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Biochemical localization of the transformation-sensitive 52 kDa (p52) protein to the substratum contact regions of cultured rat fibroblasts

Butyrate induction, characterization, and quantification of p52 in v-*ras* transformed cells

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A 52 kDa protein (p52) was identified, using differential extraction and electrophoretic criteria, as a major extracellular and substrate-associated component of normal rat kidney (NRK) fibroblasts. Cells transformed with Kirsten murine sarcoma virus (KNRK cells) did not express p52 constitutively, but were inducible for both p52 production and its substrate association during culture in sodium butyrate (NaB)-supplemented growth medium. Comparative analysis of the relative molecular mass, subcellular distribution, and isoelectric complexity (five variants ranging in pI from 5.4 to 6.2) of the 52 kDa species constitutively and inducibly expressed by NRK and KNRK/NaB cells respectively, indicated that they were, indeed, the same protein. p52 selectively localized to cellular fractions enriched in substrate focal contact sites and associated ventral undersurface components. NaB induction of p52 in KNRK cells occurred before cell spreading; other polar compounds, such as dimethyl sulphoxide, which did not induce KNRK cell spreading, similarly failed to elicit p52 production. p52 accumulated more rapidly in (and was quickly released from) the focal-contact-enriched protein fraction of NRK cells compared with its time course of appearance in the medium. These data collectively suggest that p52 is one of a relatively small number of proteins the synthesis of which is either involved in determination of cell shape or regulated as a consequence of cell-shape changes.

INTRODUCTION

Malignant conversion of established avian/mammalian fibroblasts by acute transforming retroviruses or by transfection of v-*onc* genes is usually associated with dramatic changes in overall cellular architecture (e.g. Wahrman *et al.*, 1985). Development of morphological anomalies probably reflects defined cytostructural alterations occurring concomitantly with, or subsequent to, cell transformation. Such events include loss of cytoplasmic stress fibres (Altenburg *et al.*, 1976; Wang & Goldberg, 1976), altered expression of specific tropomyosin isoforms (Matsumura *et al.*, 1983; Cooper *et al.*, 1985), disruption of adhesion plaques (Maness, 1981), aggregation of the microfilament/adhesion plaque-associated proteins talin, vinculin, α-actinin and fimbrin (Carley *et al.*, 1981; Stickel & Wang, 1987), and impaired cell–substratum contact (Altenburg *et al.*, 1976; Hayman *et al.*, 1981). The resultant loss of mechano-structural linkage may well underlie generation of the pleomorphic, less-well ordered, phenotype characteristic of transformed fibroblasts (Wang & Goldberg, 1976; Wahrman *et al.*, 1985).

Certain retrovirally transformed cells undergo morphological reorganization (to a phenotype approximating that of their normal counterparts) during culture in the presence of at least some chemical inducers of differentiation [e.g. sodium butyrate (NaB)] (Altenburg *et al.*, 1976; Ryan *et al.*, 1987). Exposure of Kirsten murine sarcoma virus (KiMSV)-transformed rat kidney fibroblasts (which express high levels of the v-*ras* transforming protein) to NaB thus initiates cytoskeletal reorganization as shown by augmented total cellular and cytoskeletal actin content, a restructuring of actin microfilaments, and increases in the levels of α-actinin and fibronectin (Ryan & Higgins, 1988*a*). The latter represent two proteins involved in microfilament network formation (Mangeat & Burridge, 1984; Ali *et al.*, 1977). Such induced reorganization did not reflect direct effects of NaB on actin polymerization since NaB, in the concentrations used, failed to influence either filament polymerization or nucleation reactions *in vitro* (Ryan *et al.*, 1987). Exposure of KiMSV-transformed rat fibroblasts to NaB does, however, enhance formation of contact sites between the ventral surface of individual cells and the culture-dish surface (Altenburg *et al.*, 1976). The development of such contacts reflects induced changes in glycoconjugate composition and enhanced substrate adhesion (Altenberg *et al.*, 1976; Via *et al.*, 1980). The precise relationship between such NaB-induced increases in cellular adherence and microfilament reorganization is not known. Focal contacts with the substrate, however, are known to

Abbreviations used: IEF, isoelectric focusing; NaB, sodium butyrate; KiMSV, Kirsten murine sarcoma virus; KNRK, KiMSV-transformed cells; NRK, normal rat kidney; FCS, foetal-calf serum; DMSO, dimethyl sulphoxide; HBSS, Hanks' balanced salts solution; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; SP, secreted proteins; SAM, substrate-attached material; SAP, saponin residual material; PAI, plasminogen-activator inhibitor.

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be the sites of stress fibre attachment to the plasma membrane (see Burridge, 1986, for review) and may well be involved in NaB-enhanced adhesiveness (Altenberg *et al.*, 1976).

In this paper, we characterize the complement of secreted and substrate-associated proteins of normal rat kidney (NRK) fibroblasts and their KiMSV-transformed derivatives (KNRK cells). One protein with a molecular mass of 52 kDa (p52) was found to be a major substrate-attached component of NRK cells. p52 could not be resolved in the corresponding fraction of KNRK cells, but was inducible in transformed fibroblasts by exposure to NaB, an agent which initiates cell spreading (Ryan & Higgins, 1988*a*) and substrate–contact site formation (Altenburg *et al.*, 1976).

MATERIALS AND METHODS

Cell culture

NRK fibroblasts and KNRK cells (gifts from Dr. Stuart Aaronson, National Cancer Institute, Bethesda, MD, U.S.A.) were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal-calf serum (FCS) and standard concentrations of penicillin and streptomycin (GIBCO, Grand Island, NY, U.S.A.). NaB (Pfaltz and Bauer, Waterbury, CT, U.S.A.) was prepared as a 200 mM stock in FCS-free RPMI 1640 medium and diluted 1:100 (v/v) in complete growth medium (2 mM final concentration) for addition to 75–80% confluent cell cultures; the duration of exposure to NaB was 1–3 days. Dimethyl sulphoxide (DMSO; Fisher Scientific, Fairlawn, NJ, U.S.A.) was used at a final concentration of 2% (v/v).

Metabolic labelling

Growth medium in 35 mm-diameter culture dishes of NRK, KNRK, and KNRK/NaB cells was aspirated, the monolayers washed twice with Hanks' balanced salts solution (HBSS) and 1 ml of labelling medium (FCS- and methionine-free RPMI 1640 medium containing 50 μCi of [^{35}S]methionine; sp. radioactivity 1100 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) added to each culture. Cells were returned to the incubator for an additional 6 h. At the end of the labelling period, the medium [containing secreted proteins (SP)] was aspirated, clarified by centrifugation at 13000 *g* for 5 min and the cells washed twice with HBSS for processing as described below. Cells exposed to NaB or DMSO were labelled in their presence.

Cell extraction and gel electrophoresis

Total cell extracts were prepared by scraping cells into lysis buffer [9.8 M-urea, 2% (v/v) NP-40, 2% (v/v) Ampholytes, pH 7–9, 100 mM-dithiothreitol] (Bravo, 1984), followed by vortex agitation and clarification at 13000 *g*. For preparation of the detergent-resistant cytoskeletal fraction, EDTA-released cells (0.2 g of EDTA/1 Mg^{2+} and Ca^{2+}-free HBSS) (Rosen & Culp, 1977; Rheinwald *et al.*, 1987) were collected by centrifugation and extracted in TN/Triton buffer (140 mM-NaCl, 10 mM-Tris/HCl, pH 7.6, 1% Triton X-100) (Franke *et al.*, 1981*a*,*b*; Higgins, 1986) by vortex agitation. Extracts were centrifuged at 13000 *g* and separated into the detergent-soluble and insoluble cytoskeletal fractions. Cytoskeletal residues were dissolved directly in sample buffer [50 mM-Tris/HCl, pH 6.8, 10% (v/v) glycerol, 1% SDS and 1% 2-mercaptoethanol] (Laemmli, 1970). Substrate-attached material (SAM), representing the extracellular matrix and cellular 'footpad' structures (Rosen & Culp, 1977; Lark *et al.*, 1985) remaining after EDTA-release of adherent cells, was solubilized directly in either lysis buffer (for two-dimensional gel electrophoresis) or sample buffer (for separation on one-dimensional gels). Cell–substratum contact regions were prepared with saponin [0.2% in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS)] (Neyfakh & Svitkina, 1983) and the saponin residual material (SAP) solubilized in sample buffer. For electrophoresis on two-dimensional gels (Bravo, 1984), 5×10^4–5×10^5 c.p.m. of [^{35}S]methionine-labelled protein (in lysis buffer) was loaded on to prerun 1.5 mm-diameter tube gels for isoelectric focusing (IEF) for 18 h before molecular-mass separation on 10% (w/v) SDS/polyacrylamide slab gels (SDS/PAGE) (Laemmli, 1970; Bravo, 1984). One-dimensional gel electrophoresis of 25000 c.p.m. of labelled cellular protein in sample buffer was done on 10% SDS/polyacrylamide slab gels as described in detail (Ryan *et al.*, 1987; Ryan & Higgins, 1987). Radioactive peptides were visualized in En^{3}Hance (New England Nuclear)-treated gels by fluorography. Fluorographs were scanned within the linear range of autoradiographic sensitivity using a model GS300 densitometer (Hoefer Instruments, San Francisco, CA, U.S.A.) interfaced to a Bio-Rad chart recorder (Higgins *et al.*, 1987). Total profile and individual peak areas were determined using a Zeiss MOP III digital image analyser and individual proteins were quantified as a function of c.p.m. loaded per gel. Where necessary, scan areas were normalized per 10^6 cells to calculate the relative extracellular contribution of individual protein species as a function of cell number. Trichloroacetic acid precipitation of proteins used the procedure of Bravo (1984).

Fluorescence microscopy

Mouse monoclonal antibodies (as ascites fluid) to chicken-gizzard-smooth-muscle vinculin (clone VIN-11-5) (Sigma, St. Louis, MO, U.S.A.) and fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat anti-(mouse IgG) (Kirkegaard and Perry, Gaithersburg, MD, U.S.A.) were diluted 1:10 in a modified HBSS (HBSS without $CaCl_2$ and $MgSO_4$, $7H_2O$, but containing 2 mM-EGTA/5.5 mM-$MgCl_2$/5 mM-Pipes/0.1% Tween 20). Rhodamine-conjugated phalloidin, which possesses a high affinity for F-actin microfilaments (Wulf *et al.*, 1979; Verderame *et al.*, 1980), was a gift from Dr. Th. Wieland (Max-Planck Institut, Heidelberg, Germany) and was diluted to a final contentration of 1 μg/ml in PBS. For immunolocalization of vinculin, cell monolayers were washed, fixed [10% (v/v) formalin for 5 min at 37 °C], then permeabilized (0.5% Triton X-100 for 20 min at room temperature) and washed (all steps used reagents prepared in modified HBSS without Tween 20). Free aldehyde groups were blocked by incubation in 0.1 M-glycine for 10 min. Sequential incubation of cells with anti-vinculin and FITC-goat anti-mouse antibodies was as described previously (Erlandson *et al.*, 1984). Visualization of microfilaments with rhodamine–phalloidin utilized 20% confluent cell cultures which were fixed for 20 min in 10% (v/v) formalin/PBS, rinsed, then extracted in 1% (v/v) Nonidet P-40/PBS for an additional 20 min (Wahrman *et al.*, 1985). For staining, fixed and permeabilized cells were incubated with rhod-

amine–phalloidin at 37 °C for 1 h, rinsed, and coverslips mounted in 50% (v/v) glycerol/PBS for examination with a Nikon epifluorescence microscope. SAP was fixed *in situ* using 10% (v/v) buffered formalin, washed with PBS, incubated for 2 h at 37 °C with diluted rhodamine–phalloidin and subsequently processed as described above.

RESULTS

Comparative analysis of the [^{35}S]methionine-labelled SP complement of NRK, KNRK, and KNRK/NaB cells by SDS/PAGE revealed significant differences in the extracellular level of individual protein species, in particular, those with molecular masses ranging from 31 to 70 kDa (designated p31, p41, p52, p62, and p70) (Fig. 1). Of these five major SP species, only one (p52) was negatively regulated (to virtually undetectable levels) as a consequence of KiMSV transformation and was re-expressed (in KNRK cells) during the period of NaB treatment. Under non-induced conditions, p52 represented < 1% of the total protein secreted by KNRK cells (this was below the lower limit of significant detectability, calculated to be 1% of the total attainable by digital-image analysis of densitometric profiles) (Table 1). On an equivalent cell basis, the amount of NaB-induced p52 in KNRK cell cultures approximated (or exceeded by 50%) that constitutively secreted by NRK fibroblasts (Table 2). Comparisons of such normalized data clearly indicated that the level of p52 produced by KNRK cells approached just 1.8% and 1.2% of the amount secreted by NRK and KNRK/NaB cells respectively. Induced (i.e. NaB-mediated) alterations in the pattern of SP, evident within 12 h after addition of NaB to KNRK cell cultures, were maximal by 24 h, and did not change thereafter (Fig. 2).Re-expression of p52 in KiMSV-transformants was relatively specific to NaB since the addition of other polar differentiation-inducing agents (e.g. DMSO) failed to induce the 52-kDa protein (Fig. 2). Secretion of p52 was not simply a consequence of arrested cell proliferation. Both NaB and DMSO were equally effective at restricting KNRK cell growth (results not shown), yet only NaB-treated populations produced p52 (Fig. 2).

To address the potential function of p52, it was first necessary to determine its subcellular distribution. Differential separation of KNRK/NaB cellular proteins into four operational compartments [i.e. SP, detergent soluble, detergent insoluble (cytoskeletal) and SAM] clearly indicated that p52 preferentially localized with

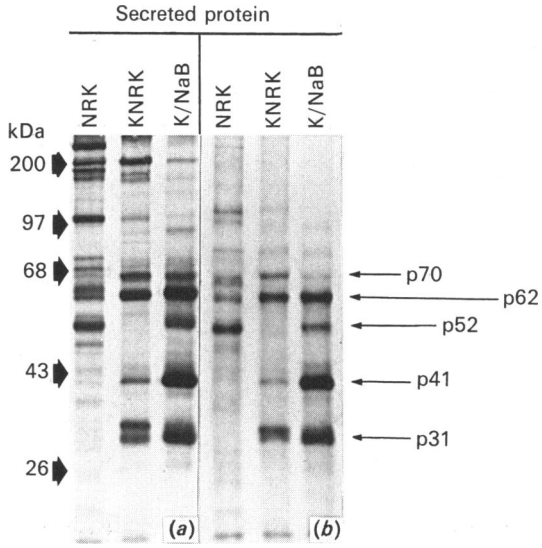

Fig. 1. Definition of the complement of SP peptide species recovered in the medium fraction of NRK, KNRK and 3-day NaB-treated (K/NaB) cells at the termination of the 6 h labelling period

Panel (*a*), lanes loaded with equivalent aliquots (50 μl) of medium (diluted 1:1 with sample buffer and boiled before electrophoresis) containing [^{35}S]methionine-labelled protein. Panel (*b*), lanes loaded with equivalent amounts (25000 c.p.m.) of trichloroacetic acid-insoluble [^{35}S]-methionine-labelled SP. The major SP species of K/NaB cells are p70, p62, p52, p41 and p31 (designations are in kDa; molecular mass calculated using standards of known molecular mass); by comparison, p31 and p41 are minor contributors to the SP fraction of NRK cells. The absence of a peptide of 52 kDa (p52) from the SP fraction of KNRK cells, which is constitutively expressed by NRK fibroblasts, and its induction as a consequence of NaB treatment are obvious.

Table 1. Individual contribution of p70, p62, p52, p41 and p31 to the total secretory protein complement of NRK, KNRK, and KNRK/NaB cells

[^{35}S]Methionine-labelled SPs were fractionated on 10% (w/v) SDS/polyacrylamide slab gels and the peptides were visualized by fluorography. Scans were generated, within the linear range of autoradiographic sensitivity, using a model GS300 scanning densitometer. Total profile area and individual protein peak areas were determined with a Zeiss MOP III digital image analyser. Data represent means ± S.D. of arbitrary densitometric units based on triplicate analyses. Percentages given in parentheses were calculated using group mean. NM = not measurable.

Cell type	Total profile area	Amount secreted (% of total)				
		p70	p62	p52	p41	p31
NRK	2540.0 ± 30.2	43.3 ± 0.6 (1.7)	212.1 ± 4.1 (8.3)	547.7 ± 5.3 (21.5)	NM (0)	NM (0)
KNRK	2959.3 ± 44.4	304.9 ± 5.1 (10.3)	650.2 ± 5.4 (22.0)	26.5 ± 2.2 (0.9)	114.6 ± 0.6 (3.8)	531.7 ± 7.2 (17.9)
KNRK/NaB	6601.8 ± 48.5	286.2 ± 3.4 (4.3)	1406.3 ± 23.8 (21.3)	508.6 ± 1.6 (7.7)	2321.5 ± 29.0 (35.2)	803.4 ± 8.6 (12.2)

Table 2. Comparison of the relative amounts of p70, p62, p52, p41 and p31 proteins secreted per 10^6 cultured cells

Individual protein scan areas were determined with the use of a Zeiss MOP III digital image analyser as detailed in the legend to Table 1. Scan areas were normalized per 10^6 cells based on mean counts of 1.10×10^6, 2.94×10^6 and 0.66×10^6 cells recoverable per 35 mm-culture dish (in triplicate assays) for NRK, KNRK and KNRK/NaB populations respectively. NM = not measurable.

	Relative extracellular content (normalized per 10^6 cells)				
Cell type	p70	p62	p52	p41	p31
NRK	39.3±0.5	192.6±3.4	498.0±4.8	NM	NM
KNRK	103.7±1.7	222.3±3.7	9.0±0.7	38.9±0.2	180.9±5.0
KNRK/NaB	433.7±5.2	2130.8±36.2	770.6±2.5	3475.3±51.1	1202.7±9.9

the SP and SAM fractions (Fig. 3). An identical selective compartmentalization of p52 also typified normal rat fibroblasts (results not shown). Previous two-dimensional electrophoretic analyses and proteolytic-digest-mapping comparisons of the secreted and SAM-associated 52 kDa proteins of NRK cells have confirmed that they are, indeed, the same protein (Ryan & Higgins, 1988*b*). While p52 was evident in the SP and SAM compartments of both NRK and KNRK/NaB cell cultures, the absence of the 52 kDa protein from the corresponding fractions of KNRK cells was obvious (Figs. 4 and 5; Table 3). As was the case for extracellular levels of p52 (e.g. Table 1), the amount of this protein in the SAM-associated fraction of KNRK fibroblasts was below the lower limit of significant detectability (i.e. < 1% of the total SAM protein) measurable by digital-image analysis. In order of decreasing p52 content, the several KNRK/NaB cellular compartments partitioned into the following sequence: SAM fraction > detergent-insoluble fraction > detergent-soluble fraction > total cellular protein (Fig. 3).

p52 in the SAM fraction of NRK cells exists as a series of five pI variants which migrate, in two-dimensional electrophoretic separations, to the basic side of actin (Fig. 6). The individual pI variants of p52 were designated 1–5, with p52-1 representing the most basic (IEF M_r [actin] = 0.67) and p52-5 the most acidic (IEF M_r [actin] = 0.94) of the isoforms (Table 4). A

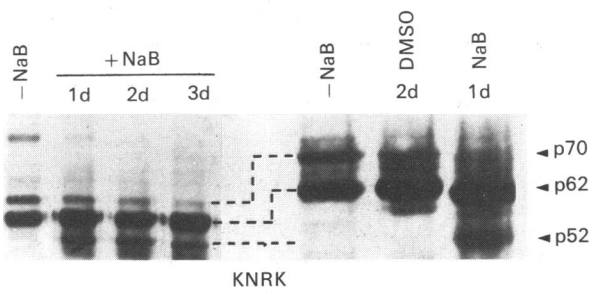

Fig. 2. Time course and specificity of p52 induction in KNRK cells

Electrophoresis of the SP fraction of KNRK cells cultured without (−) or with (+) NaB for 3 days indicated that p52 was maximally induced by 1 day (d) of exposure; the level of this protein was not significantly changed by continued culture (for 2–3 days) in the presence of NaB. Induction of p52 was relatively specific for NaB since exposure of KNRK cells to another polar differentiation-inducing agent [DMSO; 2% (v/v) final concentration for 2 days] failed to induce p52 production.

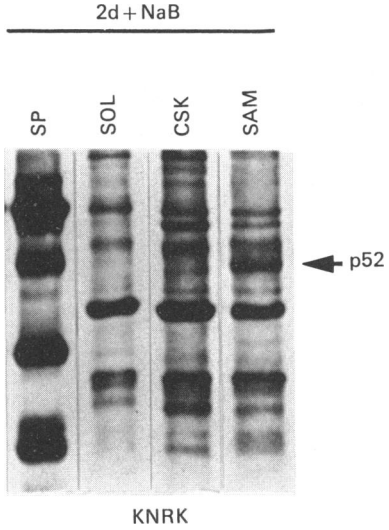

Fig. 3. Electrophoretic analysis of the distribution of p52 in several compartments of 2-day NaB-treated KNRK cell populations

Individual lanes were loaded with 25000 c.p.m. of [^{35}S]-methionine-labelled protein from the following cellular fractions: (SP) secreted proteins; (SOL) EDTA-released cells, TN/Triton X-100-soluble fraction; (CSK) EDTA-released cells, TN/Triton X-100-insoluble fraction; (SAM) EDTA-resistant substrate-associated material fraction. The position of p52 is indicated by an arrow. Previous two-dimensional electrophoretic profiling of NRK SP and SAM fractions combined with side-by-side comparisons of peptides generated by chymotrypsin and V8-protease digestion of electrophoretically purified p52 established that the 52 kDa protein found in the SP compartment is identical (in molecular mass, fragment size and IEF heterogeneity) with SAM p52 (Ryan & Higgins, 1988*b*). The progressive increase in extract p52 content through the fractionation sequence SOL-CSK-SAM is clear and was confirmed by quantitative scanning densitometry (see text for details).

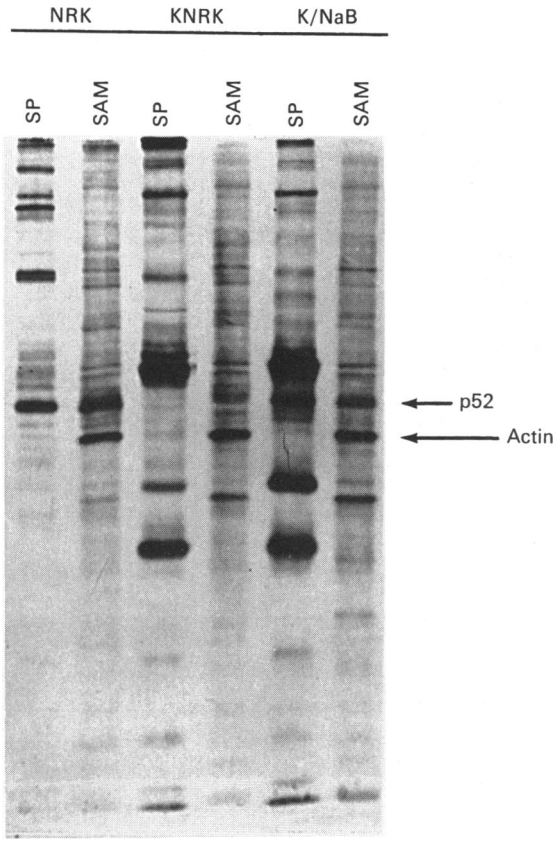

Fig. 4. Comparative side-by-side electrophoresis of proteins comprising the SP and SAM compartments of NRK, KNRK and 2-day NaB-treated KNRK (K/NaB) cell cultures

Lanes were loaded with equivalent aliquots of labelling medium (containing [^{35}S]methionine-labelled SP) or with 25000 c.p.m. of SAM-fraction protein. By using the constitutively expressed NRK SP p52 as a marker, the absence of this protein in both the SP and SAM compartments of KNRK cells and its inducibility by NaB in the corresponding fractions of K/NaB cell cultures is apparent (compare with Fig. 6). The p52 content of NRK and K/NaB SAM fractions closely approximated that of actin [which was identified using criteria described previously in detail (Ryan & Higgins, 1988*a*,*b*; Higgins *et al.*, 1987)].

corresponding series of five variants is secreted into the labelling medium by NRK cells (Ryan & Higgins, 1988*b*). The relative proportion of the individual p52 species, however, appears to be different in the two (SP or SAM) cellular compartments. Quantitative densitometric analysis indicated that the major SP forms of p52 are p52-3 and p52-4, whereas the predominant SAM p52 variants are p52-2 and p52-3 (Table 5). KNRK/NaB p52 also exhibits five basic (pI relative to actin) species in both the SP and SAM fractions. A greater proportion of total secreted KNRK/NaB p52, however, partitions into the more basic variant classes compared with the major secreted forms of NRK p52, while the major SAM variants of KNRK/NaB p52 are quite similar to the corresponding SAM p52 components of NRK cells (Table 5).

Because of the structural and biochemical complexity of the SAM fraction residue (see Discussion), attempts were made to define further the relationship between p52 and the substrate-adherent protein compartment. SAP fraction residues were particularly enriched in p52 (Fig. 7); quantitative measurements indicated that p52 was the only SAP-associated constituent to show a significant increase compared with the corresponding SAM fraction protein (Table 6). p52 additionally appeared to accumulate more quickly in the SAM fraction relative to its initial time of appearance in the labelling medium (e.g. Ryan & Higgins, 1988*b*). These data suggested that newly synthesized p52 may be deposited by cells in their immediate vicinity with subsequent release into the medium. The kinetics of p52 release from the matrix and its rate of accumulation into the culture medium were, therefore, examined in detail. Near-confluent (90%) cultures of NRK cells were labelled with [^{35}S]methionine for 6 h under standard conditions (see the Materials and methods section), the labelling was terminated by two washed in HBSS, followed by addition of serum-free growth medium (without label) for subsequent incubation at 37 °C. At specific intervals during this chase, media and SAP fractions were collected, proteins were separated by gel electrophoresis, and the p52 content was determined by densitometric analysis of the fluorographs. p52 was rapidly removed from the SAP residue; 94% of [^{35}S]methionine-labelled p52 was cleared by 4 h (Fig. 8). The reciprocal increase in the p52 content of the medium

Table 3. Quantification of p52 in the SAM fraction of NRK, KNRK, and KNRK/NaB fibroblasts

Autoradiographs were generated from electrophoretic separations of 25000 c.p.m. of [^{35}S]methionine-labelled SAM-fraction protein from the indicated cultures. Total profile and individual peak areas were determined by scanning densitometry. Percentages given in parentheses were calculated using group mean.

Cell type	Total profile area	SAM-associated p52 (% of total)	SAM-associated actin (% of total)
NRK	2107.2±1.2	338.6±6.0 (16.0)	207.9±5.7 (9.9)
KNRK	2446.6±49.1	20.2±0.46 (0.8)	294.1±2.5 (12.0)
KNRK/NaB	2235.3±45.9	221.9±0.65 (9.9)	194.6±3.1 (8.8)

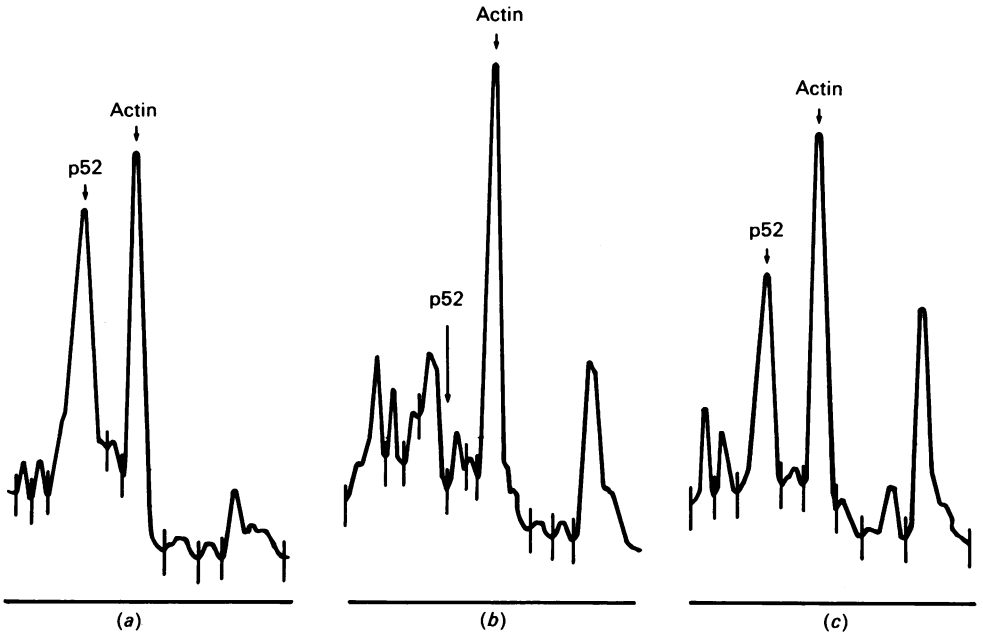

Fig. 5. Densitometric scans of fluorographs generated upon electrophoresis of 25000 c.p.m. of [^{35}S]methionine-labelled SAM fraction proteins (e.g. Fig. 4) illustrating the relative SAM content of p52 and actin in (*a*) NRK, (*b*) KNRK, and (*c*) 2-day NaB-treated KNRK cells

Only the relevant portions of the individual scans are shown.

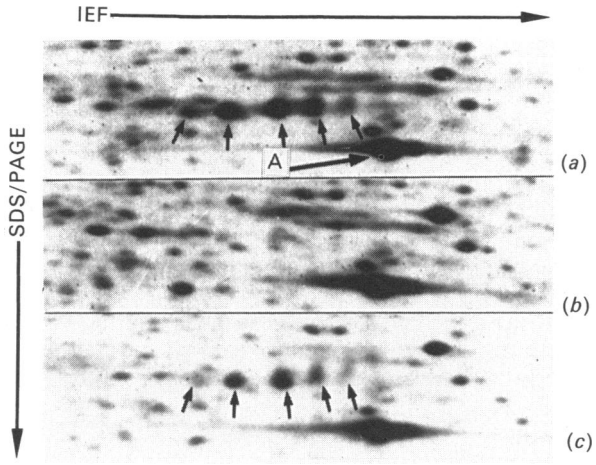

Fig. 6. Two-dimensional electrophoretic separations of 50000 c.p.m. of SAM-fraction protein from (*a*) NRK, (*b*) KNRK and (*c*) 2-day NaB-treated KNRK cells

The NaB-induced 52 kDa protein in KNRK cells (indicated by small arrows) clearly has both a mobility and complexity identical with that of the constitutively-expressed p52 protein of NRK cells. A = actin; directions of IEF and molecular sizing (SDS/PAGE) are indicated.

Table 4. Two-dimensional electrophoretic characteristics of individual SAM p52 species

Protein	Acidic mobility relative to actin (IEF M_r [actin])	Apparent pI
p52-1	0.67	6.2
p52-2	0.73	6.0
p52-3	0.82	5.8
p52-4	0.88	5.7
p52-5	0.94	5.6
Actin	1.00	5.4

suggested accumulation of SAP-released p52 into this compartment.

It was additionally necessary to identify visually (if possible) specific cellular structures resistant to saponin extraction. Rhodamine–phalloidin and monoclonal antibodies to vinculin were used as probes, therefore, to ascertain the distribution of microfilaments and their associated focal-contact termini (Burridge, 1986), respectively, in NRK cells (Figs. 9*a* and 9*b*). While saponin extracts were obviously devoid of transcytoplasmic microfilaments, the rhodamine–phalloidin probe did bind to specific structures which possessed density, length and spacing virtually identical with that of vinculin-containing focal contacts (compare Figs. 9*b* and 9*c*); such structures, however, did not contain immunodetectable levels of vinculin (see Discussion; Bayley & Rees, 1982).

DISCUSSION

Differential-extraction experiments identified the majority of p52 as belonging to the cellular SAM protein fraction. These data thus confirm a previous suggestion (Ryan & Higgins, 1988*c*) that this protein might be a component of the ventral undersurface or substrate–contact-site structures of cultured fibroblasts.

Table 5. Relative content of p52 variants in the secreted and SAM fractions of NRK and KNRK/NaB cells

The five variants of p52 are designated 1–5 with 1 being the most basic isoform and 5 the most acidic.

Cell type	Compartment	Total profile area	Contribution of individual p52 variants (% of total p52)				
			p52-1	p52-2	p52-3	p52-4	p52-5
NRK	Secreted	16378.4±69.0	940.6±13.1 (5.7)	4058.6±88.0 (24.8)	5339.9±26.5 (32.6)	5984.6±31.0 (36.5)	2935.2±53.5 (17.9)
NRK	SAM	6628.3±28.9	688.5±1.9 (10.4)	2502.6±17.8 (37.8)	2051.6±20.4 (30.8)	1090.6±1.1 (16.4)	191.2±1.5 (2.9)
KNRK/NaB	Secreted	18505.6±64.1	3469.4±34.9 (18.7)	8820.3±23.1 (47.7)	4682.0±17.5 (25.3)	2797.1±23.5 (15.1)	1151.7±16.8 (6.2)
KNRK/NaB	SAM	2542.7±11.3	101.6±0.8 (3.9)	855.1±11.3 (33.6)	938.9±4.6 (36.9)	376.4±5.1 (14.8)	126.5±3.2 (4.9)

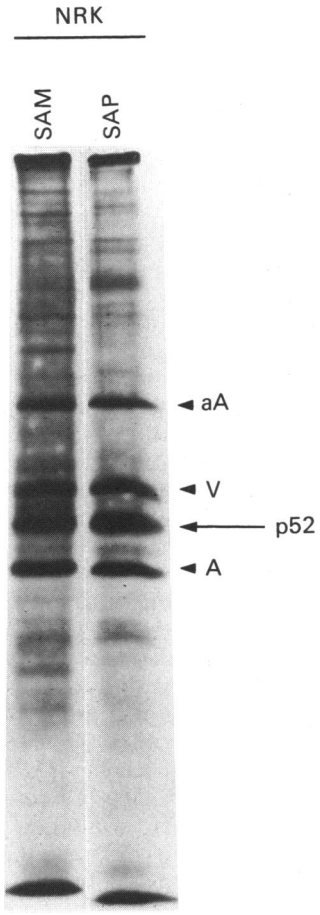

Fig. 7. Side-by-side electrophoretic comparison of [^{35}S]-methionine-labelled NRK SAM and SAP fraction proteins (25000 c.p.m./lane)

Quantitative scanning densitometry of similar fluorographs indicated a significant enrichment only for p52 in SAP preparations (e.g. Table 6). A, actin; V, vimentin; aA, α-actinin.

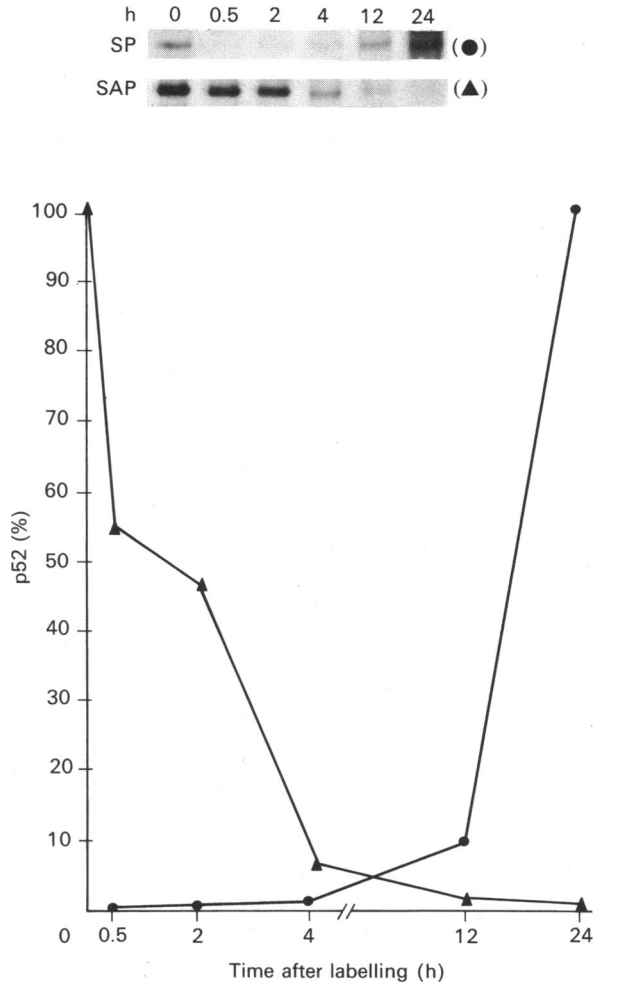

Fig. 8. Kinetics of release of p52 from the SAP fraction of NRK cells and its rate of accumulation in the culture medium

After a 6 h-labelling period, cultures were washed with HBSS and subsequently incubated with fresh FCS-free medium. At the time points indicated, media (SP) and SAP fractions were collected and analysed by gel electrophoresis (of equivalent volumes of media or 25000 c.p.m. of SAP residue); p52 content was assessed by fluorography and scanning densitometry. Results are plotted as the percentage of p52 in each compartment relative to either time 0 (SAP) or 24 h (medium) levels, which are indicated as 100%. Only the relevant p52 bands are shown in the accompanying fluorogram.

The minor, but consistently resolved, percentage of p52 associated with the cytoskeletal compartment of KNRK/NaB cells raises the possibility that, under specific inducing conditions, at least a subset of p52 molecules may function in a structural role.

SAM matrices, prepared by chelation, represent

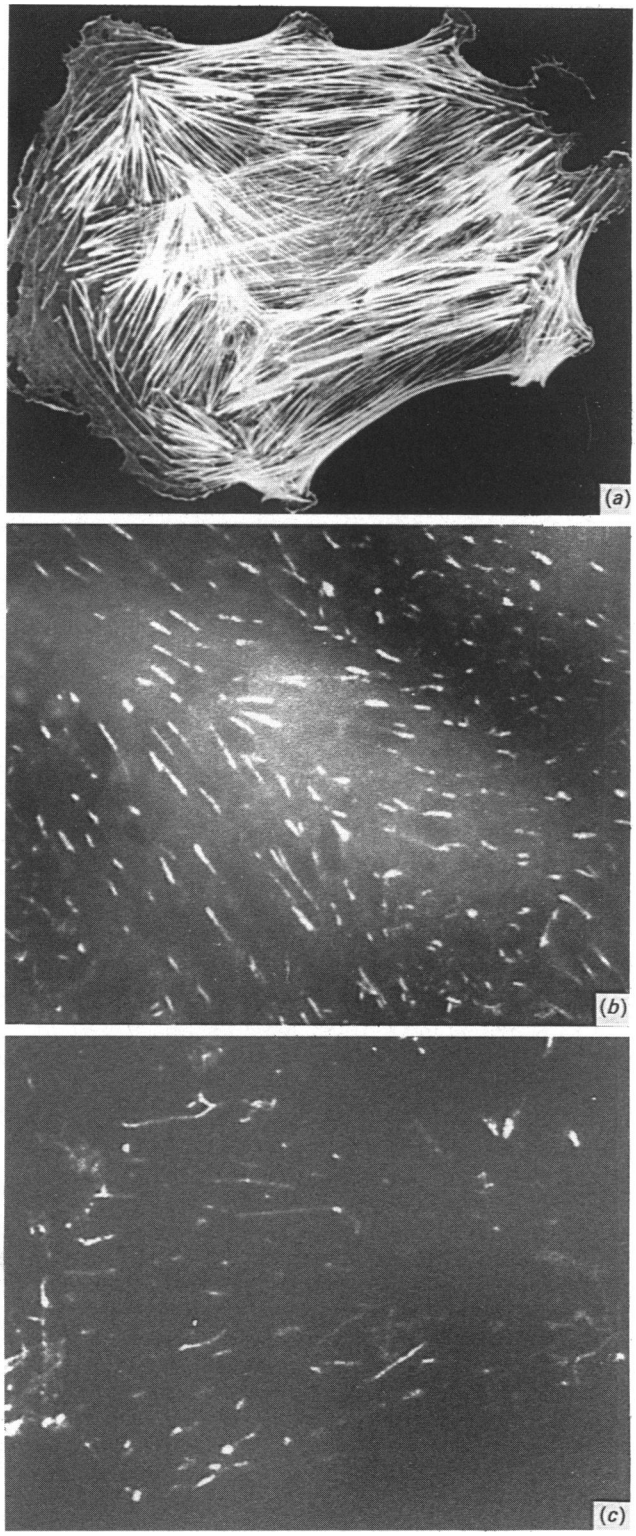

Fig. 9. Localization of structural elements in NRK fibroblasts by fluorescence microscopy

(*a*) Visualization of actin-containing microfilaments in NRK cells using the F-actin-specific probe rhodamine–phalloidin (×400). The ends of the microfilament cables (bundle termini) correspond to focal adhesive interactions with the culture substratum (see Burridge, 1986 for details). It is these ventral focal contacts which contain abundant amounts of vinculin as detected (in *b*) with monoclonal antibodies to chicken gizzard vinculin (×600). SAP material of NRK cells retains rhodamine–phalloidin-binding structures (*c*) (×400). The resolved structures (in *c*) have a similar overall pattern (spacing, frequency and orientation) to bundle termini and focal contacts (*a* and *b*).

specialized regions of the fibroblast plasma membrane–substrate contact area. This fraction comprises approx. 1% of the total cellular protein complement and is enriched in microfilaments, intermediate filaments, cellular fibronectin, hyaluronic acid and various proteoglycans (Rosen & Culp, 1977; Lark *et al.*, 1985) (e.g., Fig. 6). Structurally, SAM consists primarily of tight focal contacts and a subset of a close contacts (for review see Lark *et al.*, 1985), which are distinguishable both in proximity to the substratum surface and in protein composition (for detailed structural analysis of these contact regions see Krueger *et al.*, 1984; Ben-Ze'ev, 1985; Burridge *et al.*, 1987). Cell–substrate contact sites were also prepared in the present study using the saponin-extraction procedure. Such extractions generate peptide patterns (Pollanen *et al.*, 1987) and subcellular structures (Neyfakh & Svitkina, 1983) that are simpler than those encountered in SAM preparations and that probably represent predominantly focal-type contact regions (Neyfekh & Svitkina, 1983). Electrophoretic comparison of SAM- and SAP-fraction proteins indicated that SAP preparations of NRK cells contained augmented levels of p52 (relative to SAM-fraction p52 content) and that this enrichment appeared to be selective. Quantification of the four major protein species found in SAP preparations of NRK fibroblasts (α-actinin, vimentin, actin and p52) revealed a significant increase (165–170% of SAM fraction content) only for p52. These observations have been subsequently confirmed by two-dimensional electrophoretic mapping of the constituent SAM and SAP compartment proteins (results not shown). Other proteins with molecular masses similar to p52 have been localized to the cell–substratum contact region. Neyfakh & Svitkina (1983) identified proteins of 51 and 47 kDa as enriched components of saponin-prepared focal contacts in chick and mouse fibroblasts respectively. Bayley & Rees (1982), by using stream-dislodgement of cultured rat fibroblasts to generate focal adhesions, resolved actin, α-actinin and vimentin, in addition to a protein of 50–55 kDa, in such structures but failed to find vinculin, a major cytoplasmic-face protein of focal contacts (Burridge, 1986). These data are consistent with results obtained in the present study and suggest that vinculin is either removed from, or present at relatively low levels in, the residual structural elements left behind after physical dislodgement (Bayley & Rees, 1982) or saponin extraction of rat fibroblasts. Of considerable relevance is the finding by Pollanen *et al.* (1987) that the 50–54 kDa protein identified in focal-contact preparations of normal and transformed fibroblasts is a plasminogen-activator inhibitor (PAI). This immediately raises speculation as to the respective involvement of enzymic inhibitors in focal-contact stabilization and of (*ras*-type) oncogenes in disruption of such structures (see below). Preliminary immunoprecipitation experiments, using antisera to PAI-type 1 (generously supplied by Dr. D. Loskutoff and Dr. V. van Hinsbergh), have clearly indicated that p52 shares certain antigenic homology with human PAI (P. J. Higgins & M. P. Ryan, unpublished results). Whether p52 is actually the rat homologue of human PAI-type 1 will have to be utimately

Table 6. Enrichment of p52 in the saponin-resistant contact sites of NRK fibroblasts

NRK cells were labelled with [^{35}S]methionine for 6 h prior to preparation of either SAM or SAP fractions using the EDTA-release or saponin-extraction procedures respectively. Autoradiographs were generated from electrophoretic separations of 25000 c.p.m. of labelled proteins from the indicated fractions. Total profile and individual peak areas were determined by scanning densitometry. The identification of α-actinin, vimentin and actin in each electrophoretic profile utilized criteria described in detail previously (Ryan & Higgins, 1988*a*; Higgins *et al.*, 1987).

Preparation	Total profile area	Contribution to total			
		α-Actinin	Vimentin	p52	Actin
SAM fraction	766.2±5.2	77.6±1.6	151.3±2.4	191.1±1.6	165.7±1.1
SAP fraction	848.3±6.0	79.3±1.0	182.9±0.5	312.2±1.1	174.8±0.7

determined by sequencing of appropriate peptide fragments.

The present and previous investigations into the mechanism of action of NaB on KNRK fibroblasts have clearly indicated an association between p52 expression and induced cell spreading. Induction of p52 occurs before NaB-initiated cell spreading (Ryan & Higgins, 1988*b*); it is the only major protein obviously deficient in the SAM compartment of virus-transformed fibroblasts (e.g. Fig. 6) and plating of KNRK cells on to p52–containing matrices is followed by a marked, however transient, spreading response (Ryan Higgins, 1988*b*). Exposure of KNRK cells to other polar differentiation-including agents (e.g. DMSO), which do not initiate cell spreading, consistently failed to induce p52 expression (Fig. 2). Such augmented cell spreading in response to NaB (Altenburg *et al.*, 1976; Ryan & Higgins, 1988*a*), and concomitant increased adhesivity (Via *et al.*, 1980), may depend, at least partially, on specific alterations in membrane glycoconjugate composition (Via *et al.*, 1980). NaB [which is a potent inducer of membrane glycoconjugate formation (Via *et al.*, 1980) and p52 synthesis (this paper) in KNRK cells] and uridine diphosphoglucose [which is a weak inducer of p52 (P. J. Higgins & M. P. Ryan, unpublished observations) and, by virtue of its conversion to UDP-galactose, serves as an important glycosyl donor to complex polysaccharides and glycoproteins (Frisell, 1982)], both affect, to varying degrees, KNRK cell spreading. Furthermore, addition of tunicamycin to KNRK cell cultures inhibits NaB-induced spreading by approximately 80% (results not shown). These observations collectively indicate a necessity for production and deposition of a specific complement of post-transitionally modified, matrix-targeted proteins to achieve complete spreading of KNRK cells.

v-*ras* belongs to a particular viral oncogene group whose members exhibit certain correlative biological properties. Thus expression of v-*ras*, v-*mos*, v-*src*, v-*fes*, or v-*fms* in appropriate host cell lines results in characteristic phenotypic changes, including: (1) altered cell morphology; (2) actin microfilament disorganization; (3) tropomyosin down-regulation; and (4) acquisition of metastatic growth traits (Ryan & Higgins, 1988*a*; Egan *et al.*, 1987; Cooper *et al.*, 1985; Wahrman *et al.*, 1985). Loss of strong anchoring elements (e.g. adhesion plaques and their associated stress fibres) in KNRK (Altenberg *et al.*, 1976; Ryan & Higgins, 1988*a*) probably contributes to the construction of faulty contact-site structures, resulting in reduced substrate adherence and the development of a pleomorphic, more migratory, cellular phenotype. p52 appears to be a major constituent of this cell–substrate contact-site region. Whether p52 content or the quantitative distribution of p52 variants is altered in cells transformed with v-*mos*, v-*src*, v-*fes*, and v-*fms* oncogenes, as occurs in v-*ras*-expressing cells, will be investigated. Determination of such events would further elucidate specific pathways whereby members of this particular oncogene group influence the regulation of cell shape and associated growth traits.

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