Induction of the cholesterol metabolic pathway regulates the farnesylation of RAS in embryonic chick heart cells: a new role for Ras in regulating the expression of muscarinic receptors and G proteins

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We propose a novel mechanism for the regulation of the processing of Ras and demonstrate a new function for Ras in regulating the expression of cardiac autonomic receptors and their associated G proteins. We have demonstrated previously that induction of endogenous cholesterol synthesis in cultured cardiac myocytes resulted in a coordinated increase in expression of muscarinic receptors, the G protein α -subunit, G- α_{i2} , and the inward rectifying K⁺ channel, GIRK1. These changes in gene expression were associated with a marked increase in the response of heart cells to parasympathetic stimulation. In this study, we demonstrate that the induction of the cholesterol metabolic pathway regulates Ras processing and that Ras regulates expression of G- α_{i2} . We show that in primary cultured myocytes most of the RAS is localized to the cytoplasm in an unfarnesylated form. Induction of the cholesterol metabolic pathway results in increased farnesylation and membrane association of RAS. Studies of Ras mutants expressed in cultured heart cells demonstrate that activation of Ras by induction of the cholesterol metabolic pathway results in increased expression of G- α_{i2} mRNA. Hence farnesylation of Ras is a regulatable process that plays a novel role in the control of second messenger pathways.

Keywords: cholesterol/farnesylation/G protein/heart cells/Ras

Introduction

The relationship between the availability of low density lipoprotein (LDL) cholesterol and the control of cholesterol biosynthesis has been well established (Brown and Goldstein, 1980). We have demonstrated previously that induction of endogenous cholesterol synthesis in embryonic chick heart cells by culture in medium supplemented with lipoprotein-depleted serum (LPDS) resulted in a 40% increase in total cell cholesterol (Haigh *et al.*, 1988). This change in cholesterol was associated with profound effects on cardiac physiology and gene expression. Specifically, induction of the cholesterol metabolic pathway was associated with a 10-fold increase in the

efficacy of the muscarinic cholinergic agonist carbamylcholine in decreasing the rate of myocyte contraction and a reciprocal decrease in the ability of the β -adrenergic agonist isoproterenol to increase the force of contraction (Haigh *et al.*, 1988; Barnett *et al.*, 1989).

The increased responsiveness to carbamylcholine seen in heart cells following induction of cholesterol biosynthesis was associated with an increase in expression of components of the parasympathetic pathway, i.e. high affinity muscarinic receptors and the G protein α -subunit G- α_{i2} (Haigh *et al.*, 1988), and an increase in levels of mRNAs coding for the M₂ muscarinic receptor, G- α_{i2} and the G protein-coupled inward rectifying K⁺ channel, GIRK1 (Gadbut *et al.*, 1994). The decreased responsiveness to isoproterenol following induction of cholesterol biosynthesis was associated with a coordinated decrease in components of the sympathetic response system, i.e. a decrease in β -adrenergic receptors and the G protein α -subunit G- α_s (Barnett *et al.* 1989). Mevinolin, a highly specific inhibitor of HMG CoA reductase (Figure 1), reversed all the effects of induction of endogenous cholesterol biosynthesis on cardiac autonomic physiology and receptor, G protein and K⁺ channel expression (Haigh *et al.*, 1988; Barnett *et al.*, 1989; Gadbut *et al.*, 1994). These findings suggested the existence of a program of gene expression in which components of second messenger pathways are regulated by sterol metabolism. The experiments outlined in this study attempt to determine the mechanism by which regulation of the cholesterol metabolic pathway regulates expression of these genes.

Farnesylpyrophosphate (FPP) is a cholesterol precursor as well as an intermediate in four other isoprenoid-dependent metabolic pathways. One of these FPP-dependent pathways is responsible for the post-translational modification and membrane localization of proteins such as Ras, Rac1 and nuclear lamin A and B (Figure 1; Hancock *et al.*, 1989; for review, see Clarke, 1992). The requirement of FPP for the processing of these proteins has established a new link between cholesterol metabolism and the activity of these molecules.

The farnesylation of Ras is catalyzed by farnesyltransferase (FPTase), a cytoplasmic heterodimer composed of a 48 kDa α -subunit and a 46 kDa β -subunit (Reiss *et al.*, 1991). FPTase catalyzes the transfer of the 15 carbon farnesyl moiety of FPP to the carboxy-terminal cysteine of proteins expressing a CAAX motif, where C is cysteine, A is an aliphatic amino acid and X is either serine, glutamine or methionine. Proteins ending in CAAX, where X is leucine, are substrates for another enzyme, geranyl-geranyl transferase, which catalyzes the covalent linkage of a 20 carbon geranyl-geranyl moiety (Figure 1) to the carboxy-terminal cysteine (for review, see Zhang and Casey, 1996). Following farnesylation of Ras, the

Fig. 1. Cholesterol metabolic pathway. The schematic representation of the cholesterol biosynthetic pathway includes a number of cholesterol by-products including dolichol and ubiquinone. Note the sites of action of mevinolin, BZA and TMD.

terminal tripeptide is cleaved and the resulting carboxy-terminal cysteine is carboxymethylated (Gutierrez *et al.*, 1989). The farnesylated form of Ras serves as substrate for palmitoyl-thiotransferase, which catalyzes the binding of palmitic acid via a relatively labile thioester linkage to cysteines at positions 181 and 184 in H-Ras (Hancock *et al.*, 1989). Although farnesylation is necessary for the association of Ras with the membrane, palmitoylation is required for efficient membrane binding (Casey *et al.*, 1989; Gutierrez *et al.*, 1989; Dudler and Gelb, 1996). Palmitoylation, which takes place at the membrane, has been shown to be a rapidly reversible and regulatable process (Milligan *et al.*, 1995; Wedegaertner *et al.*, 1996). Until now, farnesylation of Ras, which involves the formation of a stable thioether linkage and takes place in the cytoplasm, has been presumed to rapidly follow protein synthesis and to be essentially complete and irreversible, followed by rapid association of Ras with the membrane. These observations supported the conclusion that farnesylation is not a regulatable process (Kohl *et al.*, 1993). However, most studies of Ras processing have been carried out in rapidly dividing transformed cells. Recently, Agarwal *et al.* (1993) demonstrated that in non-transformed murine epidermal cells, Ras may be found in the cytoplasm. If the processing of a cytoplasmic Ras is limiting in non-transformed cells, regulation of cholesterol metabolism might play a role in controlling the farnesylation of cytoplasmic Ras and its localization to the membrane.

In this study, we will demonstrate that in a primary cultured cardiac myocyte, a large fraction of Ras is found unprocessed in the cytoplasm. The data further suggest that farnesylation and membrane association of Ras are

regulatable under the control of the cholesterol biosynthetic pathway. We will demonstrate further that regulation of Ras processing represents a novel mechanism for the control of expression of genes coding for proteins involved in the modulation of the parasympathetic response of the heart.

Results

Determination of the mevalonate-dependent pathway responsible for increased expression of mRNAs coding for M_2 and $G\alpha_{i2}$ following induction of cholesterol synthesis

To determine whether expression of mRNAs coding for M_2 muscarinic receptors and $G\alpha_{i2}$ was coordinately regulated by induction of the cholesterol metabolic pathway, total RNA from atrial myocytes cultured in medium supplemented with fetal calf serum (FCS) or LPDS was analyzed by RNase protection. Growth with LPDS resulted in a coordinated increase in levels of mRNAs coding for both M_2 and $G\alpha_{i2}$, compared with those in cells cultured in FCS (Figure 2a, lanes 2 and 3). Mevinolin, an inhibitor of HMG CoA reductase, reversed the increase in levels of mRNA coding for M_2 and $G\alpha_{i2}$ due to growth with LPDS to levels below those seen in cells grown in medium supplemented by FCS (Figure 2a, lane 4). These data are typical of five other experiments.

The cholesterol metabolic pathway gives rise to FPP, which serves as a precursor to cholesterol and at least four other products (Clarke, 1992; Figure 1). Since mevinolin inhibits the synthesis of cholesterol at the level of mevalonate, it does not differentiate between any of the FPP-dependent pathways. To determine which of the FPP-dependent pathways was responsible for the increased expression of mRNAs coding for M_2 and $G\alpha_{i2}$, atrial myocytes were cultured in the presence of LPDS plus inhibitors of two of these pathways: TMD, an inhibitor of 2,3 oxido-squalene cyclase (Chang *et al.*, 1979), which catalyzes the conversion of squalene to lanosterol; and BZA-5B, a specific inhibitor of FPTase (James *et al.*, 1993), the enzyme which catalyzes the covalent binding of a farnesyl moiety to cellular proteins ending in the CAAX motif (Figure 1).

TMD had no effect on levels of mRNAs coding for M_2 and $G\alpha_{i2}$ in cells cultured with LPDS (Figure 2a, column 5). This suggested that either the cholesterol branch of the pathway was not involved in regulating M_2 and $G\alpha_{i2}$ mRNA expression, or that TMD was incapable of inhibiting 2,3 oxido-squalene cyclase in these cells. However, while growth of cells in LPDS resulted in at least a 30% increase in total cell cholesterol compared with cells grown with FCS (Figure 2b, bars 1 and 2), TMD decreased cholesterol by 70% in these cells (Figure 2b, bars 3 and 4). Hence the increase in levels of mRNAs coding for M_2 and $G\alpha_{i2}$ following induction of the cholesterol metabolic pathway was not dependent on the level of cellular cholesterol.

In cells grown in the presence of LPDS plus 50 μ M BZA-5B, levels of mRNAs coding for M_2 and $G\alpha_{i2}$ decreased below those seen in control cells (Figure 2a, lane 6). These data suggested that a farnesylated protein was critical for the increased expression of $G\alpha_{i2}$ and

Fig. 2. (a) Effects of growth in medium supplemented with LPDS, LPDS + BZA-5B or LPDS + TMD on levels of mRNA coding for M₂ and G-α_{i2}. Atrial cells from hearts 14 days *in ovo* were cultured for 3 days in medium supplemented with FCS (lane 2), LPDS (lane 3), LPDS + 30 μM mevillin (lane 4), LPDS + 50 μM TMD (lane 5) or LPDS + 50 μM BZA-5B (lane 6), added on the second day of culture. Antisense riboprobes were either undigested (lane 1) or hybridized to total RNA from cultures (lanes 2–6) or to tRNA (lane 7). In samples hybridized to M₂ and G-α_{i2} riboprobes, 15 μg of total RNA were used. The GAPDH riboprobe was hybridized with 10 μg of total RNA. Radiographic exposure time for M₂ and G-α_{i2} was 3 days and exposure time for GAPDH was 6 h. The intensity of the bands protected by the antisense riboprobe to GAPDH was identical for cells grown in FCS and LPDS, indicating equal loading of RNA.

(b) Effects of TMD on levels of total cell cholesterol in cells grown in FCS and LPDS. Atrial cells were incubated in medium supplemented with FCS (bar 1), LPDS (bar 2). LPDS + 2 μg/ml TMD (bar 3) or LPDS + 500 ng/ml TMD (bar 4). TMD was added on the second day of culture. Levels of total cell cholesterol were determined by the method of Heider and Boyett (1978) on cells harvested on the third day of culture. Data are typical of two studies.

M₂ mRNAs in response to induction of the cholesterol metabolic pathway.

Effect of induction of the cholesterol metabolic pathway on post-translational modification and cellular distribution of Ras

Of those proteins which require farnesylation for their association with the membrane, we reasoned that Ras was the protein most likely to play a role in the regulation of second messenger pathways. Since farnesylation of Ras has been shown to be necessary for both translocation of Ras to the cell membrane and for Ras activity (Hancock *et al.*, 1989; Lowy and Willumsen, 1993), we further reasoned that if BZA-5B decreased the expression of M₂ and G-α_{i2} mRNAs via inhibition of FPTase, then induction of the cholesterol metabolic pathway might increase the expression of M₂ and G-α_{i2} mRNA via an increase in the farnesylation of Ras. To test this hypothesis, we first determined the effect of stimulation of the cholesterol pathway on the processing and localization of Ras.

Analysis of the distribution of Ras between cytoplasmic and membrane fractions demonstrated that most of the Ras from cells cultured in FCS was found in the cytoplasm and migrated as a doublet on polyacrylamide gel electrophoresis (PAGE) (Figure 3a, lane A). The small amount of Ras found in the membrane fraction (Figure 3a, lane B) migrated faster than that in the cytoplasm and also often migrated as a doublet. Since Ras antibody Y-238 used in these studies cross-reacts with both H- and K-Ras (Shen *et al.*, 1987), the cytoplasmic doublet in Figure 3a could represent unfarnesylated H- and K-Ras. Several alternative explanations are possible, however, Western blot analysis of cytoplasmic extracts with commercially available K-Ras (B) antibody did not give interpretable results. It has been demonstrated that on PAGE, farnesylated Ras migrated ahead of unprocessed Ras. Hence, the doublet could represent processed and unprocessed Ras. Furthermore, partial palmitoylation of cytoplasmic Ras might also lead to a doublet on PAGE. Finally, several studies have suggested that treatment of cells with phorbol esters might lead to the generation of a phosphorylated Ras, which migrated behind unphosphorylated Ras (Ballester *et al.*, 1987; Jeng *et al.*, 1987).

In cells cultured in LPDS, membrane-associated Ras was markedly increased compared with that in membranes from cells cultured in FCS (Figure 3a, compare lanes B and D). One interpretation of these findings is that in cultured chick atrial cells, unprocessed Ras accumulates in the cytoplasm and the induction of the cholesterol metabolic pathway results in increased farnesylation and membrane association of cytoplasmic Ras. If such a mechanism for the regulation of Ras localization did exist, then cytoplasmic Ras must be unprocessed and farnesylatable, and membrane-associated Ras must be farnesylated.

Processing of cytoplasmic and membrane-associated Ras

If membrane-associated Ras in chick atrial cells is farnesylated, then inhibition of FPTase by BZA-5B should result in a decrease in the Ras localized to the membrane. Incubation of cells in LPDS plus 50 μM BZA-5B resulted in the almost complete disappearance of membrane-associ-

Fig. 3. (a) Effects of LPDS on the farnesylation and localization of Ras. Atrial cells were cultured for 2 days in medium supplemented with either FCS (lanes A and B), LPDS (lanes C and D) or LPDS + 50 μ M BZA-5B (lanes E and F). On the second day of culture, cells were metabolically labeled with [³⁵S]methionine for 16 h. On the third day, cells were homogenized, and membrane and cytoplasmic fractions prepared as described in Materials and methods. Extracts were immunoprecipitated with monoclonal antibody Y-238 specific for H-Ras, but which also cross-reacted with K-Ras. As a control, homogenates were incubated with an equal volume of DMEM supplemented with 6% FCS (data not shown). Samples were analyzed by PAGE followed by autoradiography. Between 21 and 35 kDa, only two bands were precipitated specifically by Y-238. This gel is representative of five separate experiments. Lanes A, C and E are from the cytoplasmic fraction; lanes B, D and F are from the membrane fraction. **(b)** *In vivo* labeling of Ras by [³H]mevalonic acid. Embryonic chick atrial cells 14 days *in ovo* were cultured for 2 days in medium supplemented with FCS. On the second day of culture, medium was made 0.9 mCi/ml in [³H]mevalonate. On the third day, cells were washed, and cytoplasm and membrane were prepared followed by immunoprecipitation, PAGE and autoradiography as described in Materials and methods. Lane A, cytoplasm; lane B, membrane. Data are typical of three experiments.

ated Ras and a marked increase in cytoplasmic Ras (Figure 3a, lanes E and F). The Ras in the cytoplasm of BZA-5B-treated cells also migrated as a doublet. However, these bands appeared to migrate more slowly than those in control cells and in cells incubated with LPDS (Figure 3a, compare lane E with lanes A and C).

Fig. 4. (a) *In vitro* farnesylation of atrial cell cytoplasmic Ras. Cytoplasm was prepared from atrial cells 14 days *in ovo* cultured in medium supplemented with FCS. Cytoplasm was dialyzed and concentrated on a Centriprep-10 microconcentrator (Amicon, Beverly, MA) and 88 μ g of cytoplasmic protein from heart cell or NIH-3T3 cell cytoplasm were incubated with [³H]FPP (22 Ci/mM, Dupont NEN) and purified FPTase for 30 min at 37°C, followed by PAGE and autoradiography. Lane A, 25 ng of purified Ras; lane B, chick atrial cytoplasm; lane C, 3T3 cell cytoplasm. **(b)** Distribution of [³H]palmitate-labeled Ras between cytoplasm and membrane. Embryonic chick atrial cells were incubated in medium supplemented with FCS. On the second day of culture, medium was made 0.5 mCi/ml in [³H]palmitate. On the third day, cells were harvested, membrane and cytoplasm prepared and Ras immunoprecipitated followed by PAGE and autoradiography. Lane A, cytoplasm; lane B, membrane fraction. Data are typical of three similar experiments.

Cytoplasmic Ras in cultured atrial cells is unprocessed and farnesylatable

The finding that inhibition of FPTase with BZA-5B did not decrease the level of cytoplasmic Ras, but rather caused it to increase (Figure 3a, lanes E and F), supports the conclusion that cytoplasmic Ras is unfarnesylated. To test directly the hypothesis that cytoplasmic Ras is unfarnesylated, cells were incubated for 16 h in FCS plus 0.9 mCi/ml of 5' [³H]mevalonate. Fractionation of cells into membrane and cytoplasm followed by immunoprecipitation with antibody Y-238 demonstrated that >95% of the [³H]mevalonate incorporated into Ras was found in the membrane fraction (Figure 3b). Furthermore, in cells labeled with [³H]mevalonate and incubated in LPDS plus 50 μ M BZA-5B, no [³H]mevalonate was incorporated into cytoplasmic Ras (data not shown).

To determine further whether Ras accumulated in the cytoplasm of cultured atrial myocytes in an unprocessed and farnesylatable form, we studied the ability of cytoplasmic Ras to serve as a substrate for FPTase. Equal amounts of cytoplasmic protein from cultured atrial cells or NIH-3T3 cells were incubated with purified FPTase and [³H]FPP followed by PAGE and autoradiography (Figure 4a). Atrial cell cytoplasmic extracts demonstrated a radiolabeled band which migrated in parallel with

purified Ras radiolabeled with [^3H]FPP (Figure 4a, lanes A and B). No Ras band was detected in NIH-3T3 cell cytoplasm (Figure 4a, lane C), a cell in which Ras is in high abundance and almost fully farnesylated (Kohl *et al.*, 1993). Since FPTase might catalyze the farnesylation of other low molecular weight proteins, the Ras from [^3H]FPP-labeled cytoplasm was immunoprecipitated with antibody Y-238 followed by PAGE and autoradiography. Radiolabeled bands migrated as expected for Ras (data not shown). A farnesylatable Ras could also be detected in the cytoplasm of atrial cells cultured in medium supplemented with LPDS (data not shown). Although it was not possible to quantitate the fraction of total atrial cytoplasmic Ras which was farnesylatable, these data demonstrate that a significant fraction of cytoplasmic Ras in cultured atrial cells was in the native unfarnesylated form.

Cytoplasmic Ras is neither palmitoylated nor phosphorylated

The absence of [^3H]mevalonate incorporation into cytoplasmic Ras strongly supports the conclusion that the cytoplasmic Ras doublet demonstrated in Figure 3a does not represent a mixture of farnesylated and unfarnesylated Ras or processed Ras which had become depalmitoylated and cycled back to the cytoplasm. To rule out further the possibility that cytoplasmic Ras represents a partially processed form of Ras, a further series of experiments was carried out.

Cultured atrial cells were incubated overnight with [^3H]palmitate, and incorporation into cytoplasmic and membrane-associated Ras was determined. Data summarized in Figure 4b demonstrate that all of the palmitate incorporated into Ras was found in the membrane fraction. To rule out the possibility that the cytoplasmic doublet represents phosphorylated and unphosphorylated Ras, cells were metabolically labeled with either [^{35}S]methionine or $^{32}\text{PO}_4$, followed by immunoprecipitation with antibody Y-238. Although membrane and cytoplasmic Ras were labeled with [^{35}S]methionine, no phosphorylated Ras could be detected in either the cytoplasmic or membrane fraction (data not shown). The finding that cytoplasmic Ras in BZA-5B-treated cells appeared to migrate more slowly than Ras from the cytoplasm of control cells or cells incubated with LPDS (Figure 3a, lane E) suggested that BZA-5B treatment might result in the accumulation of a processed form of Ras in the cytoplasm. As described above, no [^3H]mevalonate-labeled Ras could be detected in the cytoplasm of cells incubated in LPDS plus BZA-5B. Furthermore, incubation of cells with BZA-5B and [^3H]palmitate or $^{32}\text{PO}_4$ did not result in the appearance of either farnesylated, palmitoylated or phosphorylated Ras in the cytoplasm (data not shown). Thus, the shift in electrophoretic mobility in Ras from the cytoplasm of BZA-5B-treated cells is not due to any known modification of Ras.

These data support the conclusion that most of the Ras in atrial cytoplasm represents two species which are neither farnesylated, palmitoylated nor phosphorylated. At least a portion of this cytoplasmic Ras may serve as a substrate for FPTase. The finding that no farnesylatable Ras could be found in the cytoplasm of rapidly dividing NIH-3T3 cells suggested that unfarnesylated Ras might

Fig. 5. Effect of LPDS and Ras mutants on G- α_{i2} promoter activity. Embryonic chick atrial cells were co-transfected with α_{i2} -Luc and a human placental alkaline phosphatase (PAP) with or without Val12 Ras or Asn17 Ras. On the third day of culture, cells were harvested and luciferase activity was determined and normalized to human PAP activity. Data are reported as the ratio of luciferase activity to PAP with cells cultured in FCS taken as 1. Bars: 1, cells cultured in FCS; 2, cells cultured in LPDS; 3, cells cultured in FCS and transfected with Val12 Ras and inhibited with 50 μM BZA-5B added 16 h before harvesting; 4, cells cultured in FCS and transfected with Val12 Ras; 5, cells cultured with LPDS and transfected with Asn17 Ras.

not be found in rapidly dividing cells. The finding that Ras accumulated in an unfarnesylated form in the cytoplasm of primary cultured heart cells and that induction of the cholesterol metabolic pathway resulted in an increase in the level of farnesylated membrane-associated Ras supported the conclusion that the cholesterol metabolic pathway may control Ras activity by regulating the processing of cytoplasmic Ras.

Regulation of G- α_{i2} promoter activity by induction of the cholesterol metabolic pathway

Data presented in Figures 2a and 3a demonstrated a striking parallel between changes in expression of mRNAs coding for M₂ and G- α_{i2} and changes in the membrane association of Ras in response to both induction of the cholesterol metabolic pathway and to inhibition of FPTase activity by BZA-5B. To determine whether an increase in membrane-associated Ras actually played a role in the increased expression of M₂ and G- α_{i2} , the effect of LPDS and Ras mutants on G- α_{i2} promoter activity in atrial myocytes was studied.

Cultured chick atrial cells 14 days *in ovo* were transfected with a construct containing 1.8 kb of 5' flanking sequence of chick G- α_{i2} genomic DNA ligated to a luciferase reporter (α_{i2} -Luc). Growth of these cells in medium supplemented with LPDS resulted in a 2.5 ± 0.5 ($\pm$ SEM, $n = 7$) fold increase in luciferase activity compared with cells grown in the presence of FCS (Figure 5, bars 1 and 2). Luciferase activity in cells transfected with a promoterless luciferase was no different from baseline in either FCS or LPDS. Hence, induction of the cholesterol metabolic pathway increased G- α_{i2} expression at least in part by an effect on the G- α_{i2} promoter.

Effect of Ras mutants on G- α_{i2} promoter activity

In order to determine whether the stimulation of the G- α_{i2} promoter by LPDS was dependent on Ras, cells co-transfected with α_{i2} -Luc and a dominant-negative Ras mutant (Asn17 Ras, Feig and Cooper, 1988) were cultured in LPDS. Co-expression of α_{i2} -Luc plus Asn17 Ras

resulted in a decrease in luciferase activity to the levels seen in cells grown in FCS. Hence expression of Asn17 Ras reversed the effect of induction of the cholesterol metabolic pathway on stimulation of G- α_{i2} promoter activity (Figure 5, bars 1, 2 and 5). Co-expression of Asn17 Ras and α_{i2} -Luc in cells cultured in medium supplemented with FCS resulted in a 20% decrease in luciferase activity compared with cells transfected with α_{i2} -Luc alone (data not shown). These data suggest that only a fraction of basal G α_{i2} promoter activity is Ras dependent.

In order to determine whether Ras mimicked the effect of growth in LPDS on G- α_{i2} promoter-driven luciferase activity, cells were cultured in FCS and co-transfected with α_{i2} -Luc plus a dominant active Ras (Val12 Ras; Lowy and Willumsen, 1993). Expression of Val12 Ras increased luciferase activity 2.4 ± 0.3 - ($\pm$ SEM, $n = 5$) fold compared with cells transfected with α_{i2} -Luc alone (Figure 5, bar 4). Hence, Val12 Ras mimicked the effect of growth in LPDS on regulation of the G- α_{i2} promoter. Co-transfection of atrial cells cultured in medium supplemented with FCS with α_{i2} -Luc plus Val12 Ras followed by an overnight incubation with 50 μ M BZA-5B resulted in a 70% decrease in Val12 Ras-stimulated luciferase activity (Figure 5, bar 3). Although it is not possible quantitatively to compare steady-state levels of α_{i2} mRNA and transcription rates as measured by α_{i2} promoter-driven Luciferase activity, these data support the conclusion that like LPDS-stimulated α_{i2} production, the stimulation of α_{i2} -Luc in response to Val12 Ras was also BZA-5B inhibitable and hence dependent on FPTase activity and hence must require membrane association of the mutant Ras. Co-transfection of cells cultured in LPDS with both Val12 Ras and α_{i2} -Luc did not increase luciferase activity significantly above that in cells cultured with LPDS and transfected with α_{i2} -Luc alone (data not shown). Since the effects of LPDS and Ras were not additive, this further supported the conclusion that both LPDS and Ras acted via the same pathway.

Effect of Val12 Ras on expression of G- α_{i2} in atrial myocytes

In order to determine directly whether Ras played a role in regulating the expression of mRNA coding for G- α_{i2} , we expressed an activated mutant of Ras (Val12 Ras) in cultured atrial cells and determined its effect on expression of G- α_{i2} . Since transfection efficiency, as measured by co-transfection of cells with α_{i2} -Luc and a β -gal SV40 construct, was <5%, we used an avian retrovirus containing the Val12 Ras to express this mutant in cultured atrial cells. Staining for the viral specific Gag protein in control cells and cells infected with RCAS virus demonstrated that ~66% of total cells stained for Gag (Figure 6a).

When atrial cells cultured in FCS were infected with either control replication competent avian sarcoma (RCAS) virus or RCAS virus expressing Val12 Ras, Western blot analysis demonstrated a 3-fold increase in G- α_{i2} expression in cells infected with RCAS virus expressing Val12 (Figure 6b). Furthermore, infection with control virus had no effect on levels of G- α_{i2} compared with uninfected heart cells grown in medium supplemented with FCS (data not shown).

In order to determine whether levels of mRNA coding for G- α_{i2} were also increased by an activating Ras mutant, levels of G- α_{i2} mRNA were compared in cells infected with native RCAS virus or with RCAS virus expressing Val12 Ras. RNase protection studies demonstrated that expression of Val12 Ras markedly increased levels of mRNA coding for G- α_{i2} (Figure 6c). Although primary heart cell cultures are mixtures of myocytes and fibroblasts, and the RCAS virus infects both cell types, our cultures contained <4% fibroblasts at the time of plating. Hence, fibroblast contamination would have a minor effect on Western blot or RNase protection data.

These data demonstrate that an activating Ras mutant (Val12 Ras) mimicked the effect of growth in LPDS on G- α_{i2} expression (see Figure 2, lanes 2 and 3). A dominant-negative Ras mutant reversed the effect of induction of the cholesterol metabolic pathway on G- α_{i2} promoter activity. Taken together, these data suggest that an increase in Ras activity is both necessary and sufficient for the increased expression of G- α_{i2} in response to induction of the cholesterol metabolic pathway.

Discussion

These data demonstrate a new link between cholesterol metabolism, Ras processing and the regulation of genes which play an important role in signal transduction. The data suggest that in primary cultured heart cells, a significant pool of unfarnesylated Ras is found in the cytoplasm. The data further demonstrate that induction of the cholesterol metabolic pathway results in a dramatic increase in farnesylated and membrane-associated Ras. Since Ras activity requires association with the membrane, these data suggest that the regulation of Ras processing plays an important role in the control of Ras activity. Finally, the data support the conclusion that increased Ras activity following induction of the cholesterol metabolic pathway regulates the expression of G- α_{i2} and other proteins which play a critical role in the control of signal transduction and cardiac excitability.

Unprocessed Ras accumulates in the cytoplasm of atrial myocytes

A series of elegant studies have demonstrated that the post-translational modification of Ras involves four sequential steps: the farnesylation of the carboxy-terminal cysteine (Hancock *et al.*, 1989), proteolysis of the three carboxy-terminal amino acids (Gutierrez *et al.*, 1989), carboxy-methylation of the C-terminal cysteine (Clarke *et al.*, 1988) and palmitoylation of Cys181 and Cys184 in H-Ras and Cys181 in K-Ras 4A (Buss and Sefton, 1986). Farnesylation alone has been shown to be insufficient for association of Ras with the membrane (Hancock *et al.*, 1989). Thus for H- and K- (A) Ras, palmitoylation appears to be required for efficient membrane association and physiologic functions such as meiotic maturation in *Xenopus* oocytes (Dudler and Gelb, 1996) while K-Ras 4B does not require palmitoylation, but does contain a polylysine region which enhances Ras association with the membrane.

In transformed cells, nearly all of the Ras is membrane associated (Sinensky *et al.*, 1990; Kohl *et al.*, 1993). The accumulation of Ras in the cytoplasm of primary cultured

Fig. 6. Infection of chick atrial cells with RCAS containing Val12 Ras. **(a)** Immunofluorescence studies of chick atrial cells infected with RCAS virus. Atrial cells were cultured as described in Materials and methods. At the time of plating, cells were infected with an avian retrovirus, RCAS; 4.8×10^6 heart cells were mixed with 5.0×10^6 infectious RCAS particles (Morgan and Fekete, 1996). Following 3 days in culture, cells were fixed and stained for the viral-specific Gag protein. (i), atrial myocytes infected with RCAS virus; (ii), uninfected cells. Approximately 66% of total cells are stained for Gag protein. **(b)** Western blot analysis of G- α_{i2} in cells infected with Val12 Ras RCAS. Atrial cells were cultured as described in Materials and methods. At the time of plating, cells were mixed at a 1:1 ratio with either RCAS virus or RCAS virus containing the coding region for Val12 Ras. After 3 days in culture, cells were washed and proteins prepared as described in Materials and methods. Relative levels of G- α_{i2} were determined by Western blot analysis. Lane 1, G- α_{i2} expression in cells infected with control RCAS; lane 2, G- α_{i2} expression in cells infected with Val12 Ras RCAS virus. **(c)** Effect of infection of atrial cells 14 days *in ovo* with Val12 Ras RCAS virus on levels of mRNA coding for G- α_{i2} . Atrial cells were infected with either RCAS virus or RCAS virus containing the coding region for Val12 Ras. After 3 days in culture, cells were washed, total RNA prepared and relative levels of G- α_{i2} were determined by RNase protection. Lane 1, undigested probe; lane 2, RNA from cells infected with RCAS alone; lane 3, RNA from cells infected with RCAS + Val12 Ras; lane 4, tRNA control.

atrial myocytes could reflect the fact that in non-dividing cells one or more of the four steps in the processing of Ras has become rate limiting. Accumulation of unfarnesylated Ras in the cytoplasm would suggest that the FPTase reaction is limiting, while the accumulation of farnesylated Ras in the cytoplasm would suggest that one or more of the steps following farnesylation is limiting. Data presented here support the conclusion that most of the cytoplasmic Ras is unfarnesylated. Specifically, it was demonstrated that cytoplasmic Ras was capable of serving as a substrate for purified FPTase. Since only unprocessed Ras will serve as a substrate for FPTase, these data suggest that at least a fraction of total cytoplasmic Ras must be in the native unprocessed form.

The finding that [^{3} H]mevalonate was incorporated exclusively into membrane-associated Ras further supported the conclusion that cytoplasmic Ras is unprocessed. Furthermore, inhibition of FPTase with BZA-5B resulted in the disappearance of membrane-associated Ras and an increase in cytoplasmic Ras, consistent with the conclusion that cytoplasmic Ras is not farnesylated. Finally, the

finding that only membrane-associated Ras was labeled with [^{3} H]palmitate ruled out the possibility that cytoplasmic Ras was partially palmitoylated.

In chick atrial cells, cytoplasmic Ras usually migrated as a doublet on PAGE. Experiments cited above rule out partial farnesylation or partial palmitoylation as the source of this doublet. Several studies have suggested that activation of protein kinase C may result in the phosphorylation of Ras at a serine site in either K-Ras *in vivo* or H-Ras *in vitro* (Ballester *et al.*, 1987; Jeng *et al.*, 1987; Saikumar *et al.*, 1988). Hence, another possible explanation for the cytoplasmic doublet is that it represents phosphorylated and unphosphorylated Ras. However, metabolic labeling of cells with $^{32}\text{PO}_4$ followed by immunoprecipitation with antibody Y-238 did not demonstrate a phosphorylated species of Ras in the cytoplasm. In cells cultured in LPDS plus BZA-5B, nearly all of the Ras appeared in the cytoplasm as a doublet. Comparison between the doublet from the cytoplasm of BZA-5B-treated cells and the cytoplasm of control and LPDS-treated cells suggested that the doublet from the cytoplasm of BZA-5B-treated

cells migrated differently. However, this band was neither farnesylated, palmitoylated nor phosphorylated. The difference in migration could be due to the markedly higher levels of these forms of Ras in the BZA-5B-treated cytoplasm or the less likely possibility that treatment with BZA-5B results in a unique previously undescribed modification of Ras. Since antibody Y-238 used for immunoprecipitation in these studies cross-reacts with H- and K-Ras, one interpretation of this doublet is that it represents H- and K-Ras. Immunoblotting analysis with commercially available K-Ras antibody did not result in an interpretable signal in chick atrial extracts. However, this could be due to lack of cross-reactivity with chick K-Ras.

Mechanisms by which induction of the cholesterol metabolic pathway might regulate farnesylation of Ras

Several mechanisms might explain the accumulation of unprocessed Ras in the cytoplasm of atrial myocytes. These include a modification of Ras which prevents it from serving as a substrate for FPTase, limiting levels of FPTase activity in heart cell cultures or limiting levels of the FPTase substrate, FPP, in cultured heart cells. The finding that a portion of cytoplasmic Ras in primary cultures of myocytes is farnesylatable argues against a modification of Ras which interferes with the farnesylation reaction. However, a recent study has suggested that cellular transformation may be associated with an increase in FPTase activity. Agarwal *et al.* (1993) demonstrated that Ras is found in the cytoplasm of epidermal cells. Transformation of these cells resulted in an increase in both cytoplasmic and membrane-associated Ras and an increase in FPTase activity. Goalstone and Draznin (1996) suggested that insulin was capable of stimulating FPTase activity in 3T3-L1 adipocytes. They further suggested that this increase in FPTase activity was associated with an increase in the amount of farnesylated Ras. These studies suggested that limiting levels of FPTase activity could be responsible for the accumulation of unprocessed Ras in the cytoplasm. Hence, induction of the cholesterol metabolic pathway could increase the level of farnesylated and membrane-associated Ras by inducing an increase in FPTase activity.

The availability of the substrate FPP could also regulate the farnesylation of Ras. Bruenger and Rilling (1988) have presented data which suggest that the intracellular concentration of FPP may be as high as 1 μ M, which would be saturating for FPTase (Furfine *et al.*, 1995). However, recent data support the hypothesis that FPP might be compartmentalized within the cell (Tschantz *et al.*, 1997). These authors demonstrated that FPP was required for release of the farnesylated Ras from the enzyme. Hence, the measurement of total FPP might not reflect the concentration of FPP at its site of interaction with the farnesyl-Ras-FPTase complex. Recently we studied the level of FPP using HPLC analysis of basic hot ethanol extracts of chick atrial cells cultured in FCS and LPDS. We demonstrated a 1.5-fold increase in the level of FPP in cells cultured in LPDS compared with control cells cultured in FCS (J.A. Watson and J.B. Galper, unpublished observation). If FPP is compartmentalized in

the cell, a 1.5-fold increase in FPP concentration could result in increased FPTase activity.

Finally, the finding that farnesylated Ras does not accumulate in the cytoplasm suggests that the accumulation of Ras in the cytoplasm is not due to limiting levels of the proteolysis, carboxymethylation or palmitoylation of Ras. However, since membrane association requires farnesylation as well as all three of these subsequent steps, the data presented here do not rule out the possibility that induction of the cholesterol metabolic pathway also increases the level of proteolysis, carboxymethylation and palmitoylation of Ras.

Ras is both necessary and sufficient to mediate the effect of induction of the cholesterol metabolic pathway on G- α_{i2} expression

Although muscarinic stimulation via a pertussis toxin-sensitive G protein-coupled pathway has been shown to be associated with the activation of Ras and the tyrosine kinase cascade (Winitz *et al.*, 1993), activation of Ras had not been shown to regulate expression of either muscarinic receptors or pertussis toxin-sensitive G proteins. The close parallel between the induction of the cholesterol metabolic pathway and the increase in membrane association of Ras with the increased expression of mRNAs coding for M₂ and G- α_{i2} suggested that Ras might be involved directly in the coordinate regulation of M₂ and G- α_{i2} expression in response to changes in cholesterol metabolism.

The increased level of G- α_{i2} promoter-driven luciferase activity in response to LPDS or Val12 Ras demonstrates that in atrial cells a G- α_{i2} promoter element can be regulated by Ras and by induction of the cholesterol metabolic pathway. Furthermore, the effect of Val12 Ras on levels of G- α_{i2} protein and mRNA in the absence of LPDS suggests a direct role for Ras in the regulation of G- α_{i2} in cells which have not been perturbed by changes in cholesterol metabolism. The finding that both induction of expression of α_{i2} mRNA by growth in LPDS and the increase in α_{i2} -Luc activity in cells grown in LPDS are sensitive to inhibition by BZA-5B support the conclusion that both processes depend on FPTase activity. Moreover, Asn17 Ras, a dominant-negative Ras mutant, is capable of reversing the effects of the induction of the cholesterol pathway on G- α_{i2} promoter-driven luciferase activity, indicating that the response to LPDS requires a functional Ras. Taken together, these data demonstrate that an increase in the farnesylation of Ras in cells grown in LPDS is capable of exerting an effect on G- α_{i2} expression. Further deletional analysis of the 5' flanking region of G- α_{i2} should help identify *cis*-acting elements involved in this regulation.

Regulation of the farnesylation of Ras may constitute a unique mechanism for the regulation of a program of gene expression

The data presented here and in prior studies demonstrate that induction of the cholesterol metabolic pathway increases expression of G- α_{i2} , M₂ and GIRK1 (Haigh *et al.*, 1988; Gadbut *et al.*, 1994). Ligand-binding data and Western blot analysis have suggested that levels of β -adrenergic receptors and G- α_s are coordinately decreased by induction of the cholesterol pathway (Barnett *et al.*, 1989). The role of Ras in the regulation of G- α_s and β_1 -

adrenergic receptor expression has not yet been demonstrated. However, since HMGCoA reductase inhibitors also reverse the effects of LPDS on the expression of β -adrenergic receptors and $G\text{-}\alpha_s$, these data taken together suggest that the control of FPTase and membrane association of Ras might regulate a program of gene expression which coordinately regulates expression of receptors and their associated G proteins and reciprocally regulates receptors and G proteins which exert opposing effects on second messenger pathways.

The concept of cross-regulation of genes mediating opposing functions has been described in other systems. Many of these studies have dealt with the effects of prolonged agonist exposure on the down-regulation of receptors and G proteins which are related at least in part to changes in cAMP levels. Parsons and Stiles (1987) demonstrated that chronic administration of adenosine agonists to rats resulted in a decrease in the level of adenosine receptors and $G\text{-}\alpha_i$ and an increase in the levels of $G\text{-}\alpha_s$ in adipocytes. Hadcock *et al.* (1990) demonstrated that stimulation of S49 lymphoma cells with forskolin or isoproterenol enhanced somatostatin inhibition of adenylyl cyclase activity, increased levels of $G\text{-}\alpha_{i2}$ and decreased levels of mRNA coding for $G\text{-}\alpha_s$. This program for cross-regulation of gene expression is significantly different from that described here. Growth of chick heart cells in LPDS does not require agonist binding and does not result in a change in resting cAMP levels (Tan *et al.*, 1993). However, since muscarinic stimulation and stimulation of other receptors have been shown to enhance Ras activity directly (Winitz *et al.*, 1993), it is intriguing to speculate that the stimulation of Ras in response to hormone receptor interaction might utilize the same program of gene expression as that described for LPDS to reciprocally regulate receptor G protein-coupled systems.

Conclusions

The data presented here suggest the following mechanism. In the presence of LDL cholesterol, when the endogenous cholesterol metabolic pathway is suppressed, a large fraction of unprocessed Ras is localized to the cytoplasm. Induction of the cholesterol metabolic pathway following removal of LDL from the serum results in an increase in farnesylated membrane-associated Ras. This increase in processed Ras is both necessary and sufficient to increase expression of $G\text{-}\alpha_{i2}$ and may also increase levels of the M_2 muscarinic receptor and GIRK1. These data describe a new pathway by which induction of cholesterol biosynthesis regulates post-translational modification of proteins which in turn establishes the level of membrane-associated Ras. This constitutes a new pathway for the regulation of Ras. The finding that muscarinic receptors, $G\text{-}\alpha_{i2}$ and GIRK1 are coordinately increased while β_1 -adrenergic receptors and $G\text{-}\alpha_s$ are coordinately decreased by induction of the cholesterol metabolic pathway suggests the intriguing hypothesis that Ras may reciprocally regulate levels of receptors, G proteins and effectors involved in opposing second messenger pathways. In the heart, Ras may regulate the balance between sympathetic and parasympathetic responsiveness and hence may play a role in modulating cardiac excitability.

Materials and methods

Cell culture

Chick atrial myocyte cultures were prepared by a modification of the method of DeHaan (1967) as described previously (Gadbut *et al.*, 1994).

Preparation of lipoprotein-depleted serum (LPDS)

LPDS was prepared as described previously (Gadbut *et al.*, 1994). Briefly, FCS was overlaid with a solution of KBr with a final density of 1.21 g/ml followed by centrifugation in a Ti-70 rotor at 265 000 g for 16 h at 10°C. The resultant LPDS was dialyzed against a solution containing 0.15 M NaCl and 4 mM Na_2HPO_4 pH 7.4.

RNase protection analyses of M_2 and $G\text{-}\alpha_{i2}$

RNase protection probes for M_2 and $G\text{-}\alpha_{i2}$ have been described previously (Gadbut and Galper, 1994; Kilbourne and Galper, 1994). RNase protection was carried out as described previously (Gadbut and Galper, 1994). Briefly, total RNA was isolated from cultures of chick atrial cells 14 days *in ovo* using guanidinium CsCl_2 centrifugation as described (Sambrook *et al.*, 1990). Riboprobes were hybridized to 15 μg of total RNA from cultures of atrial cells. The samples were treated with RNase and analyzed by PAGE on 6% gels containing urea followed by autoradiography.

Immunoprecipitation of metabolically labeled Ras

Chick atrial myocytes 14 days *in ovo* were cultured on 100 mm dishes for 2 days in medium supplemented either with 6% FCS or LPDS. On the second day, cells were washed for 15 min with methionine-minus Dulbecco's modified Eagle's medium (DMEM) (Gibco) followed by incubation in 6 ml of a 1:40 mixture of fresh DMEM and fresh methionine-minus DMEM supplemented with 6% FCS or LPDS. The medium was made 94 $\mu\text{Ci}/\text{ml}$ in Trans [^{35}S]methionine (1083 Ci/mM, ICN, Irvine, CA) and, where indicated, incubated in 50 μM BZA-5B, (a gift of James Marsters, Genentech, South San Francisco, CA), an inhibitor of FPTase. Immunoprecipitation followed by PAGE was carried out as described (Qiu *et al.*, 1991) using purified monoclonal Ras p21 antibody Y-238.

Cellular localization of Ras

These experiments were carried out as described above except that three 100 mm dishes were used for each condition. Cells were metabolically labeled with [^{35}S]methionine as described above, washed three times with phosphate-buffered saline (PBS), scraped into a 5 ml tube and spun for 5 min at 1000 g at 4°C. The pellets were resuspended in 800 μl of buffer B [5 mM MgCl_2 , 10 mM HEPES pH 7.3, 0.1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 0.7 $\mu\text{g}/\text{ml}$ pepstatin], divided into two aliquots and sonicated on ice three times for 10 s. After centrifugation at 1000 r.p.m. in a microfuge at 4°C for 5 min to remove nuclei and other cellular debris, the supernatant was spun at 100 000 g in a Beckman TL-100 micro-ultracentrifuge for 40 min at 4°C. The supernatant was diluted 1:1 with 2 $\times$ PBS-TDS (150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.2% NaN_3 , 10 mM sodium phosphate pH 7.3, 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM EDTA, 0.7 mg/ml pepstatin, 0.2 mM PMSF) and the pellet resuspended in 1 ml of 1 $\times$ PBS-TDS by passage through a 22 gauge needle. Immunoprecipitation was carried out as described above. Aliquots were taken for TCA precipitation to determine total radioactivity.

In vitro farnesylation of cytoplasmic Ras

Cytoplasm was prepared as described above. The extract was dialyzed for 2 h against two changes of buffer C (40 mM Tris pH 7.5, 1 mM EDTA, 5 mM MgCl_2 , 5 mM ZnCl_2 , 1 mM DTT), the sample concentrated on a Centricon-10 filter (Amicon, Beverly, MA) and 100 μg of protein, as determined by the method of Lowry *et al.* (1951), were incubated at 30°C for 40 min with 2.5 μCi of [^3H]FPP (Dupont NEN) and 3.5 μg of purified FPTase (a gift from Patrick Casey) in a total volume of 50 μl of buffer C. Sample buffer (3 $\times$) was added to the reaction and the sample subjected to PAGE on a 15% acrylamide gel followed by autoradiography.

Preparation of [^3H]mevalonolactone

[^3H]Mevalonolactone was prepared by the method of Keller (1986) as modified by Havel *et al.* (1992). Mevaldic acid precursor was obtained from Sigma. $\text{NaB}[^3\text{H}]_4$ (15 Ci/mM) was obtained from ICN (Costa Mesa, CA). The resulting [^3H]mevalonolactone had a specific activity of 3.75 mCi/mM. For metabolic labeling of cultured chick atrial cells,

the stock solution of [^3H]mevalonolactone was diluted 1:20 or 1:30 with growth medium to a final concentration of 0.35–0.23 mM. The medium was incubated for 30 min at 37°C at a pH >8. Cells were incubated for 16 h with [^3H]mevalonate followed by immunoprecipitation, PAGE and autoradiography as described above. Most films were developed after a 14 day exposure.

Labeling of embryonic chick atrial with [^3H]palmitic acid

Cells were incubated overnight in growth medium made 0.5 mCi/ml in [^3H]palmitate (30 Ci/mM, Dupont New England Nuclear) followed by immunoprecipitation, PAGE and autoradiography as described above.

Phosphorylation of Ras

Chick atrial cells 14 days *in ovo* were cultured on 100 mm dishes for 2 days in medium supplemented with either 6% FCS or LPDS. On the third day of culture, cells were washed for 15 min with phosphate-free medium (Gibco, Grand Island, NY) followed by incubation for 2 h in fresh phosphate-free medium. The medium was made 0.5 mCi/ml in $^{32}\text{PO}_4$ (ICN, Costa Mesa, CA) and incubation continued for 2 h. Cells were washed and harvested, the cytoplasm and membrane prepared, and Ras immunoprecipitated, followed by PAGE and autoradiography as described above. Replicate plates were metabolically labeled with [^{35}S]methionine as described above. Samples labeled with either ^{35}S or $^{32}\text{PO}_4$ were analyzed in parallel by PAGE and autoradiography. An intense doublet migrating at 21 kDa was observed in the ^{35}S lane while no signal was observed in the ^{32}P lane.

Western blotting

Polyclonal (rabbit) antiserum raised against the carboxy-terminal decapeptide from rat G- α_{12} , which is identical to the carboxy-terminus of chick G- α_{11} and G- α_{12} , was a gift of David Manning. Cultured chick atrial cells 14 days *in ovo* were grown for 2 days in FCS or LPDS either as controls or following infection with Val12 Ras RCAS virus. Cells were washed in PBS and harvested in a Tris buffer (100 mM Tris, pH 7.2, 5 mM MgCl_2 , 1 mM EDTA, 4 mM PMSF), frozen and thawed twice, and Dounce homogenized. Protein concentration was determined by the method of Lowry *et al.* (1951). An aliquot containing 50 μg of membrane protein was diluted with 3 $\times$ SDS-Laemmli buffer followed by polyacrylamide gel electrophoresis on a 4–15% gradient gel (Bio-Rad, Hercules, CA). Immunoblotting was carried out using the ECL Western blotting analysis system (Amersham Corp. Buckinghamshire, UK) as described (Gadbut *et al.*, 1994).

Cholesterol assay

Cholesterol was extracted from the cell pellet by sonication in the presence of 1 ml of 1-propanol. Total cell cholesterol was assayed according to the method of Heider and Boyett (1978).

Characterization of genomic clones and generation of luciferase constructs for G- α_{12}

A chick genomic library was screened with a fragment generated from the 5' end of the coding region of a chick G- α_{12} cDNA. We obtained five clones which were positive by Southern blot analysis. A 4.5 kb fragment of one of these genomic clones was sequenced using an oligonucleotide primer from the 3' end of exon I. Sequencing demonstrated that the fragment contained the first 40 bases of the 5' end of exon I. To date, we have sequenced 600 bp of the 5' flanking region, which was 74% identical to the 5' flanking region of porcine α_{12} reported by Holtzman *et al.* (1991). More recently, RNase protection demonstrated a transcription start site –35 bp from the initiation codon in exon I.

Digestion of the 4.5 kb fragment with *NcoI* gave a 1.8 kb fragment beginning just 5' of the translation initiating ATG of exon I. This fragment was ligated to a promoterless luciferase reporter to generate α_{12} -Luc. Analysis of the available sequence of chick G- α_{12} 5' flanking region demonstrated three SRE-like sites, one in the reverse orientation; four SP1 sites; and one EGR-1 site at –300. These data suggested a high degree of homology between the chick and porcine 5' flanking regions.

Luciferase and alkaline phosphatase assay

Chick heart cells were cultured in the presence of either FCS or LPDS. On the second day of culture, 15 μg of α_{12} -Luc (1.8) and 4 μg of a human PAP under the control of an SV40 promoter (pSV2Apap, a gift of L.Ercolani) were transfected into heart cells by a modification of the method of Xu *et al.* (1992).

At 72 h after transfection, cells were washed in PBS and solubilized in lysis buffer II, 250 μl /plate (24 mM glycyl-glycine, 15 mM MgSO_4 , 4 mM EGTA, 1% Triton X-100 and 1 mM DTT). The extract was

sonicated three times for 10 s, centrifuged at 13 000 g for 3 min at 4°C and the supernatant assayed for luciferase and alkaline phosphatase activity as described (Holtzman *et al.*, 1991). In some experiments, cells transfected as described above were co-transfected with either 15 μg of a Val12 Ras in pRCAS under the control of a long terminal repeat promoter or 15 μg of an Asn17 Ras in pSVN17 under the control of an SV40 promoter (gifts of S.Hughes and G.Cooper respectively).

Infection of chick atrial cells with RCAS virus containing Val12 Ras

In order to generate RCAS virus, chick embryo fibroblasts were grown to 80% confluence, harvested and diluted 1:5 as described (Morgan and Fekete, 1996). After 1 day in culture, cells were transfected with 20 μg of Val12 pRCAS provirus or control pRCAS provirus (a gift of S.Hughes) by the calcium phosphate method. After each passage, the level of viral expression was determined by Western blot analysis of cell homogenates using a 1:250 dilution of an anti-Gag antibody (a gift of Volker Voit). Once infection was maximal and cells were confluent, medium was replaced with growth medium plus 6% new serum (Sigma). After 24 h, media were removed and concentrated by ultracentrifugation at 50 000 g for 2 h. Viral titer was determined as described (Morgan and Fekete, 1993).

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References

- Agarwal,R., Khan,S.G., Athar,M., Zaidi,S.I.A., Bickers,D.R. and Mukhtar,H. (1993) RAS protein p21 processing enzyme farnesyltransferase in chemical carcinogen-induced murine skin tumors. *Mol. Carcinogen.*, **81**, 290–298.
- Ballester,R., Furth,M.E. and Rosen,O.M. (1987) Phorbol ester- and protein kinase C-mediated phosphorylation of the cellular kirsten Ras gene product. *J. Biol. Chem.*, **262**, 2688–2695.
- Barnett,J.V., Haigh,L.S., Marsh,J.D. and Galper,J.B. (1989) Effects of low density lipoproteins and mevinolin on sympathetic responsiveness in cultured chick atrial cells. *J. Biol. Chem.*, **264**, 10779–10786.
- Brown,M.S. and Goldstein,J.L. (1980) Multivalent feedback regulation of HMGCoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J. Lipid Res.*, **21**, 505–517.
- Bruenger,E. and Rilling,H.C. (1988) Determination of isopentenyl diphosphate and farnesyl diphosphate in tissue samples with a comment on secondary regulation of polyisoprenoid biosynthesis. *Anal. Biochem.*, **173**, 321–327.
- Buss,J.E. and Sefton,B.M. (1986) Direct identification of palmitic acid as the lipid attached to p21. *Mol. Cell. Biol.*, **6**, 116–122.
- Casey,P.J., Solski,P.A., Der,C.J. and Buss,J.E. (1989) p21ras is modified by a farnesyl isoprenoid. *Proc. Natl Acad. Sci. USA*, **80**, 8323–8327.
- Chang,T.Y., Schiavoni,E.S., McCrae,K.R., Nelson,J.A. and Spencer,T.A. (1979) Inhibition of cholesterol biosynthesis in Chinese hamster ovary cells by 4,4,100-trimethyl-trans-decal-30-ol. *J. Biol. Chem.*, **254**, 11258–11263.
- Clarke,S. (1992) Protein isoprenylation and methylation of carboxyl-terminal cysteine residues. *Annu. Rev. Biochem.*, **61**, 355–3860.
- Clarke,S., Vogel,J.P., Deschenes,R.J. and Stock,J. (1988) Posttranslational modification of the Ha-Ras oncogene protein: evidence of a third class of protein carboxyl methyltransferase. *Proc. Natl Acad. Sci. USA*, **85**, 4647–4653.
- DeHaan,R.L. (1967) Regulation of spontaneous activity and growth of embryonic chick heart cells in tissue culture. *Dev. Biol.*, **16**, 216.
- Dudler,T. and Gelb,M.H. (1996) Palmitoylation of Ha-Ras facilitates membrane binding, activation of downstream effectors, and meiotic maturation in *Xenopus* oocytes. *J. Biol. Chem.*, **271**, 11541–11547.
- Feig,L.A. and Cooper,G.M. (1988) Inhibition of NIH 3T3 cell proliferation by a mutant RAS protein with preferential affinity for GDP. *Mol. Cell. Biol.*, **8**, 3235–3243.

- Furfine, E.S., Leban, J.J., Landavazo, A., Moomaw, J.F. and Casey, P.J. (1995) Protein farnesyltransferase: kinetics of farnesylpyrophosphate binding and product release. *Biochemistry*, **34**, 6857–6862.
- Gadbut, A.P. and Galper, J.B. (1994) A novel M3 muscarinic acetylcholine receptor is expressed in chick atrium and ventricle. *J. Biol. Chem.*, **269**, 25823–25829.
- Gadbut, A.P., Toupin, D.K., Kilbourne, E.J. and Galper, J.B. (1994) Low density lipoproteins induce parasympathetic responsiveness in embryonic chick ventricular myocytes in parallel with a coordinate increase in expression of genes coding for the M₂ muscarinic receptor, G α_2 and the acetylcholine-sensitive K⁺ channel. *J. Biol. Chem.*, **269**, 30707–30712.
- Goalstone, M.L. and Draznin, B. (1996) Effect of insulin on farnesyltransferase activity in 3T3-L1 adipocytes. *J. Biol. Chem.*, **271**, 27585–27589.
- Gutierrez, L., Magee, A.I., Marshall, C.J. and Hancock, J.F. (1989) Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J.*, **81**, 1093–1098.
- Hadcock, J.R., Ross, M., Watkins, D.C. and Malbon, C.C. (1990) Cross-regulation between G-protein-mediated pathways. *J. Biol. Chem.*, **265**, 14784–14790.
- Haigh, L.S., Leatherman, G.F., O'Hara, D.S., Smith, T.W. and Galper, J.B. (1988) Effect of low density lipoproteins and mevinolin on cholesterol content and muscarinic cholinergic responsiveness in cultured chick atrial cells. *J. Biol. Chem.*, **263**, 15608–15618.
- Hancock, J.F., Magee, A.I., Childs, J.E. and Marshall, C. (1989) All RAS proteins are polyisoprenylated but only some are palmitoylated. *Cell*, **57**, 1167–1177.
- Havel, C.M., Fisher, P. and Watson, J.A. (1992) Isopentenoid synthesis in isolated embryonic *Drosophila* cells: absolute, basal mevalonate synthesis rate determination. *Arch. Biochem. Biophys.*, **294**, 639–646.
- Heider, J.G. and Boyett, R.L. (1978) The picomole determination of free cholesterol in cells in culture. *J. Lipid Res.*, **49**, 514–518.
- Holtzman, E.J., Soper, B.W., Stow, J.L., Ausiello, D.A. and Ercolani, L. (1991) Regulation of the G-protein α_{i2} subunit gene in LLC-PK1 renal cells and isolation of porcine genomic clones encoding the gene promoter. *J. Biol. Chem.*, **266**, 1763–1771.
- James, G.L. *et al.* (1993) Benzodiazepine peptidomimetics: potent inhibitors of RAS farnesylation in animal cells. *Science*, **260**, 1937–1942.
- Jeng, A.Y., Srivastava, S.K., Lacal, J.C. and Blumberg, P.M. (1987) Phosphorylation of ras oncogene product by protein kinase C. *Biochem. Biophys. Res. Commun.*, **145**, 782–78.
- Keller, R.K. (1986) The mechanism and regulation of dolichyl phosphate biosynthesis in rat liver. *Biol. Chem.*, **261**, 12053–12059.
- Kilbourne, E.J. and Galper, J.B. (1994) Cloning of cDNAs coding for the G- α_{i1} and G- α_{i2} G-proteins from chick brain. *Gene*, **150**, 341–344.
- Kohl, N.E. *et al.* (1993) Selective inhibition of ras dependent transformation by a farnesyltransferase inhibitor. *Science*, **260**, 1934–1937.
- Lowy, D.R. and Willumsen, B.M. (1993) Function and regulation of RAS. *Annu. Rev. Biochem.*, **62**, 851–891.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- Milligan, G., Parenti, M. and Magee, A.I. (1995) The dynamic role of palmitoylation in signal transduction. *Trends Biochem. Sci.*, **20**, 181–186.
- Morgan, B.A. and Fekete, D.M. (1996) Manipulating gene expression with replication competent retroviruses. *Methods Cell Biol.*, **51**, 185–218.
- Parsons, W.J. and Stiles, G.L. (1987) Heterologous desensitization of the inhibitory A₁ adenosine receptor-adenylate cyclase system in rat adipocytes. *J. Biol. Chem.*, **262**, 841–847.
- Qiu, M.-S., Pitts, A.F., Winters, T.R., Green, S.H. (1991) RAS isoprenylation is required for RAS-induced but not for NGF induced neuronal differentiation of PC12 cells. *J. Cell Biol.*, **115**, 795–808.
- Reiss, Y., Seabra, M.C., Armstrong, S.A., Slaughter, C.A., Goldstein, J.L. and Brown, M.S. (1991) Nonidentical subunits of p21 H-Ras farnesyltransferase. *J. Biol. Chem.*, **266**, 10672–10677.
- Saikumar, P., Ulsch, L.S., Clanton, D.J., Huang, K.-P. and Shih, T.Y. (1988) Novel phosphorylation of c-ras p21 by protein kinases. *Oncogene Res.*, **3**, 213–222.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1990) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shen, W.P.V., Aldrich, T.H., Venta-Perez, G. and Franza, B.R. (1987) Expression of normal and mutant ras proteins in human acute leukemia. *Oncogene*, **2**, 157–165.
- Sinensky, M., Beck, L.A., Leonard, S. and Evans, R. (1990) Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis. *J. Biol. Chem.*, **265**, 19937–19941.
- Szeberenyi, J., Cai, H. and Cooper, G.M. (1990) Effect of a dominant inhibitory Ha-ras mutation on neuronal differentiation of PC12 cells. *Mol. Cell. Biol.*, **10**, 5324–5332.
- Tan, W., Barnett, J.V., Peirobon, D., Hehn, G., Greiser, C., Marsh, J.D. and Galper, J.B. (1993) Effect of low density lipoproteins, mevinolin, and G-proteins on Ca response in cultured chick atrial cells. *Am. J. Physiol.*, **265**, H191–H197a.
- Tschantz, W.R., Furfine, E.S. and Casey, P.J. (1997) Substrate binding is required for release of product from mammalian protein farnesyltransferase. *J. Biol. Chem.*, **272**, 9989–9993.
- Wedegaertner, P.B., Bourne, H.R. and von Zastrow, M. (1996) Activation-induced subcellular redistribution of G- α_s . *Mol. Biol. Cell*, **7**, 1225–1233.
- Winitz, S., Russell, M., Qian, N.-X., Gardner, A., Dwyer, L. and Johnson, G.L. (1993). Involvement of RAS and Raf in the G_i-coupled acetylcholine muscarinic M₂ receptor activation of mitogen activated protein (MAP) kinase and MAP kinase. *J. Biol. Chem.*, **268**, 19196–19199.
- Xu, H., Miller, J. and Liang, B.T. (1992) High-efficiency gene transfer into cardiac myocytes. *Nucleic Acids Res.*, **20**, 6425–6426.
- Zhang, F.L. and Casey, P.J. (1996) Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.*, **65**, 241–269.

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