

Recombinant $G_q\alpha$

MUTATIONAL ACTIVATION AND COUPLING TO RECEPTORS AND PHOSPHOLIPASE C*

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G_q mediates hormonal stimulation of phosphoinositide-specific phospholipase C (PI-PLC). We mutated the α subunit of G_q (α_q) to replace arginine 183 with cysteine. Mutations that substitute cysteine for the corresponding arginine residues of α_s and α_{12} constitutively activate their respective effector pathways, creating the *gsp* and *gip2* oncogenes. Transient expression of α_q -R183C in COS-7 and HEK-293 cells constitutively activates PI-PLC, but wild type (WT) α_q does not. This suggests that the mutated arginines in α_s , α_{12} , and α_q share a common function in regulating the active state of these proteins and that the α_q gene may serve as a target for oncogenic mutations in human tumors. In an attempt to develop an assay for receptor stimulation of recombinant α_q , we co-expressed receptors with α_q -WT. We found that the α_2 -adrenoceptor stimulates PI-PLC activation in HEK-293 cells in a fashion that depends completely on co-expression of α_q -WT. These findings create an experimental model, similar to that provided for α_s by S49 *cyc*⁻ cells, that should make it possible to analyze receptor and effector coupling by mutant α_q against a null background.

Heterotrimeric G proteins¹ transduce extracellular signals detected by a variety of cell surface receptors into altered activities of specific effector proteins, including adenylyl cyclase, cGMP phosphodiesterase, phospholipase C, phospholipase A₂, and ion channels (1-4). In most cases (but probably not all), the GTP-bound α subunit of the G protein regulates the effector. Agonist-bound cell surface receptors activate G

proteins by catalyzing release of GDP from the α subunit and its replacement by GTP. In this process the α chain separates from the $\beta\gamma$ subunit. The α subunit then turns itself off by hydrolyzing bound GTP. By abrogating this turn-off mechanism, GTPase-inhibiting mutations in genes encoding the α subunits of G_s and G_{12} cause, respectively, constitutive activation of adenylyl cyclase (5-8) and constitutive inhibition of cAMP accumulation (9). Such mutations in the same genes create oncogenes that are found in human endocrine tumors of the pituitary, thyroid, ovary, and adrenal (6, 10).

A recently cloned (11) G protein α chain, α_q , stimulates phosphoinositide-specific phospholipase C (PI-PLC) (12, 13). Two other recently cloned α chains, α_{11} (11) and α_{1L} (14), share greater than 80% homology with α_q and, like α_q , lack the cysteine residue required for ADP ribosylation by pertussis toxin. A mixture of α_q and α_{11} purified from bovine brain (12) and liver (13) activates PI-PLC in lipid vesicles. Antibodies directed against the carboxyl-terminal 11 amino acids of α_q and α_{11} immunoprecipitate a GTP-dependent factor that stimulates PI-PLC in turkey erythrocyte membranes (15); such antibodies also block GTPase stimulation by thromboxane A₂ in platelet membranes (16).

This report describes the effects of transient expression of wild type and mutant α_q in COS-7 and human embryonic kidney (HEK-293) cells. Mutational substitution of cysteine for arginine 183 in α_q (Fig. 1), imitating cognate activating mutations in α_s and α_{12} (6, 7, 9), creates a mutant α_q protein (α_q -R183C) that constitutively activates the appropriate effector, PI-PLC. Over-expression of wild type α_q (α_q -WT) enables a co-expressed α_2 -adrenoceptor (α_2 -AR) to mediate agonist stimulation of PI-PLC. These observations define experimental models for testing interactions of recombinant α_q with both effectors and receptors, without interference from endogenous G_q-like proteins.

EXPERIMENTAL PROCEDURES

Materials—cDNAs used in these experiments were generous gifts from Melvin I. Simon of the California Institute of Technology (murine α_q in the Bluescript vector, Ref. 11), Wolfgang Sadee of this institution, (human m1-muscarinic receptor in the CDM8 vector), Lee E. Limbird of Vanderbilt (porcine α_2 -AR, subtype 2A, in the pCMV4 vector, Ref. 17), and Deborah Segaloff of the University of Iowa (human lutropin receptor in pCIS vector, Ref. 18). UK-14304 was obtained from Research Biochemicals Inc., as part of the National Institute of Mental Health Chemical Synthesis Program Contract 278-90-0007(BS). Other items included: pcDNA-I (Invitrogen); Muta-gene kit and AG1-8X Dowex columns (Bio-Rad); human chorionadotropin (the National Pituitary Agency); plasmid purification columns (Qiagen); restriction enzymes (Bethesda Research Labs and New England Biolabs); HEK-293 cells (American Type Culture Collection CRL-1573); [³H]inositol and [³H]adenine (Amersham Corp.). COS-7 cells were a gift from Zach Hall of this institution. All other reagents were obtained from Sigma.

Plasmid Construction and Transfection— α_q -WT cDNA was excised from Bluescript and subcloned into pcDNA-I using *Hind*III linkers. The cDNA was then mutagenized as described (19) using the mutagenic primer ACA CAA CAA GAC GTG CTA CGC GTT TGT GTC CCC ACT ACA GGG. The resulting mutant DNA (α_q -R183C) has a new silent *Mlu*I restriction enzyme site. Mutations were confirmed by DNA sequencing. COS-7 cells were propagated in Dulbecco's modified Eagles medium with 10% fetal calf serum, in 5% CO₂. DNAs were transfected into COS-7 cells (10⁶ cells/60-mm dish) in the presence of DEAE-dextran (250 μ g/ml) and chloroquine (100 μ M) for 4 h. Cells were shocked with phosphate-buffered saline containing

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¹ The abbreviations used are: G protein, guanine nucleotide-binding regulatory protein; PI-PLC, phosphoinositide-specific phospholipase C; IP, inositol phosphate, regardless of the number of phosphate groups; α_q , α subunit of the G protein G_q ; WT, wild type; α_q -R183C, α_q with arginine at position 183 changed to cysteine; HEK-293 cells, human embryonic kidney cells; α_2 -AR, α_2 -adrenoceptor; mAChR, m1-acetylcholine receptor; LHR, lutropin receptor; UK-14304, 5-bromo-6-(imidazolin-2-ylamino) quinoxaline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

10% dimethyl sulfoxide for 2 min, then washed once with phosphate-buffered saline and maintained in growth media for 18–24 h before re-seeding in 24-well plates. HEK-293 cells were maintained in minimum essential medium (Earle's) containing 10% fetal calf serum. The HEK-293 transfection procedure was identical to that used with COS-7 cells, except that the transfection was stopped after 2 h.

Inositol Phosphate (IP) Formation Assay—24 h after transfection each 60-mm dish of COS-7 cells was split into 12 wells of a 24-well plate and incubated in medium containing [3 H]inositol (2 μ Ci/ml). 18–24 h later the cells were washed with 1 ml of assay medium (20 mM HEPES-buffered Dulbecco's modified Eagle's medium without bicarbonate) and incubated at 37° for 1 h with 1 ml of assay medium containing 5 mM LiCl and the indicated agonist. IP formation was assayed as described (20), with minor modifications. After incubation with drugs, medium was aspirated and cells were lysed by addition of 0.75 ml of ice-cold 20 mM formic acid (30 min). Supernatant fractions were loaded on to AG1-8X Dowex columns, followed by immediate addition of 3 ml of 50 mM NH_4OH (^3H]inositol fraction). The columns were then washed with 4 ml of 40 mM ammonium formate, followed by 5 ml of 2 M ammonium formate (^3H]IP fraction). Combined radioactivity in these two fractions correlated directly with the number of cells in each well. Accordingly, data are presented as the quotient of [^3H]IP divided by [^3H]inositol plus [^3H]IP. The IP formation assay for HEK-293 cells was identical to that used for the COS-7 cells, except that after transfection each 60-mm dish was split into 6 wells of a 24-well dish because of the slower growth rate of HEK-293 cells.

cAMP Assay—24 h after transfection each 60-mm dish of HEK-293 cells was split into 6 wells of a 12-well plate and incubated in medium containing [^3H]adenine (2 μ Ci/ml). 18–24 h later, the HEK-293 cells were washed once with 1 ml of assay medium followed by 1 ml of assay medium containing 1 mM 1-methyl-3-isobutylxanthine and other drugs as indicated. Intracellular [^3H]cAMP accumulation was estimated by determining the ratios of cAMP to total ATP and ADP pools as described (9).

RESULTS

Carbachol, a muscarinic agonist, stimulated IP formation in COS-7 cells expressing the muscarinic m1-acetylcholine receptor (m1AChR) (Fig. 2). The effect of carbachol on m1AChR-transfected COS cells is not blocked by treatment with pertussis toxin (21), suggesting that it was mediated by endogenous G_q or a G_q -like protein. Co-expression of α_q -WT with the m1AChR failed to alter either the maximal stimulation of PI-PLC by carbachol (Fig. 2A) or the EC_{50} of carbachol's effect (Fig. 2B); from three separate experiments, the mean EC_{50} values were 13 ± 5 and 11 ± 7 μM , respectively, with and without co-transfected α_q -WT.

Expression of α_q -R183C caused constitutive elevation of IP synthesis in COS-7 (Fig. 2A) and HEK-293 cells (Fig. 3C). Activation of the co-expressed m1AChR consistently augmented PI-PLC activity, even in the presence of the activated mutant α_q (Fig. 2A).

The α_2 AR is known to stimulate PI-PLC in COS-7 cells (22). Co-expression of α_q -WT with the α_2 -AR markedly increased the ability of UK-14304, an α_2 -AR agonist, to stimulate PI-PLC in these cells (Fig. 3A). In HEK-293 cells, agonist stimulation of the α_2 -AR had no effect on PI-PLC activity unless α_q -WT was co-expressed with the receptor; the effect of agonist increased progressively with increasing amounts of transfected α_q -WT DNA (Fig. 3B).

It is pertinent to ask whether expression of recombinant α_q controls other effector pathways, in addition to PI-PLC.

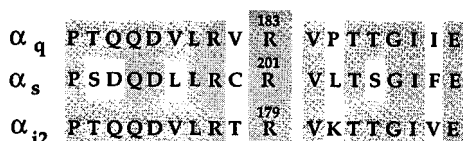


FIG. 1. Conservation of α chain amino acid sequences surrounding a key arginine residue.

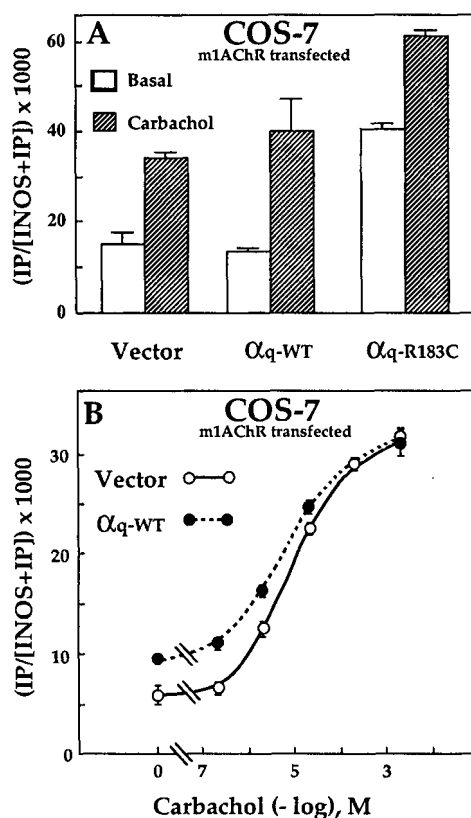


FIG. 2. Co-transfection for α_q and m1AChR DNA in COS-7 cells. A, IP formation was measured as described under "Experimental Procedures" for 1 h in the presence or absence of 200 μM carbachol as indicated. Cells were transfected with 1 μg of m1AChR-CDM1 DNA plus 1 μg of either pcDNA-I alone (vector control) or pcDNA-I containing α_q -WT or α_q -R183C. B, carbachol concentration-effect curves, performed in cells transfected with m1AChR DNA, with or without α_q -WT DNA, as indicated. Data points in each panel represent the mean \pm S.D. of triplicate measurements in a single experiment. For both panels A and B, three or more separate experiments yielded results similar to those shown.

Expression of mutationally activated α_s stimulates cAMP accumulation in HEK-293 cells; conversely, expression of mutationally activated α_{i2} inhibits the cAMP accumulation triggered by agonist stimulation of the lutropin receptor (LHR) (Fig. 4 and Ref. 9). Neither basal cAMP (not shown) or LHR-stimulated cAMP accumulation (Fig. 4) was affected by expression of either α_q -WT or α_q -R183C in HEK-293 cells.

DISCUSSION

The present observations bear on four issues in the investigation of G protein function.

First, the effect of the R183C mutation on α_q confirms the functional importance of this conserved arginine residue in a shared structural motif that is postulated (23) to be involved in the hydrolysis of GTP by all G protein α chains. Side chains of cognate arginines in α_i and α_s serve as targets for ADP-ribosylation by cholera toxin (23, 24) a covalent modification that activates the corresponding G proteins by inhibiting GTP hydrolysis. Similarly, mutational replacements of this arginine in α_s to cysteine, alanine, glutamic acid, or lysine inhibit GTPase activity and lead to constitutive signaling by the mutant α_s (6, 7). Although the precise chemical role of this arginine is unknown, it is conserved in all known α chains (Fig. 1 shows α_s , α_{i2} , and α_q) and located in a predicted loop (23) homologous to the region of p21^{ras} that is regulated by GTPase activating protein.

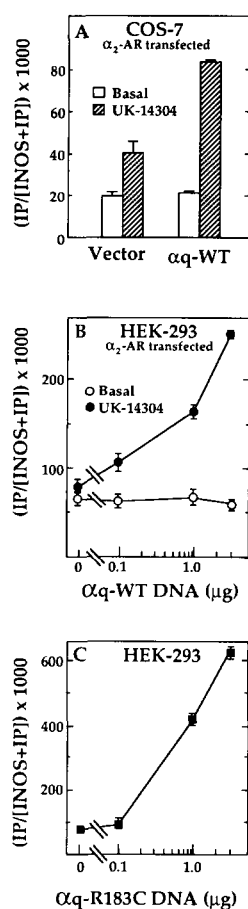


FIG. 3. Co-transfection of α_q and α_2 -AR DNA. COS-7 and HEK-293 cells were transfected with 1 μ g α_2 -AR-pCMV4 DNA plus either pcDNA-I alone (vector control) or pcDNA-I containing α_q -WT or α_q -R183C; the amounts of these additional DNAs were 1 μ g (panel A) or as indicated (panels B and C). Cells were treated with or without 10 μ M UK-14304 for 1 h and IP formation measured as indicated. Data points in each panel represent the mean \pm S.D. of triplicate measurements in a single experiment. For all three panels, two or more separate experiments yielded results similar to those shown. COS-7 and HEK-293 cells transfected with α_q -WT but not the α_2 -AR showed no response to 10 μ M UK-14304 (not shown).

Second, transiently expressed α_q -R183C can activate PI-PLC independently of endogenous α_q or receptors coupled to α_q . This should make it possible to use site-directed mutations and chimeric proteins constructed from α_q and other α chains to map regions of the α_q molecule responsible for specific interaction with PI-PLC. The transient expression model makes such experiments much easier and faster. Instead of working for weeks to establish a cell line stably expressing a single mutant protein, the experimenter can test large numbers of mutant proteins, alone and in combination with other recombinant proteins, in a few days.

Third, HEK-293 cells provide a model of receptor-mediated stimulation of PI-PLC in which both the receptor and the G protein α subunit are supplied by transient transfection of recombinant DNAs. In such an experimental system it will be easy to manipulate the amount or primary structure of either the receptor or the α chain and to study their functional interactions without having to select cell lines stably expressing two recombinant signaling molecules. This system should be especially useful for molecular genetic analysis, because it provides a null background (*i.e.* no response in the absence of exogenous receptor or α_q) analogous to the null cAMP background of S49 *cyc*⁻ cells genetically lacking α_s (25).

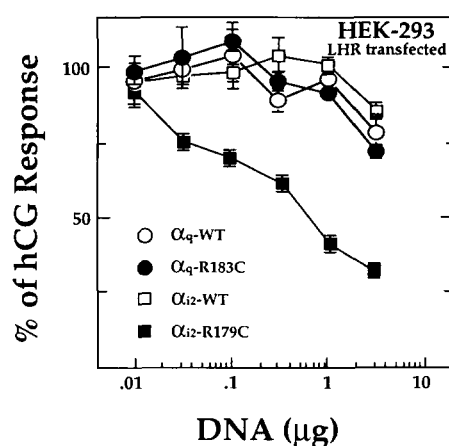


FIG. 4. α_q does not inhibit adenyllylcyclase. HEK-293 cells were transfected with 0.5 μ g of LHR-pCIS DNA and varying amounts of vector DNA containing α_q -WT, α_q -R183C, or α_{i2} -R179C (9), as indicated. cAMP accumulation was assayed in the presence of 1 mM 1-methyl-3-isobutylxanthine and human chorionadotropin (*hCG*, 5 ng/ml) as described (9). Results are expressed as a percentage of human chorionadotropin-stimulated cAMP accumulation in cells transfected with LHR alone. The data represent triplicate determinations in a single experiment; two additional experiments gave similar results.

Carbachol can stimulate PI-PLC in HEK-293 cells via endogenous muscarinic receptors (not shown). Thus, in contrast to the *cyc*⁻ precedent, these cells do not lack endogenous α_q (or an α_q -like protein). Why, then, do recombinant α_2 -ARs and mAChRs differ so strikingly in their abilities to couple to the endogenous α_q -like proteins of HEK-293 and COS-7 cells (Figs. 2 and 3)? The answer will require quantifying relative amounts of endogenous α_q -like proteins in the two cell types and measuring their relative affinities for interacting with the mAChR *versus* the α_2 -AR.

Finally, ectopic expression or prolonged stimulation of receptors that stimulate PI-PLC, probably via α_q , can exert mitogenic and even tumorigenic effects in certain cell types (26, 27). Accordingly, we predict that PI-PLC stimulation caused by expression of α_q -R183C in appropriate cultured cell lines will also induce increased proliferation and, possibly, neoplastic transformation. If so, mutations that activate α_q in such cells may create α_q oncogenes in human tumors, in parallel with the α_s and α_{i2} oncogenes already described (6, 10, 28–30).

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