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ACKNOWLEDGEMENTS. We thank L. Robertson and A. Efstratiadis for the IGF-II gene knockout mice; L. Robertson for continuing advice and encouragement; I. Herskowitz, P. Howely, E. Wagner, K. Yamamoto and members of the Hanahan laboratory for comments on the manuscript; K. Smith, R. Hirose and J. Arbeit for technical advice; and J. Karrim, F. Loeffler and A. Neill for technical assistance. This research was supported by a grant from the NCI and benefited from core support for the transgenic mouse facility from the Markey Charitable Trust. G.C. was supported by an American Heart Association Postdoctoral Fellowship.

Ras-dependent activation of MAP kinase pathway mediated by G-protein βγ subunits

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MITOGEN-ACTIVATED protein kinases, MAP kinases or ERKs (extracellular signal-regulated kinases) are rapidly stimulated by growth-promoting factors acting on a variety of cell-surface receptors[1,2]. In turn, ERKs phosphorylate and regulate key intracellular enzymes and transcription factors involved in the control of cellular proliferation[3,4]. The tyrosine-kinase class of growth-factor receptors transmits signals to ERKs in a multistep process that involves Ras and a limited number of defined molecules[5]. In contrast, ERK activation by G-protein-coupled receptors is poorly understood[3,6], as is the role of *ras* in this signalling pathway[7,8]. We have explored in COS-7 cells the mechanism of ERKs activation by m1 and m2 muscarinic receptors, typical examples of receptors coupled through Gq proteins to induce phosphatidylinositol hydrolysis and to G_i proteins to inhibit adenylyl cyclase, respectively[9]. Here we present evidence that ERK activation is mediated by βγ subunits of heterotrimeric G proteins acting on a *ras*-dependent pathway.

Receptors m1 and m2 were expressed in COS-7 cells together with an epitope-tagged-ERK2 (HA-ERK2)[10] (Fig. 1*a*). In cells expressing either muscarinic receptor the cholinergic agonist carbachol induced a dose-dependent increase in HA-ERK2 activity (Fig. 1*b*). Treatment with pertussis toxin (PTX), which uncouples certain G proteins by ADP-ribosylation[11], nearly abolished ERK2 activation mediated by m2 receptors, but not by m1 or EGF receptors (Fig. 1*c*). Thus, activation of ERK2 through m2 but not m1 or EGF receptors involves a PTX-sensitive G protein, presumably a member of the G_i family.

To investigate how G proteins signal to ERKs, we coexpressed HA-ERK2 together with wild-type or GTPase-deficient mutants of α subunits from G_q, G_{i2}, and G_{13}[12–16]. All transfected α subunits were detectably expressed (Fig. 2*a*). Activated α_q and α_{i2}, as expected, potently induced phosphatidylinositol hydrolysis or inhibited adenylyl cyclase, respectively (refs 6, 14, 15 and data not shown), mimicking the effect of m1 or m2 stimulation. However, cells expressing either wild-type or activated forms of these $G\alpha$ subunits did not display significantly enhanced HA-ERK2 phosphorylating activity (Fig. 2*b*). Furthermore, ERK2 was not significantly activated on expression of GTPase-deficient mutants representing each of the four known classes of G protein α-subunits, alone or in all possible combinations (Fig. 2*b*). In contrast, v-*ras* (ref. 17) effectively activated ERK2 under identical experimental conditions. Thus, molecules in addition to α subunits of G proteins must be involved in stimulating ERK activity.

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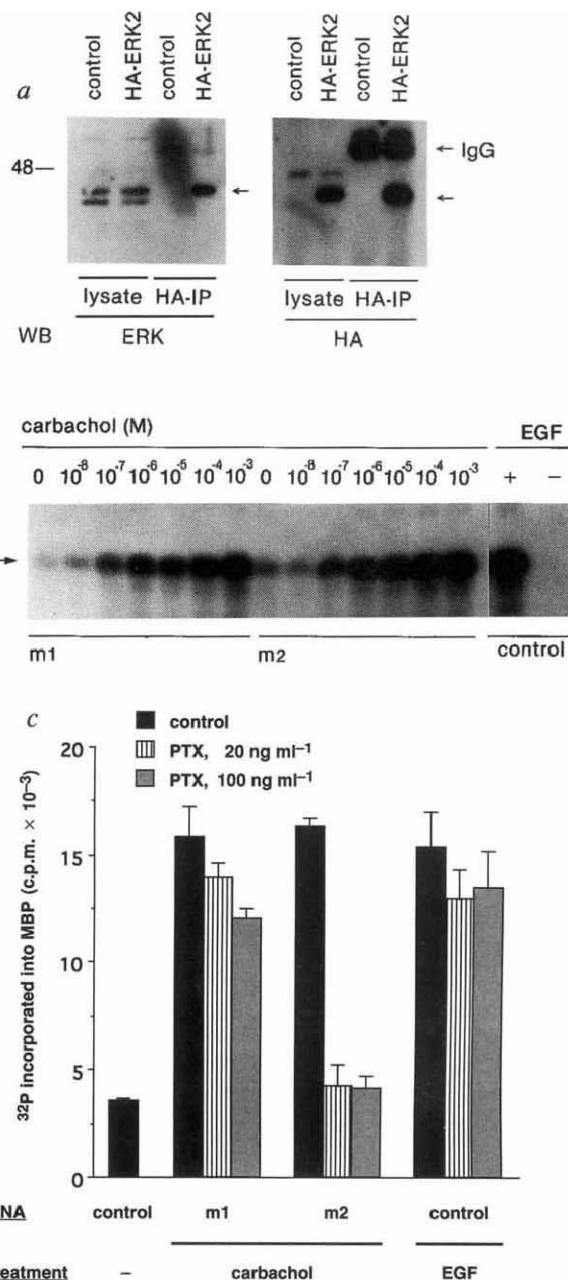

FIG. 1 Cholinergic agonist, carbachol, increases the enzymatic activity of an epitope-tagged ERK2 when coexpressed with m1 or m2 muscarinic receptors in COS-7 cells. *a*, Lysates containing 40 µg of protein from COS-7 cells transfected with the pCDNA I vector (control) or with an expression plasmid containing the epitope-tagged murine ERK2 cDNA[10] (provided by M. J. Weber) (HA-ERK2, 1 µg per plate) were subjected to western blot (WB) analysis with a C-terminal anti-ERK serum (UBI; ERK), or with epitope-tag-specific 12CA5 antibody (Babco) and a secondary rabbit anti-mouse serum (Cappel; HA). Parallel samples containing about 800 µg of cellular protein were first immunoprecipitated with the 12CA5 antibody (HA-IP). Bands were visualized with ^{125}I-Protein A. The protein product of the HA-ERK2 construct is indicated with an arrow. *b*, COS-7 cells transfected with vector (control), m1 or m2 were serum starved overnight, lysed on stimulation with the indicated concentrations of carbachol or EGF (100 ng ml^{-1}) for 5 min, and immunoprecipitated with the 12CA5 antibody (2 µg). ERK kinase activity was determined in immunoprecipitates in a reaction buffer containing 1 µCi[γ-^{32}P]ATP, 20 µM ATP and 1.5 mg ml^{-1} myelin basic protein (MBP; Sigma) for 20 min at 30 °C, essentially as described[10]. Phosphorylated MBP was visualized by autoradiography after PAGE. Similar results were obtained in 3 independent experiments. *c*, Effect of pertussis toxin (PTX) on the activation of ERK2 by agonist in control, m1 or m2 transfected COS-7 cells. Untreated cells or cells treated for 18 h with 20 or 100 ng ml^{-1} PTX were exposed to vehicle alone (minus), carbachol (100 µM) or EGF (100 ng ml^{-1}) for 5 min, and processed as above. Data represent average ±s.e.m. of radioactivity incorporated in MBP in triplicate samples. Similar results were obtained in 3 independent experiments.

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When activated, receptors coupled to G proteins catalyse the replacement of GDP by GTP bound to the α subunit. This exchange induces dissociation of α-GTP from $\beta\gamma$ dimers[12,13,18]. To investigate whether $\beta\gamma$ complexes participate in the activation of ERKs, we took advantage of the finding that overexpression of the α subunit of transducin (α_t) blocks $\beta\gamma$-dependent pathways, probably by sequestering free $\beta\gamma$ dimers[19]. As shown in Fig. 3*a*, expression of α_t did not affect the response of ERK2 to EGF but inhibited its activation when mediated by either muscarinic receptor, suggesting that $\beta\gamma$ dimers play a role in signalling from G-protein-coupled receptors to ERKs.

We next determined whether overexpression of $\beta\gamma$ subunits activates ERK2. In addition to the wild-type construct we used an altered γ subunit lacking an isoprenylation signal, designated γ^*, which dimerizes with β subunits but fails to associated with the plasma membrane[20,21]. Only cells cotransfected with both β and γ (or γ^*) complementary DNAs expressed elevated levels of their respective protein products (Fig. 3*b*), probably because complex formation between β and γ is required for the stability of either G-protein subunit[20-23]. Coexpression of $\beta\gamma$ but not of $\beta\gamma^*$ led to a dramatic increase in ERK2 phosphorylating activity (Fig. 3*c*), demonstrating that $\beta\gamma$-heterodimers can directly

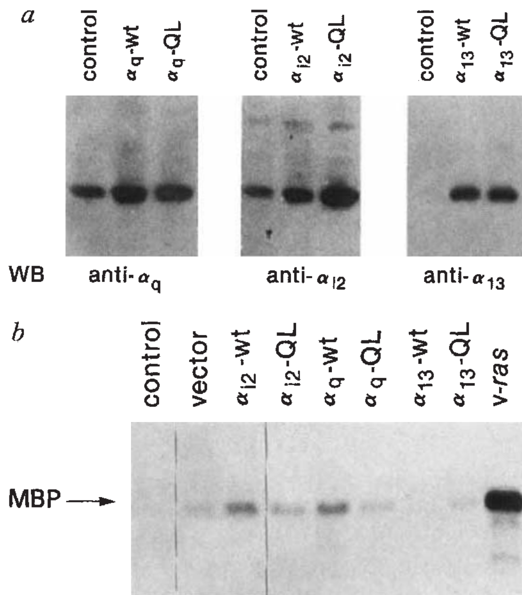
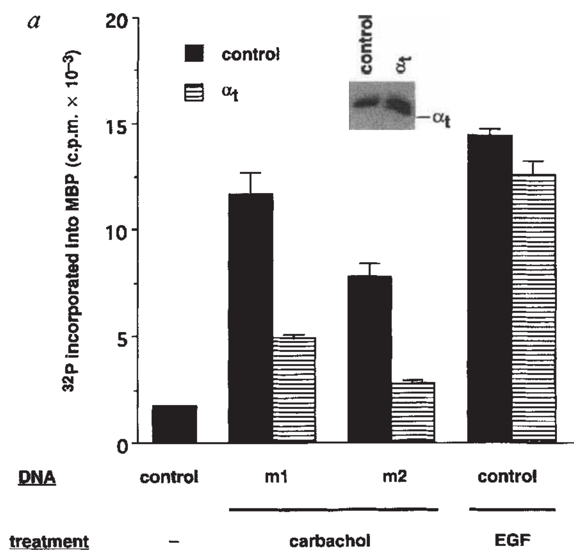


FIG. 2 Highly expressed wild-type or activated mutants of G-protein α subunits fail to stimulate ERK2 enzymatic activity significantly. COS-7 cells were transfected with the pCDNA I vector (control) or the same vector carrying cDNAs for v-*ras* (ref. 17), wild-type murine α_q, α_{i2}, α_{13}, or for their corresponding activated mutants α_q Q209L (ref. 15), α_{i2} Q207L (ref. 14) or α_{13} Q226L (ref. 16 and N.X. *et al.*, manuscript in preparation) (5 µg per plate in each case), together with the HA-ERK2 expression plasmid (1 µg per plate). *a*, Lysates containing 40 µg total cellular proteins were subjected to western blot analysis using subunit specific antisera, as indicated. *b*, HA-ERK2 kinase activity was determined in anti-HA immunoprecipitates from the corresponding cellular lysates. Control cells were not transfected with HA-ERK2 plasmid. Small changes in ^{32}P-labelled MBP in G-protein-transfected cells were not consistently observed, and therefore represent background variations. Similar results were obtained in 5 independent experiments. Similar lack of demonstrable ERK2-activation was observed on transfection of activated mutants of $G\alpha_s$ or $G\alpha_{12}$, or when the different activated $G\alpha$ subunits were coexpressed in all possible combinations (data not shown).



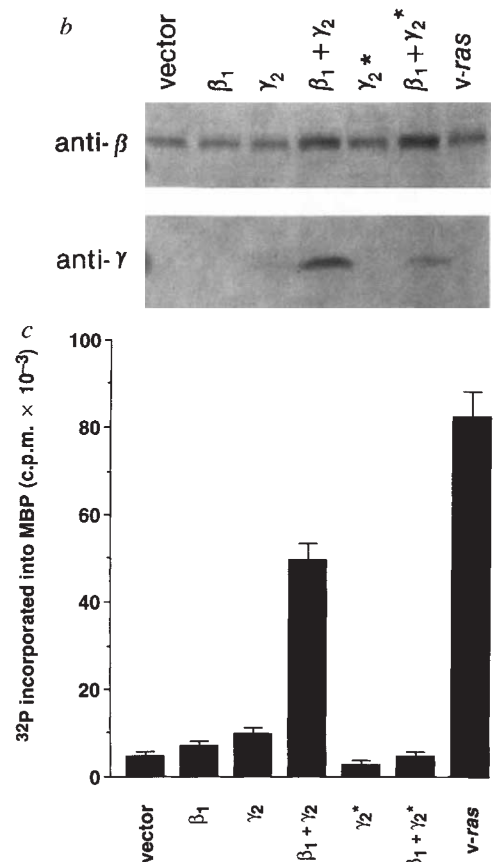


FIG. 3 *a*, α_t coexpression blocks agonist-induced activation of ERK2 in m1 or m2 transfected COS-7 cells. Plasmids containing m1 and m2 (1 µg per plate) or the pcDNA I vector (control) were cotransfected with an expression vector carrying α_t cDNA (α_t, 5 µg per plate) or with equal amount of vector. Cells were exposed to carbachol (100 µM) or EGF (100 ng ml^{-1}) for 5 min, lysed and ERK activity assayed on anti-HA immunoprecipitates as above. After autoradiography, gels were sliced and radioactivity incorporated in MBP counted. Data represent average ±s.e.m. of triplicate samples. Similar results were obtained in 3 independent experiments. Inset, Cells transfected with the indicated plasmid DNA were analysed by western blotting with the C-terminal α_t/α_i antisera AS (ref. 20). The protein product of the transfected α_t gene is indicated. The upper band present in both lysates represents endogeneous α_i proteins. *b*, *c*, Overexpression of G protein β and γ subunits stimulates ERK2 enzymatic activity. COS-7 cells were transfected with the pcDM8-1 vector (control) or the same vector carrying cDNAs for the β_1 subunit, the wild-type γ_2 subunit or an isoprenylation deficient mutant of $\gamma_2(\gamma_2^*)^{20}$ (5 µg per plate in each case) together with pcDNA-HA-ERK2 plasmid (1 µg per plate). Cotransfection of pcDNA-v-*ras* (5 µg per plate) with the HA-ERK2-DNA was used as a control. *b*, 40 µg total lysate proteins were subjected to western blot analysis using subunit specific antisera (C-terminal peptide β and γ_2 antisera, SW and EDPL, respectively; W.S., manuscript in preparation), as indicated. *c*, HA-ERK2 kinase activity was determined in anti-HA immunoprecipitates obtained from cotransfected cells as shown. After autoradiography, gels were sliced and radioactivity incorporated in MBP counted. Data present average ±s.e.m. of triplicate samples. Similar results were obtained in 6 independent experiments.

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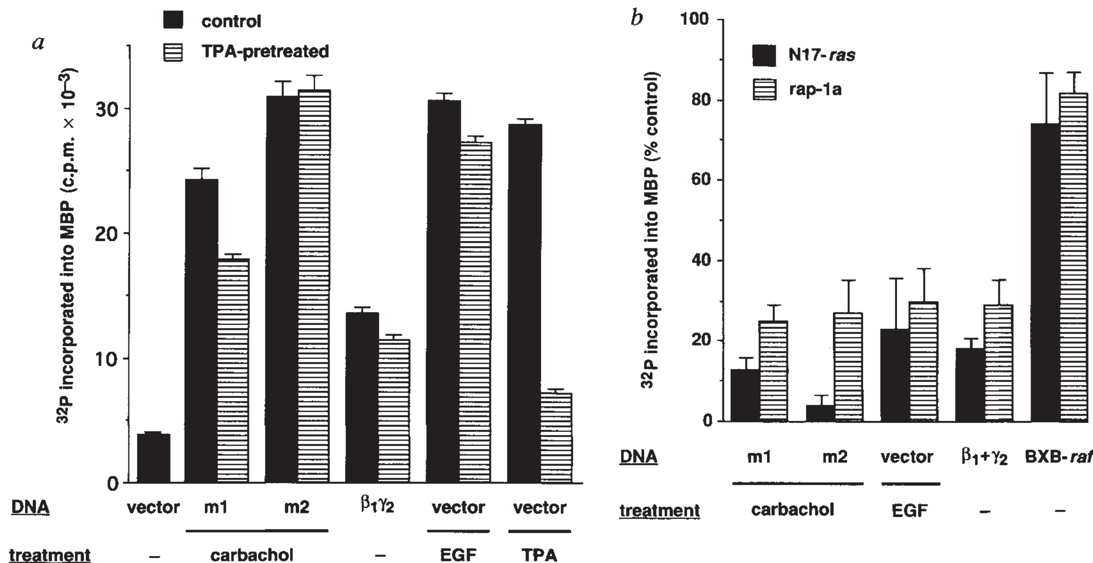

FIG. 4 *a*, Effect of protein kinase C downregulation on agonist or $\beta\gamma$-induced activation of ERK2. Cells were transfected with the indicated expression plasmids together with pcDNA-HA-ERK2. Two days latter, cells were serum starved and left untreated (control) or were treated with phorbol 12-myristate 13-acetate (TPA) (1 μg ml^{-1}) (TPA-pretreated). After 18 h, cells were untreated or exposed to carbachol (100 μM), EGF (100 ng ml^{-1}), or TPA (50 ng ml^{-1}) as indicated for 5 min, lysed and ERK activity assayed on anti-HA immunoprecipitates as described in Fig. 1. Protein kinase C downregulation by the 18-h treatment with TPA was confirmed by western blot analysis (data not shown). Data represent average ±s.e.m. of triplicate samples. Similar results were obtained in 3 independent experiments. *b*, Effect of *ras*-inhibitory proteins on agonist-induced activation of ERK2. pcDNA (vector), m1, m2, $\beta_1 + \gamma_2$ or activated *raf*(BXB-*raf*, ref. 30) containing plasmids (5 μg per plate) were cotransfected with an expression vector carrying a dominant negative mutant of Ras (N17-*ras*)[25], or rap-1a (ref. 26), 5 μg per plate, as indicated. Cells were untreated or exposed to carbachol (100 μM) or EGF (100 ng ml^{-1}) for 5 min, lysed and ERK activity assayed on anti-HA immunoprecipitates as described in Fig. 1. Data represent the mean ±s.e. of 3–5 independent experiments, expressed as a percentage of radioactivity incorporated into MBP as compared to cells transfected with vector DNA (100%). These same *ras*-inhibiting DNAs did not affect expression of HA-ERK2, m1 or m2 receptors, nor did they inhibit coupling to PI–PLC (data not shown).

stimulate biochemical pathways leading to ERK activation. Membrane association appears to be a requisite for this function.

We investigated whether protein kinase C (PKC) mediates ERK activation by G proteins in cells depleted of PKC by prolonged treatment with a high concentration of phorbol ester[23]. As shown in Fig. 4*a*, this procedure abolished ERK2 activation in response to a subsequent challenge with phorbol ester. In contrast, PKC downregulation only partially diminished activation of ERK2 mediated by m1 receptors, a finding consistent with previous reports[6,24]. Furthermore, we observed that PKC downregulation had little effect on ERK2 activation elicited by m2 or EGF receptors, or by overexpressed $\beta\gamma$ subunits. Taken together these data demonstrate a PKC-independent pathway mediating signalling from G proteins to ERKs.

To explore the role of *ras* in ERK2-activation by heterotrimeric G proteins, we cotransfected COS-7 cells with m1, m2, or $\beta\gamma$ cDNAs together with plasmids expressing *ras*-inhibitory proteins such as a dominant negative mutant of Ras (N17-*ras*)[25] and rap-1a (ref. 26). As shown in Fig. 4*b*, cotransfection of each *ras*-inhibiting construct nearly abolished ERK2 activation when induced through EGF, m1 and m2 receptors, or by $\beta\gamma$ dimer overexpression, but had little effect on stimulation of ERK2 by activated *raf*. These findings provide strong evidence that *ras* function is required for transducing signals from G proteins to ERK2.

On the basis of our present study, activation of ERKs can be added to the growing list of effector functions mediated by $\beta\gamma$-heterodimers[18]. Furthermore, our findings also provide strong evidence that at least in COS-7 cells, free $\beta\gamma$ dimers rather than activated α subunits transfer signals from G-protein-linked receptors to ERKs. $\beta\gamma$ subunits have been previously shown to mediate pheromone receptor activation of ERKs in the budding yeast *Saccharomyces cerevisiae*, although in this case the pathway is *ras*-independent[27]. In mammalian cells, however, signalling from $\beta\gamma$ complexes to ERKs appears to require Ras, thus raising a mechanistic question regarding communication between $\beta\gamma$ subunits and *ras*. In this regard, a recent finding has shown that β ardrenergic-receptor kinase binds free $\beta\gamma$ heterodimers through a region that contains a pleckstrin homology (PH) domain[28]. The majority of proteins known to regulate *ras* activity also contain a PH domain[29]. Thus, we propose that free $\beta\gamma$ subunits might affect functioning of Ras by binding the PH element of one or more *ras*-regulatory proteins. Such a hypothesis, linking G-protein-coupled receptors to small GTP-binding proteins, is testable and will be investigated in the future. □

Received 30 December 1993; accepted 2 April 1994.

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ACKNOWLEDGEMENTS. We thank M. Weber for the gift of HA-ERK2 cDNA; M. Simon for the gift of α_{13} cDNA; U. Rapp for the gift of BXB-*raf* cDNA; H. Bourne, T. Vonyo-Yasenetskaya and B. Conklin for the gift of α_{13}-QL cDNA and for suggesting the use of α_t as well as providing an α_t expression plasmid and K. C. Robbins for support and counsel. W.F.S. acknowledges support from A. M. Spiegel. P. C. was supported by a Fullbright Fellowship from the Government of Spain.

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