Stimulation of gene expression in neonatal rat ventricular myocytes by Ras is mediated by Ral guanine nucleotide dissociation stimulator (Ral.GDS) and phosphatidylinositol 3-kinase in addition to Raf

Stephen J. FULLER*[1], Stephen G. FINN*, Julian DOWNWARD† and Peter H. SUGDEN*

*NHLI Division (Cardiac Medicine), Imperial College School of Medicine, London SW3 6LY, U.K., and †Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, U.K.

Treatment of cultured neonatal ventricular myocytes with oncogenic Ras increases their size and stimulates the re-expression of genes which are normally restricted to the fetal stage of ventricular development, including atrial natriuretic factor (ANF) and skeletal muscle (SkM)-α-actin. To determine which signalling pathways mediate these responses, myocytes were transfected with oncogenic (V12) Ras mutants which interact selectively with different effectors and their effects on luciferase (LUX) reporter plasmids were examined. V12 human Ras (V12HRas), itself, activated ANF–LUX 9.6-fold, whereas mutants of V12HRas, which selectively stimulate Ral guanine nucleotide dissociation stimulator (Ral.GDS) (E37G), c-Raf (D38E) and phosphatidylinositol 3-kinase (PI-3-K; Y40C) enhanced ANF–LUX expression 3.0-, 3.7- and 1.7-fold respectively. The full response of ANF–LUX to V12HRas was restored by using a combination of the individual effector domain mutants. Likewise, SkM-α-actin–LUX expression was activated 12.0-, 3.5-, 4.5- and 3.0-fold by V12HRas, E37G, D38E and Y40C respectively, and a similar pattern of activation was also observed using a c-*fos* serum-response element–LUX reporter gene. Cell size was also increased by each of the mutants, but simultaneous expression of all three mutant constructs was needed to reconstitute the full effect of V12HRas on cell size (50% increase). Transfection with a constitutively active mutant of PI-3-K (p110K227E) stimulated ANF–LUX, SkM-α-actin–LUX, c-*fos*-serum-response element–LUX and Rous sarcoma virus–LUX by 3.1-, 3.2-, 2.1- and 2.9-fold respectively, but the co-transfected cytomegalovirus-β-galactosidase reporter gene was activated to a similar extent (1.9-fold). These results suggest that Raf, Ral.GDS and PI-3-K can all transduce transcriptional responses to V12HRas, but that the specific induction of genes associated with the hypertrophic response is not mediated through PI-3-K.

INTRODUCTION

In response to a variety of stimuli (α_1-adrenergic agents, endothelin-1, peptide growth factors and stretch), cultured neonatal rat ventricular myocytes exhibit a hypertrophic phenotype. This is characterized by an increase in cell size and protein synthesis, enhanced myofibrillar organization and specific alterations in gene expression (reviewed in [1]). The transcriptional responses encompass the transient activation of immediate early genes (e.g. c-*fos*, c-*jun* and *egr-1*), re-expression of embryonic genes [e.g. atrial natriuretic factor (ANF), skeletal muscle (SkM) α-actin and β-myosin heavy-chain] and upregulation of contractile protein genes such as ventricular myosin light-chain-2. Although it is the subject of much research activity, the precise intracellular signalling pathways through which these hypertrophic changes are mediated have yet to be fully defined. It is clear, however, that activation of the small GTP-binding protein Ras is a cardinal feature of the hypertrophic response in cardiac myocytes. Microinjection of oncogenic Ras recapitulates the transcriptional and morphological changes elicited by hypertrophic agonists, whereas a dominant-negative Ras mutant blocks the response to the α_1-adrenergic agonist phenylephrine (PE [2]). In addition, when expressed in the ventricles of transgenic mice, oncogenic Ras induces pathological cardiac hypertrophy [3].

Despite the general consensus about the involvement of Ras, there is considerable debate as to the subsequent signalling mechanisms through which activated Ras exerts its pleiotrophic effects on gene expression and morphology. We [4–7] and others [8–10] have proposed that the Raf → mitogen activated protein kinase (MAPK) kinase (MEK) → extracellular signal-related protein kinase (ERK) pathway may be important in this respect, on the basis that hypertrophic agonists activate this pathway and overexpression of individual components of the pathway elicits appropriate transcriptional responses. However, other evidence indicates that activation of ERKs alone is insufficient to initiate a hypertrophic response and may, indeed, be inhibitory [11]. Thus, ERK1 and ERK2 can be activated in myocytes by bradykinin, carbachol and ATP, agonists which are poor activators of ANF expression [12,13]. More recently, two other Ras effectors, Ras.GAP, which stimulates Ras GTPase activity, and MEK kinase (MEKK) have been proposed as transducers of the transcriptional responses in cardiac myocytes [14,15].

In common with the mechanism by which Ras transforms permissive cells [16,17], it is probable that more than one effector

Abbreviations used: ANF, atrial natriuretic factor; β-gal, β-galactosidase; CMV, cytomegalovirus; ERK, extracellular signal-regulated kinase; GAP, GTPase-activating protein; JNK, c-Jun N-terminal kinase; LUX, luciferase; MAPK, mitogen-activated protein kinase; MEK, MAPK (or ERK) kinase; MEKK, MEK kinase; PBS, Dulbecco's Ca^{2+}/Mg^{2+}-free PBS; PE, phenylephrine; PI-3-K, phosphatidylinositol 3-kinase; Ral.GDS, Ral guanine nucleotide dissociation stimulator; RSV, Rous sarcoma virus; SkM-α-actin, skeletal muscle α-actin; SRE, serum responsive element; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; the one letter symbols for amino acids are used.

[1] To whom correspondence should be addressed (e-mail stephen.fuller@ic.ac.uk).

of Ras may be required to induce the full hypertrophic response in cardiac myocytes. Once activated, Ras interacts with a growing list of well-established or potential effector molecules including Raf, MEKK, phosphatidylinositol 3-kinase (PI-3-K), Ras.GAP, Ral guanine nucleotide dissociation stimulator (Ral.GDS), RGL (Ral.GDS-like) and protein kinase C ζ (reviewed in [18]). Essential to the interaction of Ras with these target molecules is a short sequence of amino acids (residues 32–40) termed the Ras effector domain. Mutations in this region impair the binding of Ras-GTP to its target proteins. Of particular interest is the finding that substitution of various amino acids within the effector domain alters the binding and activation of different effectors to different extents [19]. This phenomenon can been exploited to determine which downstream effectors may be important for particular functions of Ras. Here we have used effector-domain mutants of oncogenic V12 human Ras (V12HRas) which, of the known effectors, interact selectively with Ral.GDS (E37G), Raf (D38E) and PI-3-K (Y40C), to determine which of these effectors are able to initiate a hypertrophic response. We propose that Ral.GDS and Raf are mediators of specific hypertrophic transcriptional responses, whereas PI-3-K elicits a global effect on gene expression in cardiac myocytes.

EXPERIMENTAL

General materials

Sprague–Dawley rats were bred within the NHLI Division (Imperial College School of Medicine, London, U.K.) Culture medium and other reagents were from Sigma Chemical Co. (Poole, Dorset, U.K.), Marathon Laboratory Supplies (London, U.K.) or Merck (Lutterworth, Leics., U.K.), as described previously [7]. PE was prepared freshly on the day of use.

Expression and reporter plasmids

The ANF-luciferase (ANF–LUX) reporter construct pANF(-638)LΔ5′ [20], Rous sarcoma virus (RSV)–LUX and pON249 [21], in which β-galactosidase (β-gal) is expressed from a constitutively active cytomegalovirus (CMV) promoter, were provided by Dr. K. R. Chien (Department of Medicine, University of California San Diego, CA, U.S.A.). The LUX reporter constructs for SkM-α-actin [22–24] and the c-*fos* serum responsive element (SRE) [25] were gifts from Dr. M. D. Schneider (Molecular Cardiology Unit, Baylor College of Medicine, Houston, TX, U.S.A.) and have been described in detail previously [6]. Constructs expressing V12HRas and the effector domain mutants V12HRasE37G, V12HRasD38E and V12HRasY40C have been described elsewhere [19]. Plasmids were purified by poly(ethylene glycol) precipitation [26].

Transient transfection of cultured neonatal rat ventricular myocytes

Ventricular myocytes were isolated from the hearts of 1–2-day old rats by a modification of the method of Iwaki et al. [27] as described previously [6]. After a 30 min pre-plating to decrease fibroblast contamination, myocytes were plated on gelatin-coated 60 mm tissue-culture dishes at a density of 1×10^6 cells per dish (350 cells/mm^2) for reporter gene experiments, or at a density of 5×10^5 cells per 60 mm dish for assessment of cell size. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air. At 20 h after plating and 4 h before transfection, medium was changed to 4 % (v/v) horse serum in maintenance medium (Dulbecco's modified Eagle's medium and medium 199 in a 4:1 ratio). Plasmids were diluted in 0.25 M $CaCl_2$ and an equal volume of 50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (pH 7.1)/280 mM NaCl/1.5 mM Na_2HPO_4 was added. After 20 min, 0.5 or 1 ml of this suspension (containing 3 or 5 μg of LUX reporter plasmid, 1 or 2 μg of pON249 and 0.2–5 μg of test plasmid, as indicated) was added to 4 ml of medium on each plate. After overnight transfection, cells were washed once with 10 % (v/v) horse serum in maintenance medium and twice with maintenance medium before being incubated in maintenance medium. Transfection efficiency assessed by (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (X-gal)-staining of cells and light microscopy is routinely 1–2 % by this method [6] but was not assayed here. After 48 h, myocytes were washed twice with ice-cold PBS and extracted on ice with 0.1 M potassium phosphate (pH 7.9)/0.5 % (v/v) Triton X-100/1 mM dithiothreitol (0.4 ml) for 15 min. Assays for LUX and β-gal were performed as detailed previously [6].

Determination of myocyte size

To assess the area of transfected myocytes, cells were transfected as above, except that myocytes were plated at a density of 5×10^5 cells per dish to facilitate identification of individual cells. Cells were washed three times with ice-cold PBS, fixed with 4 % formaldehyde in PBS for 10 min, re-washed three times with PBS and then stained with 0.2 mg/ml X-gal/5 mM $K_4Fe(CN)_6$/5 mM $K_3Fe(CN)_6$/2 mM $MgCl_2$ in PBS. Transfected (blue) cells were randomly selected from all areas of the dishes and video hardcopies taken. Cell area was determined by planimetry.

Expression of results and statistical analysis

Results are expressed as the ratio of LUX reporter gene expression in the presence of constitutively active mutants of V12HRas or PI-3-K relative to empty vector controls. This facilitates comparison of the relative potencies of the expression plasmids. Because V12HRas enhances expression of the co-transfected CMV–β-gal plasmid, results are presented as means ± S.E.M. of non-normalized data for the number of separate myocyte preparations shown in the Figure legends. The data normalized for β-gal expression are included in the Figure legends. Statistical significance between conditions was assessed by using an unpaired Student's *t*-test, except for comparison with controls (set at 1) which used a paired *t*-test. A significant difference was taken as being established at $P < 0.05$.

RESULTS

Stimulation of a hypertrophic pattern of gene expression by V12HRas is mediated through Ral.GDS and Raf, but not PI-3-K

Oncogenic Ras is a powerful hypertrophic agent and recent data using an R12HRasE63K mutant, which impairs the binding of guanine nucleotide exchange factors to Ras, has suggested a role for Ras.GAP in mediating transcriptional responses to Ras in cardiac myocytes [14]. To determine whether other Ras effectors may also be important in the control of transcription in cardiac myocytes, we have used oncogenic V12HRas containing a series of effector-domain mutants which selectively activate Ral.GDS (E37G), Raf (D38E) and PI-3-K (Y40C) of the known Ras effectors [19]. The effects of these mutations on the ability of V12HRas to stimulate gene expression was determined using LUX constructs under the control of promoters for ANF, SkM-α-actin and the c-*fos*-SRE, whose specific induction is a hallmark of the hypertrophic response in these cells [28]. Transfection

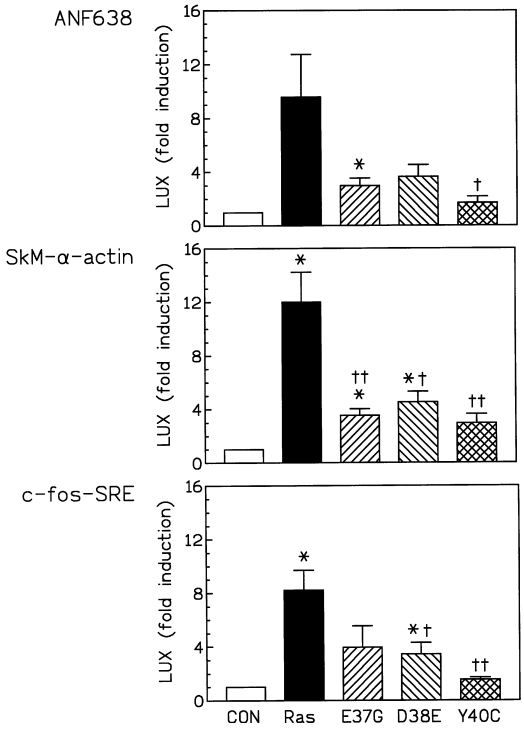

Figure 1 Stimulation of ANF–LUX, SkM-α-actin–LUX and c-*fos*-SRE–LUX expression by effector domain mutants of V12HRas

Neonatal cardiac myocytes were transfected with ANF–LUX, SkM-α-actin–LUX or c-*fos*-SRE–LUX (5 μg/dish), pON249 (1 μg/dish) and 1 μg of V12HRas, V12HRasE37G (E37G), V12HRasD38E (D38E), V12HRasY40C (Y40C) or pcDNA3 backbone vector (CON) as appropriate, as described in the Experimental section. After a further 48 h the cells were extracted and assayed for LUX and β-gal expression. The results are the means ± S.E.M. from four (ANF and c-*fos*-SRE) or five (SkM-α-actin) separate myocyte preparations and are expressed as fold induction relative to transfections with the control vector pcDNA3. Fold induction normalized for co-transfected β-gal and expressed relative to transfections with pcDNA3 for V12HRas, E37G, D38E and Y40C respectively, was as follows: ANF: 2.48 ± 0.22**, 2.05 ± 0.18*, 2.13 ± 0.17**, 1.33 ± 0.37; SkM-α-actin: 3.35 ± 0.15***, 2.15 ± 0.22**, 2.41 ± 0.19**, 1.47 ± 0.18; c-*fos*-SRE: 2.40 ± 0.39*, 1.73 ± 0.47, 1.64 ± 0.19*, 0.90 ± 0.06. Statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with control; †$P < 0.05$, ††$P < 0.01$ compared with V12HRas.

of myocytes with V12HRas induced an 8- to 12-fold activation of each of the LUX reporter genes (Figure 1). A smaller degree of activation of these reporter genes (3- to 4-fold) was seen with mutations permitting only selective activation of Ral.GDS (E37G) or Raf (D38E), but V12HRasY40C, which selectively activated PI-3-K, was much less effective. V12HRas also enhanced the expression of the co-transfected CMV–β-gal vector to a much greater extent than the E37G and D38E mutants. This probably reflects an effect of V12HRas on transcription/translation in general, since the CMV–β-gal vector is constitutively active. Hence, when normalized to β-gal expression, the effects of the E37G and D38E mutants on ANF-, SkM-α-actin- and c-*fos*-SRE-LUX expression were of a similar magnitude to those of V12HRas (results in legend to Figure 1). These results suggest that V12HRas has two components to its effects on gene expression, one which is a general effect on the transcriptional/translational machinery, leading to enhanced expression of all genes, and a second which leads to the specific activation of genes associated with the hypertrophic phenotype in these cells and which may be transduced through Ral.GDS and Raf, but not PI-3-K.

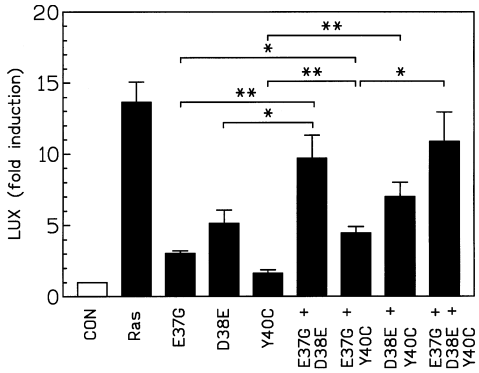

Figure 2 Additivity of the stimulation of ANF–LUX expression by effector domain mutants of V12HRas

Neonatal cardiac myocytes were transfected with ANF–LUX (5 μg/dish), pON249 (1 μg/dish) and 1 μg of V12HRas, V12HRasE37G, V12HRasD38E, V12HRasY40C either alone or in combination as shown, as described in the Experimental section. The total concentration of test DNA was kept constant at 3 μg by the inclusion of pcDNA3 backbone vector (CON) as appropriate. After a further 48 h the cells were extracted and assayed for LUX and β-gal expression. The results are the means ± S.E.M. from four separate myocyte preparations and are expressed as fold induction relative to transfections with the control vector pcDNA3. Fold induction normalized for co-transfected β-gal and expressed relative to transfections with pcDNA3 was as follows: V12HRas 3.75 ± 0.45; E37G 2.33 ± 0.31; D38E 3.73 ± 0.94; Y40C 1.41 ± 0.16; E37G + D38E 4.65 ± 0.53; E37G + Y40C 2.60 ± 0.40; D38E + Y40C 3.09 ± 0.38; E37G + D38E + Y40C 3.80 ± 0.57. Statistical significance: $*P < 0.05$, $**P < 0.01$.

We next determined whether the effects of the Ras mutants were additive (Figure 2). In this series of experiments, V12HRas activated ANF–LUX to 13.7-fold of controls and the fold inductions of ANF–LUX by the E37G, D38E and Y40C mutations were 3.03 ± 0.17, 5.13 ± 0.94 and 1.63 ± 0.25 respectively. In combination, the effects of the mutants were equivalent to the sum of their individual effects: 9.69 ± 1.61-fold (E37G + D38E); 4.44 ± 0.45-fold (D37G + Y40C); 6.99 ± 1.01-fold (D38E + Y40C) and 10.85 ± 2.08-fold (E37G + D38E + Y40C). In addition, neither the stimulation of ANF–LUX by the combination of all three mutants, nor that in response to E37G + D38E, were significantly different from that of V12HRas itself. Thus, the effects of V12HRas on ANF–LUX induction can be fully accounted for by signalling through Raf and Ral.GDS.

Meaningful comparison of the different Ras mutants assumes that they are equally expressed. The low transfection efficiency by calcium phosphate precipitation in cultured myocytes [29] makes it difficult to determine the relative levels of transfected vectors reliably and to verify this assumption. However, comparison of the effects of the mutants over a 33-fold range of concentrations (0.3–10 μg) did not alter ANF–LUX expression significantly, suggesting that each was exerting a maximal effect (results not shown). These results suggest that the reduced efficacy of the mutants compared with V12HRas in stimulating ANF–LUX expression is unlikely to be due to differences in the levels of their expression, but that the E37G and D38E mutants are unable to elicit the full response of ANF–LUX expression to V12HRas individually.

Effector domain mutations reduce the increase in myocyte size elicited by V12HRas

Another characteristic feature of ventricular myocytes treated with oncogenic Ras is an increase in cell size [2,30]. To determine

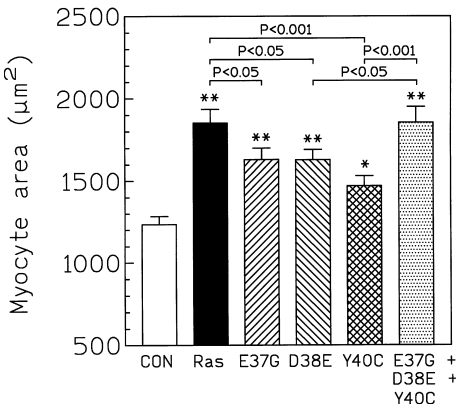

Figure 3 Effects of V12HRas effector domain mutants on myocyte size

Neonatal cardiac myocytes (5×10^5 cells per 60 mm culture dish) were transfected with pON249 (2 μg/dish) and 1 μg of V12HRas (Ras), V12HRasE37G (E37G), V12HRasD38E (D38E), V12HRasY40C (Y40C), alone or in combination as shown, as described in the Experimental section. Control transfections (CON) contained 3 μg of pcDNA3 vector, and 2 μg of pcDNA3 vector was also added to transfections with Ras, E37G, D38E and Y40C to maintain a constant vector content. After a further 48 h, the cells were fixed and stained with X-gal and cell areas were measured by planimetry. Results are means $\pm$ S.E.M. from 96–110 randomly chosen transfected cells. Statistical significance: $^*P < 0.01$, $^{**}P < 0.001$ compared with control.

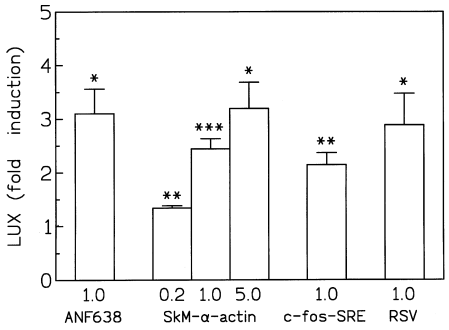

Figure 4 Stimulation of ANF–LUX, SkM-α-actin–LUX, c-*fos*-SRE–LUX and RSV–LUX expression by constitutively active p110K227E

Neonatal cardiac myocytes were transfected with ANF–LUX, SkM–LUX, c-*fos*-SRE–LUX or RSV–LUX (3 μg/dish), pON249 (1 μg/dish) and with 0.2, 1 or 5 μg of p110K227E or pSG5 backbone vector as indicated, as described in the Experimental section. After a further 48 h the cells were extracted and assayed for LUX and β-gal expression. The results are the means $\pm$ S.E.M. of four or five separate myocyte preparations and are expressed as LUX activities relative to transfections with the control vector pSG5. Fold induction normalized for co-transfected β-gal and expressed relative to transfections with pSG5 was as follows: ANF $1.97 \pm 0.29^*$; SkM-α-actin 0.98 ± 0.06 (0.2 μg), 1.10 ± 0.19 (1 μg), 1.21 ± 0.18 (5 μg); c-*fos*-SRE 1.08 ± 0.13; RSV 1.25 ± 0.25. Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with pSG5 control.

which effector-functions of V12HRas are important for this aspect of the hypertrophic response, myocytes were transfected with the double mutants and cell area was determined (Figure 3). V12HRas increased myocyte area by 50 %, which was similar to the increase elicited by 100 μM PE (60 %, results not shown). The E37G and D38E mutants each increased myocyte area by 32 %, significantly less than the effect of V12HRas. The Y40C mutant was even less effective in altering cell size (19 % increase), but the combination of all three mutant plasmids fully restored the hypertrophic response to that seen with V12HRas. Analysis of the effects of the mutants on the cell area/perimeter ratio, which is a crude index of cell shape [7], resulted in similar conclusions (results not shown). Thus, like its effects on transcription, the effects of V12HRas on cell size appear to be mediated predominantly through activation of Ral.GDS and Raf.

PI-3-K initiates a general increase in gene expression in cardiac myocytes

Although the V12HRasY40C mutant was the least effective in stimulating LUX reporter gene expression, it did significantly enhance expression of the co-transfected CMV–β-gal vector (1.69 ± 0.17; $P < 0.01$). This suggests that Ras may promote a general increase in gene expression by signalling through PI-3-K. To determine whether PI-3-K is able to fulfil such a role, the effects of a constitutively active p110 catalytic subunit of PI-3-K (p110K227E) on ANF–LUX, SkM-α-actin–LUX, c-*fos*-SRE–LUX and RSV–LUX expression were assessed (Figure 4). As expected, p110K227E significantly stimulated the activity of all of the reporter genes, including the nominally constitutively active RSV–LUX transgene. Also in keeping with an effect on overall gene expression, p110K227E significantly increased CMV–β-gal expression in these experiments (1.9 ± 0.1-fold of control; $P < 0.001$). Thus, there was no stimulation of expression of SkM-α-actin–LUX, c-*fos*-SRE–LUX or RSV-LUX over and above that for CMV–β-gal, and only a modest extra stimulation of ANF–LUX (Figure 4).

DISCUSSION

The importance of Ras in controlling cell growth is highlighted by the observation that mutations in Ras are the most frequently identified aberration in cancerous cells, being found in nearly one-third of all human cancers [31]. Oncogenic Ras is able to transform many cells in culture and to cause quiescient cells to re-enter the cell cycle [16,17]. In cultured cardiac myocytes, which have permanently withdrawn from the cell cycle, microinjection of oncogenic Ras induces hypertrophic growth, as determined by activation of c-*fos* and ANF expression and by characteristic changes in cell morphology [2]. By targeting V12HRas to the heart using the rat ventricular myosin light-chain-2 promoter, oncogenic Ras has also been shown to be capable of inducing cardiac hypertrophy in transgenic mice [3]. Mice homozygous for the V12Ras transgene exhibited a 57.5 % increase in left ventricular mass/body mass ratio, a 22 % increase in myocyte cross-sectional area, a profound upregulation of ANF mRNA and diastolic dysfunction associated with a hypertrophic cardiomyopathic phenotype [3]. These *in vitro* and *in vivo* studies define a role for Ras-dependent pathways in the development of cardiac hypertrophy and motivate the understanding of the signalling mechanisms through which this is brought about.

The ability of Ras to control cell growth is underpinned by its interaction with many different regulatory components (reviewed in [18]). Ras is activated following stimulation of both receptor protein tyrosine kinases and G-protein-coupled receptors and mediates its effects through multiple downstream components. Of the known Ras effectors, the best established is Raf, which couples activated Ras to stimulation of the ERK cascade [32]. In cultured ventricular myocytes, direct activation of c-Raf induces the transcriptional changes, but not the morphological changes, associated with the hypertrophic phenotype [8], as does transfection with constitutively active MEK and, in combination with wild-type ERK, the kinases immediately downstream of Raf [6].

Since oncogenic Ras is reported to induce both transcriptional and morphological changes [2], this implies that additional Ras effectors must be involved for the full hypertrophic response. One additional effector is MEKK, which has been suggested to mediate ANF gene expression following Ras activation by PE in cardiac myocytes [15]. Herein we have provided evidence for a role for two other Ras effectors, Ral.GDS and PI-3-K, in the regulation of cardiac gene expression.

There are two components to the transcriptional/translational responses to oncogenic Ras in cardiac myocytes. The first is a generalized increase in the transcription/translation of all genes, which presumably results from an increase in the quantity of the transcriptional and/or translational machinery or the efficiency with which they operate. This response also underpins the general anabolic effects of growth factors for which activation of Ras is a key early step. In addition to this general effect, oncogenic Ras also selectively activates genes associated with the hypertrophic phenotype in cardiac myocytes ([2,33] and herein). These specific effects result from the activation of particular combinations of transcription factors which bind to the cognate *cis*-elements in the promoters of these 'hypertrophic' genes. In the current experiments, ANF, SkM-α-actin and c-*fos*-SRE induction was greatest with V12HRas mutants which selectively activated Ral.GDS and Raf. Stimulation of transcription through Raf involves activation of ERKs and phosphorylation of transcription factors such as Elk-1 and Sap1A [34,35]. For Ral.GDS, the events subsequent to its activation by Ras are much less clearly defined. Ral.GDS stimulates GTP/GDP exchange on Ral A and Ral B, which are small G-proteins that share 58% homology with Ras. Ral A has been shown to bind to phospholipase D in NIH 3T3 cells [36] and Ral binding protein-1 (RalBP1), an effector protein of Ral, exhibits guanine nucleotide exchange activity for cdc42 and Rac [37–39]. Hence, transmission of the effects of Ras through Ral.GDS may involve regulation of cdc42, Rac or phospholipase D.

For the V12HRasY40C mutant, activation of ANF, SkM-α-actin and c-*fos*-SRE was similar in magnitude to activation of the co-transfected β-gal, suggesting that Ras signalling through PI-3-K exerts a predominantly general effect on gene expression. Such a conclusion is re-inforced by the observation that transfection with a constitutively active PI-3-K mutant also stimulated β-gal expression to a similar extent as ANF, SkM-α-actin and c-*fos*-SRE. The mechanism through which activation of PI-3-K induces a general increase in gene expression is also unknown, but may involve effects on either transcription or translation. Downstream targets of PI-3-K of potential significance are $p70^{S6K}$ [40], which may exert an effect on protein synthesis, protein kinase C ζ [41], which is able to induce ANF expression when a mutationally activated form is transfected into myocytes [42], protein kinase B (also called Akt) [43–45] and the rac-c-*Jun* N-terminal kinase (JNK) signalling pathway which has recently been shown to be involved in PI-3-K-induced process outgrowth in rat PC12 cells [46]. The current observations are potentially significant, because in some studies oncogenic Ras exhibits only a global effect on transcription in myocytes [14,22], whereas in others specific effects are also observed ([2,33] and herein). Whether or not specific effects are observed may depend on the extent to which different Ras effectors are activated under different experimental conditions. Oncogenic Ras with an effector domain substitution (P34R) which abolished GAP binding produced only a general effect in cardiac myocytes and led to the proposal for a role for Ras.GAP in the stimulation of global gene expression [14]. Hence, if Ras.GAP or PI-3-K are the preferred effectors then a global effect may predominate, whereas specific transcriptional effects may be observed if Raf or Ral.GDS are affected most. Another possibility is that global gene expression might require the co-operative activation of many or all of the pathways emanating from Ras. In support of this, V12HRas had a much greater effect than any of the dual mutants on the upregulation of the nominally constitutively active CMV–β-gal vector.

The growing list of effectors through which Ras can transduce signals in cardiac myocytes poses the question as to which may be the most significant. We [4–7] and others [8–10] have proposed a role for activation of the ERK cascade in the hypertrophic response. This is based on the observations that hypertrophic agents activate ERKs in myocytes [4,5] and that overexpression of constitutively activated MEK1 in combination with wild-type ERK2 stimulates a hypertrophic pattern of gene expression [6]. However, not all agents that activate ERKs are hypertrophic [12,13], suggesting that other signalling pathways may be equally or more important. The recent demonstration that the MEKK-JNK pathway is stimulated by Ras activation in myocytes [15] and that overexpression of constitutively active MEKK elicits a hypertrophic phenotype [15,30] is indicative of a role for this parallel MAPK cascade. This conclusion is reinforced by the observations that cardiac hypertrophy is induced by MKK7, a specific activator of JNKs in ventricular myocytes [47], and that the JNK cascade is activated in the hearts of transgenic mice overexpressing oncogenic Ras [15]. In all likelihood, activation of several downstream Ras effectors may be required to elicit a maximum hypertrophic response. From our present results it would appear that simultaneous activation of both Raf and Ral.GDS is sufficient to elicit the full transcriptional response of V12HRas (Figure 2). In this regard, it is noteworthy that these same two Ras effectors have recently been demonstrated to co-operate in the formation of foci and in the morphological transformation of NIH 3T3 cells [17]. However, co-operation between Raf and PI-3-K and between Ral.GDS and PI-3-K has also been shown to be effective in transforming NIH 3T3 cells [19].

Each of the Ras effector mutants was able to induce an increase in myocyte size, but the full hypertrophic effect of V12HRas was only recapitulated in the presence of all three mutants. The E37G and D38E mutants were more effective than Y40C in increasing cell size, in parallel with their respective effects on transcription. It has previously been reported that c-Raf kinase activity is insufficient alone to initiate the morphological changes (increased cell size and stimulation of myofibrillogenesis) associated with cardiac hypertrophy [8]. Recent evidence suggests that Rho A, a small G-protein which is involved in the organization of the actin cytoskeleton in many cell types, acts synergistically in parallel with Ras to promote a hypertrophic response in cardiac myocytes [48,49]. In agreement with this, although oncogenic Ras has been reported to induce organization of the contractile apparatus when introduced into myocytes by microinjection [2] or by viral infection [49], we have consistently failed to see any such effect when a plasmid encoding oncogenic Ras is transfected alone into these cells (S. J. Fuller, J. Gillespie-Brown and P. H. Sugden, unpublished work). On balance, the major morphological response to activated Ras alone appears to be an increase in cell size and, from the present studies, most likely requires activation of more than one downstream effector for a maximum response.

In summary, these studies have shown that Ral.GDS and PI-3-K should be added to the list of Ras effector molecules, which already includes c-Raf [8], Ras.GAP [14] and MEKK [15], which may be involved in mediating the hypertrophic response. It is likely that the different morphological and transcriptional aspects of the hypertrophic response will depend on these various

effector molecules to different extents. The challenge will be to understand which combinations subserve individual functions.

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