Regulation of Apoptosis by α-Subunits of G12 and G13 Proteins via Apoptosis Signal-regulating Kinase-1*

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Many growth factors and G protein-coupled receptors activate mitogen-activated protein (MAP) kinase pathways. The MAP kinase pathways are involved in the regulation of the ubiquitous process of apoptosis or programmed cell death. Two related MAP kinase kinase kinases, apoptosis-signal regulating kinase 1 (ASK1) and MAP kinase kinase kinase 1 (MEKK1), stimulate c-Jun kinase (JNK) activity and induce apoptosis. Transient transfection of dominant negative and constitutively active components of the JNK pathway in COS-7 cells showed that two G protein subunits, Ga12 and Ga13, stimulated the JNK pathway in a ASK1- and MEKK1-dependent manner. Moreover, the mutationally activated Ga12 and Ga13 stimulated the kinase activity of ASK1. Both Ga12 and Ga13 employ small GTPases, Cdc42 and Rac1, to transduce signal to MEKK1 and, subsequently, to JNK. However, activation of JNK by Cdc42 and Rac1 did not require ASK1. Additionally, ASK1 and MEKK1 are involved in the apoptosis induced by Ga12 and Ga13. We conclude that Ga12 and Ga13 can induce apoptosis using two separate MAP kinase pathways; one is initiated by ASK1, and the other is initiated by MEKK1. Furthermore, Bcl-2 can block apoptosis induced by Ga12 and Ga13. This death-sparing function was associated with increased Bcl-2 phosphorylation, suggesting that phosphorylation of Bcl-2 may be a critical mechanism protecting cells from Ga12- and Ga13-induced apoptosis.

Mitogen-activated protein (MAP)1 kinases serve as a point of convergence for growth signals, including those generated from G protein-coupled receptors (1). The MAP kinase signaling pathway consists of three distinct members of the protein kinase family, including MAP kinase (MAPK), MAPK kinase (MAPKK), and MAPKK kinase (2). MAPK kinase phosphorylates and activates MAPKK, and the activated form of MAPKK in turn phosphorylates and activates MAPK. Activated MAPK may translocate to the cell nucleus and regulate the activities of transcription factors, thereby controlling gene expression. At least two defined MAP kinase signaling modules function in mammalian cells: the Raf-MEK-ERK, or ERK, pathway (3, 4) and the MEKK1-JNK kinase-JNK, or JNK, pathway (5).

Apoptosis or programmed cell death is a highly conserved active cellular mechanism characterized by cell shrinkage, chromatin condensation, and nuclear fragmentation (6). Apoptosis occurs in many physiological and pathophysiological conditions and is a fundamental process for the normal development of multicellular organisms (6–8). Apoptosis is controlled in part by a family of proteins whose prototype is Bcl-2. Bcl-2 protein blocks apoptosis mediated by many but not all mammalian physiological cell death stimuli (9). Phosphorylation is one of the mechanisms that may regulate the antiapoptotic function of Bcl-2 (10, 11). Interestingly, Bcl-2 undergoes phosphorylation by JNK in the presence of constitutively activated member of Rho family G proteins, Rac1 (12).

The MAP kinase pathways, in particular the JNK pathway, participate in stress responses and apoptosis (13); the apoptosis signal-regulating kinase 1 (ASK1) corresponding to MAPKK kinase has been recently identified (14). ASK1 induces apoptosis and regulates the activity of JNK and p38 MAPK (14). Activated MEKK1 similarly induces apoptosis (15).

Among the important functions of G proteins, their involvement in the regulation of cell growth and mitogenesis contrasts with their signaling of apoptosis. Thus, activating mutations in Gαs and Gαi2 genes are found in specific subsets of human tumors (16, 17). Additionally, mutationally activated α-subunits of G12 and G13 induce mitogenesis and neoplastic transformation in NIH3T3 cells and Rat-1 cells (18, 19). In contrast, recent evidence demonstrates that heterotrimeric G proteins may be involved primarily in the signaling pathways that regulate apoptosis (20, 21). In this study, we have analyzed the molecular mechanisms of signaling of apoptosis induced by Gα12 and Gα13. We found that both activation of JNK and apoptosis induced by Gα12 and Gα13 are mediated by two MAPKK kinases, MEKK1 and ASK1. We also observed that Bcl-2 inhibited apoptosis induced by Gα12 and Gα13. The results suggest that phosphorylation of Bcl-2 may be involved in the protective effect against apoptosis induced by Gα12 and Gα13.

EXPERIMENTAL PROCEDURES

Materials—Protein A-agarose was obtained from Life Technologies, Inc. Antibodies to Bcl-2, Gα12, Gα13, MEKK1, and 9E10 antibody to c-Myc epitope were obtained from Santa Cruz Biotechnology, Inc. Antibodies to Gα12, Gα13, MEKK1, and 9E10 antibody to c-Myc epitope were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Iγ-PiATP and FmAdenine were obtained from NEI Life Science Products. Polyclonal β-galactosidase antibody and rabbit rhodamine-conjugated IgG were purchased from Cappel and Pierce, respectively. The Apoptag Plus kit was purchased from Oncor. The production of ASK1 antibody (DAV) was described previously (22).
Mounting medium with 4',6-diamidino-2-phenylindole was obtained from Vector Laboratories. The ECL kit was obtained from Amersham Pharmacia Biotech. All other reagents were obtained from Sigma.

**DNA Constructs—**Plasmids used in this work are as follows. Murine Ge12 and Ge13 in the Bluescript vector were gifts from M. Simon (Caltech, CA). Constructs of constitutively active mutants of Go12-(Q229L), Go13(Q226L), Go13(Q227L), hemagglutinin-tagged JNK (JNK-HA), and MEKK1 were described previously (23). Myc-Cdc42(V12) and Myc-Rac(V12) were provided by M. Symons (Onyx, CA). Wild type and dominant negative ASK1 and MKK6 were previously described (14). Bcl-2 was a gift from David Ucker (University of Illinois, Chicago). The C terminus of ASK1 was tagged with HA epitope. Briefly, ASK1 was excised from pcDNA3 using a XhoI restriction enzyme. Next, purified ASK1 DNA fragment was ligated with pcDNA3 containing the following oligonucleotides encoding the HA epitope: 5′-GGCTCGAGAAGCATAATCAGGAACATCATAACTT (lower strand) and 3′-TCTAGATCCTATCTTTATGATGTTCCTGATTATGCTTGAT (upper strand). The sequence of annealed oligonucleotides encoding the HA epitope was as follows: CGAGGTTATGTAGTTCCTGATATGCTGT (upper strand) and CTAGATCAGGCATACATGAGGACATCACTT (lower strand). The sequence of the resulting construct was confirmed using restriction and sequencing analysis.

**Cell Culture and DNA Transfection—**COS-7 cells and human kidney embryonic 293 (HEK293) cells were propagated and transfected using the DEAE-dextran/chloroquine method, as described previously (23, 24) with an efficiency of transfection of 60–70%. To maintain uniform expression of transfected proteins as described under “Results,” transient transfection of either wild type ASK1 or HA-tagged ASK1 (data not shown). Aliquots of whole cell lysates from the same experiments were subjected to immunoblotting analysis to confirm the appropriate expression of transfected proteins as described under “Results.”

**Bcl-2 Phosphorylation—**Bcl-2 was transfected in the presence of various cDNA constructs as described under “Results,” and each transfection was performed on 2 × 10⁶ cells in a 60-mm dish. Twenty-four hours after transfection, the cells were transferred into two wells of a six-well plate. For each transfection, one well was used for Bcl-2 phosphorylation assay, and the other was used for immunoblotting analysis to confirm the appropriate expression of transfected proteins. Cells were labeled with 0.5 μCi/ml [32P]orthophosphoric acid in phosphate-free medium for 16 h. Cells were lysed with lysis buffer, and Bcl-2 protein was immunoprecipitated using 5 μg of Bcl-2 antibody for 4 h at 4 °C. The proteins were separated on SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and visualized by autoradiography, and radioactivity was quantitated with a Molecular Imager System (Bio-Rad). The same blot was then probed with Bcl-2 antibody and developed using the ECL kit.

**Analysis of Apoptotic Phenotype—**For apoptosis analysis, cells were co-transfected with cDNA encoding β-galactosidase. Twenty-four hours after transfection the cells were transferred into two wells of a six-well plate. Cells were grown on coverslips and fixed 48 h after transfection with 2% paraformaldehyde for 15 min. Cells were then permeabilized with 0.1% Triton X-100 for 10 min. Staining for fragmented genomic DNA with the Apoptag Plus kit was performed according to manufac-
The presence of GASK1(K709R) proteins was determined by immunoblotting with ASK1 determined with HA antibody 12CA5. The presence of ASK1 and using recombinant c-Jun as a substrate. The presence of JNK-HA was precipitated with 12CA5 antibody, and kinase activity was measured.

RESULTS
Ga12 and Ga13 Stimulate JNK via ASK1—We and others (23, 25, 26) have shown previously that mutationally activated Ga12 and Ga13 stimulate JNK activity. In addition, both $\alpha$-subunits employ small GTPases, Cdc42 and Rac (23, 26), and serine–threonine kinase MEKK1 (23) to transduce signal to JNK.

To examine if, in addition to MEKK1, other members of the MAP kinase family can mediate the regulation of JNK activity by Ga12 and Ga13, we used expression vectors encoding wild type and dominant negative ASK1. The substitution of Lys$^{709}$ to arginine makes ASK1 dominant negative; this mutant can prevent both activation of JNK and apoptosis induced by tumor necrosis factor-$\alpha$ and actinomycin D in lung epithelial cells (14).

To investigate the involvement of ASK1 in the signaling pathways regulated by Ga12 and Ga13, JNK-HA activity was measured in COS-7 cells expressing different amounts of wild type ASK1 and ASK1(K709R). JNK activity was not changed with 200 ng of ASK1(K709R) cDNA. A further increase of ASK1(K709R) cDNA resulted in a modest activation of JNK (Fig. 1). Titration experiments indicated that a 5-fold stimulation of JNK activity was observed with 100 ng of ASK1 plasmid.

Ga12 activity was expressed as -fold increase over control. The control level of phosphorylation was defined as the amount of [γ-32P]ATP incorporated into the MKK6 by ASK1-HA expressed alone. Data represent mean ± S.E. of triplicate determinations.

Graphs, a representative experiment. Cell lysates were divided and immunoprecipitated with 12CA5 antibody, and kinase activity was measured using recombinant c-Jun as a substrate. The presence of JNK-HA was determined with HA antibody 12CA5. The presence of ASK1 and ASK1(K709R) proteins was determined by immunoblotting with ASK1 antibody DAV. The presence of Ga12 or Ga13 proteins was determined by immunoblotting with Ga12 or Ga13 antibody, respectively. Two additional experiments gave similar results.
To evaluate how ASK1 is involved in the activation of JNK by Ga12 and Ga13, we measured JNK-HA activity in cells expressing wild type and constitutively activated Ga12 and Ga13 and wild type and dominant negative ASK1 (Fig. 2). Consistent with previous results showing that Ga12 and Ga13 activate the JNK pathway (23, 25, 26), constitutively activated forms of both proteins stimulated JNK-catalyzed phosphorylation of c-Jun in COS-7 cells (Fig. 2). Co-expression of the α-subunits with ASK1 resulted in additive increases in JNK activity. In contrast, dominant negative ASK1(K709R) inhibited JNK activation induced by both Ga12 and Ga13 (Fig. 2, graphs). Expression of JNK-HA protein remained unchanged in all experimental conditions as judged by the immunoblotting analysis (Fig. 2, graphs). This result indicates that changes in the JNK-HA activity are not due to changes in JNK-HA expression. Similarly, expression of Ga12, Ga13, ASK1, or ASK1(K709R) proteins remained unchanged after co-expression with indicated cDNA constructs as it was determined by the immunoblotting analysis with polyclonal antibodies to Ga12, Ga13, or ASK1 (Fig. 2, graphs). Thus, in addition to MEKK1 (23) Ga12 and Ga13 appear to stimulate JNK via ASK1.

To examine how Ga12 and Ga13 affect the kinase activity of ASK1, COS-7 cells were transiently transfected with ASK1 tagged with the HA epitope (ASK1-HA) at the C terminus. After immunoprecipitation with 12CA5 antibody and kinase assay with an exogenous substrate, we found that constitutively activated forms of both G proteins stimulated ASK1-catalyzed phosphorylation of MKK6 in COS-7 cells (Fig. 3, A and B). In agreement with previous observations (14), stimulation of the cells with tumor necrosis factor-α for 30 min also resulted in ASK1 activation. Dominant negative ASK1(K709R) inhibited ASK1 activation induced by Ga12, Ga13, and tumor necrosis factor-α (Fig. 3). Control immunoblotting from the same lysates confirmed appropriate expression from transfected plasmids (Fig. 3), indicating that Ga12 and Ga13 stimulate kinase activity of ASK1 in vivo.

Cdc42 and Rac1 Stimulate JNK via MEKK1 but Not via ASK1—Small GTPases, Rac1, Cdc42, and RhoA regulate the activity of the JNK pathway (27, 28). Because Ga12 and Ga13 regulate JNK activity via Cdc42 and Rac1 (23, 26), we investigated if Cdc42 and Rac1 employ the serine/threonine kinase ASK1 to stimulate JNK. Co-expression of constitutively activated Cdc42(V12) with ASK1 resulted in a synergistic increase of JNK activity (Fig. 4A). In addition, dominant negative ASK1(K709R) did not affect JNK activation induced by Cdc42(V12) (Fig. 4A). Similarly, co-expression of constitutively activated Rac1(V12) with ASK1 resulted in a synergistic increase of the JNK activity (Fig. 4A). Finally, dominant negative ASK1(K709R) did not affect the JNK activation induced by Rac1(V12) (Fig. 4A). The expression of JNK-HA protein remained unchanged in all experimental conditions as it was judged by immunoblotting analysis (Fig. 4A, graph), indicating that observed changes in the JNK-HA activity are not due to changes in JNK-HA expression. Control immunoblotting from the same lysates confirmed appropriate expression of the wild type and mutant ASK1, Cdc42(V12), and Rac1(V12) from transfected Cdc42 and Rac1 Stimulate JNK via MEKK1 but Not via ASK1—Small GTPases, Rac1, Cdc42, and RhoA regulate the activity of the JNK pathway (27, 28). Because Ga12 and Ga13 regulate JNK activity via Cdc42 and Rac1 (23, 26), we investigated if Cdc42 and Rac1 employ the serine/threonine kinase ASK1 to stimulate JNK. Co-expression of constitutively activated Cdc42(V12) with ASK1 resulted in a synergistic increase of JNK activity (Fig. 4A). In addition, dominant negative ASK1(K709R) did not affect JNK activation induced by Cdc42(V12) (Fig. 4A). Similarly, co-expression of constitutively activated Rac1(V12) with ASK1 resulted in a synergistic increase of the JNK activity (Fig. 4A). Finally, dominant negative ASK1(K709R) did not affect the JNK activation induced by Rac1(V12) (Fig. 4A). The expression of JNK-HA protein remained unchanged in all experimental conditions as it was judged by immunoblotting analysis (Fig. 4A, graph), indicating that observed changes in the JNK-HA activity are not due to changes in JNK-HA expression. Control immunoblotting from the same lysates confirmed appropriate expression of the wild type and mutant ASK1, Cdc42(V12), and Rac1(V12) from transfected plasmids (Fig. 3), indicating that Ga12 and Ga13 stimulate kinase activity of ASK1 in vivo.

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Heterotrimeric G Proteins and Apoptosis

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Fected plasmids (Fig. 4A, graph), suggesting that small G proteins Cdc42 and Rac1 and serine/threonine kinase ASK1 use different pathways to stimulate JNK.

To confirm that ASK1 is not involved in the pathway that leads from Cdc42 and Rac to stimulation of JNK, the ASK1 activity was measured in the cells expressing activated Cdc42 and Rac (Fig. 4B). Our results showed that co-expression of either Cdc42(V12) or Rac(V12) with ASK1-HA did not result in increased catalytic activity of the tested kinase (Fig. 4B). At the same time, immunoblotting analysis of the total cell lysates showed the appropriate expression of the proteins from the transfected plasmids (Fig. 4B, graph).

At the same time, dominant negative MEKK1(K432A) inhibited JNK activation induced by both Cdc42(V12) and Rac(V12) (Fig. 4C). Finally, expression of Cdc42(V12) or Rac(V12) proteins remained unchanged after co-expression with ASK1 or ASK1(K709R) as determined by the immunoblotting analysis with 910E antibody (Fig. 4C, graph). Taken together, these results indicate that Cdc42 and Rac1 stimulate JNK via MEKK1 but not via ASK1.

Apoptosis Induced by Ga12 and Ga13 Is Mediated by both ASK1 and MEKK1—To study the apoptosis induced by Ga12 and Ga13, β-galactosidase cDNA was co-transfected with the various α-subunits. Twenty-four and 48 h after transfection, the extent of cell death was estimated measuring the number of cells with fragmented DNA using the Apoptag Plus kit (Fig. 5, A–C). Constitutively activated Ga12 and Ga13 induced apoptosis in 25–30% of the cells (Fig. 5D). Apoptosis induced by wild type Ga12 and Ga13 was less pronounced. To study the specificity of apoptotic response induced by Ga12 and Ga13, constitutively activated Gas(Q227L) was used. Gas(Q227L) did not induce apoptosis when transiently transfected into COS-7 cells (Fig. 5D). In the same cells, Gas(Q227L) induced a 6-fold increase in cAMP accumulation (Fig. 5E), confirming the functional activity of the expressed protein.

We next examined if serine/threonine kinases ASK1 and MEKK1 are involved in the apoptosis induced by Ga12 and Ga13. Co-expression of the tested α-subunits with ASK1 resulted in an additive increase in apoptosis (Fig. 6, A and B). Moreover, dominant negative ASK1(K709R) inhibited apoptosis induced by Ga12 and Ga13 by 50–60% (Fig. 6, A and B). Similarly, co-expression of the tested α-subunits with MEKK1 resulted in additive increase in apoptosis (Fig. 6, A and B). Additionally, dominant negative MEKK1(K432A) inhibited apoptosis induced by Ga12 and Ga13 by 50–60% (Fig. 6, A and B). Immunoblotting analysis from the same lysates confirmed appropriate expression of the proteins from transfected plasmids (Fig. 6, A and B, graphs), indicating that apoptosis induced by Ga12 and Ga13 is mediated by both ASK1 and MEKK1.

Bel-2 Prevents Apoptosis and Undergoes Phosphorylation in Cells Expressing Ga12 and Ga13.—The protooncogene Bel-2 functions as an apoptosis suppressor in many systems (29–33), although its mechanism of action is not yet known. Emerging data imply that members of the Bel-2 gene family are targets for phosphorylation, suggesting one potential mechanism of control (10, 12). Co-expression of Bel-2 with constitutively activated Ga12 and Ga13 almost completely abolished apoptosis induced by these α-subunits (Fig. 7A). Immunoblotting analysis revealed endogenously expressed Ga12 and Ga13 in COS-7

COS-7 cells (data not shown). E, cAMP accumulation in the cells expressing Gas(Q227L). After transfection, cells were divided for apoptosis and cAMP assays. The data represent the mean ± S.E. of triplicate determinations in a representative experiment; two additional experiments gave similar results.
cells. The amount of the G protein α-subunits was apparently increased in the cells transfected with cDNAs encoding Gα12 or Gα13. At the same, the commercial antiserum for Bcl-2 failed to detect endogenous Bcl-2 protein; however, the transfected Bcl-2 protein was readily detectable (Fig. 7A, graph).

We investigated if the phosphorylation state of Bcl-2 was affected by Gα12 and Gα13. Following metabolic labeling with inorganic 32P, Bcl-2 could be identified as a phosphoprotein (Fig. 7B). Incorporation of labeled phosphate into Bcl-2 in the cells expressing Gα12 and Gα13 was significantly higher. However, the amount of Bcl-2 protein expressed in the same experimental conditions remained unchanged as tested with Western blotting analysis (Fig. 7B), indicating a net increase in Bcl-2 phosphorylation. These data show that G protein-induced apoptosis was suppressed by Bcl-2 and that this sparing effect was associated with Bcl-2 phosphorylation.

**DISCUSSION**

Our results allow us to draw three new conclusions with respect to the effects of Gα12 and Gα13 on downstream signaling pathways. These proteins (a) activate MAPK kinase ASK1 in a Cdc42- and Rac-l-independent manner; (b) induce apoptosis by a mechanism that involves MEKK1 and ASK1; and (c) induce the phosphorylation of the ant apoptotic Bcl-2 protein.

**Activation of ASK1 by Gα12 and Gα13**—In the present investigation, we characterized a new signaling pathway that is regulated by Gα12 and Gα13. Recently identified MAPK kinase ASK1 activates MKK4 and MKK6, which in turn activate JNK and p38 MAPK, respectively (14). In addition, ASK1 is activated by tumor necrosis factor-α and induces apoptosis (14). We tested if ASK1 is regulated by α-subunits of heterotrimeric G12 and G13 proteins. Our data show that in transient transfection experiments, constitutively activated mutants of Gα12 and Gα13 proteins stimulate the kinase activity of ASK1 (Figs. 3 and 8). In our previous work, we showed that Gα12 and Gα13 stimulate JNK via MEKK1 (23). We now show that Gα12 and Gα13 stimulate JNK via ASK1 in addition to MEKK1 (Figs. 2 and 8).

The MAP kinase pathways (ERK and JNK) are typically regulated by small G proteins: Ras in the case of the ERK pathway (34, 35) and Cdc42, Rac, and Rho (27, 28) in the case of the JNK pathway. Moreover, regulation of these MAP kinase pathways by heterotrimeric G proteins seems to require activation of the small G proteins mentioned above. Thus, G12-dependent activation of ERK requires Cdc42 and Rac1 stimulate JNK via ASK1 independently of Cdc42 or Rac1 (Fig. 4). Finally, stimulation of the JNK by Cdc42 and Rac1 occurs by a mechanism that requires MEKK1 but not ASK1 (Figs. 4 and 8). Whether small G proteins can regulate ASK1 activity remains to be investigated.

**Ga12 and Ga13 Induce Apoptosis by a Mechanism That Involves MEKK1 and ASK1**—Several lines of evidence suggest...
that heterotrimeric G proteins are involved in the regulation of apoptosis in mammalian cells as follows. (a) G protein-coupled receptors can either prevent or induce apoptosis. Somatostatin receptor type 3 and purinergic receptor P2Y2 induces apoptosis in different cell systems (37, 38). In contrast, activation of muscarinic cholinergic receptors blocks apoptosis of cultured cerebellar neurons (39). (b) Small G proteins, such as Ha-Ras, R-Ras, Rho, and Rac, which are regulated by heterotrimeric G proteins (among other stimuli), induce apoptosis in certain cell systems. For example, two small G proteins, R-Ras and Rho, induce apoptosis in cultured cells via a Bel-2-suppressible mechanism (40, 41). Additionally, in human leukemic Jurkat cells, apoptosis induced by ceramide and Fas ligand was blocked after inhibition of Ras, Rac1, or JNK/p38 MAPK (42, 43). Finally, genetic inhibition of Ras and Rac by dominant negative N17Ras and N17Rac, antisense oligonucleotides to Rac, or cellular treatment with Botulinus C3 exoenzyme prevent Ras stimulation as well as apoptosis after Fas receptor stimulation (43). (c) The JNK/p38 MAPK pathways, which are regulated by heterotrimeric G proteins (among other stimuli), cause apoptosis in certain cell systems. Thus, persistent activation of JNK is involved in the initiation of the apoptosis (13, 44). (d) Apoptosis induced by familial Alzheimer’s disease-associated mutants of the amyloid precursor protein is mediated by the Go protein (20). A recent report by Althoefer (21) shows that Gαq and Gα13 induce apoptosis that is Bcl-2 supressible and does not require serum deprivation (21).

In our experiments, we found that constitutively activated Gα12 and Gα13 induce apoptosis in COS-7 (Fig. 5) and HEK293 cells (data not shown). In addition, we found that apoptosis induced by Gα12 and Gα13 was executed by a MEKK1- and ASK1-dependent mechanism (Fig. 6). We also found that ASK1 is a more potent inducer of apoptosis than MEKK1 (Fig. 6); to induce an equal amount of apoptotic cells, 10 times more MEKK1 was required than ASK1 (Fig. 6). However, the mechanism by which ASK1 induces apoptosis is unknown. One possibility is that, similarly to MEKK1 (45), ASK1 activation by caspase cleavage will be required for apoptotic response.

**Fig. 7.** Apoptosis induced by Gα12 and Gα13 is inhibited by Bel-2. A, Bel-2 inhibited apoptosis induced by Gα12 and Gα13. COS-7 cells were transiently transfected with 1 μg of α-subunits and 1 μg of Bel-2 as indicated. The number of cells that underwent apoptosis was estimated 48 h after transfection. In each experiment, 400 cells were scored. Data represent mean ± S.E. of triplicate independent experiments. Graph, expression of the transfected cDNAs. After transfection, cells were divided for apoptosis and immunoblotting assays. Immunoblotting of aliquots of whole cell lysate with Gα12 (top) or Gα13 (middle) antibody is shown. Expression of Bel-2 protein was determined by immunoblotting with Bel-2 antibody (bottom). B, incorporation of inorganic 32P into Bel-2 in cells overexpressing Gα12 and Gα13. After transfection, cells were divided for phosphorylation and immunoblotting assays. Immunoblotting of aliquots of whole cell lysate with Bel-2, Gα12, or Gα13 antibody is shown. The experiment was performed twice with similar results.

**Fig. 8.** Signaling pathways connecting Gα12 and Gα13 with activation of JNK (A) and apoptosis (B). Gα12 and Gα13 stimulate JNK using two independent pathways; one pathway connects Go subunits to JNK via small G proteins, Cdc42 and Rac, and kinase MEKK1. Another pathway is using ASK1 to stimulate JNK. However, both MEKK1 and ASK1 are involved in the apoptosis induced by Gα12 and Gα13 proteins. Question marks show unknown participants in the Gα12 and Gα13 signaling pathway. JNKK, JNK kinase.
esis or apoptosis (18, 19, 21, 26). One possibility is that different composition of signaling components that can be regulated by Gα12 and Gα13 will determine the cellular response initiated by these G proteins.

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REFERENCES
1. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) Science 260, 315–319
2. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89
3. Davis, R. J. (1994) Trends Biochem. Sci. 19, 470–473
4. Waskiewicz, A. J., and Cooper, J. A. (1995) Curr. Opin. Cell Biol. 7, 798–805
5. Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Science 267, 682–685
6. Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980) Int. Rev. Cytol. 68, 251–306
7. Raff, M. C. (1992) Nature 356, 397–400
8. Cohen, J. J. (1993) Science 260, 6511–6519
9. Ucker, D. S. (1997) J. Biol. Chem. 272, 11671–11673
10. Chen, C.-Y., and Faller, D. V. (1997) J. Biol. Chem. 272, 2376–2379
11. Maundrell, K., Antonsson, B., Magnenat, E., Camps, M., Muda, M., Chabert, C., Gillieron, C., Boschet, U., Vial-Knecht, E., Martinou, J.-C., and Arkinstall, S. (1997) J. Biol. Chem. 272, 25238–25242
12. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) Science 270, 1326–1331
13. Ichijo, H., Nishida, E., Irie, K., Dijke, P., Takagi, M., Matsumoto, K., K., M., and Gotoh, Y. (1997) Science 275, 90–94
14. Johnson, N. L., Gardner, A. M., Diener, K. M., Lange-Carter, C. A., Glevay, J., Jarbe, M. B., Minden, A., Karin, M., Zon, L. I., and Johnson, G. L. (1996) J. Biol. Chem. 271, 3229–3237
15. Lyons, J., Landis, C. A., Harsh, G., Vallar, L., Grunewald, K., Feichtinger, H., Duh, Q. Y., Clark, O. H., Kawasaki, E., Bourne, H. R., and McCormick, F. (1990) Science 249, 655–659
16. Landsis, C. A., Masters, S. B., Spoda, A., Page, A. M., Bourne, H. R., and Vallar, L. (1989) Nature 340, 692–696
17. Xu, N., Bradlet, L., Ambdukar, I., and Gutkind, J. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6741–6745
18. Voyno-Yasenetskaya, T. A., F., A. P., and Bourne, H. R. (1994) Oncogene 9, 2559–2565
19. Yamatsuji, T., Matsui, T., Okamoto, T., Komatsuaki, K., Takeda, S., Nakamoto, H., Iwasaki, T., Suzuki, N., Azami-Okada, A., Ireland, S., Kinane, T. B., Giambarella, U., and Nishimoto, I. (1996) Science 272, 1349–1352
20. Althoefer, H., Eversole-Cire, P., and Simon, M. I. (1997) J. Biol. Chem. 272, 24380–24386
21. Ichijo, H., Nishida, E., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., Ichijo, H. (1998) EMBO J. 17, 2596–2606
22. Voyno-Yasenetskaya, T. A., Faure, M., A., Ahn, N., and Bourne, H. R. (1996) J. Biol. Chem. 271, 21081–21087
23. Faure, M., Voyno-Yasenetskaya, T. A., and Bourne, H. R. (1994) J. Biol. Chem. 269, 7851–7854
24. Prasad, M. V. V. S., Dermott, J. M., Heasley, L. E., Johnson, G. L., and Dhanasekaran, N. (1995) J. Biol. Chem. 270, 18655–18659
25. Collins, L. R., Minden, A., Karin, M., and Brown, J. H. (1996) J. Biol. Chem. 271, 17349–17353
26. Yoon, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) Cell 81, 1137–1146
27. Teramoto, H., Crespo, P., Yoon, O. A., Ijichi, T., Xu, N., and Gutkind, J. S. (1996) J. Biol. Chem. 271, 25731–25734
28. Ucker, D. S., Faure, M., and Bourne, H. R. (1996) Mol. Cell Biol. 1688–1696
29. Wacker, A. F., Small, M. B., and Hay, N. (1993) Mol. Cell Biol. 13, 2432–2440
30. Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Millman, C. L., and Korsmeyer, S. J. (1993) Cell 75, 241–251
31. Kane, D. J., Sarafian, T. A., Anten, R., Hahn, H., Grolla, E. B., Valentine, J. S., Ord, T., and Brederson, D. E. (1993) Science 262, 1274–1277
32. Mosch, S. A., Williams, B. M., Weber, M. J., and Wolfman, A. (1993) Science 260, 1568–1611
33. van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6213–6217
34. Pace, A. M., Faure, M. and Bourne, H. R. (1995) Mol. Biol. Cell 6, 1685–1695
35. Sharma, K., Patel, Y. C., and Srikant, C. B. (1996) Mol. Endocrinol. 10, 1688–1696
36. Apasov, S. G., Koshiha, M., Chused, T. M., and Sitkovsky, M. V. (1997) J. Immunol. 158, 5095–5105
37. Yan, G. M., Lin, S. Z., Irwin, B. P., and Paul, S. M. (1995) Mol. Pharmacol. 47, 248–257
38. Jimenez, B., Arrendas, M., Esteve, P., Perona, R., Sanchez, R., Cai, S. R., Wyllie, A., and Alcali, J. C. (1995) Oncogene 10, 811–816
39. Wang, H. G., Millan, J. A., Cox, A. D., Der, C. J., Rapp, U. R., Beck, T., Zha, H., and Reed, J. C. (1995) J. Cell Biol. 129, 1103–1114
40. Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F., and Gulbins, E. (1997) J. Biol. Chem. 272, 22173–22181
41. Gulbins, E., Coggeshall, M. B., Brenner, B., Schlottmann, K., Linderkamp, O., and Lang, F. (1996) J. Biol. Chem. 271, 26389–26394
42. Chen, Y. R., Meyer, C. F., and Tan, T. H. (1996) J. Biol. Chem. 271, 631–634
43. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frish, S. M. (1997) Cell 80, 315–323
44. Yang, E., and Korsmeyer, S. J. (1996) Blood 88, 386–401
45. Farrow, S. N., and Brown, R. (1996) Curr. Opin. Genet. Dev. 6, 45–49
46. Oltvai, Z. N., Millman, C. S., and Korsmeyer, S. J. (1993) Cell 74, 699–619