition sequence (Satake *et al.*, 1989). The rat PAI-1 gene additionally contains multiple recognition sites for non-AP-1 (i.e. glucocorticoid) transcriptional effectors (Bruzdzinski *et al.*, 1990). Such sequences may also be negatively regulated as a function of *ras* transformation (Jaggi *et al.*, 1988). It remains to be determined whether AP-1/glucocorticoid response elements effectively regulate p52 (PAI-1) expression in the NRK cell system via *ras*-linked signal-transduction pathways.

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