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Constitutively Active Gαq and Gα13 Trigger Apoptosis through Different Pathways*

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Henning Althoefer, Pamela Eversole-Cire, Melvin I. Simon‡

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

We investigated the effect of expression of constitutively active Gα mutants on cell survival. Transfection of constitutively active Gαq and Gα13 in two different cell lines caused condensation of genomic DNA and nuclear fragmentation. Endonuclease cleavage of genomic DNA was followed by labeling the DNA fragments and subsequent flow cytometric analysis. The observed cellular phenotype was identical to the phenotype displayed by cells undergoing apoptosis. To distinguish between the apoptosis-inducing ability of the two Gα-subunits, the signaling pathways involved in this cellular function were investigated. Whereas Gαq induced apoptosis via a protein kinaseC-dependent pathway, Gα13 caused programmed cell death through a pathway involving the activation of the small G-protein Rho. Both of the pathways leading to apoptosis were blocked by overexpression of bcl-2. In contrast to other apoptosis-inducing systems, expression of constitutively active Gαq and Gα13 triggered apoptosis in high serum as well as in defined medium.

There are two distinct modes of death in cellular systems. In one, an insult leading to irreversible cellular injury causes a phenotype called necrosis that is manifested by the rupture of the cell membrane. In the other, under certain physiological situations, death of cells is triggered by metabolic or developmentally programmed events and may be required for the organisms survival or for differentiation (1). This programmed cell death (apoptosis) occurs when a cell dies by a mechanism initiated by proteins encoded by its own genome.

Apoptosis can be distinguished from necrosis through morphological characteristics including cell shrinkage, chromatin condensation, activation of specific proteases and endonucleases, and fragmentation of genomic DNA (2). Apoptosis has been implicated in many important biological processes including immune defense, growth control, and development (3). In addition some events leading to human disease involve apoptosis (4, 5).

The execution of apoptosis is triggered by intrinsic signal transduction events that link changes in physiological conditions to the cell death machinery. Signal transduction through seven pass membrane receptors represents a common mechanism of eukaryotic signaling and physiological control. During this process heterotrimeric G-proteins are responsible for transducing a ligand binding event at the membrane into a cellular response (6, 7). There are four different classes of Gα-subunits that contribute to heterotrimeric G-proteins mediated responses (8). In addition to tight regulation of activation of signal transduction pathways the cell also contains a carefully regulated system to shut down signaling after prolonged exposure to ligands. This desensitization is primarily achieved by phosphorylation induced inactivation of the receptor and subsequent internalization of the phoshorylated receptor (9). Recently a desensitization mechanism directly acting at the α-subunits was discovered. GAP proteins were cloned that accelerate the intrinsic GTPase activity of α-subunits thereby shutting off the signal (10).

In some situations permanently elevated levels of second messengers are observed inside the cells. It is believed that these elevated activities contribute to the execution of pathological phenotypes (11). Recent data point to the involvement of the Gα-subunit, Gαo, in the execution of apoptosis triggered by a mutated and presumably constitutively active amyloid precursor protein (APP) in Alzheimer's disease (12).

To investigate the effect of other constitutively active G-protein subunits, we examined their ability to cause apoptosis *in vivo*. Constitutively active mutants of Gαq, Gα12, Gα13, and Gαi2 were expressed in CHO[1] and COS-7 cells, and the resulting cellular phenotypes were observed.

EXPERIMENTAL PROCEDURES

Materials—The dye Hoechst 33258 was obtained from Sigma. The Texas red conjugated goat antibodies to rabbit immunoglobulin G (IgG) and the polyclonal antibodies to β-galactosidase were received from Cappel and 5 Prime → 3 Prime, Inc., Boulder, CO, respectively. Profectin $Ca_3(PO_4)_2$ Transfection kit was purchased from Promega. Protein kinase C inhibitors and EGTA/AM were received from Calbiochem. Cell culture and transfection reagents including DMEM, FBS, serum free medium (Optimem), LipofectAMINE, and trypsin/EDTA were purchased from Life Technologies, Inc., whereas Ham's F-12 was received from Irvine Scientific. The Apo-Direct kit was obtained from Phoenix Flow Systems. The peptide inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.fmk), was purchased from Enzyme Systems Products. The *in situ* cell death detection kit was obtained from Boehringer Mannheim, and the Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole was from Vector Laboratories.

Cell Culture and Maintenance—Cells were grown in 5% CO_2 and DMEM, 10% FBS (COS-7), or Ham's F-12, 10% FBS (CHO-K1), respectively. Transfection protocols for COS-7 and CHO-K1 cells with LipofectAMINE were described previously (13). Cells were kept in serum-free medium during LipofectAMINE transfection for 5 h and followed by the addition of an equal volume of DMEM, 20% FBS. Transfection of COS-7 cells with the Perfectin kit was performed according to the manufacturer's instructions in DMEM, 10% FBS. Treatment of COS-7 cells with z-VAD.fmk was performed according to the peptide manufacturer's directions. Immediately after transfection of cells using lipo-

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‡ To whom correspondence should be addressed: Div. of Biology, 147-75, California Institute of Technology, Pasadena, CA 91125. Tel.: 818-395-3944; Fax: 818-796-7066; E-mail: simonm@starbase1.caltech.edu.

[1] The abbreviations used are: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; G-protein, guanosine nucleotide-binding protein; PKC, protein kinase C; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

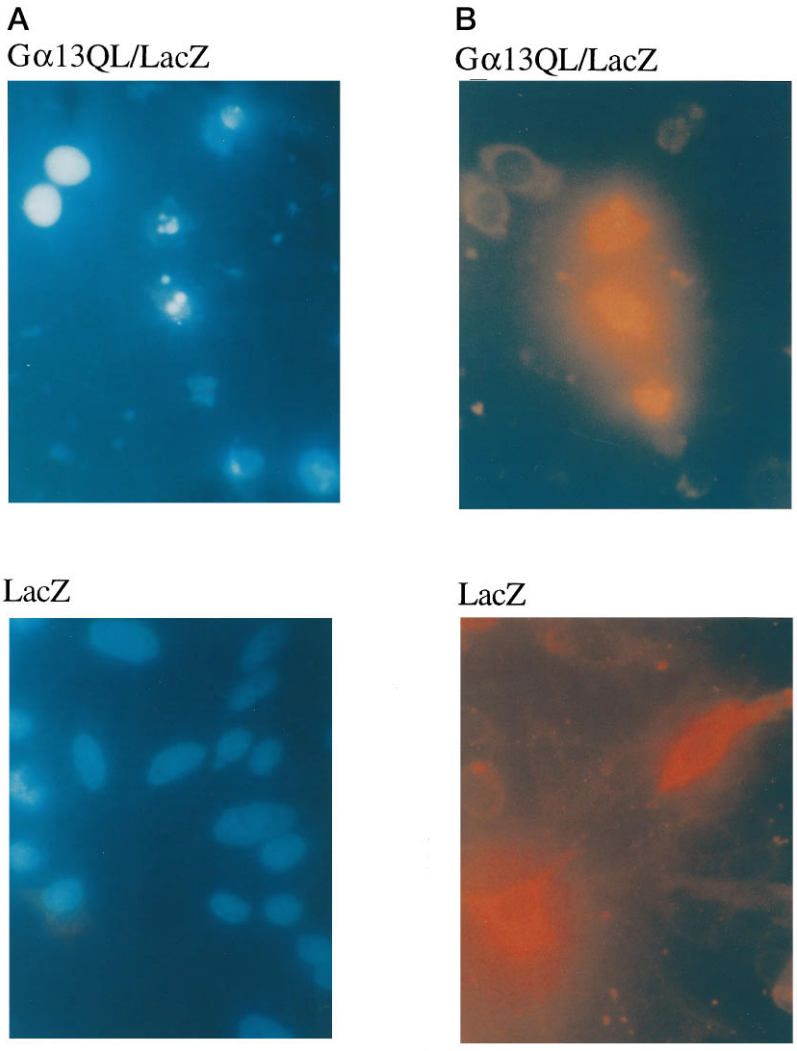

FIG. 1. **Nuclear fragmentation caused by expression of constitutively active Gα-subunits.** Approximately 1×10^5 CHO and COS-7 cells were transiently cotransfected with 0.5 μg of pCisLacZ and pCisGα13 or pCis. After 48 h, cells were fixed and stained as described under "Experimental Procedures." A representative field is shown through the respective filters. *A*, Hoechst staining of cell nuclei; *B*, Texas Red staining of LacZ (Gα13QL/LacZ) expressing cells. Cells expressing Gα13QL display a fragmented nucleus and condensed DNA, whereas only LacZ transfected cells do not. Expression of GαqRC in CHO cells or Gα13QL and GαqRC expression in COS-7 cells causes a similar phenotype (data not shown). Transfections were performed with LipofectAMINE.

fectAMINE, z-VAD.fmk was added to a final concentration of 50 μM. Cells were maintained in medium containing 50 μM z-VAD.fmk for approximately 41 h. New aliquots of the peptide inhibitor were added 4 times during the course of the incubation period to ensure that the effectiveness of the inhibitor was maximized.

Expression Plasmids—Expression plasmids for Gα13QL, GαqRC, LacZ, rasN19, and rhoAN17 were previously described (14–16). The cDNA of the constitutively active Gαi2QL mutant or of bcl-2 was inserted into the pcDNA expression vector (Invitrogen) or the Pac expression vector to yield an expression plasmid for Gαi2QL or bcl-2, respectively (17).

Analysis of Apoptotic Phenotypes—Cells were grown on glass coverslips and fixed at 48 h after transfection by incubation in methanol/acetone for 2 min. Immunostaining of β-galactosidase and staining of the genomic DNA were performed as described (18). Cells expressing LacZ were counted using a Zeiss Axiovert 35 fluorescence microscope. 200–1200 cells were counted in each experiment. Preparation of COS-7 cells for flow cytometric analysis were done with the Apo-Direct kit following the manufacturer's instructions. The total cell population (transfected and non-transfected cells) was used for the flow cytometric analysis. The total amount of DNA used in the assays was kept constant. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay was performed as described in the *in situ* detection kit manufacturers' instructions. Coverslips were mounted on glass slides using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole to visualize the nuclear morphology of the cells.

RESULTS

Expression of Constitutively Active Gα13 and Gαq but not Gαi2 Triggers Apoptosis—Mutations in the catalytic domain of Gα-subunits have been described which inhibit their intrinsic GTPase activity and therefore convert these proteins into constitutively active α-subunits (19). To investigate the phenotype of cells transfected with constitutively active Gα-subunits, the nuclear morphology of the transfected cells was analyzed. The characteristic nuclear phenotype exhibited by apoptotic cells (nuclear fragmentation and condensation of genomic DNA) was used to distinguish between normal and apoptotic cells (Fig. 1). To eliminate the background of non-transfected cells, cells were cotransfected with expression plasmids for the constitutively active Gα-subunit and LacZ. Only cells expressing LacZ were counted during the experiment. Expression of the various proteins was verified by Western blot analysis (data not shown). In general, a large fraction of singly transfected cells are also cotransfected with a second plasmid. Thus, there is a high probability that LacZ positive cells are also expressing the cotransfected G-protein expression plasmid.

Expression of constitutively active Gαi2QL in COS-7 cells does not increase the number of apoptotic cells over that observed in control experiments where LacZ alone was expressed. In contrast, expression of constitutively active GαqRC and Gα13QL increased the appearance of the apoptotic phenotype dramatically. An approximate three-fold rise in the percentage of apoptotic cells was observed. Both GαqRC and Gα13QL expression triggered programmed cell death in nearly 30% of the transfected cells (Fig. 2A). Varying the amount of expression plasmid (0.05–1 μg) revealed no differences in the occurrence of the apoptotic phenotype in our assays (data not shown). Thus, differences in the ability to trigger apoptosis does not appear to be dependent on expression levels of the

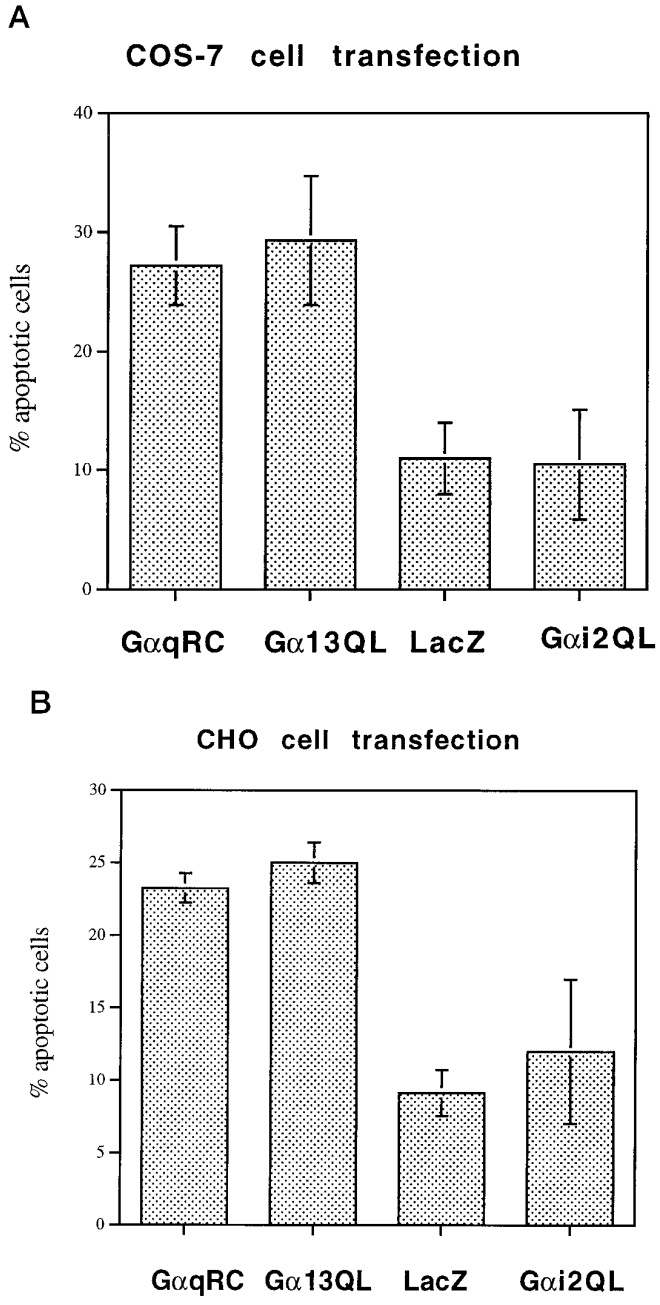

FIG. 2. **GαqRC and Gα13QL expression triggers programmed cell death in transiently transfected COS-7 and CHO cells.** Cells were cotransfected with GαqRC, Gα13QL, or Gαi2QL and LacZ. 48 h after LipofectAMINE transfection, cells were stained as described under "Experimental Procedures." Cells expressing LacZ and cells expressing both LacZ and showing an apoptotic nuclear phenotype were counted. The percentage of transfected cells showing fragmented nuclei are presented in these figures. *Vertical bars* represent ± S.D. *A*, transfection of COS-7 cells; *B*, transfection of CHO cells.

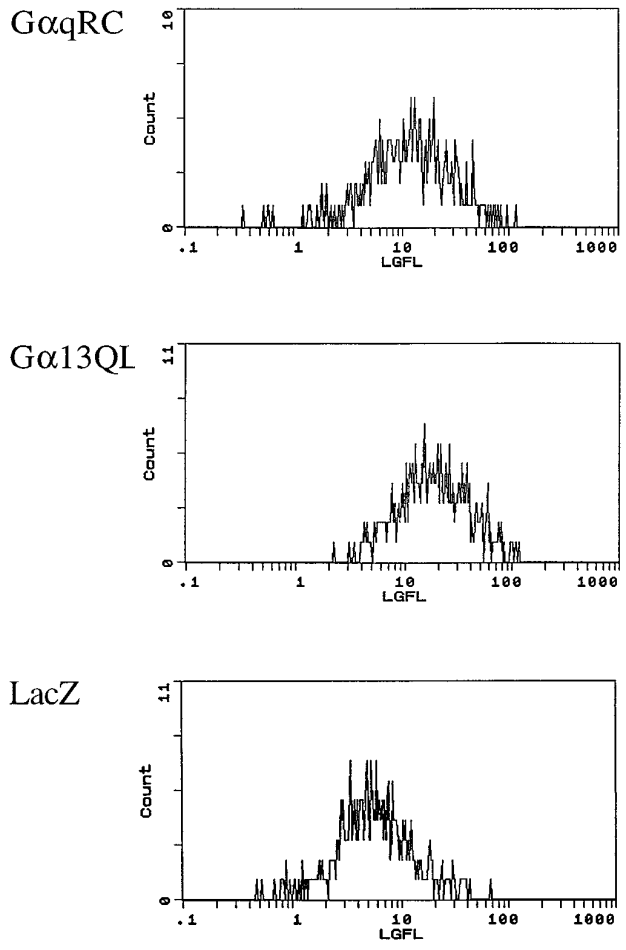

FIG. 3. **Increased fragmentation of genomic DNA due to GαqRC and Gα13QL expression.** COS-7 were transfected with GαqRC, Gα13QL, or LacZ. 48 h after transfection with LipofectAMINE, cells were fixed and treated according to the Apo-Direct staining protocol. Treated cells were analyzed in the Coulter Elite flow cytometer at 488 nm excitation. Data were plotted as a histogram. Relative fluorescence intensities are marked on the *x* axis.

different Gα-subunits.

To rule out the effect of cell line specific mutations that could lead to differences in cell survival, CHO cells were also transfected with expression plasmids for GαqRC, Gα13QL, and Gαi2QL. Again in the CHO cell system the expression of GαqRC and Gα13QL led to significant apoptotic cell death, whereas Gαi2QL expression did not (Fig. 2*B*). Thus, the results with the CHO cells correspond to the data obtained using the COS-7 cells and, together they suggest that the ability of Gα13 and Gαq to induce programmed cell death in tissue culture is cell type independent.

A second assay system was employed to further confirm the correlation between the expression of GαqRC and Gα13QL and apoptosis. The Apo-Direct assay (Phoenix Flow Systems) was used to detect increased activity of an endonuclease, an additional characteristic of apoptotic cell death, in the transfected cells. In this assay fluorescently labeled nucleotides are incorporated into the ends of the fragmented, genomic DNA via terminal transferase. Therefore an increase in fluorescence intensity indicates an increase in the amount of apoptotic cells. COS-7 cells expressing GαqRC and Gα13QL exhibit an increase in incorporation of fluorescently labeled nucleotides (Fig. 3). This is consistent with the observed morphological nuclear changes in the transfected cells and confirms the notion that Gα13QL and GαqRC expression causes apoptosis.

Induction of Apoptosis by GαqRC and Gα13QL Occurs in High Serum—Most proteins involved in both apoptotic and non-apoptotic signaling trigger an apoptotic phenotype only in low serum. This is thought to be caused by the imbalance of proliferative signals given by overexpression of these proteins and by cell cycle arrest imposed by serum starvation (20). Whereas activation of Gαq causes a strong proliferative response, Gα13 has only a modest effect on cell proliferation (21). LipofectAMINE transfection requires incubation of the transfection mixture for 5 h in serum-free medium. Although 5 h in serum-free medium is not sufficient for the cells to cease progression through the cell cycle, another transfection protocol

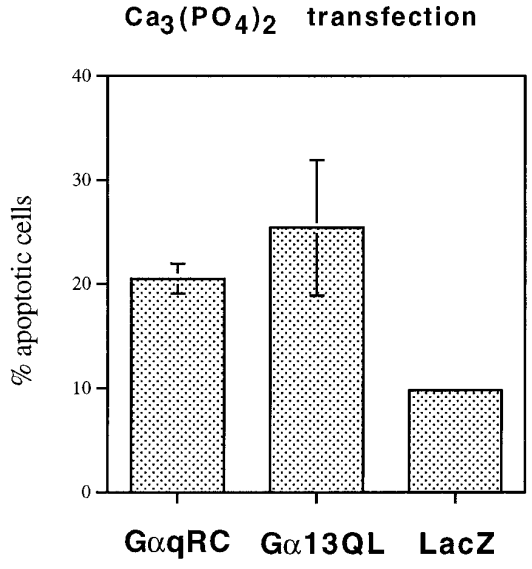

FIG. 4. **Expression of GαqRC and Gα13QL triggers apoptosis also via $Ca_3(PO_4)_2$ transfection in high serum.** COS-7 cells were cotransfected with pCisLacZ and the expression plasmid for the constitutively active Gα-subunits or an empty expression vector (total 1.5 μg DNA, 1×10^5 cells). Transfection was performed with $Ca_3(PO_4)_2$ and continuous culture in DMEM with 10% FBS as noted under "Experimental Procedures." 48 h after transfection, transfected cells were counted, and the percentage of cells displaying an apoptotic nuclear phenotype was determined.

was used to keep the cells in high serum throughout the experiment. Therefore, to determine if the expression of Gα13QL and GαqRC also cause an imbalance in cell cycle signaling, transfections were performed with $Ca_3(PO_4)_2$ in COS-7 cells in high serum. Even at continuous culture in 10% FBS, expression of Gα13QL and GαqRC increased the appearance of the apoptotic nuclear phenotype 2–3- fold compared with the amounts observed in control experiments (Fig. 4). These data confirm that constitutive activation of Gα13 and Gαq can activate pathways directly targeting the cell death machinery.

Signaling Pathways Activated by Gαq and Gα13 Leading to Apoptosis Are Different but Converge at a Step Controlled by bcl-2—To investigate the signaling mechanism connecting the expression of Gα13QL and GαqRC to apoptosis, the effects of inhibitors of different pathways on apoptotic cell morphology were examined in COS-7 cells. Although some of the cellular responses caused by activation of Gα13 and Gαq are similar, Gαq and Gα13 activate different pathways. Whereas the target of Gαq signaling is the family of phospholipase C-β enzymes, Gα13 does not activate this class of enzymes (21–24). In contrast, several groups have established that a major target of Gα13 signaling is the small G-protein Rho (25). Therefore, to test the involvement of the Rho and the phospholipase C-β pathways in the induction of apoptosis by GαqRC and Gα13QL, two different approaches were employed. First cells were cotransfected with expression plasmids for Gα13QL or GαqRC and a dominant interfering RhoA mutant (RhoAN19) (Fig. 5*A*). Coexpression of RhoAN19 with Gα13QL compared with Gα13QL expression yielded a significant reduction in the amount of transfected cells displaying an apoptotic nuclear phenotype. This reduction was not observed when GαqRC and RhoAN19 were coexpressed (Fig. 5*A*).

To rule out a general effect of the expression of dominant negative small G-proteins, the effect of coexpression of a dominant negative Ras mutant (RasN17) in this assay system was investigated (19). Neither GαqRC- nor Gα13QL-induced apoptosis was reduced by coexpression of RasN17 demonstrating that the effect of RhoAN19 on Gα13QL-triggered apoptosis was specific for Rho.

In the second approach toward analyzing these pathways, protein kinase C inhibitors were added to cells expressing constitutively active GαqRC and Gα13QL mutants. Calphostin C and Ro31–8220 were shown to inhibit all isoforms of PKC at IC_{50} of 50 or 10 nM, respectively. Thus, 50–100-fold higher concentrations of the respective inhibitors were used in these assays. Inhibition of other kinases by these inhibitors is effective only at approximately 500–1000-fold higher concentrations (26, 27). Whereas both Calphostin C and Ro31–8220 diminished the amount of apoptotic cells caused by GαqRC expression 2–3-fold, Gα13QL-induced programmed cell death was not significantly altered by the addition of the PKC inhibitors (Fig. 5*B*). This indicates that the PKC inhibitors specifically interfere with Gαq signaling which leads to apoptosis.

Although Gα13 and Gαq obviously activate different signaling mechanisms, these pathways may converge to initiate the apoptotic cellular response. Signal transduction pathways triggering the activation of the cell death machinery share common steps, which in many cases are inhibited by expression of bcl-2 (28, 29). Expression of bcl-2 was shown to inhibit apoptosis in several cell systems by sequestering molecules of the apoptotic pathway. To test the effect of bcl-2 on Gαq and Gα13 activated pathways, COS-7 cells were cotransfected with expression plasmids for Gα13QL or GαqRC and bcl-2. Both Gα13QL- and GαqRC-induced apoptosis were greatly reduced by overexpressed bcl-2. The observed 3-fold reduction again confirms the connection of pathways activated by Gα13 and Gαq to the cell death machinery (Fig. 6). This also reveals that both pathways must converge upstream of a point controlled by bcl-2 (Fig. 7).

Activation of the interleukin-1β-converting enzyme-like family of cysteine proteases is thought to play a central role in the programmed cell death pathway (for review see Ref. 5). Proteolysis of their many target substrates may be responsible for the eventual demise of the cell. The interleukin-1β-converting enzyme-like family of proteases or caspases can be inhibited by specific peptide inhibitors (30). To determine if activation of caspases is involved in the observed increase in cell death induced by Gα13QL and GαqRC, COS-7 cells were cotransfected with LacZ and Gα13QL or GαqRC in the presence of 50 μM of z-VAD.fmk, an irreversible inhibitor of the caspase family of cysteine proteases. The presence of the peptide inhibitor prevented the induction of cell death by both Gα13QL and GαqRC when measured using the TUNEL assay, supporting the notion that activation of a caspase(s) is involved in Gα13QL- and GαqRC-induced apoptosis (Table I). Although, the peptide inhibitor prevented DNA fragmentation, nuclear integrity still seemed to be affected. Many cells appeared to display some of the morphological characteristics of apoptosis, nuclear fragmentation and DNA condensation. These results are similar to findings previously reported demonstrating that in BAX-induced programmed cell death inhibition of interleukin-1β-converting enzyme-like proteases with z-VAD.fmk prevented DNA fragmentation but not many of the other morphological changes associated with apoptosis (31).

DISCUSSION

Collectively, our results suggest that expression of constitutively active Gα13QL or GαqRC mutants trigger apoptosis in higher eukaryotic cell lines. Different cell lines contain different mutations that interfere with the cell death machinery and render these lines immortal. Gα13QL and GαqRC expression caused programmed cell death in two different cell lines, thus affecting a common pathway leading to apoptosis. In addition, the observed increase in apoptotic cell number correlates with data obtained with the expression of other proteins or with extracellular stimuli causing apoptosis, *e.g.* with expression of

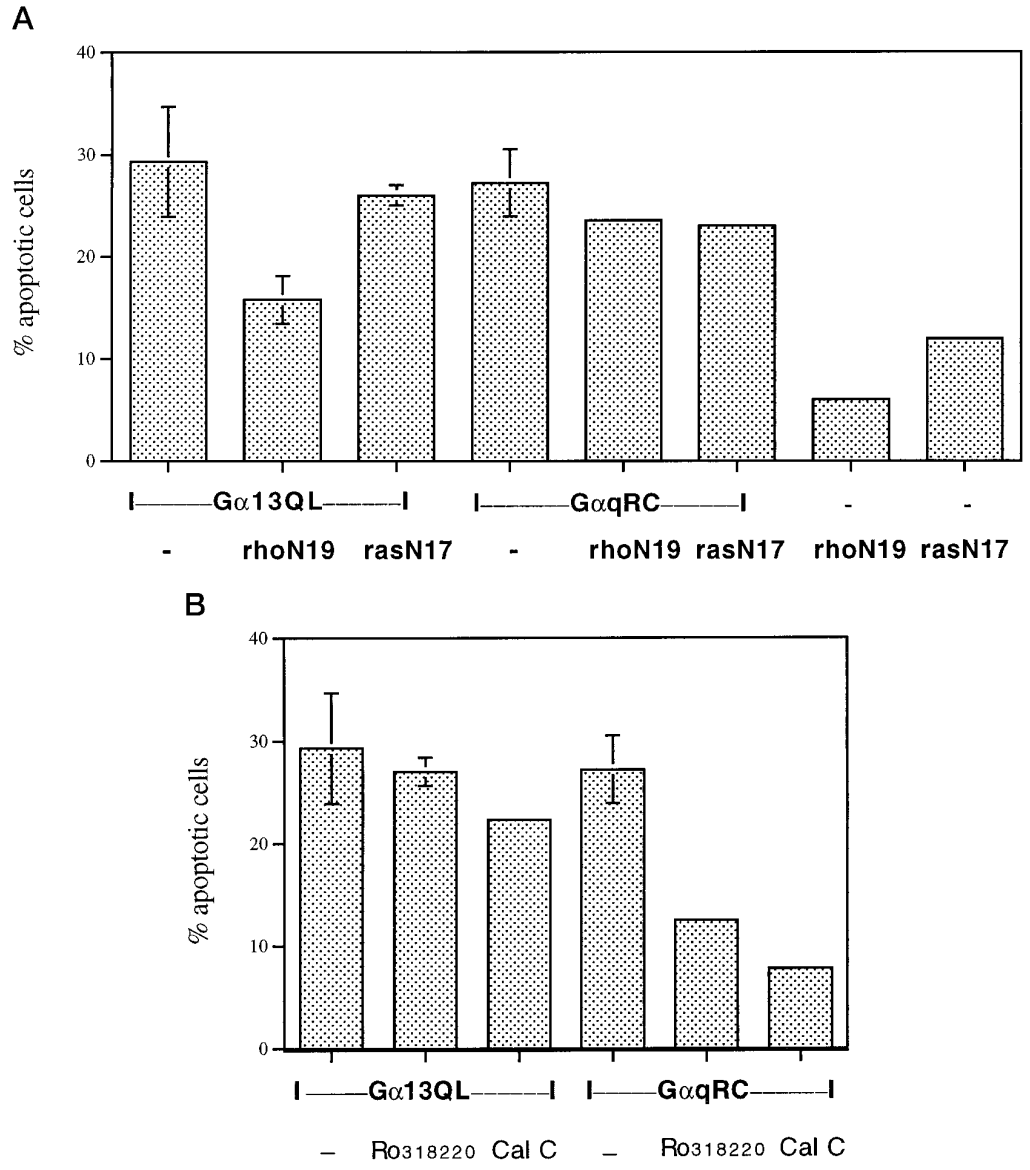

FIG. 5. **Investigation of signaling pathways connecting Gα activation with the cell death machinery.** COS-7 cells were cotransfected with 0.5 μg of LacZ, a constitutively active Gα-subunit and a dominant interfering small G-protein or an equal amount of empty vector (*A*). Cells expressing LacZ and cells expressing LacZ and showing an apoptotic nuclear phenotype were counted after 48 h. The percentage of transfected cells undergoing nuclear fragmentation is displayed. COS-7 cells cotransfected with a constitutively active Gα-subunit and LacZ were treated with PKC inhibitors or with an equal volume of inhibitor solvent as a control (*B*). Calphostin C (*Cal C*) and Ro31–8220 were added to a final concentration of 2 and 1 μM, respectively. Cell morphology was analyzed 48 h after transfection. Transfections were performed with LipofectAMINE.

MEKK1 in PC12 cells or tumor necrosis factor-α stimulus in U937 cells (18, 32). The ability of GαqRC and Gα13QL to activate the cell death machinery also in high serum distinguish our data from other studies where apoptosis was only achieved by simultaneous expression of the respective protein and incubation in low serum. Therefore, Gα13QL and GαqRC seem to activate signaling pathways directly linked to the cell death machinery.

Activation of Gαq can be correlated with an increase in PKC activity and elevation of internal Ca^{2+} concentration as a result of phospholipase C-β activation. The role of PKC activation in apoptosis is not completely clear. In some cell systems PKC activation inhibits apoptosis, whereas in others PKC activation can be correlated with the onset of apoptosis (33–35). In our assay system, the PKC inhibitors totally abolished the apoptosis-inducing ability of Gαq. This reveals that PKC signaling is an essential step in Gαq triggered apoptosis. Elevation of internal Ca^{2+} levels is often observed in cells dying from apoptosis (36). Therefore, to determine if GαqRC induced an increase in cytoplasmic Ca^{2+} levels that may be associated with apoptosis, COS-7 cells expressing GαqRC were incubated with the Ca^{2+} chelator EGTA/AM. Incubation with 10 μM of this Ca^{2+} chelator provokes only a slight decrease in the number of apoptotic cells, presumably due to the low concentration of EGTA/AM used (data not shown). Higher concentrations of the Ca^{2+} chelator could not be used because of its cytotoxic action at higher doses during prolonged incubation (37). Interestingly, a neural disorder, amyotrophic lateral sclerosis, causing neural cell death presumably by apoptosis is accompanied by an elevation in PKC activity and cytoplasmic Ca^{2+} levels (11). The cause of this disease is currently unknown.

Sustained activation of Gαq was recently shown to cause transformation of NIH3T3 cells (38). This study also showed that a large fraction of the transfected cells died due to expression of constitutively active Gαq. These data can now be explained by the correlation between GαqRC and apoptosis that we found. The transformed phenotype might occur in cells that accumulate mutations affecting the cell death machinery.

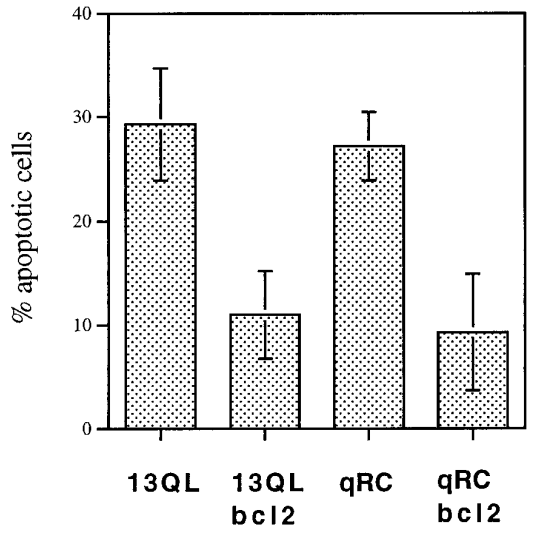

FIG. 6. **Overexpression of bcl-2 blocks apoptosis triggered by Gα13QL and GαqRC.** 0.5 μg of constitutively active Gα-subunit expression plasmid and 0.5 μg of bcl-2 expression plasmid or the equal amount of the empty expression vector were cotransfected into COS-7 cells. 48 h after transfection with LipofectAMINE, transfected cells were counted, and the percentage of cells displaying fragmented nuclei was determined.

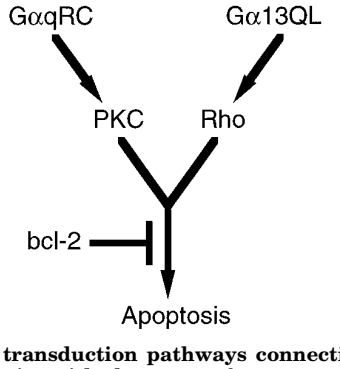

FIG. 7. **Signal transduction pathways connecting the activation of Gα-subunits with the onset of programmed cell death.** Expression of constitutively active Gαq and Gα13 trigger different pathways that lead to activation of apoptosis. However, these pathways converge upstream of a signaling event controlled by bcl-2 before initiation of the cell death program.

Recently it was demonstrated that sustained activation of Rho triggers programmed cell death in low serum (39). In contrast, our data show that coexpression of RhoAN19 and Gα13QL lead to a significant but not total reduction of apoptosis to background levels in the cell population (Fig. 5*A*). Therefore, it is likely that RhoAN19 expression levels are too low to completely prevent Rho-induced apoptosis. On the other hand, Gα13 might activate an additional pathway that contributes to the Rho signaling in inducing apoptosis in high serum.

Our data clearly demonstrate that Gα13QL- and GαqRC-triggered apoptosis can be blocked by overexpression of bcl-2. Thus, these pathways enter the cell death signaling cascade upstream of a point controlled by bcl-2. In conclusion, our results demonstrate the ability of Gα-subunits to induce programmed cell death through different pathways that, however, converge before the final execution of cell death.

We also performed experiments where constitutively active Gα12QL was expressed in COS-7 cells. A significant fraction of the cells showed the apoptotic phenotype, however, the amount was well below the fraction of apoptotic cells obtained with expression of GαqRC and Gα13QL (20% ± 6.08; data not shown). Recently it was demonstrated that Gα13 and Gα12 recruit different signaling pathways to activate Na^+/H^+ exchangers (40). In addition, null mutation of Gα13 in mice displays a distinct phenotype, which also argues against redundant functions of Gα13 and Gα12 (41). Therefore, it is likely that activated Gα13 transmits signals to the cell death machinery through pathways not recruited by Gα12.

TABLE I
Gα13QL- and GαqRC-induced apoptosis is inhibited by treatment with z-VAD.fmk

COS-7 cells were transfected with LacZ and Gα13QL or GαqRC with and without the addition of 50 μM of z-VAD.fmk. z-VAD.fmk was added four times over the course of the incubation period to maximize the effectiveness of the protease inhibitor. DNA fragmentation was assessed using the TUNEL assay to identify cells undergoing apoptosis. The percentage of TUNEL positive cells was calculated from the total number of cells transfected as determined by antibody staining of β-galactosidase. Results represent duplicate samples for each condition.

Cell transfection and treatment	Apoptotic Cells (% of Transfected Cells)
LacZ	10.3
	13.1
LacZ/Gα13QL	32.0
	36.6
LacZ/GαqRC	31.6
	37.9
LacZ (z-VAD.fmk)	5.4
	4.4
LacZ/Gα13QL (z-VAD.fmk)	12.8
	12.0
LacZ/GαqRC (z-VAD.fmk)	5.3
	10.0

Our study clearly demonstrates that sustained activation of Gαq or Gα13 can trigger apoptosis in different cell systems. Several human diseases are thought to be caused by a sustained stimulus that cannot be shut down through desensitization processes. The role of sustained signaling through Gαq or Gα13 in development and in disease remains to be more clearly defined.

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