Protein turnover in 3T3 cells transformed with the oncogene c-H-ras1

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We have examined protein turnover, growth, DNA synthesis and proliferation in three independent clones of 3T3-NR6 cells transformed with the oncogene c-H-ras1. We find that, firstly, the half-maximum concentration of serum and insulin regulating protein turnover in *ras*-transformed cells is significantly reduced from 0.5 to 0.3% for serum and from 4 nM to 0.5 nM for insulin, and, secondly, *ras*-transformed cells consistently have lower rates of protein degradation. The catabolic effect of conditioned medium or serum withdrawal is attenuated in transformed lines by maintaining lower basal rates of protein breakdown and higher basal rates of DNA and protein synthesis. Serum stimulation of growth in transformed cells is achieved in the short term by lower rates of protein breakdown rather than higher rates of protein synthesis: rates of protein synthesis become significantly higher 24 h after serum stimulation. Therefore transformed cells have higher rates of proliferation and grow to higher densities, but display characteristics common to normal cells because rates of protein synthesis decrease and protein degradation increase as a function of cell density. We conclude that higher basal rates of protein synthesis and growth with retention of the normal proliferative response to serum result from the pleiotropic nature of *ras* transformation, whereas lower rates of protein degradation and increased sensitivity to serum and insulin imply a direct regulatory role for *ras*.

INTRODUCTION

High rates of protein turnover, growth and proliferation are characteristic features of cancer cells when they are compared with their normal counterparts in culture. The changes in protein turnover arise because transformed cells have higher basal rates of synthesis often (but not always) accompanied by lower basal rates of degradation (Ballard *et al.*, 1980; Cockle & Dean, 1982a, 1984; Lockwood & Minassian, 1982; Ballard & Gunn, 1985), and because both pathways have an increased sensitivity to serum growth factors such as insulin, epidermal growth factor (EGF) and insulin-like growth factor-2 (IGF-2) (Gunn *et al.*, 1977, 1983; Ballard *et al.*, 1980; Ballard & Gunn, 1985). As a group, growth factors act firstly to inhibit autophagic vacuole formation, thereby rapidly and co-ordinately decreasing rates of bulk protein breakdown occurring via the lysosomal pathway of protein degradation (Ballard, 1987; Marzella & Glaumann, 1987), and secondly to increase rates of protein synthesis (James & Bradshaw, 1984). When placed on top of the altered basal rates found in transformed cells, these two regulatory effects lead to higher rates of protein accumulation and explain higher rates of cell proliferation.

Some proto-oncogene gene products are regulatory proteins of the normal mitogenic pathway and their mutation often leads to enhanced growth in transformed cells (Bishop, 1987). The effects of a single, specific oncogene product on a metabolic pathway, with its ability to augment or even to override the normal regulation of the pathway, offers a simple and attractive way of probing the relationship between oncogene expression, growth-factor response and metabolic control. The absolute requirement for growth-factor-regulated protein turnover in cell proliferation (Baserga, 1985) and the involvement of p21^{ras} proteins in the

signal-transduction pathways of some growth factors (Barbacid, 1987; Hall, 1990) prompted us to examine the effect of C-H-ras1 expression on rates of protein turnover and the regulation of protein turnover by serum and insulin.

MATERIALS AND METHODS

Cell culture

The NR6 clone of Swiss 3T3 cells was provided by Dr. Harvey Herschman, University of California at Los Angeles, Los Angeles, CA, U.S.A. The cells were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% (v/v) calf serum. To condition serum, the growth medium of confluent cultures of NR6 cells in T-75 flasks was refreshed and the cells incubated for 3 days. The medium then was removed, filtered, and diluted 10-fold with serum-free medium as required. Serum (100 ml) was dialysed at 4 °C for 24 h against two changes, each 1 litre, of Hanks salts using Spectra/Por membranes with a nominal *M_r* retention ≥ 3500 (Spectrum, Houston, TX, U.S.A.). Tissue-culture medium was purchased from Irvine Scientific, bovine calf serum from Hyclone Laboratories, and tissue-culture flasks and dishes were from Costar. Insulin was Actrapid from Novo, L-[4,5-³H]leucine (40–60 Ci/mmol) and [methyl-³H]-thymidine (40–60 Ci/mmol) were from the Amersham Corporation, a labelling mixture of [³⁵S]methionine and [³⁵S]cysteine (> 1000 Ci/mmol) was from ICN Biomedicals, and all other reagents were from Sigma.

Transfection and analysis of *ras* expression

The 6.6 kb endonuclease-*Bam*H1 fragment of pUC EJ 6.6 (Shih & Weinberg, 1982), purchased from the American Type Culture Collection, containing the c-H-ras1 gene isolated from

Abbreviations used: DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulphoxide; DegMEM, DMEM containing 2 mM-leucine; PBS, phosphate-buffered saline (20 mM-sodium phosphate/0.9% NaCl, pH 7.3); Cl₃Ac, trichloroacetic acid; PMA, phorbol 12-myristate 13-acetate ('TPA'); ANOVA, analysis of variance; ED₅₀, concentration causing 50% of maximum effect; EGF, epidermal growth factor; IGF, insulin-like growth factor; SV40, simian virus 40; TGF, transforming growth factor.

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the EJ human bladder carcinoma, was inserted into the *Bam*HI site of plasmid pSV2neo (Southern & Berg, 1982). Transfection of NR6 cells with 1 µg of carrier-free pSV2neo-*ras* DNA was by the calcium phosphate-precipitation technique (Howley *et al.*, 1983) with a 10% (v/v) dimethyl sulphoxide (DMSO) shock at 4 h. After 2 days, selection medium containing G418 (a neomycin analogue) (400 µg/ml) was added, and 14 days later, four independent clones, designated *rasA*–*D*, were isolated and expanded.

For Southern analysis (Wahl *et al.*, 1987), the genomic DNA of each clone was restricted with *Eco*R1 and probed with the nick-translated *Bam*HI fragment of pUC EJ 6.6. Expression of the c-H-*ras*1 gene product was measured by precipitation of ³⁵S-labelled cells with a monoclonal antibody, clone Y13-259, following the procedure recommended by the vendor, Oncogene Science. The antigen antibody complexes were analysed by gel electrophoresis and autoradiography.

Measurement of cell growth

Cells were plated at 1×10^5 cells/well into six-well dishes in DMEM containing 10% calf serum and 4 h later the medium was changed to DMEM containing 1% conditioned serum. After 18 h (day 0) the medium was changed to DMEM containing either 1% or 10% calf serum. At daily intervals triplicate monolayers was dispersed with EDTA/trypsin and the cell number was determined with a Coulter counter.

Measurement of DNA labelling

The medium on subconfluent cells in 24-well dishes was changed to 900 µl of DMEM containing 1% conditioned serum. After 18 h, 100 µl of DMEM containing serum and [³H]thymidine (1 µCi, 5 µM) was added. The final serum concentration was either 1% conditioned, or 1% or 10% fresh, calf serum. After a 24 h pulse, the monolayer was rinsed twice with cold PBS, twice with cold 5% (w/v) trichloroacetic acid (Cl₃Ac), once with cold water and was dissolved in 0.5 ml of 0.1 M-NaOH/0.1% Triton X-100. Duplicate aliquots were taken for scintillation counting and protein determination (Smith *et al.*, 1985).

Measurement of protein turnover

Rates of intracellular protein turnover were measured by a pulse-chase technique (Ballard & Gunn, 1985). Briefly, growth of cells in 24-well dishes was arrested by incubating in leucine-free DMEM containing 1% conditioned serum and 1 µCi of [³H]leucine/ml. After 18 h, the monolayers were rinsed twice with Earle's Salts containing 2 mM-leucine and incubated in DMEM containing 2 mM-leucine (DegMEM) and 1% conditioned serum for a further 2 h to allow for the breakdown of rapidly degraded proteins. Then the medium was changed to DegMEM alone or DegMEM containing either 1% conditioned serum or 1% or 10% fresh dialysed serum as described, and the cells incubated for a 4 h measurement period. In other experiments the cells either were incubated with 1% serum replacing conditioned serum to measure the density-dependent changes in protein turnover (Fig. 1 below), or were incubated in DegMEM alone for the 2 h chase period to remove the influence of serum before measuring the concentration effects of dialysed serum and insulin on protein turnover (Fig. 2 below). Rates of protein degradation (% in 4 h) are reported as the percentage of radioactivity soluble in 10% Cl₃Ac.

Rates of protein synthesis were measured by using exactly the same protocol except that [³H]leucine (2 µCi/ml) was added during the 4 h measurement period. Leucine incorporation from the medium is measured as nmol/4 h per mg. From the time course of [³H]leucine incorporation measured over a 72 h labelling period we calculated the maximum incorporation of

leucine from the medium (sp. radioactivity 1 µCi/µmol) to be 562 ± 11 nmol/mg of protein in NR6, 633 ± 39 in *rasA*, 642 ± 35 in *rasB*, 601 ± 12 in *rasC* and 557 ± 38 in *rasD*. The values are less than the leucine content of cell protein (Ballard & Gunn, 1985), presumably because unlabelled leucine is present in cell protein at the start of the labelling period, variable amounts of leucine are reincorporated and unlabelled leucine is derived by proteolysis of serum proteins. To correct for such variables and at the same time express synthesis in the same units as degradation, rates of protein synthesis for each cell line are reported as the percentage synthesized in 4 h relative to these maximum incorporation values (taken as 100%).

The fractional rates of protein accumulation or protein growth (percentage in 4 h) are calculated as the difference between percentage synthesized and percentage degraded. It should be noted that degradation is expressed as a fractional rate relative to the labelled proteins present at the start, and synthesis as a fractional rate relative to the labelled proteins present at the end of the 4 h period. Hence synthesis is underestimated relative to degradation. However the error is small unless the monolayers are accumulating significant amounts of protein over the 4 h period or, in the case of comparisons between cell lines, one line is accumulating significantly more protein than the other. Because synthesis and degradation are measured in separate multi-well dishes, there are two or three times fewer independent calculations for accumulation than there are measurements for synthesis and degradation.

Statistical analysis

Analysis of variance (ANOVA) procedures at the 95% confidence level on all main effect means were performed using the Kramer (1956) modification of Duncan's New Multiple Range Test to compare differences between cell lines in rates of protein synthesis and degradation. Duncan's procedure is a multiple *t* test that makes all pairwise comparisons, and Kramer's modification allows comparison of samples with unequal numbers. Calculated rates of protein accumulation are inherently less accurate because they are dependent on measured rates of synthesis and degradation and because a smaller number of values is involved. Therefore differences between cell lines in rates of protein accumulation were compared by using Fisher's Protected LSD procedure, a multiple *t* test that is a more liberal *post hoc* procedure than Duncan's. Linear-regression analysis and a *t* test were used to determine the difference in proliferation rates of the cell lines and difference in the mid-points of the serum and insulin concentration curves. The statistical program was Super-ANOVA v1.1 (Abacus Concepts, Berkeley, CA, U.S.A.) run on a Macintosh SE/30 computer.

RESULTS

We transfected 3T3-NR6 cells with a pSV2neo construct containing the c-H-*ras*1 oncogene from human bladder carcinoma T24 (Shih & Weinberg, 1982) and cloned four independent cell lines by selection with the neomycin analogue G418. Southern analysis of genomic DNA indicated that all four lines contained the oncogene, although the higher mobility of the *Eco*R1 restriction fragment in clone *rasD* indicated a different site of integration. Immunoprecipitation of ³⁵S-labelled cell proteins confirmed that all four clones and NR6 synthesized the normal p21^{ras}, and that only lines *rasA*, *rasB* and *rasC*, but not *rasD*, synthesized the oncogenic p21^{H-ras} and were transformed (results not shown). Therefore we used NR6 cells as the parental control, *rasD* cells as the transfection-negative control and *rasA*, *rasB* and *rasC* cells as transformed lines in measuring the effect of *ras* transformation on growth and proliferation.

Table 1. Doubling times for *ras*-transformed NR6-3T3 cells

Growth curves for cells in medium containing either 1% or 10% calf serum were measured over 3 days as described in the Materials and methods section. Doubling times in days are reported as means $\pm$ S.E.M. ($n = 6$) and were calculated from the slope of a plot of log(cell number) versus time determined by linear-regression analysis. *indicates significantly different from 3T3-NR6 ($P < 0.01$).

Cells	Serum...	Doubling time (days)	
		1 %	10 %
3T3-NR6		1.91 $\pm$ 0.11	0.79 $\pm$ 0.04
NR6- <i>rasA</i>		1.15 $\pm$ 0.09*	0.67 $\pm$ 0.03*
NR6- <i>rasB</i>		1.14 $\pm$ 0.07*	0.60 $\pm$ 0.02*
NR6- <i>rasC</i>		1.07 $\pm$ 0.12*	0.67 $\pm$ 0.04*
NR6- <i>rasD</i>		1.99 $\pm$ 0.13	0.81 $\pm$ 0.05

transformed lines grew to much higher densities and maintained high rates of proliferation, even at cell densities at which the normal cells were contact-inhibited. This is illustrated in Fig. 1, where we measured rates of protein turnover in *rasB* and *rasD* cells as a function of protein content. Rates of protein synthesis are high in sparse cultures and decline as the cells approach confluency, whereas rates of protein degradation are low in sparse cultures and increase at confluency. Consequently rates of protein growth or accumulation (i.e. rates of synthesis minus degradation) progressively decrease as a function of density. In confluent cultures, rates of protein synthesis are approximately equal to rates of protein breakdown, so that rates of accumulation approach zero. At all stages of the growth curve up to confluency, the *rasB* line had slightly higher rates of protein synthesis, but much lower rates of protein breakdown and consequently greater rates of protein accumulation or growth. Note that, in these experiments, we measure the effect of cell density on protein turnover and avoid the influence of depleted serum growth factors by refreshing the medium daily and during the chase and measurement periods. The higher rates of proliferation of the *ras*-transformed lines therefore correlate with alterations in rates of protein turnover.

Differences in rates of proliferation also correlated well with rates of DNA labelling measured over the first 24 h after serum stimulation (Table 2). In sparse cultures at protein densities producing rates of protein accumulation of $\leq 6\%/4$ h in *rasD* and $8\%/4$ h in *rasB* (Fig. 1), rates of DNA labelling after addition of 1% serum were approx. 50% higher in *ras*-transformed lines than in normal cells (the data for *rasA* and *rasC* were the same as for *rasB*). Rates were further stimulated in NR6 and *rasD* by addition of 10% serum, whereas in transformed lines, such as *rasB*, 10% serum had no further stimulatory effect. However, the most remarkable difference between the cell lines was the 2-fold higher rate of DNA labelling measured for *ras*-transformed cells in the presence of conditioned serum. In dense cultures (at protein accumulation rates $\leq 2\%/4$ h; see Fig. 1) this difference was over 4-fold (results not shown). In general, rates of DNA labelling for transformed cells in conditioned serum were equivalent to those for normal cells in fresh 1% serum. Taken together, these data indicate the *ras*-transformed cells have a lower serum requirement for growth and proliferation, i.e. rates of protein turnover, DNA labelling

Rates of proliferation in medium containing either 1% or 10% serum are significantly faster for *rasA*, *rasB* and *rasC* than for NR6 and *rasD* (Table 1). Both NR6 and *rasD* had doubling times of approx. 2 days in 1% serum and 0.8 day in 10% serum,

Table 2. DNA labelling in *ras*-transformed NR6 cells

Rates of DNA labelling (d.p.m./24 h per μ g of protein) were measured as described in the Materials and methods section. Results are the mean $\pm$ S.E.M. of eight values calculated using the protein content of the control cells in conditioned (Cond.) serum. These values were 24.2, 18.4 and 23.0 μ g/well for NR6, *rasD* and *rasB* respectively. Values in parentheses are the fold increases above the rate in conditioned serum for *rasD*. The data for *rasA* and *rasC* were similar to those for *rasB*.

Cell type	Addition	Rate (d.p.m./24 h per μ g)
3T3-NR6	Cond. serum	28.24 $\pm$ 2.07
	1 % Serum	64.39 $\pm$ 1.17
	10 % Serum	121.12 $\pm$ 1.32
NR6- <i>rasD</i>	Cond. serum	33.52 $\pm$ 1.20
	1 % Serum	69.61 $\pm$ 2.12 (2.08)
	10 % Serum	121.36 $\pm$ 1.82 (3.62)
NR6- <i>rasB</i>	Cond. serum	73.08 $\pm$ 2.12 (2.18)
	1 % Serum	93.28 $\pm$ 4.83 (2.78)
	10 % Serum	99.88 $\pm$ 2.77 (2.98)

Fig. 1. Density-dependent changes in protein turnover

NR6-*rasB* (closed symbols) and *rasD* (open symbols) were plated at low density in medium containing 1% calf serum. The medium was changed daily. Rates of protein synthesis (○, ●), degradation (□, ■) and accumulation (△, ▲) in DegMEM containing 1% dialysed serum were measured as described in the Materials and methods section. Each point is the average for three determinations, and two separate experiments are shown.

Table 3. Protein turnover in *ras*-transformed NR6 cells

Rates of protein turnover (%/4 h) were determined as described in the Materials and methods section. For the 4 h measurement period the cells were switched from DegMEM containing 1 % conditioned (Cond.) serum to the medium indicated. Results are the means $\pm$ S.E.M. for seven to ten determinations for synthesis and degradation and two or three determinations of accumulation. The protein content is that used for determining rates of synthesis. Means with different superscript letters are significantly different ($P < 0.05$). The data for *rasA* and *rasC* are similar to *rasB* and for NR6 are similar to those for *rasD*.

Cell type	Addition	Protein (μ g/well)	Synthesis	Degradation	Accumulation
NR6- <i>rasD</i>	DegMEM	18.6 $\pm$ 0.6	7.63 $\pm$ 0.32 ^a	12.17 $\pm$ 0.08 ^a	-4.50 $\pm$ 0.66 ^a
	Cond. serum	24.2 $\pm$ 0.3	11.77 $\pm$ 0.20 ^b	9.53 $\pm$ 0.12 ^b	2.34 $\pm$ 0.66 ^b
	1 % Serum	25.0 $\pm$ 1.0	13.90 $\pm$ 0.24 ^c	9.14 $\pm$ 0.09 ^{bc}	4.86 $\pm$ 0.51 ^c
	10 % Serum	26.9 $\pm$ 0.9	13.42 $\pm$ 0.12 ^c	8.42 $\pm$ 0.09 ^d	5.05 $\pm$ 0.30 ^c
NR6- <i>rasB</i>	DegMEM	85.8 $\pm$ 2.8	11.45 $\pm$ 0.24 ^b	8.99 $\pm$ 0.18 ^c	2.46 $\pm$ 0.58 ^b
	Cond. serum	88.8 $\pm$ 3.4	13.81 $\pm$ 0.16 ^c	6.93 $\pm$ 0.29 ^c	6.88 $\pm$ 0.35 ^d
	1 % Serum	96.1 $\pm$ 3.4	13.93 $\pm$ 0.14 ^c	6.75 $\pm$ 0.21 ^c	7.18 $\pm$ 0.26 ^d
	10 % Serum	99.8 $\pm$ 6.3	13.89 $\pm$ 0.15 ^c	6.04 $\pm$ 0.16 ^d	7.85 $\pm$ 0.27 ^d

whereas the three transformed lines had doubling times of ≤ 1.15 day in 1 % and ≤ 0.67 day in 10 % serum. In addition, the and proliferation in medium containing 1 % serum are all greater for *rasA*, *rasB* and *rasC* than for NR6 or *rasD*.

We measured rates of protein turnover for sub-confluent cultures at the beginning of the DNA labelling period, that is, for the 4 h period immediately after addition of fresh serum. In these experiments we have compared rates in conditioned medium with those either in fresh serum or the absence of serum. Because of the density-dependent changes in rates of protein turnover (Fig. 1), we were careful to compare cells at similar stages of the growth cycle, i.e. at $< 30 \mu$ g/well (2 cm^2) for normal and $< 100 \mu$ g/well for transformed, densities which are about one-third confluent. The data for *rasB* and *rasD* are shown in Table 3; the data for *rasA* and *rasC* are similar to *rasB*, and for NR6 are similar to *rasD*. Qualitatively similar responses are seen in normal and transformed cells: addition of fresh serum decreases rates of protein degradation and increases rates of protein synthesis and accumulation, whereas removal of serum has exactly opposite effects on each rate. Quantitatively a comparison of the main effect means for transformed (*rasA*, *rasB* and *rasC*) versus normal (NR6, *rasD*) cells shows that average rates of protein degradation are significantly lower and rates of protein synthesis and accumulation significantly higher for *ras*-transformed cells.

During the first 4 h after addition of fresh serum, rates of protein synthesis are the same for normal and transformed cells (Table 3). They differ significantly in the higher rates exhibited by *ras*-transformed cells in conditioned medium and under step-down conditions in DegMEM. Indeed, rates of protein synthesis for *rasB* cells in conditioned serum were identical with those for normal cells in fresh serum, a finding consistent with the effect of conditioned serum on DNA labelling (Table 2). The major distinction between *rasD* and *rasB* cells is that rates of protein breakdown are significantly lower in the transformed line under all conditions. The higher basal rates of protein synthesis and lower basal rates of protein degradation in the transformed lines has two consequences: firstly, the response to serum is reduced from $\geq 2.5\%/4 \text{ h}$ to $\leq 1.5\%/4 \text{ h}$, and, secondly, rates of protein accumulation are significantly higher for the transformed lines than for NR6 or *rasD* [rates of protein accumulation correlate with the doubling times in 1 % serum ($r = -0.97$) and in 10 % serum ($r = -0.91$)]. Under step-down conditions in serum-free medium the normal cells display a dramatic decrease in rates of protein synthesis and an increase in rates of protein breakdown, resulting in a net negative nitrogen balance. In contrast the

Table 4. Protein synthesis in NR6-*rasB* and NR6-*rasD* cells 24 h after serum addition

Rates of protein synthesis (%/4 h) were measured as described in the Materials and methods section. At zero time the medium was changed to DegMEM containing either 1 % conditioned (Cond.) serum or fresh serum as indicated. After 20 h the medium was changed to one of exactly the same composition but also containing [³H]leucine, and the cells were pulse-labelled for 4 h. Results are means $\pm$ S.E.M. for four to seven determinations. Means with different superscript letters are significantly different ($P < 0.05$). The data for *rasA* and *rasC* are similar to those for *rasB* and for NR6 are similar to those for *rasD*.

Cell type	Addition	Protein (μ g/well)	Synthesis
NR6- <i>rasD</i>	Cond. serum	25.6 $\pm$ 2.8	10.29 $\pm$ 0.55 ^a
	1 % Serum	39.6 $\pm$ 1.9	11.97 $\pm$ 0.40 ^b
	10 % Serum	71.5 $\pm$ 1.1	13.53 $\pm$ 0.28 ^c
NR6- <i>rasB</i>	Cond. serum	107.6 $\pm$ 2.8	11.21 $\pm$ 0.68 ^{ab}
	1 % Serum	145.8 $\pm$ 2.6	13.87 $\pm$ 0.40 ^c
	10 % Serum	172.6 $\pm$ 6.0	15.61 $\pm$ 0.16 ^d

responses in *ras*-transformed cells are much smaller and a net positive nitrogen balance is maintained.

The high and identical rates of protein synthesis for *rasD* and *rasB* cells in 1 % and 10 % serum was surprising, because we anticipated that *rasB* would have higher rates than *rasD* and that 10 % serum would produce higher rates than 1 % serum. In Table 4 we report rates of protein synthesis at 20–24 h after serum stimulation for cells starting at densities similar to those in Table 3. Compared with conditioned serum, fresh serum significantly increases protein synthesis in both normal and transformed cells and rates are significantly higher in 10 % than in 1 % serum. In addition, rates are significantly higher for *rasB* than for *rasD*, with the effect of conditioned serum and 1 % serum in *rasB* being equivalent to 1 % and 10 % serum respectively in *rasD*. A comparison of Tables 3 and 4 shows that rates of protein synthesis in 1 % serum were maintained in *rasB*, but fell in *rasD*. Thus serum stimulation of cells lowers rates of protein breakdown and raises rates of protein synthesis in both normal and transformed cells, but the serum-independence of the later allows them to maintain the high rates of protein synthesis even at low serum concentrations.

The higher rates of proliferation, DNA labelling and protein turnover at low serum concentrations and in conditioned medium

Fig. 2. Effects of insulin and serum concentration on protein turnover

Rates of protein synthesis ($\circ$, $\bullet$), degradation ($\square$, $\blacksquare$) and accumulation ($\triangle$, $\blacktriangle$) in DegMEM containing dialysed calf serum (a and b) or insulin (c and d) were measured in NR6-*ras*B (closed symbols) and *ras*D (open symbols) cells as described in the Materials and methods section. One of two separate experiments is reported. Here cell densities (μ g of protein/well) were 58 (a and b) and 95 (c and d) for *ras*B, and 29 (a and b) and 21 (c and d) for *ras*D. Each point is the mean $\pm$ S.E.M. for four determinations for synthesis and degradation, and for a single determination for accumulation.

Table 5. Half-maximum concentrations (ED_{50}) of serum and insulin regulating rates of protein turnover in NR6 and NR6-*ras* cells

The data in Fig. 2 and from three other experiments were re-plotted as $\log[\text{rate}/(\text{maximum rate} - \text{rate})]$ versus $\log[\text{serum}]$ or $\log[\text{insulin}]$ (i.e. in the logarithmic form of the Hill equation) and are reported as the mean $\pm$ S.E.M. of the intercept on the x-axis ($\log ED_{50}$), calculated by linear regression, and as the mid-point of the dose-response curve (ED_{50}). Means with different superscript letters are significantly different ($P < 0.01$ for serum and $P < 0.001$ for insulin).

Cell type	Serum		Insulin	
	ED_{50} (%)	$\log ED_{50}$	ED_{50} (nM)	$\log ED_{50}$
3T3-NR6	0.48	-0.32 ± 0.04^a	2.95	-8.53 ± 0.07^a
NR6- <i>ras</i> D	0.47	-0.33 ± 0.02^a	4.84	-8.32 ± 0.16^a
NR6- <i>ras</i> B	0.30	-0.53 ± 0.06^b	0.53	-9.27 ± 0.12^b

indicate that *ras* transformation of NR6 cells evokes greater sensitivity, but a smaller response, to serum growth factors as a consequence of serum-independence. Serum and insulin increase rates of protein synthesis and decrease rates of protein breakdown in a concentration-dependent manner (Fig. 2). Under the conditions of this experiment, which differ from those reported previously in having a 2 h chase in DegMEM alone to remove

the influence of serum, rates of protein synthesis were always higher, and rates of protein degradation always lower, in *ras*B cells for any given concentration of serum or insulin. A positive nitrogen balance for *ras*D was not achieved in the short term at < 10 nM-insulin or $< 0.6\%$ serum, whereas *ras*B maintained a positive nitrogen balance even in the absence of serum or insulin. From a Hill plot of the data (Table 5), we calculate a significant decrease in the half-maximum concentrations (ED_{50}) of serum and insulin affecting protein turnover in *ras*B compared with the parental NR6 or *ras*D. For serum the change was from 0.5% to 0.3%, and for insulin from 4 nM to 0.5 nM. In addition, the smaller changes in the dose-response curves for protein accumulation clearly demonstrate that the absolute changes in rates of protein synthesis and degradation are reduced in *ras*B compared with *ras*D, a reflection of a reduced response to serum growth factors in the transformed cells.

DISCUSSION

The *ras* proto-oncogene family codes for several small GTP-binding proteins thought to be integral components of the signal-transduction system regulated by serum growth factors that leads to growth and proliferation (Barbacid, 1987; Hall, 1990). Mutation of the GTPase function of $p21^{ras}$ produces cell transformation, suggesting that it acts as a proliferative signal. The 3T3-NR6 clone used here lacks functional EGF receptors, has no proliferative response to EGF and grows to lower saturation densities relative to 3T3 cells (Pruss & Herschman, 1977). Therefore we anticipated normal cells would have a low functional response to serum, whereas transformed cells would have increased basal rates of protein turnover coupled with a greater functional response to serum because of the perturbation of the signal-transduction pathways by $p21^{H-ras}$. This is indeed the case. Compared with normal cells the *ras*-transformed lines have a remarkably high rate of DNA labelling and protein turnover measured in conditioned serum with equivalent rates of protein turnover, growth, DNA labelling and proliferation now occurring at lower concentrations of serum growth factors.

The *ras*-transformed cells have attenuated the catabolic response to conditioned serum and to serum withdrawal by establishing higher basal rates of protein synthesis and lower basal rates of protein breakdown (Fig. 2, Table 3). Rates of DNA labelling, protein synthesis and protein breakdown measured in transformed cells after more than 18 h in conditioned medium were the same as those measured in normal cells after addition of fresh serum. Recovery from serum withdrawal is known to be rapid in transformed cells, but delayed in normal cells (Baserga, 1985). Here we show that this is principally due to a delay in recovering normal rates of protein synthesis (cf. Fig. 2 and Table 3). Thus, in normal cells, rates of protein synthesis measured 4 h after serum addition to cells previously exposed to serum-free medium (Fig. 2) remain profoundly different from those in transformed cells, whereas after exposure to conditioned serum (Table 3) rates of protein synthesis are the same for normal and transformed. Rat 3T3 cells transfected with the c-H-*ras*1 gene are known to produce transforming growth factor- β (TGF- β) (Stern *et al.*, 1986), and part of the enhanced response to conditioned serum and the reduced response to serum withdrawal in transformed cells may reflect TGF- β or other autocrine factors that remain bound to cell-surface receptors or are secreted during the experiment.

Such high basal rates of protein synthesis and low basal rates of protein degradation in *ras*B compared with *ras*D mean that overall rates of metabolism, stimulated by a given concentration of serum growth factor, are enhanced, but, paradoxically, the incremental response to serum growth factors is often reduced.

However, the smaller incremental effects of growth factors are simply additive to higher basal rates of protein synthesis and lower basal rates of protein degradation. So the first effect of *ras* transformation is that maximum (or minimum) flux rates through the pathways are achieved at lower concentrations of growth factor. The second remarkable effect of *ras* transformation is the significant reduction in the mid-points of the dose-response curves for serum growth factors. This represents amplification of the receptor-ligand interaction by p21^{H-ras} acting distal to the receptor in accord with a hypothesis (Ellis *et al.*, 1990) of receptor tyrosine kinase-p21^{H-ras} interaction as part of the signal-transduction mechanism. We have previously reported (Gunn *et al.*, 1977, 1983; Ballard *et al.*, 1980; Ballard & Gunn, 1985; Ballard, 1987) a similar shift in the dose-response curves for insulin, EGF and IGF-2, as well as altered rates of protein metabolism in cultured tumour cells. In this regard it is noteworthy that two very different oncogenes, p21^{H-ras} and simian-virus-40 (SV40) large T antigen (in the SV40 transformation of mouse 3T3 cells or human diploid fibroblasts) both alter rates of protein turnover and both increase the sensitivity of the transformed cell to serum growth factors. Such enhanced sensitivity could be due either to secretion of an autocrine factor which, because the effects of different serum growth factors on protein turnover are additive (Gunn *et al.*, 1983; Ballard, 1987), will result in a shift in the dose-response curve, or to intracellular regulation of the signal-transduction pathway, or to both mechanisms.

Both normal and transformed cells have the same high rates of protein synthesis 4 h after serum stimulation of cells in conditioned medium (Table 3), but the transformed cells have significantly lower rates of protein breakdown. Therefore the short-term effects of growth stimulation in *ras*-transformed cells compared with normal cells arise because, firstly, the initial basal rates of protein synthesis and degradation are more anabolic, and, secondly, serum stimulation produces a significantly lower rate of protein breakdown in the transformed cells. By 24 h (Table 4), which is still the first cell cycle for cells in 1% serum, but the second for cells in 10% serum, the emphasis has shifted to include the maintenance of significantly higher rates of protein synthesis in the transformed cells. Thus small, but significant, differences in rates of protein synthesis and degradation for *ras*-transformed cells lead to more rapid rates of protein accumulation and turnover that correlate with increased rates of cell proliferation.

Such seemingly small differences in protein turnover are present throughout the growth cycle (Fig. 1). The *ras*-transformed cells retained the density-dependent changes in rates of both protein synthesis and breakdown characteristic of normal cells (Cockle & Dean, 1982a,b, 1984; Lockwood & Minassian, 1982; Lockwood *et al.*, 1982; Ballard, 1987). However, they readily grow to densities some three or four times greater than normal cells. This suggests that p21^{H-ras} overrides the normal signals for cessation of growth by contact inhibition, but that such autocrine or paracrine events arising from cell-cell interactions eventually act to modulate the pathways of protein turnover. Thus contact inhibition is muted, but not entirely lost, in *ras* transformants.

Taken together these data indicate that *ras* transformation has modulated a host of pleiotropic responses. Besides the altered rates of protein turnover reported here, rates of hexose transport, concomitant with increased levels of the glucose-transporter mRNA and protein (Flier *et al.*, 1987), glycolysis and amino acid transport (Racker *et al.*, 1985) also are increased in *ras*-transformed cells. Overall the enhanced growth and proliferation of *ras*-transformed cells is a result of altered rates of metabolic pathways and an increased sensitivity to regulation of the pathway flux by growth factors. Unfortunately the pleiotropic

nature of transformation makes it difficult to distinguish between direct effects of p21^{H-ras} on a metabolic pathway and indirect effects arising from the proliferative response to serum growth factors. We consider the higher basal rates of protein synthesis and growth with retention of the normal proliferative response to serum as part of the pleiotropic effect of transformation. However, the lower rates of protein breakdown measured in *ras*-transformed cells under all conditions strongly suggests a direct effect of p21^{H-ras} on the degradative pathways.

Transformation by *ras* increases the level of 1,2-diacylglycerol (Lacal *et al.*, 1987; Wolfman & Macara, 1987) with down-regulation of protein kinase C (Wolfman *et al.*, 1987) although the source of the diacylglycerol and the effect of p21^{H-ras} on phospholipid metabolism remains unclear (Price *et al.*, 1989; Lacal, 1990). The phorbol ester phorbol 12-myristate 13-acetate (PMA) mimics transformation by c-H-*ras*1 by increasing rates of DNA and protein synthesis and decreasing rates of protein breakdown in *ras*D cells. Conversely, depletion of protein kinase C by overnight exposure to PMA attenuates transformation in *ras*B cells by decreasing rates of DNA and protein synthesis and increasing rates of protein breakdown (J. M. Gunn, unpublished work). Thus alterations in protein turnover in 3T3 cells transformed by c-H-*ras*1 are regulated distal to p21^{H-ras} and may involve protein kinase C. Expression of *ras* is essential during the first 8 h of the G₁ phase of the cell cycle (Mulcahy *et al.*, 1985), and it is during this phase that the major difference between normal and *ras*-transformed cells is in rates of protein breakdown (Table 3). Throughout the growth cycle, rates of protein breakdown in *ras*-transformed cells remain lower than in normal cells, even at confluency, and even though the normal density-dependent changes in protein turnover are maintained (Fig. 1). Under step-down conditions, removal of serum causes the same relative increase (~30%) in rates of protein breakdown in normal and transformed cells, suggesting that the pleiotropic response is maintained, but that absolute differences in basal rates between the cell types are fixed by the expression of c-H-*ras*1. Thus regulation of the lysosomal pathway for bulk protein degradation by regulation of autophagic vacuole formation may be directly influenced by p21^{H-ras}.

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