**Gαq** Acts as an Adaptor Protein in Protein Kinase Cζ (PKCζ)-mediated ERK5 Activation by G Protein-coupled Receptors (GPCR)†§*

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Carlota García-Hoz†§, Guzmán Sánchez-Fernández‡§, María Teresa Díaz-Meco§, Jorge Moscat§, Federico Mayor§†1, and Catalina Ribas†§2

*From the 1Departamento de Biología Molecular and the 2Centro de Biología Molecular “Severo Ochoa,” Consejo Superior de Investigaciones Científicas (CSIC)-Universidad Autónoma de Madrid (UAM), Universidad Autónoma de Madrid, 28049 Madrid, Spain and the 3Department of Genome Science, Genome Research Institute, University of Cincinnati, Cincinnati, Ohio 45221

Gαq-coupled G protein-coupled receptors (GPCR) mediate the actions of a variety of messengers that are key regulators of different cellular functions. These receptors can regulate a highly interconnected network of biochemical routes that control the activity of several members of the mitogen-activated protein kinase (MAPK) family. The ERK5 MAPK has been shown to be activated by Gαq-coupled GPCR via unknown mechanisms. We find that the atypical protein kinase C (PKCζ), previously reported to interact with the ERK5 activator MEK5 and to be involved in epidermal growth factor-mediated ERK5 stimulation, plays a crucial role in the activation of the ERK5 pathway by Gαq-coupled GPCR. Stimulation of ERK5 by Gαq-coupled GPCR is abolished upon pharmacological inhibition of PKCζ as well as in embryonic fibroblasts obtained from PKCζ-deficient mice. Both PKCζ and MEK5 associate to Gαq upon activation of GPCR, thus forming a ternary complex that seems essential for the activation of ERK5. These data put forward a novel function of Gαq as a scaffold protein involved in the modulation of the ERK5 cascade by GPCR that could be relevant in Gαq-mediated physiological functions.

The activation of the mitogen-activated protein kinase (MAPK) family superfamily plays an important role in a wide variety of signaling pathways involved in embryogenesis, cell proliferation, differentiation, migration, apoptosis, and gene expression. The MAPK superfamily includes the well-known extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK1–3), and p38 (α, β, γ, and δ) families.

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the ability to interact with PLCβ (Goα12, Q209L/R256A/T257A (Q209L-AA)) was provided by Dr. Richard Lin (Stony Brook University, NY). The cDNAs encoding HA-ERK5, GST-MEK5, GST-MEK5ΔPB1, GST-PKCζ/Y261, HA-PKCζ, and HA-PKCa and the purification of recombinant full-length His-PKCζ have been previously described by our laboratories (10). The Goα12, Goα13, and Goαq constructs were purchased from the Missouri S&T cDNA Resource Center. Goαq recombinant protein, purified from baculovirus-infected Sf9 insect cells, was kindly provided by Dr. Elliot Ross (University of Texas Southwestern Medical Center, Dallas, TX). GST-MEK5 recombinant protein was purchased from Abnova (Walnut, CA). COS-7 cells were from the American Type Culture Collection (Manassas, VA), and the NIH 3T3 fibroblasts expressing ~20,000 human m1-muscarinic receptors per cell, designated 3T3-m1 cells, were kindly provided by J. S. Gutkind (National Institutes of Health, Bethesda, MD). Culture media and Lipofectamine were kindly provided by J. S. Gutkind (National Institutes of Health, Bethesda, MD). Culture media and Lipofectamine were purchased from Invitrogen. Pertussis toxin was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). The affinity-purified rabbit polyclonal antibodies Goαq1/1 (C19), Goα1 (K20), Goα1 (S-20), Goα1 (I-20), hemagglutinin (HA) (Y-11), or PKCζ (C-20), as well as the mouse monoclonal antibody (H1) raised against the carboxy terminus of PKCζ and the affinity-purified rabbit polyclonal antibody against GST (Z5), were all purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal 12CA5 anti-HA antibody was from Roche Applied Science. The rabbit polyclonal antibody that recognizes ERK5 was from Upstate Biotech Millipore (Lake Placid, NY). The MEK5 polyclonal antibody was purchased from Abcam (Cambridge, UK). Polyclonal C-16 and C-14 antibodies that recognize ERK1 and ERK2 were obtained from Santa Cruz Biotechnology. The anti-phospho-ERK1/2 polyclonal antibody was from Cell Signaling Technologies (Beverly, MA). Mouse monoclonal anti-His tag clone HIS1, EGF, sphingosine 1 phosphate, and carbachol were obtained from Sigma. Different anti-phospho-ERK5 antibodies were purchased from Invitrogen, Abcam, Cell Signaling, Santa Cruz Biotechnology, or Upstate Biotech Millipore. The Src inhibitor PP2 and the EGF receptor-specific tyrosine kinase inhibitor AG1478 were obtained from Calbiochem. Myristoylated PKCζ pseudosubstrate peptide (Myr-SIYRGGARRWRKL) was obtained from BIOSOURCE (Camarillo, CA). G protein-Sepharose and ProBond resins were obtained from Invitrogen. Pertussis toxin was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). All other reagents were of the highest commercially available grades.

**Cell Culture and Treatment**—COS-7 and NIH 3T3-ml cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich) or newborn serum (Invitrogen), respectively, at 37 °C in a humidified 5% CO2 atmosphere. Mouse embryonic fibroblasts (MEFs) obtained from wild-type or PKCζ−/− mice were cultured as described previously (18). The desired cell type was stimulated with carbachol (10 μM) or sphingosine-1-phosphate (100 nm) at 37 °C in serum-free Dulbecco’s modified Eagle’s medium during the indicated time periods. The cells were serum-starved for 5–6 h before ligand addition to minimize basal kinase activity. Treatments with the Src inhibitor PP2 (10 μM), AG1478 (250 nm), the PLCβ inhibitor U73122 (10 μM), or the PKCζ pseudosubstrate inhibitor (10 μM) were initiated 30 min before agonist stimulation. For the inactivation of Gi, proteins, cells were pretreated with pertussis toxin (100 ng/ml) for 16 h. COS-7 or 3T3 cells (70–80% confluent monolayers in 60- or 100-mm dishes) were transiently transected with the desired combinations of cDNA constructs using the Lipofectamine Plus method, following the manufacturer’s instructions. Empty vector was added to keep the total amount of DNA per dish constant. Assays were performed 48 h after transfection. Transient expression of the desired proteins was confirmed by immunoblot analysis of whole-cell lysates using specific antisera, as described below.

**Determination of MAPK Stimulation**—The activation state of ERK1/2 and ERK5 was measured by Western blot analysis of cell lysates by using anti-phospho-ERK1/2 (1:500) as reported previously (19) or anti-ERK5 (1:500) antibodies, respectively. In the latter case, the stimulation of ERK5 can be detected by the presence of a band with slower electrophoretic mobility that represents the active, phosphorylated form of the protein (20) or by using specific anti-phospho ERK5 antibodies. To obtain cell lysates, cells were washed with ice-cold phosphate-buffered saline buffer plus 1 mM sodium orthovanadate and subsequently solubilized in lysis buffer (50 mm Tris-HCl, 150 mm NaCl, 1% (w/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 1 mM NaF, supplemented with 1 mM sodium orthovanadate plus a mixture of protease inhibitors). Lysates were resolved by 6–10% SDS-PAGE and subjected to immunoblot analysis as described (19). Bands were quantified by laser-scanner densitometry, and the amount of phosphorylated ERK1/2 or phosphorylated ERK5 protein was normalized to the amount of the total ERK1/2 or ERK5 protein, as assessed by the specific antibodies. Statistical analysis was performed using the two-tailed Student’s t test, as indicated.

**Immunoprecipitation**—Immunoprecipitation assays of co-transfected proteins were performed 48 h after transfection. Cells were scraped and washed twice with ice-cold phosphate-buffered saline, solubilized in 500 μl/100-mm dish of radioimmunoprecipitate buffer (50 mm Tris, pH 7.5, 150 mm NaCl, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% SDS) supplemented with a mixture of protease inhibitors. The lysates were clarified by centrifugation, and an aliquot (30 μl) was used to assess protein expression levels. The immunoprecipitation reactions were performed by incubating the supernatants with 1 mg/ml bovine serum albumin and the specific antibodies for HA (12CA5, 4 μg), Goαq (C19, 2 μg), PKCζ (H1, 0.6 μg), or GST (Z5, 2 μg) at 4 °C overnight followed by reincubation with protein G-Sepharose for 1 h, as reported previously (21). For immunoprecipitation of endogenous proteins, 80% confluent monolayers from two 100-mm dishes of cultured cells were used. Cell lysates were tested for protein expression by using the required specific antibodies. Additionally, to identify MEK5 interaction partners, lysates from cells expressing GST-MEK5 (or GST alone as a negative control) were subjected to GST pulldown assays with glutathione-Sepharose 4B as reported previously (21). All blots were developed using the chemiluminescence method (ECL, Amersham Biosciences). When required, bands were quantified by laser-scanner densitometry, and the amount of co-precipitated protein was normalized to the amount of the immunoprecipitated protein, as
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FIGURE 1. ERK5 pathway activation by Gαq-coupled GPCR. A, NIH 3T3-m1R cells, stably expressing the human m1-muscarinic acetylcholine receptor, were incubated with 10 μM carbachol for the indicated times, and endogenous ERK5 activation was determined with an antibody that recognizes both the phosphorylated (P-ERK5) and the unphosphorylated forms of ERK5 and analyzed as detailed under “Experimental Procedures.” The band of slower electrophoretic mobility corresponds to the stimulated kinase. Band densities were measured by laser-scanner densitometry, and data were expressed as the percentage of activated kinase (P-ERK5) versus total ERK5. Data are mean ± S.E. of 3 independent experiments. *, p < 0.05 when compared with 0 min. B, NIH 3T3-m1 cells were incubated with the PLCβ inhibitor U73122 (10 μM) or vehicle prior to stimulation with carbachol. The pattern of ERK5 activation by carbachol is not affected by this inhibitor (upper panel), whereas ERK1/2 stimulation is clearly impaired (lower panel). C, NIH 3T3 cells were transiently transfected with a plasmid encoding HA-tagged ERK5 and with constitutively active Gαq mutants able (Gαq, R183C) or unable (Gαq, Q209L-AA) to interact with the Gαq-effector PLCβ. Then, HA-ERK5 was immunoprecipitated (IP) and ERK5 activation was assessed with an ERK5-phosphospecific antibody. The normalized fold stimulation of ERK5 activity versus control conditions is indicated above the representative blot. Gαq and HA-ERK5 expression was monitored by immunoblot analysis (WB) of cell lysates (lower panel). D, endogenous ERK5 activation in 3T3-m1R cells is induced upon overexpression of either Gαq, R183C or Gαq, Q209L-AA. Migration of unphosphorylated and phosphorylated forms of ERK5 or ERK1/2 and of molecular weight markers is indicated in all panels. Blots are representative of at least 3 independent experiments.

Assessed by the specific antibodies. Statistical analyses were performed using the two-tailed Student’s t test, as indicated.

Protein Interaction Assays—Purified recombinant Gαq (10–20 nm) was incubated at 4 °C with purified His-PKCζ (20 nm) or GST-MEK5 (100 nm) fusion proteins (or GST 100 nm as a negative control) in a final volume of 100 μl of binding buffer (50 mM Tris-HCl, pH 7.9, 70 mM NaCl, 0.6 mM EDTA, 0.01% Lubrol plus a mixture of protease inhibitors). Subsequently, ProBond (for His PKCζ) or glutathione- Sepharose 4B (for GST-MEK5) resins was added for 2 h at 4 °C, after which the affinity matrix was pelleted and washed four times with 500 μl of ice-cold binding buffer (in the presence of 10 mM imidazole in experiments involving His-PKCζ). Proteins retained on the matrix were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Western blot analysis was then performed with the anti-Gαq (C-19, 1:1000), anti-histidine (1:1000), or anti-GST (1:500) antibodies, depending on the experiment.

RESULTS

Stimulation of Gαq-coupled GPCR Promotes ERK5 Activation in NIH 3T3 Cells—Previous studies indicated that GPCR that can couple to the Gαq family of heterotrimeric G proteins, such as m1-muscarinic and thrombin receptors, were able to promote ERK5 activation in COS-7 or NIH 3T3 cells (15, 17). To explore the mechanisms involved, NIH 3T3 cells stably expressing the human m1-muscarinic acetylcholine receptor (NIH 3T3-m1R) (22) were stimulated with carbachol for different periods of time. This agonist promoted a clear, time-dependent increase in endogenous ERK5 activation (Fig. 1A) that can be detected by immunoblot analysis with an ERK5 antibody by the appearance of a band of slower electrophoretic mobility (corresponding to the phosphorylated, stimulated kinase), which comigrated with the band detected with different ERK5-phosphospecific antibodies (supplemental Fig. S1). Because the latter method was, in our hands, less sensitive (supplemental Fig. S1, lower panel), the band-shift method was routinely used to assess ERK5 stimulation.

Activation of ERK5 by different mitogens may involve Src tyrosine kinase (9) and can also be triggered by EGF (10). However, stimulation of ERK5 by muscarinic agonists was not affected in the presence of the Src inhibitor PP2 (supplemental Fig. S2A) or the EGF receptor tyrosine kinase inhibitor AG1478 (supplemental Fig. S2B). On the other hand, ERK5 activation by carbachol was not affected by the presence of the PLCβ inhibitor U73122, whereas ERK1/2 stimulation was markedly decreased (Fig. 1B). Moreover, expression of a GTPase-deficient, constitutively active Gαq mutant (GαqR183C) mimicked ERK5 activation by Gαq-coupled GPCR in NIH 3T3-m1R cells, and the same was true for the other constitutively active construct (Gαq, Q209L-AA), previously shown to be unable to interact with the known Gαq effector PLCβ (Fig. 1, C and D) (23). Overall, these data suggested that Gαq-coupled GPCR trigger the stimulation of the ERK5 cascade by biochemical routes involving Gαq but not its classical effector PLCβ, nor cytoplasmic tyrosine kinases nor EGF receptor transactivation.

PKCζ Is Required for ERK5 Activation by Gαq-coupled GPCR—It has been previously shown that the atypical PKC isoform PKCζ interacts with MEK5 in a growth factor-induc-
Scaffold Function of $G_{\alpha_q}$ in ERK5 Signaling

The fact that the $G_{\alpha_q}$/PKCζ association was markedly increased (from 2.5- to 4-fold over control, Fig. 3A) when expressing the active $G_{\alpha_q}$ mutant when compared with wild-type $G_{\alpha_q}$ subunit suggested that $G_{\alpha_q}$/PKCζ co-immunoprecipitation would be regulated upon $G_{\alpha_q}$ protein activation by GPCR, as is the case for other $G_{\alpha_q}$ protein subunit effectors. Consistently, carbachol stimulation of $G_{\alpha_q}$-coupled m1-muscarinic receptors promoted a clear increase in the association of either co-transfected (not shown) or endogenous PKCζ and $G_{\alpha_q}$ (3.1–5-fold over basal conditions at 5 min of agonist challenge, Fig. 3C), indicating that the functional interaction between these proteins takes place in physiological conditions upon activation of GPCR.

To determine whether the $G_{\alpha_q}$/PKCζ association was direct or mediated by other cellular proteins, we performed an “in vitro” binding assay using purified recombinant $G_{\alpha_q}$ and a His-PKCζ fusion protein. Fig. 3D shows a clear, direct interaction between both proteins.

$G_{\alpha_q}$ Interacts with MEK5—In agreement with the notion that the $G_{\alpha_q}$/PKCζ pathway is relevant for the activation of the ERK5 cascade, we were able to detect the presence of MEK5, the well known PKCζ interactor and upstream activator of ERK5 (7, 10), in $G_{\alpha_q}$ immunocomplexes (Fig. 4A) upon co-expression of active $G_{\alpha_q}$, HA-PKCζ, and GST-MEK5 constructs in COS-7...
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In principle, the observed association between Gαq and MEK5 could be either direct or mediated by endogenous PKCζ, able to interact with both proteins. To discriminate between these possibilities, we carried out similar co-immunoprecipitation assays in MEFs obtained from PKCζ knock-out mice (Fig. 5B). Under these conditions, a clear association between Gαq and MEK5 was also observed, indicating that this process does not strictly require PKCζ. Consistently, an in vitro binding assay using purified recombinant Gαq and a GST-MEK5 fusion protein shows a clear, direct interaction between both proteins (Fig. 5C). However, it is interesting to note that in the cell milieu, active and wild-type Gαq display a similar association to MEK5 in the absence of PKCζ (Fig. 5B), suggesting that PKCζ may facilitate the binding of MEK5 to active Gαq. In agreement with these observations, we found that MEK5 was also able to interact with both proteins, as observed in MEFs transfected with co-expressed Gαq/PKCζ and Gαq/MEK5 association (Fig. 4A).

FIGURE 3. Gαq associates with PKCζ. A, COS-7 cells were transiently transfected with the indicated combinations of plasmids encoding HA-tagged-PKCζ wild-type Gαq (WT) or a constitutively active Gαq mutant (Gαq R183C). Expression of the different proteins was confirmed by immunoblot analysis of cell lysates (lower panel). Cell lysates were subjected to immunoprecipitation (IP) with an anti-HA monoclonal antibody or an anti-Gαq polyclonal antibody as indicated. Immunoprecipitates were resolved by SDS-PAGE, and the presence of PKCζ and Gαq in the immunocomplexes was determined by Western blot analysis with specific antibodies. To compare the association of PKCζ with WT Gαq and Gαq R183C, band quantification was normalized by total HA-PKCζ (upper panel) or total Gαq (middle panel), and the PKCζ/Gαq association was taken as control conditions. Data are mean ± S.E. of 3–4 independent experiments. Representative blots are shown. B, COS-7 cells were transiently transfected with the indicated combinations of plasmids encoding HA-tagged-PKCζ WT Gαq or a constitutively active Gαq mutant unable to activate and interact with PLCβ (Gαq Q209L-AA). Immunoprecipitation and SDS-PAGE procedures were carried out as in panel A. C, Stimulation of Gαq-coupled GPCR promotes the association between endogenous Gαq and PKCζ proteins. NIH 3T3-m1R cells were challenged with 10 μM carbachol for different times as in Fig. 1A, and endogenous Gαq/PKCζ co-immunoprecipitation was assessed with specific antibodies. The normalized fold-stimulation of co-immunoprecipitation versus basal conditions is indicated above the representative blot. In 3 independent experiments, an average stimulation of association of 3.44 ± 1.5- and 2.06 ± 0.36-fold over basal at 5 and 10 min after carbachol challenge, respectively, was obtained. D, direct interaction between Gαq and PKCζ. Purified recombinant Gαq (20 nm) was incubated in the absence or presence of purified His-tagged PKCζ fusion protein (20 nm), and the mixture was subjected to affinity chromatography using a ProBond nickel resin. Proteins retained in the matrix were resolved by SDS-PAGE, and the presence of His-PKCζ or Gαq was analyzed by immunoblot analysis with specific antibodies, including the input (20%) of Gαq as a control. This experiment was repeated twice with similar results.

Surprisingly, MEK5 co-immunoprecipitated with Gαq even in the absence of co-expressed PKCζ, whereas the presence of extra MEK5 decreased the extent to which PKCζ associated with Gαq (Fig. 4A). The same was observed when PKCζ immunocomplexes were analyzed in similar assays using a HA immunoprecipitating antibody (Fig. 4B). However, the presence of extra Gαq does not reduce, but appears to even enhance Gαq/PKCζ interaction (Fig. 4B). To further characterize such Gαq/MEK5 functional interaction, we performed co-immunoprecipitation assays using an anti-GST-MEK5 polyclonal antibody. Gαq/MEK5 association was clearly detected and markedly increased (4-fold over control, Fig. 5A) when expressing an active Gαq mutant when compared with wild-type Gαq subunit, consistent with a stimulus-dependent interaction.
with this notion, stimulation of G\(\alpha\)-coupled m1-muscarinic receptors promoted a clear increase in the association of endogenous MEK5 and G\(\alpha\)q (Fig. 5D) with a time course slightly retarded when compared with the G\(\alpha\)q/PKC\(\beta\)/H9256 association (Fig. 3C).

**Dynamic G\(\alpha\)q-PKC\(\beta\)/H9256-MEK5 Complexes Are Essential for ERK5 Activation by GPCR**—Overall, our data suggested that by recruiting both PKC\(\beta\)/H9256 and MEK5 to the same macromolecular complex, G\(\alpha\)q would lead to ERK5 activation by GPCR. In such a model, receptor stimulation would promote association of activated G\(\alpha\)q to PKC\(\beta\), which would in turn facilitate MEK5 binding to G\(\alpha\)q and the subsequent formation of a PKC\(\beta\)-MEK5 complex, resulting in ERK5 stimulation (Fig. 6A).

The association between PKC\(\beta\) and MEK5 has been reported to involve PB1 domains in both proteins (11, 30), and the MEK5ΔPB1 mutant (which does not have a functional PB1 domain) has been shown to be unable to interact with PKC\(\beta\) upon EGF stimulation (10). We thus used this mutant to further dissect the dynamics of the G\(\alpha\)q-PKC\(\beta\)-MEK5 complexes.

Consistent with the notion that G\(\alpha\)q associates to MEK5 independently of PKC\(\beta\), MEK5ΔPB1 was detected in G\(\alpha\)q immunocomplexes (Fig. 6B, lane 4). Interestingly, although wild-type MEK5 appears to "displace" PKC\(\beta\) from G\(\alpha\)q, this effect was not observed with MEK5ΔPB1, which even increases the extent of PKC\(\beta\)/G\(\alpha\)q association (Fig. 6B, compare lanes 2, 3, and 6), as predicted by our model. Accordingly, Fig. 6C shows that when analyzing PKC\(\beta\) immunocomplex in such experimental conditions, the lack of association between MEK5ΔPB1 and PKC\(\beta\) (Fig. 6C, lane 5) can be “rescued” in the presence of extra G\(\alpha\)q (Fig. 6C, lane 3) in line with a scaffold role for G\(\alpha\)q in this process. The inability of MEK5ΔPB1 to associate to PKC\(\beta\) would stabilize the usually transient MEK5-G\(\alpha\)q-PKC\(\beta\) complex and therefore block activation of GPCR-mediated ERK5. Consistent with this notion, overexpression of MEK5ΔPB1 completely abrogates carbachol-mediated endogenous ERK5 stimulation in cells (Fig. 6D). The same effect is observed upon expression of an independent GST-PKC\(\beta\) PB1 construct, known to inhibit PKC\(\beta\)/MEK5 association (Fig. 6E).
**Scaffold Function of Gq in ERK5 Signaling**

**FIGURE 5.** Gq associates with MEK5. A, COS-7 cells were transiently transfected with the indicated combinations of plasmids encoding GST-MEK5, GST, wild-type Gq (WT), or a constitutively active Gq mutant (GqR183C). Cell lysates were subjected to pulldown (PD) with glutathione-Sepharose 4B resin as detailed under “Experimental Procedures.” Proteins retained in the matrix were resolved by SDS-PAGE, and the presence of GST, GST-MEK5, and Gq was determined by Western blot analysis with specific antibodies. To compare the association of MEK5 to WT Gq and GqR183C, blot bands were quantified and normalized to total GST-MEK5. The MEK5/WT Gq association was taken as the control condition. Data are mean ± S.E. of 3–4 independent experiments. Representative blots are shown. B, MEFs obtained from PKCζ−/− mice were transiently transfected with the indicated combinations of plasmids, and cell lysates were analyzed using a pulldown assay as in the previous panel. C, direct interaction between Gq and GST-MEK5. Purified recombinant Gq (10 nM) was incubated in the absence or presence of purified GST-MEK5 fusion protein (100 nM), and the mixture was subjected to affinity chromatography using a glutathione-Sepharose 4B resin. Proteins retained in the matrix were resolved by SDS-PAGE, and the presence of GST, GST-MEK5, and Gq was determined by Western blot analysis with specific antibodies. Data are mean ± S.E. of 3 independent experiments.

**DISCUSSION**

In this report, we show that PKCζ plays a key role in the activation of the ERK5 pathway by Gq-coupled GPCR in epithelial cells and that Gq displays a scaffold-like role in this process by independently interacting with both PKCζ and MEK5 (see model in Fig. 6A). This is, to our knowledge, the first demonstration that G protein α-subunits can serve as a scaffold, bringing two proteins into close proximity and proper relative orientation to promote their transient interaction and subsequent stimulation of a signal transduction cascade. Several lines of evidence support this model. First, ERK5 stimulation by carbachol does not appear to require the activity of EGF receptors or cytosolic tyrosine kinases, known to participate in ERK5 activation in response to different mitogens (7, 9, 10), thus indicating that potential GPCR/EGF receptor transactivation mechanisms (31) are not involved. Second, overexpression of a constitutively active Gq subunit mutant promotes ERK5 stimulation “per se,” independently of its ability to interact with the classical Gq effector PLCβ. Third, stimulation of ERK5 by Gq-coupled GPCR is blocked by PKCζ pharmacological inhibitors and is absent in MEFs derived from PKCζ-deficient mice. Fourth, Gq (and not other Gα subunits) associates with PKCζ in cells, and co-immunoprecipitation of these endogenous proteins can be promoted upon Gq-coupled activation of GPCR. Moreover, a direct Gq/PKCζ interaction can be observed using purified proteins. Fifth, Gq, PKCζ, and MEK5 (the upstream ERK5 activator) appear to form dynamic complexes to trigger ERK5 activation, involving direct interactions between Gq and both PKCζ and MEK5 and a PKCζ/MEK5 association mediated by their respective PB1 domains.

Previous reports have shown that GPCR able to couple to Gq proteins can regulate the activity of ERK5 in epithelial cells. This process was mimicked by expression of activated forms of Gq (but not of Gα or Gαi or upon overexpression of βγ subunits) and was independent of the activation of Ras or Rho signaling pathways (16, 17), although the mechanisms linking Gq to ERK5 were not identified. The primary downstream actions of Gq have been tied to activation of its classic effector PLCβ. However, because we find that pharmacological inhibition of PLCβ does not affect ERK5 activation by Gq-coupled GPCR and because the expression of an activated form of Gq that does not interact with PLCβ (23) is still able to stimulate ERK5, we demonstrate that PLCβ is not involved in this pathway. Instead, we show that the functional interactions of Gq...
with PKCζ and MEK5 underlie its ability to trigger the ERK5 cascade.

Consistent with the notion that PKCζ is a novel Gαq effector, agonists acting through Gαq-coupled GPCR such as angiotensin II, phenylephrine, platelet-activating-factor, or thromboxane A2 have been shown to promote PKCζ translocation and activation in several cell types (24–26, 32, 33), and PKCζ has been suggested to participate in GPCR-mediated control of cell proliferation (25–27), eosinophil degranulation (32), or smooth muscle cell adhesion, spreading, and hypertrophy (25). Several authors have suggested a role for PKCζ in ERK1/2 activation by GPCR (34, 35), although another recent report indicates that inhibition of PKCζ in adult cardiomyocytes has no effect in ERK1/2 activation by Gαq-coupled GPCR (36). However, this is the first report to show a direct link between Gαq and PKCζ and to establish a role for such association in the stimulation of the ERK5 MAPK cascade by GPCR.

The Btk and Csk kinases or the nucleotide exchange factor, p63RhoGEF, have also been reported to be PLCζ-independent Gαq effectors (23, 37, 38). Besides that effector diversity, our report puts forward a novel scaffold role for Gαq in ERK5 signaling based on its ability to directly interact with both PKCζ and MEK5. Our data suggest that activation of GPCR would first promote Gαq/PKCζ association followed by direct binding or MEK5 to Gαq, which would in turn favor PKCζ/MEK5 interaction through their respective PB1 domains (39, 40), leading to ERK5 activation (see model in Fig. 6).

**Figure 6.** The formation of dynamic Gαq–PKCζ–MEK5 complexes is essential for ERK5 activation. A, model for the proposed dynamics of the Gαq–PKCζ–MEK5 complexes. See "Results" for detailed explanation and discussion. B, the MEK5ΔPB1 mutant stabilizes PKCζ/Gαq association and impairs the formation of the MEK5–PKCζ complex. COS-7 cells were transiently transfected with the indicated combinations of plasmids encoding HA-tagged–PKCζ, the constitutively active Gαq mutant (GαqR183C), wild-type GST-MEK5, and the GST-MEK5ΔPB1 mutant. Cell lysates were subjected to immunoprecipitation (IP) with an anti-Gαq polyclonal antibody. Immunoprecipitates were resolved by SDS-PAGE, and the presence of PKCζ, MEK5, and Gαq in the immunocomplexes was determined by Western blot (WB) analysis with specific antibodies. To compare the association of HA–PKCζ with GαqR183C in the presence of MEK5WT or MEK5ΔPB1, band densities were normalized to total Gαq. Data are mean ± S.E. of 3 independent experiments. A representative blot is shown. C, the presence of extra Gαq rescues the lack of association between PKCζ and the GST-MEK5ΔPB1 mutant. Cell lysates as in panel B were subjected to immunoprecipitation with an anti-HA monoclonal antibody to analyze PKCζ complexes. D and E, effect of the expression of GST-MEK5ΔPB1 or the independent PKCζ PB1 domain on ERK5 activation by Gαq-coupled GPCR. NIH 3T3 m1R cells were transiently transfected with GST-MEK5ΔPB1 (D) or GST–PKCζ PB1 (E) and GST as a control. 48 h after transfection, cells were challenged with 10 μM carbachol for different times as in Fig. 1A. Endogenous ERK5 activation was then determined and expressed as a mean ± S.E. of 2–3 independent experiments. *, p < 0.05, analysis of variance followed by Fischer’s least significant difference.
tent with this notion, the time course of endogenous MEK5/Goαq co-immunoprecipitation is slightly delayed when compared with that of Goαq and PKCζ. The use of the MEK5ΔPB1 mutant, reported to be unable to interact with PKCζ (10), and of PKCζ-deficient cells has provided further insight into the dynamics of these complexes. On the one hand, the fact that the MEK5ΔPB1 mutant can associate to Goαq indicates that endogenous PKCζ does not act as a "bridge" between these two proteins, also showing that the MEK5 PB1 domain does not play a role in the interaction with Goαq. The ability of MEK5 to directly bind to Goαq is further established using purified proteins. Interestingly, although MEK5 and Goαq can co-immunoprecipitate in PKCζ-deficient MEFs, such association is not sufficient to trigger ERK5 activation, nor is it increased upon expression of activated when compared with wild-type Goαq, suggesting that the activated Goαq-PKCζ complex is the preferred recruiting site for MEK5.

Finally, it is worth noting that the presence of the MEK5ΔPB1 mutant (contrary to the wild-type kinase) does not displace Goαq from PKCζ and blocks GPCR-mediated ERK5 stimulation. This suggests that the stabilization of a "non-productive" Goαq-mutant MEK5-PKCζ complex is taking place instead of the transient ternary complex that would normally lead to MEK5/PKCζ association (see model in Fig. 6A). Consistent with the scaffold role of Goαq in the process, the presence of extra Goαq rescues the lack of association between PKCζ and MEK5ΔPB1.

Scaffold proteins bring together specific kinases or other components of signaling cascades for selective activation and localization. Both MEK5 and PKCζ have been shown to bind scaffold proteins such as p62 or Par-6 (12, 30). In fact, p62 has lead to MEK5/PKCζ instead of the transient ternary complex that would normally occur. The Lck-associated adaptor (LAD) may be responsible for facilitating MEKK2/MEK5 binding in the presence of extra Goαq. The ability of MEK5 to directly associate (see model in Fig. 6A). Consistent with the scaffold role of Goαq in the process, the presence of extra Goαq rescues the lack of association between PKCζ and MEK5ΔPB1.

Scaffold proteins bring together specific kinases or other components of signaling cascades for selective activation and localization. Both MEK5 and PKCζ have been shown to bind scaffold proteins such as p62 or Par-6 (12, 30). In fact, p62 has been described as an important factor in MEK5/ERK5-mediated activation of the transcription factors MEF2C and Sap1a following EGF stimulation (14). Moreover, p62 knockdown can block nerve growth factor-mediated activation of ERK5 (13).

Lamark et al. (14) have postulated that the interaction between PKCζ and MEK5 is stabilized by p62. In this context, our data strongly suggest that Goαq plays a similar scaffold role for PKCζ and MEK5 in GPCR-mediated ERK5-mediated activation. Interestingly, other routes of MEK5/ERK5 stimulation also appear to require adaptor proteins. The Lck-associated adaptor (LAD) may be responsible for facilitating MEK2/MEK5 binding and recruitment to the growth factor receptor complex (41), and Gab-1 participates in leukemia inhibitory factor-mediated ERK5 modulation (reviewed in Ref. 7).

It has been recently suggested that activated Goαq subunits would display specific membrane orientations that would unmask binding surfaces ready for the docking of structurally different effectors (38). Although a scaffold role for Goαq has not been reported to our knowledge, it is worth noting that different regions of this protein can specifically associate with the distinct Dbî homology (DH) and RGS homology (RH) domains of the Goαq effector p63RhoGEF (38). Moreover, recent studies have shown the formation of RGS-Goαq-p63RhoGEF or and RGS-Goαq-GRK2 ternary complexes (42), suggesting the occurrence of two independent binding surfaces in Goαq. In vitro studies have also shown the ability of Goαq to simultaneously bind to both PLCζ and phosphatidylinositol 3-kinase (43). Our preliminary data indicate that both Goαq/PKCζ and Goαq/MEK5 association are inhibited in the presence of the GRK2 RH domain, which has been reported to interact with Goαq and block the interaction with its effector PLCζ (21, 44, 45). However, the Goαq sites required for the interactions with both PKCζ and MEK5 appear to be different from those involved in PLCζ binding because a Goαq mutant that is unable to interact with the latter promotes ERK5 activation and readily associates to PKCζ. The detailed architecture of the Goαq-PKCζ-MEK5 complexes and the mechanisms underlying their spatial and temporal assembly await further investigation.

ERK5 has been implicated in the regulation of many cellular functions, such as differentiation, proliferation, migration, survival, and cardiovascular development (2, 7, 46, 47). The triggering of such ERK5 cascade by association of Goαq-coupled GPCR may thus play relevant roles in several cell types and physiological settings, which are being actively investigated in our laboratory. Finally, whether this novel Goαq/PKCζ interaction may also be involved in modulating signaling pathways downstream of PKCζ other than the ERK5 cascade (39) upon activation of Goαq-coupled GPCR also deserves to be explored in future studies.

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REFERENCES
1. Turjanski, A. G., Vaqué, J. P., and Gutkind, J. S. (2007) Oncogene 26, 3240–3253
2. Goldsmith, Z. G., and Dhanasekaran, D. N. (2007) Oncogene 26, 3122–3142
3. Rozengurt, E. (2007) J. Cell. Physiol. 213, 589–602
4. May, L. T., and Hill, S. J. (2008) Int. J. Biochem. Cell Biol. 40, 2013–2017
5. Gutkind, J. S. (2000) Sci. STKE 40, rel1
6. Hayashi, M., and Lee, J. D. (2004) J. Mol. Med. 82, 800–808
7. Wang, X., and Tournier, C. (2006) Cell Signal 18, 753–760
8. Ohara, Y., and Nakahata, N. (2010) Mol. Pharmacol. 77, 10–16
9. Sun, W., Kesavan, K., Schafer, B. C., Garrington, T. F., Ware, M., Johnson, N. L., Gelfand, E. W., and Johnson, G. L. (2001) J. Biol. Chem. 276, 5093–5100
10. Diaz-Meco, M. T., and Moscat, J. (2001) Mol. Cell. Biol. 21, 1218–1227
11. Sumimoto, H., Kamakura, S., and Ito, T. (2007) Sci. STKE 401, re6
12. Moscat, J., and Diaz-Meco, M. T. (2000) EMBO reports 1, 399–403
13. Geetha, T., and Wooten, M. W. (2003) J. Biol. Chem. 278, 4730–4739
14. Lamark, T., Perander, M., Outzén, H., Kristiansen, K., Øvervatn, A., Michaelsen, E., Bjorkoy, G., and Johansen, T. (2003) J. Biol. Chem. 278, 34568–34581
15. Marinissen, M. J., Chiariero, M., Pallante, M., and Gutkind, J. S. (1999) Mol. Cell. Biol. 19, 4289–4301
16. Fukuhara, S., Marinissen, M. J., Chiariero, M., and Gutkind, J. S. (2000) J. Biol. Chem. 275, 21730–21736
17. Marinissen, M. J., Servitja, J. M., Offermanns, S., Simon, M. L., and Gutkind, J. S. (2003) J. Biol. Chem. 278, 46814–46825
18. Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J. F., Camacho, F., Diaz-Meco, M. T., Rennert, P. D., and Moscat, J. (2001) Mol. Cell 8, 771–780
19. Elorza, A., Sarnago, S., and Mayor, F., Jr. (2000) Mol. Pharmacol. 57, 778–783
20. Xu, B. E., Stippec, S., Lenertz, L., Lee, B. H., Zhang, W., Lee, Y. K., and Cobb, M. H. (2004) J. Biol. Chem. 279, 7826–7831

4 C. García-Hoz, G. Sánchez, C. Ribas, and F. Mayor, manuscript in preparation.
21. Mariggiò, S., García-Hoz, C., Sarnago, S., De Blasi, A., Mayor, F., Jr., and Ribas, C. (2006) *Cell. Signal.* 18, 2004–2012
22. Crespo, P., Xu, N., Daniotti, I. L., Troppmair, J., Rapp, U. R., and Gutzkind, J. S. (1994) *J. Biol. Chem.* 269, 21103–21109
23. Fan, G., Ballou, L. M., and Lin, R. Z. (2003) *J. Biol. Chem.* 278, 52432–52436
24. Takeishi, Y., Jalili, T., Ball, N. A., and Walsh, R. A. (1999) *Circ. Res.* 85, 264–271
25. Parmentier, J. H., Zhang, C., Estes, A., Schaefer, S., and Malik, K. U. (2006) *Am. J. Physiol. Heart Circ. Physiol.* 291, H1602–H1613
26. Muscella, A., Greco, S., Elia, M. G., Storelli, C., and Marsigliante, S. (2003) *J. Cell Physiol.* 197, 61–68
27. Godeny, M. D., and Sayeski, P. P. (2006) *Am. J. Physiol. Cell Physiol.* 291, C1297–C1307
28. Parmentier, J. H., Smelcer, P., Pavicevic, Z., Basic, E., Idrizovic, A., Estes, A., and Malik, K. U. (2003) *Hypertension* 41, 794–800
29. Spiegel, S., and Milstien, S. (2003) *Nat. Rev. Mol. Cell Biol.* 4, 397–407
30. Moscat, J., Diaz-Meco, M. T., Albert, A., and Campuzano, S. (2006) *Mol. Cell* 23, 631–640
31. Liggert, S. B. (2006) *J. Clin. Invest.* 116, 875–877
32. Kato, M., Yamauchi, T., Tachibana, A., Suzuki, M., Izumi, T., Maruyama, K., Hayashi, Y., and Kimura, H. (2005) *Immunology* 116, 193–202
33. Cogolludo, A., Moreno, L., Bosca, L., Tamargo, J., and Perez-Vizcaino, F. (2003) *Circ. Res.* 93, 656–663
34. Hirai, T., and Chida, K. (2003) *J. Biochem.* 133, 1–7
35. Jiménez, E., and Montiel, M. (2005) *J. Cell Physiol.* 204, 678–686
36. Olson, E. R., Shamhart, P. E., Naugle, J. E., and Meszaros, J. G. (2008) *Hypertension* 51, 704–711
37. Ma, Y. C., and Huang, X. Y. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 12197–12201
38. Lutz, S., Shankaranarayanan, A., Coco, C., Ridilla, M., Nance, M. R., Vettel, C., Baltus, D., Evelyn, C. R., Neubig, R. R., Wieland, T., and Tesmer, J. J. (2007) *Science* 318, 1923–1927
39. Moscat, J., Rennert, P., and Diaz-Meco, M. T. (2006) *Cell Death Differ.* 13, 702–711
40. Hirano, Y., Yoshinaga, S., Ogura, K., Yokochi, M., Noda, Y., Sumimoto, H., and Inagaki, F. (2004) *J. Biol. Chem.* 279, 31883–31890
41. Sun, W., Wei, X., Kesavan, K., Garrington, T. P., Fan, R., Mei, J., Anderson, S. M., Gelfand, E. W., and Johnson, G. L. (2003) *Mol. Cell Biol.* 23, 2298–2308
42. Shankaranarayanan, A., Thal, D. M., Tesmer, V. M., Roman, D. L., Neubig, R. R., Kozasa, T., and Tesmer, J. J. (2008) *J. Biol. Chem.* 283, 34923–34934
43. Golebiewska, U., and Scarlata, S. (2008) *Biophys. J.* 95, 2575–2582
44. Penela, P., Ribas, C., and Mayor, F., Jr. (2003) *Cell. Signal.* 15, 973–981
45. Ribas, C., Penela, P., Murga, C., Salcedo, A., García-Hoz, C., Jurado-Pueyo, M., Aymerich, I., and Mayor, F., Jr. (2007) *Biochim. Biophys. Acta* 1768, 913–922
46. Wang, Y (2007) *Circulation* 116, 1413–1423
47. Nishimoto, S., and Nishida, E. (2006) *EMBO Rep.* 7, 782–786