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Protein Kinase C ζ Interacts with a Novel Binding Region of Gαq to Act as a Functional Effector*

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Guzmán Sánchez-Fernández[‡§¶1], Sofía Cabezudo[‡§1,2], Álvaro Caballero[‡§,2], Carlota García-Hoz[‡§], Gregory G. Tall[‖], Javier Klett[‡], Stephen W. Michnick[**], Federico Mayor, Jr.[‡§3], and Catalina Ribas[‡§4]

From the [‡]Departamento de Biología Molecular and Centro de Biología Molecular "Severo Ochoa," CSIC-UAM, Universidad Autónoma de Madrid, 28049-Madrid, Spain, [§]Instituto de Investigación Sanitaria La Princesa, 29006-Madrid, Spain, [¶]Department of Pharmacology, Max-Planck-Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany, [‖]Departments of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, New York 14642, and [**]Département de Biochimie, Université de Montréal, C.P. 6128, Succursale centre-ville, Montréal, Québec, H3C 3J7 Canada

Heterotrimeric G proteins play an essential role in the initiation of G protein-coupled receptor (GPCR) signaling through specific interactions with a variety of cellular effectors. We have recently reported that GPCR activation promotes a direct interaction between Gαq and protein kinase C ζ (PKCζ), leading to the stimulation of the ERK5 pathway independent of the canonical effector PLCβ. We report herein that the activation-dependent Gαq/PKCζ complex involves the basic PB1-type II domain of PKCζ and a novel interaction module in Gαq different from the classical effector-binding site. Point mutations in this Gαq region completely abrogate ERK5 phosphorylation, indicating that Gαq/PKCζ association is required for the activation of the pathway. Indeed, PKCζ was demonstrated to directly bind ERK5 thus acting as a scaffold between Gαq and ERK5 upon GPCR activation. The inhibition of these protein complexes by G protein-coupled receptor kinase 2, a known Gαq modulator, led to a complete abrogation of ERK5 stimulation. Finally, we reveal that Gαq/PKCζ complexes link Gαq to apoptotic cell death pathways. Our data suggest that the interaction between this novel region in Gαq and the effector PKCζ is a key event in Gαq signaling.

G-protein-coupled receptors (GPCRs)[5] are the largest and most versatile family of transmembrane receptors (1). Particularly, Gq-coupled GPCRs mediate the action of many hormones and neurotransmitters with a paramount role in health and disease. Gαq activates phospholipase C (PLCβ) isoforms, which hydrolyze PIP_2 leading to protein kinase C (PKC) activation and Ca^{2+} mobilization (2). However, a growing body of evidence suggests that alternative effectors underlie additional, PLCβ-independent functions of Gαq. Thus, p63RhoGEF (3) directly binds to Gαq/11 linking GPCRs and RhoA activation. The competition between PLCβ and p63RhoGEF for binding to Gαq indicates the existence of alternative and mutually exclusive Gαq-initiated pathways (4). Indeed, all characterized Gαq effectors have been shown to bind to the same region, which comprises the C-terminal half of the α2 helix (Switch II) together with the α3 helix and its junction with the β5 strand (5). Additionally, the GPCR receptor kinase (GRK) 2 acts as negative regulator of Gαq function by shielding this surface away from effectors (6).

Mitogen-activated protein kinases (MAPKs) are essential downstream targets in G protein pathways. MAPKs control key cellular functions, including proliferation, differentiation, migration and apoptosis, and participate in a number of disease states including chronic inflammation and cancer (7). Recently we have described a novel signaling axis for the activation of ERK5 MAPK by Gq-coupled GPCRs in epithelial cells that is independent of PLCβ and relies on a previously unforeseen role of Gαq as an adaptor protein through direct associations with two novel binding partners, PKCζ and MEK5 (8). Subsequently, this novel activation mechanism for ERK5 was shown to be conserved in cardiac cells and the physiological relevance of the Gq/PKCζ/ERK5 pathway in the development of cardiac hypertrophy programs was established using PKCζ-deficient mice (9). In the present work we have characterized the architecture of the Gαq/PKCζ complex in the context of the ERK5 pathway and determined that a novel interaction region underlies the ability of Gαq to trigger the PKCζ/ERK5 cascade and to promote apoptotic cell death.

Experimental Procedures

Materials—The cDNAs of Gαq, Gαq-R183C, and Gαq-Q209L were kindly provided by Dr. A. Aragay (CSIC, Barce-

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[1] Both authors contributed equally to this work.
[2] Recipients of FPU fellowships from the Spanish Ministry of Education.
[3] To whom correspondence may be addressed: Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid, 28049 Madrid, Spain. Tel.: +34-91-1964626; Fax: +34-91-964420; E-mail: fmayor@cbm.csic.es.
[4] To whom correspondence may be addressed: Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid, 28049 Madrid, Spain. Tel.: +34-91-1964640; Fax: +34-91-964420; E-mail: cribas@cbm.csic.es.

[5] The abbreviations used are: GPCR, G-protein-coupled receptors; PKC, protein kinase C; PI, propidium iodide; PCA, protein-fragment complementation assay; aa, amino acids; PLC, phospholipase C; GTPγS, guanosine 5′-*O*-γ-thio]triphosphate.

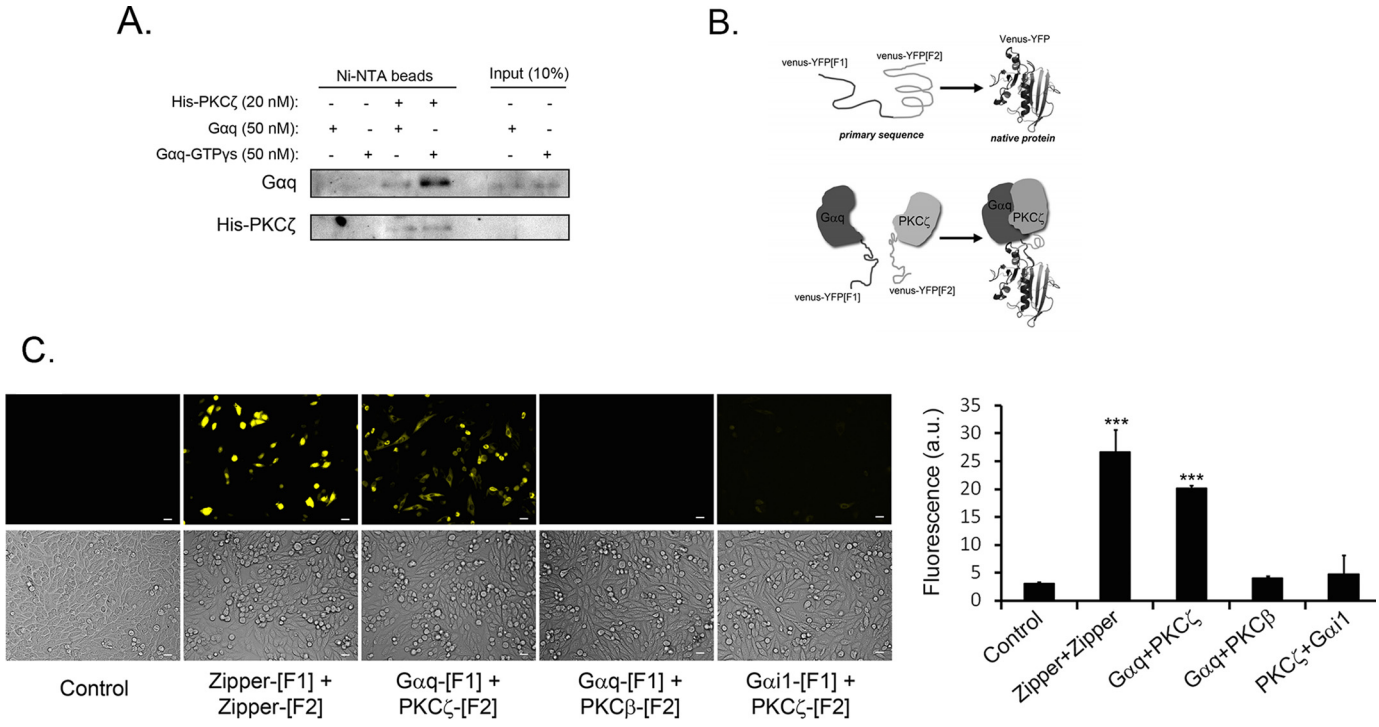

FIGURE 1. **Gαq and PKCζ interaction *in vitro* and in living cells.** *A*, PKCζ preferentially binds the GTPγS loaded form of Gαq. 20 nM of purified His-PKCζ was incubated with purified Gαq or Gαq loaded with GTPγS as detailed under "Experimental Procedures." *B* and *C*, Gαq/PKCζ complex selectivity in living cells. *B*, scheme of the Protein-fragment Complementation Assay (PCA, see "Experimental Procedures"). Fluorescence upon expression of the protein pair in living cells is a measure of the occurrence of an interaction between these proteins. The irreversible nature of fluorescent protein YFP-PCA assays allow for easy trapping and visualization of transient complexes. *C*, CHO-M3 cells were transfected with different pairs of PCA plasmids that express proteins fused to complementary N- and C-terminal fragments of Venus-YFP: Control (PKCζ-Venus YFP[F1] + pcDNA3), Zipper+Zipper (Zipper-Venus YFP[F1] + Zipper Venus YFP[F2]), Gαq + PKCζ (Gαq-Venus YFP[F1] + PKCζ-Venus YFP[F2]), Gαq+PKCβ (Gαq-Venus YFP[F1] + PKCβ-Venus YFP[F2]), PKCζ + Gαi (Gαi-Venus YFP[F1] + PKCζ-Venus YFP[F2]). Representative bright field and YFP images at 40× magnification are shown. Bar length, 25 μm. Fluorometric analysis was performed, and data (mean ± S.E. of three independent experiments) were normalized with respect to control (***, $p < 0.001$, two tailed *t* test).

lona, Spain). The constitutively active Gαq mutant protein that lacks the ability to interact with PLCβ (Gαq Q209L/R256A/T257A) was provided by Dr. Richard Lin (Stony Brook University, New York). The cDNAs encoding HA-PKCζ, GST-MEK5, and HA-ERK5 have been previously described (8). The cDNAs encoding Gαq/Gαi1 chimeras (Gαi-ctGαq, Gαq-ctGαi) were a kind gift from Dr. C. H. Berlot (Weis Center for Research). GRK2 wt and GRK2-D110A were a gift from Dr. J. L. Benovic (Thomas Jefferson University, Philadelphia, PA), GRK2-Y261F and W263D were a gift from Dr. T. Kozasa (University of Illinois at Chicago), the RH domain and RGS2/4 were from Dr. A. de Blasi (University of Rome "Sapienza", Italy), and the PKCζ-PB1 domain was described previously (8). Recombinant GST-ERK5 was obtained from Sigma-Aldrich. Recombinant His_6-PKCζ was provided by Dr. Moscat (Sanford-Burnham Medical Research Institute, La Jolla, CA). and by Dr. James Hastie (Division of Signal Transduction Therapy, School of Life Sciences, MSI/WTB/JBC Complex, University of Dundee, Scotland). PKCζ-targeting and scrambled shRNA were from Sigma-Aldrich.

CHO cells overexpressing the muscarinic M3 acetylcholine receptor, designated CHO-M3 cells, were a kind gift from Dr. A. B. Tobin (University of Leicester, UK). COS-7, HeLa, and HEK293 cells were from the American Type Culture Collection (ATCC, Manassas, VA). Culture medium and Lipofectamine were from Life Technologies Inc. (Gaithersburg, MD). The affinity-purified mouse monoclonal antibody against Gαq was from Abnova (Walnut, CA). The polyclonal antibodies against Gαq (C-19), GRK2 (C-15), ERK1 and ERK2 and GST were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal antibodies against HA tag and Glu-Glu (EE) tag were from Covance. The anti-phospho-ERK5 antibody (p-Thr218/p-Tyr220) was purchased from Invitrogen (Carlsbad, CA). Anti-ERK5 and anti-phospho ERK1/2 antibodies, anti-PKCζ, and anti-cleaved caspase-3 (Asp175) were from Cell Signaling (Beverly, MA). Anti-α-tubulin was from Sigma. The anti-GRK2 antibody that recognizes the N terminus of GRK2 was generated in our laboratory. Protein-G Sepharose was obtained from Invitrogen. Carbachol was from Sigma. All other reagents were of the highest commercially available grades.

Cell Line Culture and Treatments—CHO cells were maintained in αMEM and HeLa, COS-7, and HEK293 cells were maintained in DMEM supplemented with 10% (*v*/*v*) bovine serum (Sigma-Aldrich, St. Louis, MO) at 37 °C in a humidified 5% CO_2 atmosphere. The desired cell type was stimulated with carbachol at 37 °C in serum-free medium, at the specified doses and during the indicated time periods. The cells were serum-starved before ligand addition to minimize basal kinase activity. When required, cells (70–80% confluent monolayers in 60-mm dishes) were transiently transfected with the desired combinations of cDNA constructs using the Lipofectamine/Plus method (Invitrogen), following manufacturer's instructions. Empty vector was added to keep the total amount of DNA per dish constant. Assays were performed 24 h after

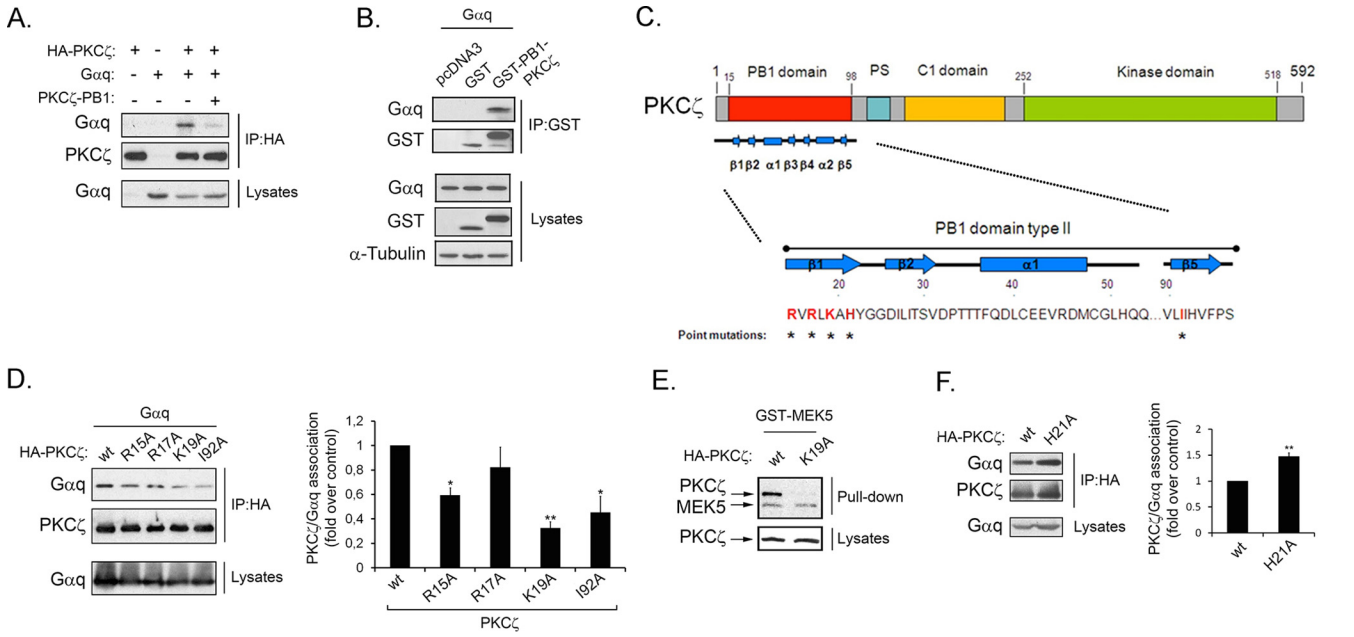

FIGURE 2. **PKCζ interacts with Gαq through its PB1-type II domain.** *A*, overexpression of PKCζ-PB1 domain interferes with Gαq/PKCζ association. *B*, Gαq co-immunoprecipitates with the PKCζ-PB1 domain. In both panels COS-7 cells were transfected with the indicated plasmids and co-immunoprecipitation assays performed as described under "Experimental Procedures". *C*, *cartoon* of the PB1 type II domain of PKCζ. Selected residues (*), homologous to interaction-driving amino acids in other PB1-type II proteins (18), were mutated to alanine. *D*, mutations in PKCζ PB1-type II domain interfere with Gαq binding. COS-7 cells were transfected with Gαq and different HA-PKCζ mutants and subjected to co-immunoprecipitation analysis as above. Data (mean ± S.E. of three independent experiments) were normalized with respect to Gαq/PKCζ wt association (*, $p < 0.05$; **, $p < 0.005$, two tailed *t* test). *E*, lysine 19 in the PB1-type II domain of PKCζ is essential for MEK5 binding. COS-7 cells transfected with different combinations of plasmids encoding MEK5-GST, HA-PKCζ, and HA-PKCζK19A and pull-downs and total lysates analyzed as in *panel B*. *F*, a mutation in the PB1-type II domain of PKCζ enhances the interaction with Gαq. COS-7 cells transfected with Gαq and wild-type or HA-PKCζ-H21A mutant constructs were analyzed as in panels above. To compare the association of Gαq with the PKCζ mutant, immunoblotted protein bands were quantified and normalized by total HA-PKCζ. Data (mean ± S.E. of three independent experiments) were normalized with respect to Gαq/PKCζ wt association. (**, $p < 0.005$, two tailed *t* test). Blots representative of three independent experiments are shown in all panels.

transfection. Transient expression of the desired proteins was confirmed by immunoblot analysis of whole-cell lysates using specific antisera.

Cloning and Mutagenesis—Venus-YFP expression constructs for the protein complementation assay (PCA) were obtained by sub-cloning *Gnaq* (mouse, accession number NM_002072) and *Prkcz* (rat, accession number NM_022507.1) into the 5′- and 3′-ends of the Venus YFP PCA fragments, referred to here as N-terminal fragment (1–158 aa; F[1]) and the C-terminal fragment (159–239 aa; F[2]), respectively, as previously described (10). PKCζ binding-deficient mutants, Gαq binding-deficient mutants and Gαq constitutively active mutants were prepared using the QuickChange® site-directed mutagenesis kit (Stratagene) following manufacturer's instructions.

Co-immunoprecipitation Assays—24–48 h after transfection, cells were scraped and washed twice with ice-cold phosphate-buffered saline, solubilized in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% (*w*/*v*) sodium deoxycholate, 1% (*w*/*v*) Triton X-100, 0.1% SDS, protease inhibitors), and clarified by centrifugation. Immunoprecipitation was performed with agarose-conjugated anti-HA antibodies (Santa Cruz Biotechnology, F-7) or, alternatively, with 1 mg/ml bovine serum albumin and anti-Gαq (Santa Cruz Biotechnology, C19) followed by re-incubation with protein G-Sepharose. All blots were developed using the chemoluminescence method and quantified by laser-scanner densitometry.

Pull-down Assays—To analyze MEK5/PKCζ binding, lysates from cells expressing GST-MEK5 (or GST alone as a negative control) were subjected to GST pull-down assays with glutathione-Sepharose 4B as previously reported (8). In the analysis of PKCζ-ERK5 binding, purified GST-ERK5 or GST were incubated overnight at 100 nM with 20 nM His-PKCζ at 4 °C in binding buffer (50 mM Tris-HCl, pH 7.9, 0.01% Lubrol, 0.6 mM, EDTA, and 70 mM NaCl) supplemented with a protease inhibitor mixture. Fusion proteins were incubated for 2 h at 4 °C with glutathione-Sepharose 4B beads and washed 8–10 times with the same buffer. To explore whether PKCζ binds to Gαq in a GTP-dependent manner, 20 nM of purified His_6-PKCζ was incubated with Ni-NTA resin (Probond) for 2 h at 4 °C in His-Binding Buffer (20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM imidazole). The mixture was then incubated with 50 nM of purified Gαq or Gαq loaded with GTPγS overnight at 4 °C in the same buffer. Recombinant protein complexes were washed 8–10 times with His-Binding Buffer supplemented with 30 mM imidazole.

Preparation of Gαq-GTPγS—Recombinant Gαq was purified as described (11). Gαq-GDP (10 μM) was incubated in a 1 ml reaction with 20 μM purified Ric-8A (12) and 100 μM GTPγS in 20 mM Hepes, pH 8.0, 100 mM NaCl, 0.05% Genapol C-100, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT for 1 h at 25 °C. The reaction was gel filtered over Superdex 75/200 columns arranged in series to separate Gαq from Ric-8A. The monomeric Gαq-GTPγS fractions were pooled, concentrated in a 10,000 MWCO Amicon Ultracentrifugal device, and stored as 20-μM aliquots at −80 °C.

Determination of ERK5 MAPK Stimulation—Lysates were resolved by 8% SDS-PAGE and subjected to immunoblot anal-

ysis as previously described (8). The activation state of ERK5 was measured by laser-scanner densitometry and expressed as the amount of phospho-ERK5 normalized to the amount of the total ERK5 protein. In CHO and HeLa cell lines HA-tagged ERK5 was transfected and immunoprecipitated with anti-HA agarose beads (Santa Cruz). Immunoprecipitates were washed 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 and phosphatase inhibitors) at 4 °C.

Protein-fragment Complementation Assays (PCA)—Protein-protein complexes can be recapitulated in living cells, by fusing protein pairs to complementary N- and C-terminal fragments of a reporter (enzyme or fluorescent protein). If the proteins interact the fragments of the reporter protein will be brought into proximity where they can spontaneously fold together and reconstitute enzymatic activity or fluorescence (10). Venus YFP-based PCA: Cells were co-transfected with the Venus YFP PCA expression vectors coding for prey-F[1] and/or bait-F[2]. Twenty-four hours after transfection, cells were subjected to fluorometric analysis and fluorescence microscopy. For the fluorometric analysis, cells were trypsinized and resuspended in PBS, transferred to 96-well black microtiter plates (Dynex; VWR Scientific, Mississauga, Ontario), and measured in a fluorometer (integration time 10 s, excitation wavelength 470 nm, emission wavelength 528 nm) (Spectra MAX GEMINI XS; Molecular Devices, Sunnyvale, CA). Background fluorescence was subtracted from fluorometric values of all of the samples. Fluorescence microscopy was performed using a Nikon Eclipse TE2000U inverted microscope with 40× objective and YFP filter cube (41028, Chroma Technologies). Images were captured with a CoolSnap CCD camera (Photometrics) using Metamorph software (Molecular Devices). When comparing different PCA pairs, identical microscopy settings were utilized, and the expression of each construct was assessed by Western blot to ensure that the differences observed in the fluorescence images was a due to a lack of interaction and not to insufficient expression of one of the reporters.

xCELLigence Measurements—The xCELLigence system RTCA SP instrument (Roche Applied Science) monitors changes in the cell index (a measure of cell attachment to the plate), which has been shown to effectively correlate to proliferation, adhesion, and viability changes (13, 14). To assess long-term viability cells were seeded in 96-well gold electrode sensor plate (E-plates) pre-coated with fibronectin (10 μM) and monitored every 15 min for at least 3 days in minimal medium (3% FBS) until an irreversible decrease (inflection point) in the cell index was recorded. Cell death was expressed as the time between the start of the experiment and the inflection point. The first 16 h after the cells were plated were excluded from each analysis as they correspond to the cell adhesion phase. In no case was cell death due to excessive confluence as confirmed by plate inspection with a microscope.

Propidium Iodide Incorporation—Cells were transiently transfected with the desired combinations of cDNA constructs and with GFP for the selection of the transfected population. Cells were cultured in 0.1% FBS DMEM for 48 h. If required, cells were treated with the PLCβ inhibitor U73122 (10 μM) or

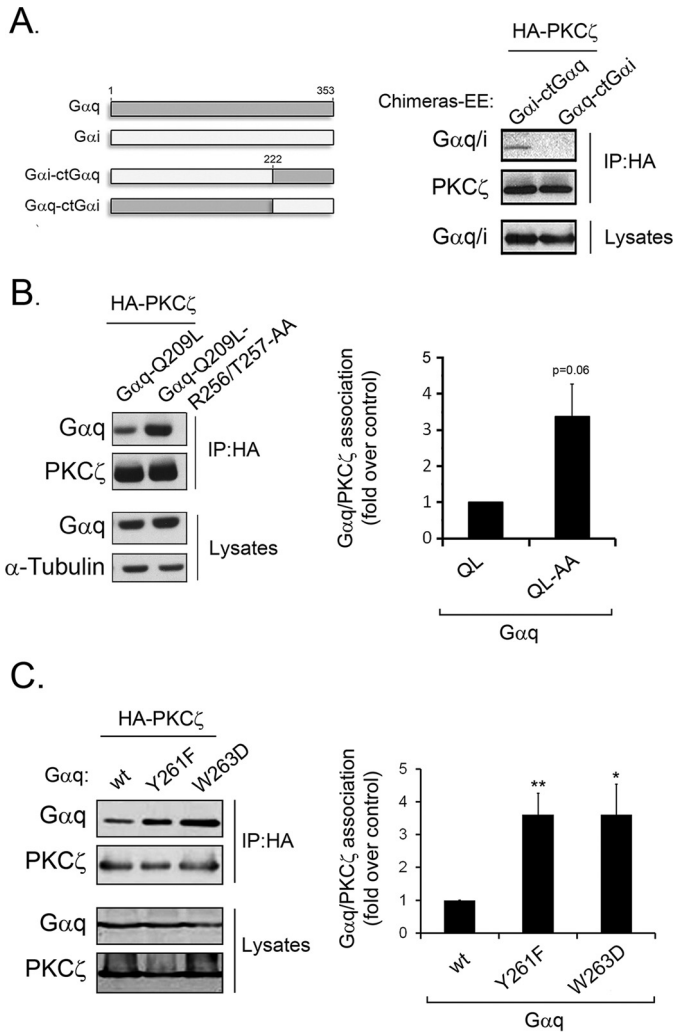

FIGURE 3. **PKCζ does not interact with the classical effector-binding region of Gαq.** *A*, C terminus of Gαq (aa222–353) is essential for the interaction with PKCζ. COS-7 cells were transfected with HA-PKCζ and two EE-tagged chimeras: Gαi-ctGαq [Gαi1(1–222)-Gαq (223–253)] and Gαq-ctGαi [Gαq (1–222)-Gαi1 (223–253)]. *B* and *C*, mutations in Gαq impairing PLCβ/p63RhoGEF/GRK2-binding enhance the interaction with PKCζ. COS-7 cells were transfected with HA-PKCζ and different association-deficient mutants of Gαq (R256A/T257A mutation disrupts PLCβ (22) and p63RhoGEF (23) binding, and either Y261F or W263D mutations disrupt GRK2 binding (24)). Cell lysates and HA-PKCζ immunoprecipitates were analyzed by Western blot as in Fig. 2. Blots representative of three independent experiments are shown in all panels. In *B* and *C*, data (mean ± S.E. of three independent experiments) were normalized with respect to control (*, $p < 0.05$; **, $p < 0.01$ two tailed *t* test).

with the ERK5 inhibitor XMD8 –92 (1 μM) 24 h before staining. Cells were washed twice with PBS and resuspended in Staining Buffer (PBS 1×, 1% BSA, 0.01% NaN_3, 1% FBS) with propidium iodide (PI) 1 μg/ml. Analysis was carried out in a BD FacsCalibur flow cytometer (BD-Bioscience) and GFP-positive and propidium iodide-positive cells were quantified using CellQuest Software (BD-Bioscience) and analyzed with the FlowJo Software. Within the GFP-positive population the percentage of PI-positive cells was calculated as a measure of cell death due to heterologous expression.

Annexin V/7-AAD Binding—To quantitatively measure apoptosis, the PE Annexin V Apoptosis Detection kit I (BD Bioscience) was utilized. Transfection and serum starving were carried out as in PI assays, after which cells were re-suspended in

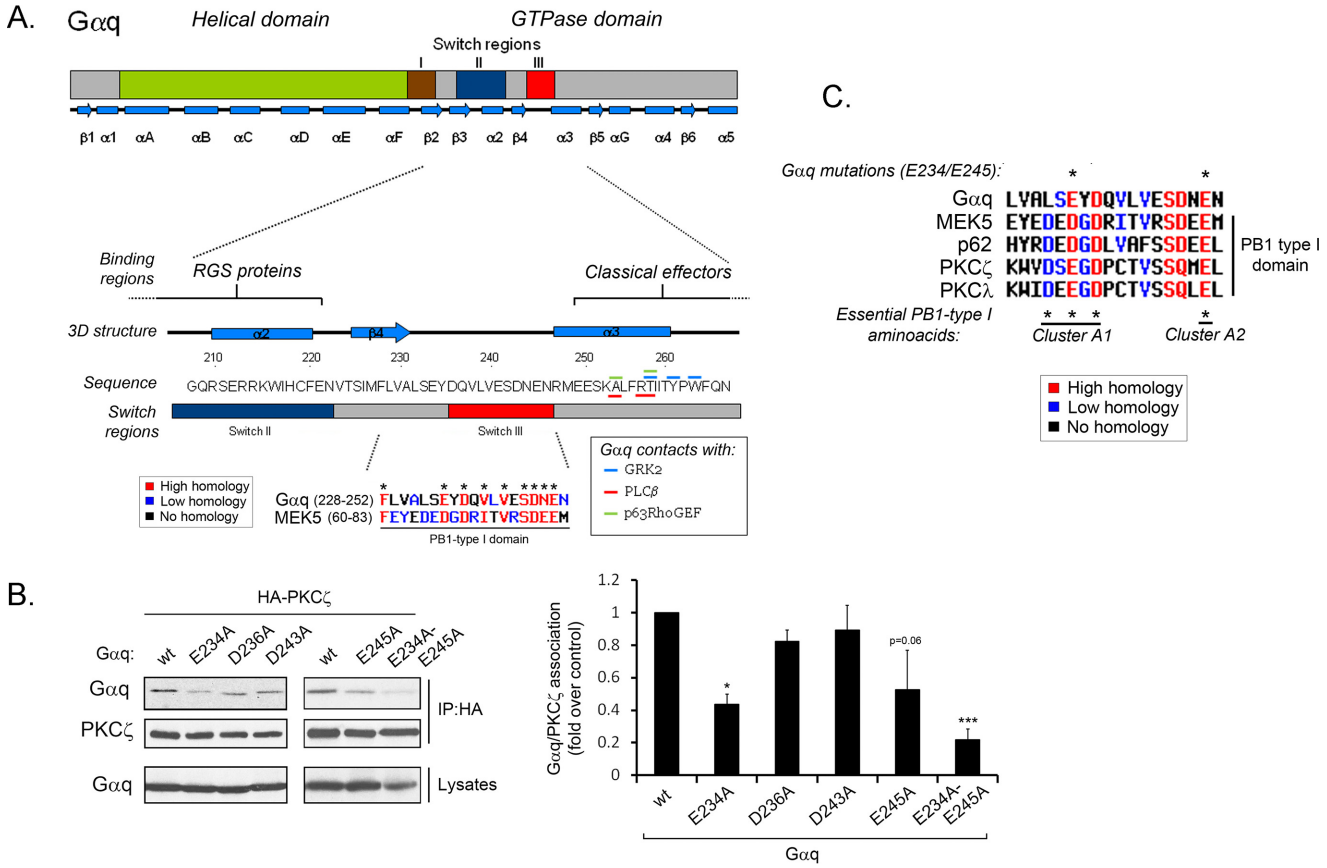

FIGURE 4. **Gαq interacts with PKCζ through a novel effector-binding region.** *A*, *cartoon* of the switch II/III region in Gαq showing the binding sites for RGS proteins and effectors. Important residues for Gαq interaction with PLCβ, GRK2, p63RhoGEF, or RGS proteins ((22–24)) are highlighted. A region with sequence similarities to the PB1 domain type I of MEK5, was identified at the β4 strand/β4-α3 loop of Gαq. Acidic residues in this region of Gαq were mutated to alanine. *B*, mutations in the (228–252) region of Gαq interfere with PKCζ binding. COS-7 cells were transfected with HA-PKCζ and the indicated Gαq mutants. Cell lysates and HA-PKCζ immunoprecipitates were analyzed as in previous figures. Data (mean ± S.E. of three independent experiments) were normalized with respect to PKCζ/Gαq wt association (*, $p < 0.05$; ***, $p < 0.001$ two tailed *t* test). *C*, sequence analysis of the PKCζ binding region in Gαq. Glutamic acids 234 and 245 (E234/E245) of Gαq align with conserved glutamic acids from PB1-type I proteins that form two clusters (A1 and A2) that are crucial for their function as a protein-protein interaction domain. Sequence alignment of different PB1-type I domains and the β4-α3 loop of Gαq were performed with Multalin software. Sequence IDs: GNAQ mouse (NP_032165), MEK5 mouse (Q62862), Sqstm1 mouse (p62, NM_011018), PKCζ mouse (NM_001039079), PKCλ/i mouse (NM_008857).

Annexin V-binding buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) at a final concentration of 1×10^5 cells. Samples were incubated with 2.5 μl of PE Annexin V and 5 μl of 7-AAD for 15 min at RT in the dark. Subsequently, 400 μl of binding buffer was added, and samples were analyzed by flow cytometry within 1 h on a BD FacsCalibur flow cytometer (BD-Bioscience). To determine the apoptotic stage of the different GFP-positive cell populations, 7-AAD- and Annexin V-positive cells were determined with the CellQuest Software (BD-Bioscience) and analyzed with the FlowJo Software. Cells treated with staurosporine (2.5 μM, 2 h) or ultraviolet irradiation (2 h), were considered the apoptotic (annexin V-positive) and necrotic (7-AAD-positive) controls, respectively. Within the GFP-positive population the percentage of annexin-positive cells was calculated as a measure of apoptotic cell death due to heterologous expression.

Statistics—Statistical analysis was performed using the two-tailed Student's *t* test, as indicated in the figure legends.

Results

Gαq/PKCζ Complex Formation in Vitro and in Living Cells—The activation of the ERK5 pathway by Gq-GPCRs appears to correlate with the formation of a transient complex between Gαq and PKCζ (8). Such interaction was suggested to be direct since these purified proteins are able to associate *in vitro*. A pull-down assay performed with purified proteins indicated that PKCζ preferentially binds the GTPγS-loaded form of Gαq (Fig. 1*A*). Further, the formation of a Gαq/PKCζ complex in living cells was assessed through a Protein-fragment Complementation Assay (PCA) (Fig. 1*B*). A clear association between PKCζ and Gαq was observed, as compared with a known high-affinity interaction (GCN4 leucine "zipper" dimerization) (Fig. 1*C*). The Gαq/PKCζ complex displays high specificity, since no association was detected between Gαq and another member of the PKC family, PKCβ, nor between PKCζ and another member of the Gα family (Gαi1) (Fig. 1*C*).

The PB1 Domain of PKCζ Is Essential for Gαq Association—PB1 domains are known protein-protein interaction domains, and this module alone accounts for the majority of the reported interactions of PKCζ (15). PKCζ-PB1 domain overexpression was shown to interfere with the formation of Gq/PKCζ complexes in cells, as assessed through co-immunoprecipitation assays (Fig. 2*A*). Indeed, the PKCζ-PB1 domain alone is able to co-immunoprecipitate with Gαq (Fig. 2*B*), thus suggesting that PKCζ might interact with Gαq through this module.

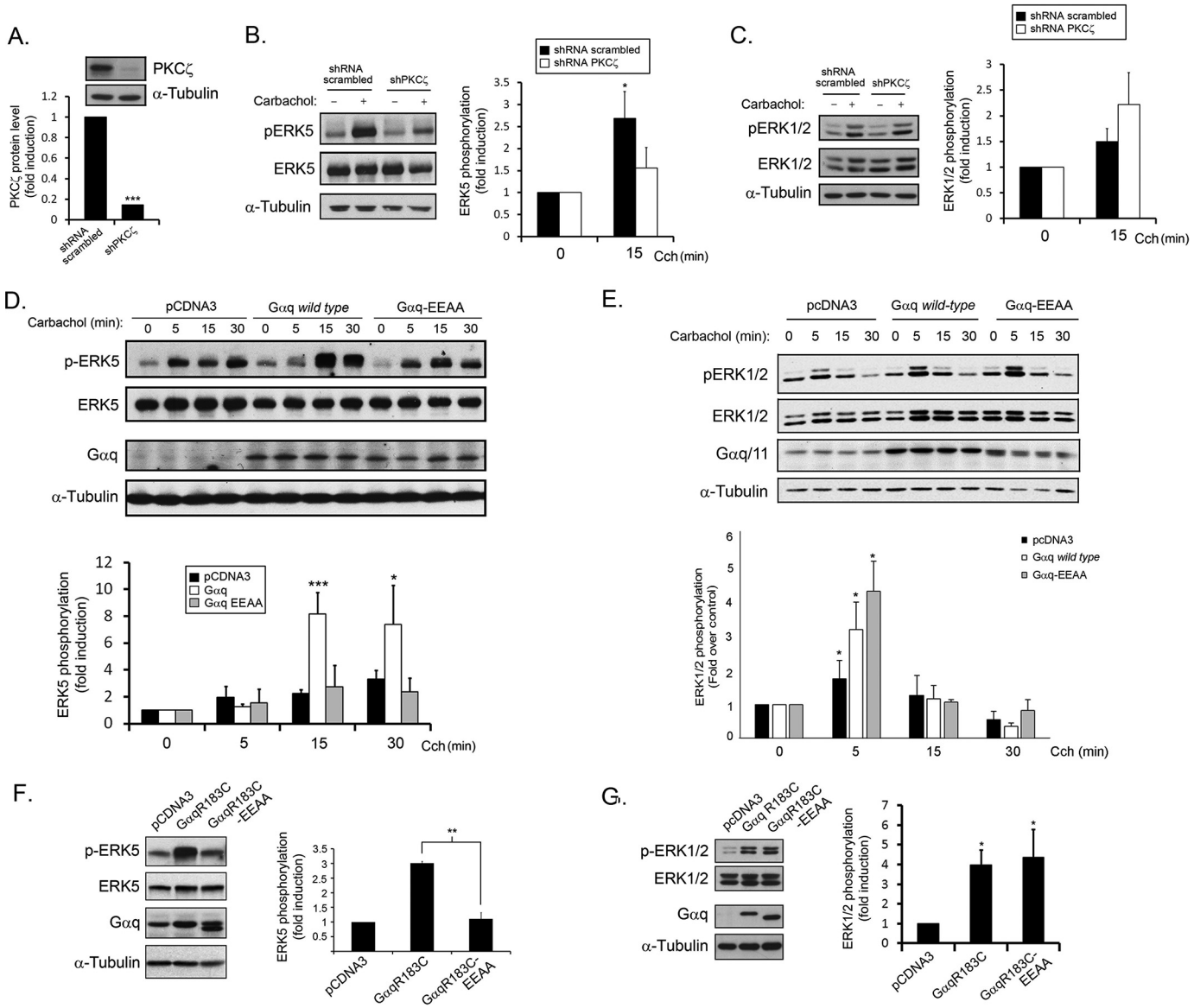

FIGURE 5. **The Gαq/PKCζ complex is involved in ERK5 activation.** *A*, knockdown efficiency upon transfection of a scrambled or a specific shRNA against PKCζ in CHO-M3 cells (see "Experimental Procedures"). *B*, PKCζ is required for ERK5 activation by a Gq-coupled GPCR. CHO-M3 cells were transfected with HA-ERK5, Gαq-wt, and either scrambled or PKCζ-targeting shRNA, serum-starved for 24 h and stimulated with carbachol (10 μM) for 15 min. ERK5-HA was immunoprecipitated and analyzed by Western blot. Data (mean ± S.E. of three independent experiments) were normalized with total ERK5 and expressed as fold-induction of ERK5 phosphorylation over control (*, $p < 0.05$; two tailed *t* test). *C*, PKCζ is not required for ERK1/2 activation by a Gq-coupled GPCR. CHO-M3 cells transfected and stimulated as in *panel B* were tested for ERK1/2 activation. Representative blot of three independent experiments is shown. *D* and *E*, PKCζ association-deficient Gαq mutant cannot activate ERK5 in response to carbachol. CHO-M3 cells were transfected with HA-ERK5, pcDNA3, Gαq or GαqE234/E245-AA (Gαq-EEAA), serum-starved for 2 h and stimulated with carbachol (10 μM). In *D*, ERK5-HA was immunoprecipitated and analyzed by Western blot. Data (mean ± S.E. of three independent experiments) were normalized with total ERK5 and expressed as fold-induction of ERK5 phosphorylation over control (*, $p < 0.05$; ***, $p < 0.001$, two tailed *t* test). In *E*, ERK1/2 activation was assessed in cell lysates as in *panel C*. Data (mean ± S.E. of three independent experiments) were normalized to pcDNA3 transfection (*, $p < 0,05$, two-tailed t-Test). *F* and *G*, a constitutively active PKCζ association-deficient Gαq mutant cannot activate ERK5 whereas it fully activates ERK1/2. CHO-M3 cells were transfected with empty vector (pcDNA3) or plasmids encoding HA-ERK5 (*F*) and the constitutively active mutants Gαq-R183C or Gαq-R183C-E234/E245-AA (Gαq-R183C-EEAA) (both *panels F* and *G*). Samples were processed and analyzed as in *D–E* (**, $p < 0.01$, two tailed *t* test; *, $p < 0.05$, two tailed *t* test).

The PB1 domain of PKCζ is composed of a PB1-type I (acidic) and a PB1-type II (basic) domain (16). Since the PB1-type II domain of PKCζ has previously been involved in ERK5 activation by the EGF receptor and in MEK5 binding (17), a strategy was designed to mutate key amino acids in this region (Fig. 2*C*). In particular, lysine 19 (K19) seems to be an invariably crucial residue in all PB1-PB1 interactions in combination with other predominantly basic residues located nearby within the three-dimensional structure (18, 19). Remarkably, different point mutations in the PB1-type II region and specially that in Lys-19 decreased the interaction with Gαq in co-immunoprecipitation experiments (Fig. 2*D*). This residue was also found to be essential for PKCζ-MEK5 binding (Fig. 2*E*), as predicted by other PB1-PB1 structures (19). Interestingly, another mutation within this domain (PKCζ-H21A) enhanced the ability of PKCζ to associate with Gαq (Fig. 2*F*). Taken together, these data indicate that the PB1 domain type II of PKCζ is crucial for binding Gαq.

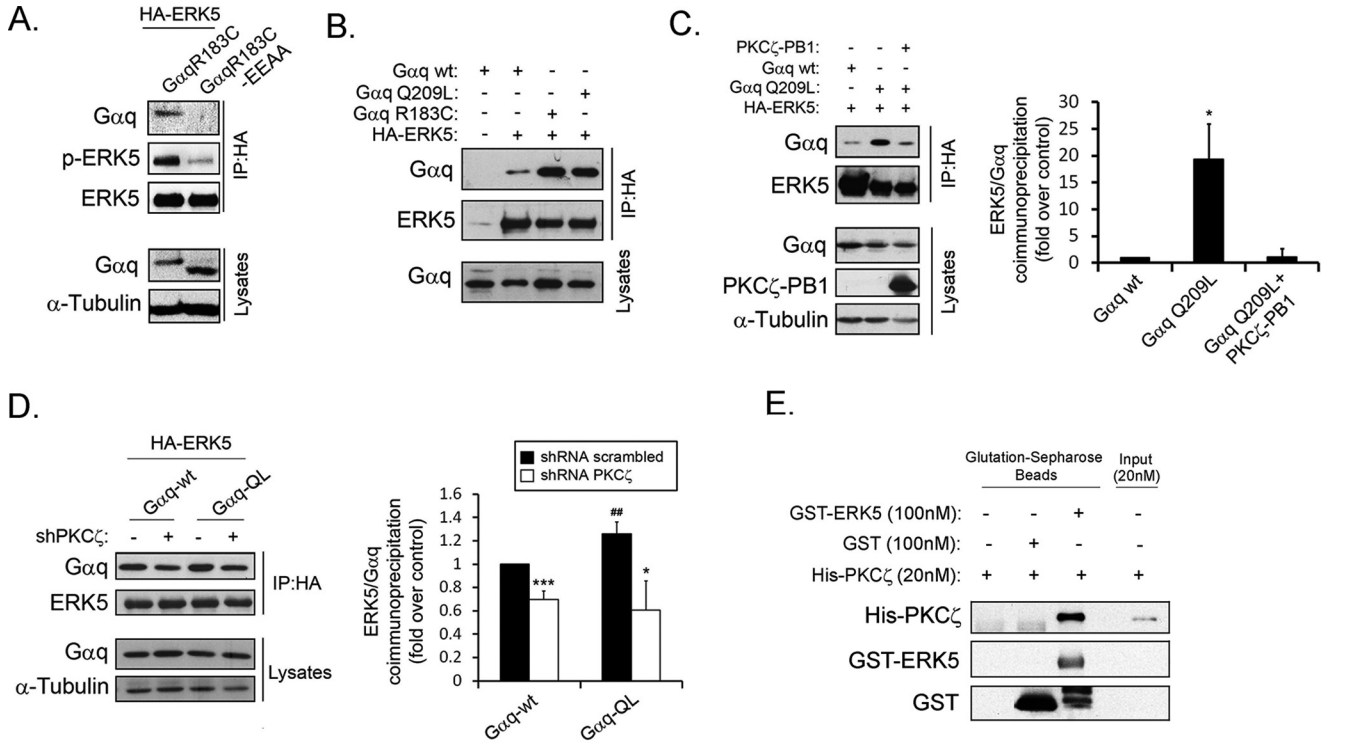

FIGURE 6. **Gαq forms an activation-dependent complex with ERK5 through PKCζ.** *A* and *B*, ERK5 preferentially co-immunoprecipitates active Gαq. CHO-M3 cells were transfected with HA-ERK5, Gαq wt, Gαq-R183C, or Gαq-Q209L (constitutively active mutants) as indicated. Cell lysates and HA-ERK5 immunoprecipitates were analyzed as in previous figures. Representative blot of three independent experiments are shown. *C*, PB1 domain of PKCζ interferes with Gαq/ERK5 complexes. CHO-M3 cells were transfected with HA-ERK5, Gαq-Q209L, and PKCζ-PB1 domain and ERK5 complexes analyzed as above (mean ± S.E. of three independent experiments) (*, $p < 0.05$). *D*, PKCζ silencing decreases the formation of Gαq/ERK5 complexes. CHO-M3 cells were transfected with HA-ERK5, Gαq Q209L, and PKCζ-targeting shRNA, and ERK5 complexes quantified as in *C*. Data (mean ± S.E. of three independent experiments) were normalized with total ERK5 and expressed as fold-induction of co-immunoprecipitated Gαq over shRNA scrambled (*, $p < 0.05$; ***, $p < 0.001$, two tailed *t* test). *E*, ERK5 interacts directly with PKCζ. Fusion proteins GST-ERK5 (100 nM) and purified GST (100 nM) as negative control were incubated with His-PKCζ (20 nM) and mixtures analyzed as detailed under "Experimental Procedures." A blot representative of two independent experiments is shown.

A Novel Region in Gαq Is Required for the Interaction with PKCζ—Since members of the Gαi family cannot interact with PKCζ (Fig. 1*C* and Ref. 8), we utilized two different chimeras in which the C terminus (aa 222–353) of either Gαq or Gαi1 had been substituted by that of Gαi1 and Gαq, respectively (20), to delineate relevant regions for PKCζ association. A Gαq chimera with the C terminus of Gαi1 was unable to interact with PKCζ when expressed in cells (Fig. 3*A*), thus suggesting that the interaction determinants are predominantly located in the C terminus of Gαq. This C-terminal stretch includes the classical effector-binding region (21). To assess whether this region is responsible for binding PKCζ, we used different Gαq mutants unable to interact with other effectors such as PLCβ and p63RhoGEF (Gαq-R256A/T257A, (22, 23)) or GRK2 (Gαq-Y261F and Gαq-W263D (24)). Surprisingly, neither mutant affected PKCζ binding but on the contrary all co-immunoprecipitated with the kinase to a greater extent than *wild-type* Gαq (Fig. 3, *B* and *C*). These data suggest that the absence of competitors on the surface of Gαq favors the interaction with PKCζ. This may indicate that PKCζ is interacting with other region close to the classical effector site. We noted that the adjacent β4-α3 loop in Gαq displays a relatively high sequence similarity with the PB1-type I domain of MEK5, a module known to interact with the PB1-type II domain of PKCζ (Fig. 4*A*). A double mutation (E234/E245-AA) in the homologous residues of Gαq in this potential interaction module significantly impaired its association with PKCζ (Fig. 4*B*). Interestingly, these amino acids were found to be homologous to highly conserved residues in several PB1-type I domain-harboring proteins as part of two major functional clusters (A1 and A2) (Fig. 4*C*) (18). Overall, these data indicate that a region of Gαq, distinct from the classical effector-binding site, is involved in the interaction with PKCζ.

An Efficient Gαq/PKCζ Association Is Required for the Activation of the ERK5 Pathway—We previously suggested that PKCζ is required for Gq-coupled GPCR activation of ERK5 (8, 9). To confirm this, we silenced PKCζ in CHO-M3 cells (Fig. 5*A*) and stimulated the cells with carbachol to reach maximum activation as previously reported (25). Activation of ERK5 was abolished in the absence of PKCζ (Fig. 5*B*), whereas ERK1/2 phosphorylation was seemingly unaffected (Fig. 5*C*). To establish whether this effect depends on the formation of a Gαq/PKCζ complex, we assessed the activation of ERK5 by the PKCζ-binding deficient mutant (Gαq-E234/E245-AA; Gαq-EEAA hereafter) in response to carbachol stimulation. Notably, overexpression of wild-type Gαq clearly enhanced ERK5 activation by GPCRs as reported (25), whereas the Gαq-EEAA mutant did not (Fig. 5*D*). In the same experimental setting the promotion of ERK1/2 activation was similar upon either wild-type Gαq or Gαq-EEAA expression (Fig. 5*E*). Consistently, the direct activation of ERK1/2 by constitutively active Gαq (R183C) was not affected by the EEAA mutation as opposed to the activation of ERK5, which was impaired (Fig. 5, *F*

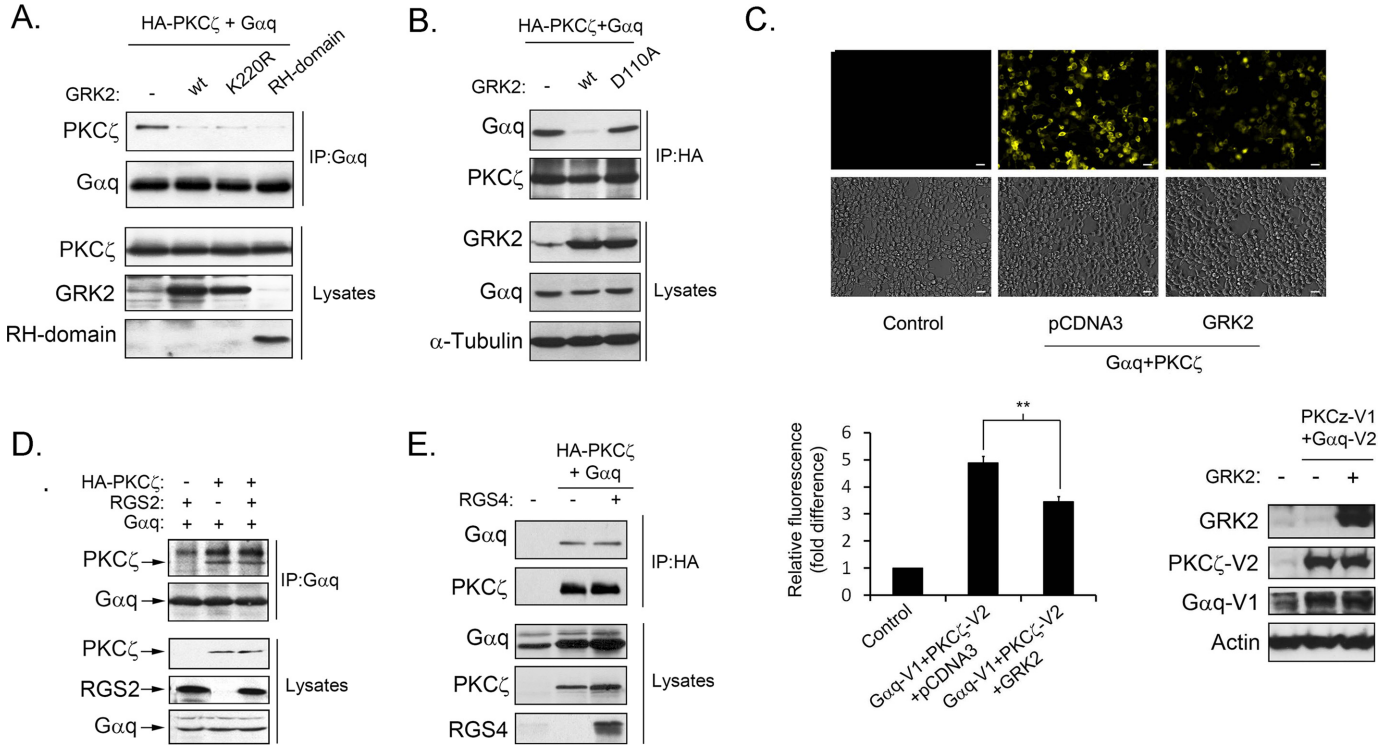

FIGURE 7. **GRK2 is a negative regulator of the Gαq/PKCζ complex.** *A*, GRK2 overexpression impairs Gαq/PKCζ association through its RH domain. COS-7 cells were transfected with HA-PKCζ and Gαq along with GRK2 wild-type, GRK2 K220R (kinase-inactive mutant) or the GRK2 RH domain. Cell lysates and Gαq immunoprecipitates were analyzed by Western blot. *B*, Gαq association-deficient GRK2 mutant does not interfere with the Gαq/PKCζ complex. COS-7 cells were transfected with Gαq, HA-PKCζ, GRK2 wt, and the GRK2 D110A mutant, which has impaired ability to bind to Gαq (25) and lysates and HA-PKCζ immunoprecipitates analyzed as in previous figures. *C*, GRK2 overexpression impairs Gαq/PKCζ association in living cells. HEK293 cells were transfected with Venus YFP PCA plasmids: Control (PKCζ-Venus YFP[F1]+pcDNA3), Gαq+PKCζ+pcDNA3 (Gαq-Venus YFP[F1]+ PKCζ-Venus YFP[F2]+pcDNA3), Gαq+PKCζ+GRK2 (Gαq-Venus YFP[F1]+ PKCζ-Venus YFP[F2]+GRK2). Data (mean ± S.E. of three independent experiments) were normalized with respect to control (**, $p < 0.005$, two tailed *t* test). Bar length, 25 μm. *D* and *E*, RGS2/4 overexpression does not alter the formation of the Gαq/PKCζ complex. COS-7 cells were transfected with combinations of plasmids encoding HA-PKCζ, Gαq, and RGS2 or RGS4. Either Gαq (*D*) or HA-PKCζ (*E*) immunoprecipitates and total lysates were analyzed as above. In all panels, blots shown are representative of 2–3 independent experiments.

and *G*). These results indicate that this mutant retains the ability to modulate the activity of other Gαq effector proteins and support the specificity of the Gαq/PKCζ axis in promoting ERK5 activation.

PKCζ Scaffolds an Activation-dependent Gαq/ERK5 Complex—Interestingly, Gαq was found to co-immunoprecipitate with the activated form of ERK5 and this was clearly decreased by the EEAA mutation (Fig. 6*A*). The formation of Gαq/ERK5 complexes was greatly favored by activating mutations in the Gα subunit (R183C or Q209L) (Fig. 6*B*), which supports the formation of the complexes upon GPCR stimulation. We hypothesized that PKCζ could be organizing a multimolecular Gαq/ERK5 complex upon G protein activation. Both the co-expression of the PKCζ-PB1 domain or the down-regulation of PKCζ expression led to a decreased formation of Gαq/ERK5 complexes (Fig. 6, *C* and *D*). To address whether PKCζ could exert a scaffold role through a direct interaction with ERK5, we performed pull-down experiments with purified proteins and found that PKCζ and ERK5 are direct binding partners (Fig. 6*E*). Although other authors have suggested the occurrence of this complex (26), we provide the first concluding evidence for a direct association. Collectively, our findings suggest that PKCζ orchestrates a ternary complex with Gαq and ERK5 that underlies the activation of the signaling cascade.

GRK2 Negatively Regulates the Gαq/PKCζ Complex and Receptor-induced ERK5 Activation—GRK2 is a negative regulator of Gαq signaling both through receptor desensitization mechanisms and direct inhibition of Gαq-effector interactions (27). Consistently, we observed that overexpression of wild-type GRK2 completely abolished Gαq association to PKCζ (Fig. 7*A*). Such effect was independent of GRK2 kinase activity and mimicked by its RH domain, a region reported to specifically interact with Gαq (28). Also, a GRK2 mutant (D110A) which is unable to interact with Gαq (28) barely interfered with formation of the Gαq/PKCζ complex (Fig. 7*B*). The negative regulation exerted by GRK2 was also detected in a natural cell milieu, as assessed through the Venus-YFP PCA (Fig. 7*C*). On the contrary, as observed for other Gαq effectors (29), PKCζ was not displaced by the Gαq regulators RGS2 or 4 (Fig. 7, *D* and *E*).

In agreement with the ability to inhibit Gαq/PKCζ interaction, enhanced GRK2 levels in CHO-M3 cells abolished carbachol-induced ERK5 activation (Fig. 8*A*). ERK5 activation was reduced to ~50% upon expression of the RH domain of GRK2 (Fig. 8*B*), whereas a kinase-inactive GRK2-K220R mutant did not disrupt ERK1/2 signaling as compared with wild-type GRK2 (Fig. 8*C*). This suggests that direct Gαq binding plays a role in the attenuation of ERK5 signaling by GRK2 in addition

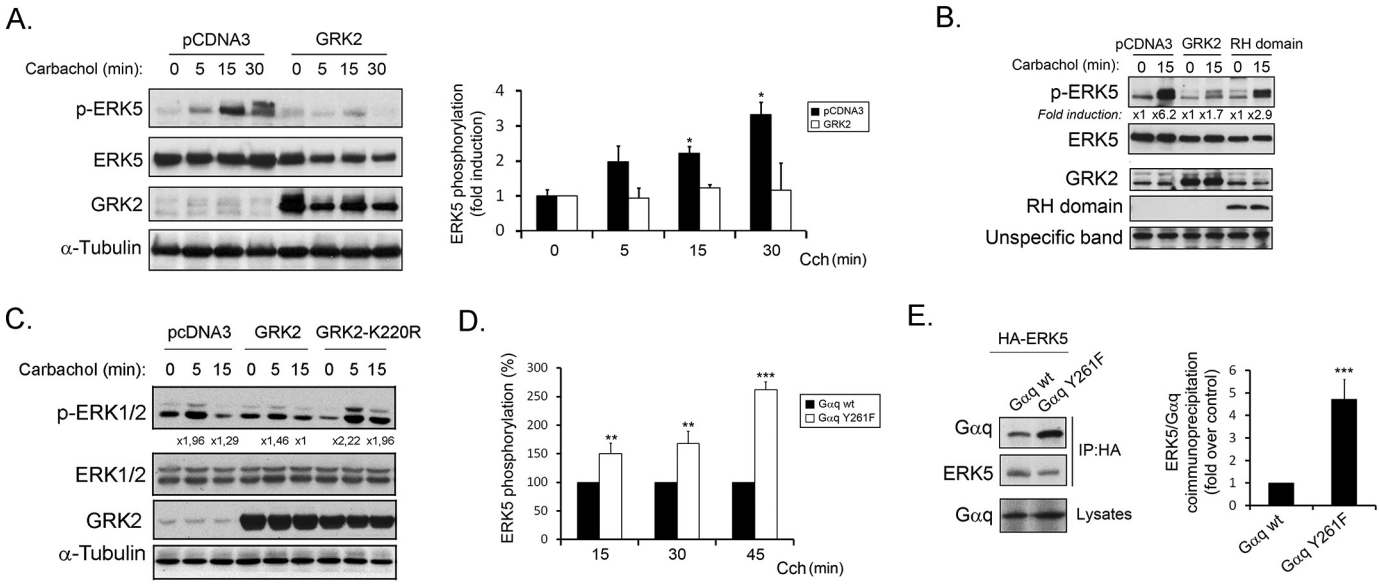

FIGURE 8. **GRK2 is a negative regulator of Gq-GPCR-mediated ERK5 activation.** *A*, GRK2 overexpression abolishes ERK5 activation by the Gq-coupled M3 receptor. CHO-M3 cells were transfected with empty vector (pcDNA3), HA-ERK5, and GRK2, serum-starved and stimulated with carbachol (10 μM). ERK5 stimulation was assessed as in Fig. 5 (mean ± S.E. of three independent experiments) (*, $p < 0.05$; two tailed *t* test). *B*, GRK2 RH domain overexpression partially abolishes ERK5 activation by the Gq-coupled M3 receptor. CHO-M3 cells were transfected with HA-ERK5 and pcDNA3, GRK2 wt or the GRK2-RH domain. Samples were processed as above for ERK5 activation. Blots shown are representative of two independent experiments and display the calculated fold-induction. *C*, Gq-GPCR-triggered ERK1/2 activation is not affected by a kinase-deficient GRK2 mutant. CHO-M3 cells were transfected with Gαq and either pcDNA3, GRK2, or GRK2-K220R, and treated as above followed by analysis of ERK1/2 activation. Data were normalized to pcDNA3 transfection. Blot is representative of four independent experiments and display the calculated fold-induction. *D*, a GRK2 association-deficient Gαq mutant enhances ERK5 activation. CHO-M3 cells were transfected with HA-ERK5 and either Gαq wt or Gαq Y261F (deficient in GRK2 binding) and processed as in *A* (**, $p < 0.005$; ***, $p < 0.001$, two tailed *t* test). *E*, Gαq mutant with diminished GRK2-association ability shows increased co-immunoprecipitation with ERK5. CHO-M3 cells were transfected with HA-ERK5 and either Gαq wt or Gαq-Y261F. Cell lysates and HA-ERK5 immunoprecipitates were analyzed. Data (mean ± S.E. of three independent experiments) were normalized with respect to ERK5/Gαq wt co-immunoprecipitation (***, $p < 0.001$).

to kinase-dependent GPCR desensitization. Consistently, the duration and amplitude of carbachol-induced ERK5 activation (Fig. 8*D*), as well as the assembly of Gαq/ERK5 multimolecular complexes (Fig. 8*E*) were markedly enhanced when expressing a GRK2 binding-deficient mutant of Gαq (Gαq-Y261F).

Gαq Is Involved in Apoptotic Cell Death Promotion via PKCζ—The description of PKCζ as an effector protein for Gαq suggested that it might underlie specific cellular functions promoted by the G protein. Since cell death promotion is a well-established Gαq-initiated process ((21) and references therein), we compared cell viability in CHO cells expressing Gαq wt or the Gαq-EEAA mutant upon long-term growth in low serum (3% FBS). Cell death took place earlier in Gαq-overexpressing cells compared with control and Gαq-EEAA populations, both of which initiated this process in a similar timeframe (Fig. 9*A*). The clear increase in cell death promoted by a constitutively active Gαq mutant (Gαq-R183C) was attenuated when introducing the EEAA mutation (which reduces the interaction with PKCζ) and, contrarily, it was enhanced by the Y261F mutation (that potentiates the PKCζ interaction) (Fig. 9*B*), consistent with a role for the Gq/PKCζ signaling axis in triggering this process. Such impaired ability of the Gαq-EEAA mutant to promote cell death was also observed in HeLa cells (data not shown). Moreover, cell death upon constitutively active Gαq overexpression in CHO cells was neither affected by a mutation that impairs PLCβ activation (R256/T257-AA Ref. 22) (Fig. 9*C*) nor by PLCβ pharmacological inhibition (Fig. 9*D*). On the other hand, either ERK5 inhibition or co-expression of the PB1 domain of PKCζ showed an inhibitory effect on Gαq-induced cell death (Fig. 9*D*), suggesting that this Gαq-initiated process is, at least in part, dependent on PKCζ-mediated activation of ERK5. The phenotype observed was determined to be apoptotic cell death, as both annexin V staining and caspase 3 cleavage were enhanced upon Gαq-R183C overexpression and abrogated by the EEAA mutation (Fig. 9, *E* and *F*). Taken together, these data reveal that the novel binding region of Gαq is involved in the promotion of apoptotic cell death via PKCζ.

Discussion

Emerging evidence indicates that activated Gαq subunits can interact with several effector proteins to trigger signaling pathways different from the canonical PLCβ cascade. Previously, we reported a direct, activation-dependent association between Gαq and PKCζ in the context of Gq-coupled GPCR-mediated activation of ERK5 (8). These data suggested a genuine G protein-effector interaction although a causal relationship between the formation of a Gαq/PKCζ complex and Gαq-dependent functional outputs remained to be established. Herein we provide conclusive evidence showing that PKCζ acts as a Gαq effector through the engagement of a novel binding region in the α subunit leading to ERK5 activation and apoptotic cell death.

First, we show that the basic PB1-type II domain of PKCζ, governed by the Lys-19 residue, is critical for the association with Gαq. This region was found to mediate protein-protein interactions of PKCζ that are involved in NFκB activation or

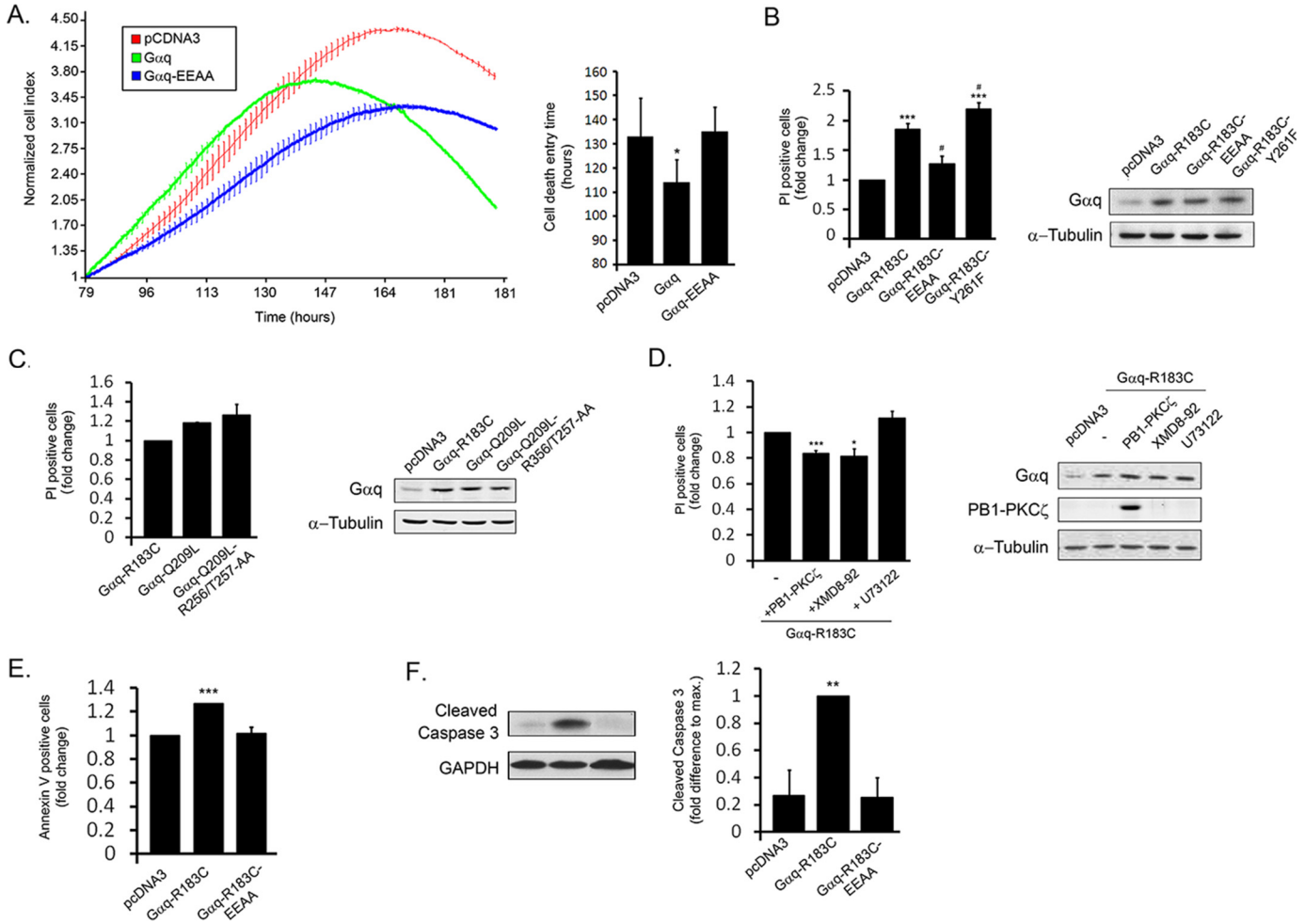

FIGURE 9. **The Gαq/PKCζ complex is involved in apoptotic cell death.** *A*, PKCζ-association impairing mutations abolish Gαq-induced cell death. CHO-M3 cells were transfected with GFP and either pcDNA3 empty vector, Gαq wild-type, or the Gαq-E234/E245-AA (Gq-EEAA) mutant. GFP-positive cells were sorted, seeded onto 96-well sensor plates and monitored with the X-Celligence system for over 140 h. Cell death entry time was determined as the inflection point at which the cell index shifts to negative values (see "Experimental Procedures"). Data were the mean ± S.E. of five independent experiments (*, $p < 0.05$, two tailed *t* test). *B*, constitutively active Gαq mutants with diminished (EEAA) or enhanced (Y261F) PKCζ association ability decrease and increase cell death, respectively. CHO-M3 cells were transfected and cultured in 0.1% FBS for 48 h. The proportion of transfectants (GFP-positive cells) that show propidium iodide-positive staining was determined through flow cytometry (mean ± S.E. of three independent experiments) (*, $p < 0.05$; ***, $p < 0.001$, two tailed *t* test over pcDNA3; #, $p < 0.05$ over Gαq-R183C). *C*, Gαq mutation that impairs PLCβ activation does not affect cell death promotion. PI-positive cells were determined in populations overexpressing constitutively active Gαq (Gαq-R183C or Gαq-Q209L) or a mutant with impaired PLCβ activation (Gαq-Q209L-R256/T257-AA) and expressed as fold over Gαq-R183C-transfected cells. *D*, Gαq-induced cell death is decreased by PKCζ-PB1 domain overexpression or by an ERK5 inhibitor (XMD8–92), but not by a PLCβ inhibitor (U73122). PI-positive cells were quantified as before in populations overexpressing Gαq-R183C and treated with the indicated modulators (*, $p < 0.05$; ***, $p < 0.001$, two tailed *t* test over Gαq-R183C). *E* and *F*, PKCζ-association impairing mutations abolish constitutively active Gαq-induced apoptosis. Assays were carried out as above. GFP-positive cells that show annexin V staining were measured by flow cytometry and the data are expressed as fold over empty vector-transfected cells (mean ± S.E. of three independent experiments) (***, $p < 0.001$; two tailed *t* test over pcDNA3). Cleaved caspase-3 was detected with a specific antibody (mean ± S.E. of three independent experiments) (**, $p < 0.005$; two tailed *t* test with respect to Gαq-R183C). Representative Western blots to confirm the expression of the different plasmids in the experiments are shown.

cell polarity establishment (30), and also in ERK5 activation by EGF (17). Our finding is consistent with the fact that independent expression of the PKCζ PB1 domain inhibited Gq-GPCR-mediated ERK5 stimulation (8). Second, we describe a novel binding region in Gαq driving the interaction with PKCζ which is different from the classical effector-binding region and shows surprising sequence similarities to PB1-type I domains.

Overall, the fact that the PKCζ interaction residues in Gαq lie in the vicinity of the classical effector-binding region, supports our conclusion that PKCζ is a *bona-fide* effector of Gαq that associates with a subset of amino acids that are distinct from the binding determinants of other Gαq binding partners (PLCβ, GRK2, and p63RhoGEF). All effectors of Gα subunits invariably associate with the extended region comprising the C-terminal half of the α2 helix, together with the α3 helix and its junction with the β5 strand, although the subsets of crucial amino acids for these associations vary with the specific effector (31). Interestingly, residues 221–245 of Gαq, which include the PKCζ-binding region but not the classical effector-binding residues, has been recently identified to mediate association with the cold-activated channel TRPM8, a novel Gαq interaction partner (32). This supports the characterization of this Gαq region as a functional module capable of binding different cellular proteins.

Our data show that Gαq strictly depends on the association with PKCζ to promote ERK5 activation. Indeed, the EEAA

mutation in Gαq abrogated both direct and receptor-induced ERK5 phosphorylation, whereas ERK1/2 activation remained unaffected. Importantly, we demonstrate that Gαq and ERK5 are found together in an activation-dependent multimolecular complex orchestrated through PKCζ scaffolding, which directly binds ERK5 and enables the stimulation of the pathway. This scaffold role was supported by the finding that Gq-coupled GPCRs do not promote phosphorylation-dependent activation of PKCζ (8). Instead we observed (data not shown) that carbachol induces dimerization of the kinase at a coincident timecourse to the Gαq-PKCζ interaction. This could be relevant since dimerization not only is a common scaffold protein mechanism but, in the case of PB1-PB1 associations, it has recently been shown to promote PKCζ activation independent of phosphorylation (33). Indeed, Par6 interaction with PKCζ induces its allosteric activation through the displacement of the PKCζ pseudo-substrate region from the active site (33). Interestingly, Gαq-mediated activation of effectors PLCβ (34) or p63RhoGEF (23) involves the allosteric relief of an auto-inhibitory loop buried within the active region. Thus, it is possible that a PB1-domain-dependent relief of pseudo-substrate autoinhibition in PKCζ could be induced upon Gαq binding or upon GPCR-induced dimerization. It is tempting to suggest that PB1-driven PKCζ scaffolding might be a cellular mechanism for imposing spatial and temporal specificity during Gαq-initiated signaling.

The regulation of Gαq/effector complexes by GRK2 is a well-established process for dampening downstream signaling. We show that GRK2 impedes the association of PKCζ with Gαq in living cells, and abrogates ERK5 activation due to G protein sequestering and receptor desensitization, as reported for other Gαq/effector complexes (35). Coincidently, we show that the impairment of the GRK2/Gαq interaction with a specific association-deficient Gαq mutant (Y261F) greatly enhances Gαq interaction with PKCζ and its presence in ERK5 complexes, thus promoting ERK5 activation. These findings strengthen the role of PKCζ as a novel Gαq effector and suggest that Gαq signaling toward the PKCζ/ERK5 pathway could be effectively modified in pathophysiological contexts where GRK2 expression and/or functionality is altered (36).

Finally, we put forward the assembly of Gαq/PKCζ complexes as an important process for the promotion of apoptotic cell death by Gαq. The increase in cell death promoted by the presence of constitutively-active Gαq was abolished by the EEAA mutation (which blocks the assembly of Gαq/PKCζ complexes), so cells expressing the Gαq-EEAA mutant displayed a higher viability than those expressing Gαq wild-type. On the contrary, the presence of the GRK2-association deficient Gαq mutation Y261F (leading to increased complex formation) potentiated cell death. This process is conserved in HeLa cells, and was characterized as apoptosis-mediated cell death, consistent with the reported role for Gαq in the promotion of apoptosis (37). In line with the notion that PKCζ is as a key effector in this process, the overexpression of the PKCζ -PB1 domain decreased Gαq-promoted cell death, whereas neither PLCβ inhibitors nor Gαq mutants that cannot activate PLCβ have an effect. These results are in agreement with previous reports showing that caspase activation and apoptosis

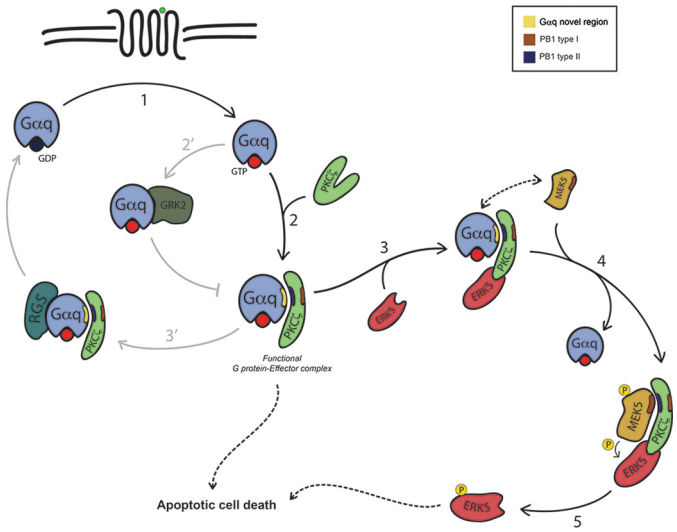

FIGURE 10. **Mechanistic model for the activation of the Gαq/PKCζ/ERK5 axis by Gq-coupled GPCRs.** Proposed sequential formation of protein complexes involved in the Gαq-ERK5 pathway. See text for detailed information.

promoted by activated Gαq is not blocked by inhibitors of IP_3- or PKC-dependent signaling (38). Also, the role of PKCζ as a pro-apoptotic protein appears to have a crucial effect on the repression of tumorigenesis in ovarian (39) and prostate cancer (40). Interestingly, pharmacological blockade of ERK5 partly inhibited cell death promotion downstream of the Gq/PKCζ axis. Although ERK5 is a well-known pro-survival factor in several contexts (41), it also has been shown to positively regulate apoptosis of medulloblastoma cells (42) and thymocytes (43). However, we cannot rule out that, alongside ERK5, other yet unidentified pathways downstream the Gαq/PKCζ axis would play a role in this process.

In sum, we propose the following mechanistic model for the Gαq/PKCζ axis (Fig. 10): Ligand binding to the receptor causes Gq activation (step 1) which, in turn, promotes the interaction between the PB1 domain type II of PKCζ and the novel effector-binding region of Gαq-GTP (step 2). This would lead to PKCζ allosteric activation, dimer/oligomerization and to the exposure of its kinase domain to interact with ERK5, which is recruited into a multimolecular complex together with Gαq (step 3). Next, MEK5 would be attracted into an intermediate signaling complex through a direct interaction with Gαq (8) which would rapidly progress into MEK5 displacing Gαq from its binding site on PKCζ (step 4). Subsequently, the interaction between MEK5 and PKCζ would favor the autophosphorylation of MEK5, which will, in turn, phosphorylate and activate ERK5 (step 5) (17). Additionally, GRK2 and RGS proteins would act as negative modulators of this cascade by sequestering Gαq away from PKCζ (step 2′), or by binding to Gαq in complex with PKCζ to promote GTPase activity and deactivation of the G α subunit (step 3′), respectively. Finally, we postulate that the promotion of apoptotic cell death may depend both on ERK5 and other yet uncharacterized targets downstream the Gαq/PKCζ complex. This model may serve as a theoretical framework for subsequent studies of this signaling axis and contribute to revise the functional consequences of Gαq activation.

Author Contributions—G. S. F., S. C., C. G. H., F. M., and C. R. designed experiments. G. S. F., S. C., A. C., G. G. T., J. K., and C. G. H. performed experiments. S. W. M. designed the PCA approach. G. S. F., F. M., and C. R. wrote the manuscript.

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Protein Kinase C ζ Interacts with a Novel Binding Region of Gαq to Act as a Functional Effector

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