Apoptosis is an essential mechanism for the maintenance of somatic tissues, and when dysregulated can lead to numerous pathological conditions. G proteins regulate apoptosis in addition to other cellular functions, but the roles of specific G proteins in apoptosis signaling are not well characterized. Gα12 stimulates protein phosphatase 2A (PP2A), a serine/threonine phosphatase that modulates essential signaling pathways, including apoptosis. Herein, we examined whether Gα12 regulates apoptosis in epithelial cells. Inducible expression of Gα12 or constitutively active (QLα12) in Madin-Darby canine kidney cells led to increased apoptosis with expression of QLα12, but not Gα12. Inducing QLα12 led to degradation of the anti-apoptotic protein Bcl-2 (via the proteasome pathway), increased JNK activity, and up-regulated IκBα protein levels, a potent stimulator of apoptosis. Furthermore, the QLα12-stimulated activation of JNK was blocked by inhibiting PP2A. To characterize endogenous Gα12 signaling pathways, non-transfected MDCK-II and HEK293 cells were stimulated with thrombin. Thrombin activated endogenous Gα12 (confirmed by GST-tetrapricopeptide repeat (TPR) pull-downs) and stimulated apoptosis in both cell types. The mechanisms of thrombin-stimulated apoptosis through endogenous Gα12 were nearly identical to the mechanisms identified in QLα12-MDCK cells and included loss of Bcl-2, JNK activation, and up-regulation of IκBα. Knockdown of the PP2A catalytic subunit in HEK293 cells inhibited thrombin-stimulated apoptosis, prevented JNK activation, and blocked Bcl-2 degradation. In summary, Gα12 has a major role in regulating epithelial cell apoptosis through PP2A and JNK activation leading to loss of Bcl-2 protein expression. Targeting these pathways in vivo may lead to new therapeutic strategies for a variety of disease processes.

Signaling through G proteins is an important mechanism regulating apoptosis, a highly conserved process of programmed cell death fundamental to normal development and somatic maintenance in all multicellular organisms. Changes in apoptosis signaling pathways contribute to major disease processes including cancer, degenerative neurological diseases, such as Parkinsonism and Alzheimer, and kidney failure from numerous etiologies including polycystic kidney disease. There are two major signaling pathways leading to apoptosis. The intrinsic pathway is mediated by the permeabilization of mitochondria and caspase-9 activation and is a general response to cell damage or stress. The extrinsic pathway is activated by stimulation from specific death inducing factors, such as Fas (apoptosis-stimulating fragment) and tumor necrosis factor and is mediated by caspase-8 (1). In the intrinsic pathway, the Bcl family (including pro- and anti-apoptotic proteins) is central to determining whether cells will undergo apoptosis (reviewed in Ref. 2). Bcl-2 is localized in the outer mitochondrial, endoplasmic reticulum, and perinuclear membranes, and under proliferative and anti-apoptotic (pro-survival) conditions, Bcl-2 heterodimerizes with pro-apoptotic protein BAX to form a stable, non-conductive complex (2). With an apoptotic stimulus, Bcl-2 is phosphorylated; this prevents dimerization with Bax and also leads to Bcl-2 degradation, thus permitting BAX to homodimerize and catalyze the formation of the mitochondrial apoptosis-induced channel. This channel allows the efflux of cytochrome c, triggering Apaf-1 and the caspase cascade leading to apoptosis (2, 3).

Heterotrimeric G proteins regulate numerous cellular processes including proliferation, differentiation, junctional assembly, and apoptosis. G proteins consist of Gα and Gβγ subunits, and form a stable heterotrimeric complex with GDP bound to the α subunit in the resting state. In canonical G protein signaling, ligand binding to a seven-transmembrane domain receptor results in conformational changes in Gα that lead to dissociation of GDP and separation from Gβγ. GTP subsequently binds to Gα, and signal transduction occurs through Gα and Gβγ subunits until the intrinsic Gα GTPase activity hydrolyzes GTP to GDP. It is now appreciated that G protein signaling is quite complex with the identification of G proteins in subcellular microdomains and in interactions with numerous regulatory and scaffolding proteins. Several studies have implicated signaling through each of the four heterotrimeric G protein families (Gα16, Gα16/13, Gα14/11, and Gα12/13) to...
regulate apoptosis, but the mechanism(s) are not well defined (4–9).

Gα_{12} and Gα_{13} regulate fundamental cellular processes that include proliferation (10), transformation (11), tight junction assembly (12, 13), directed cell migration (14), and regulation of the actin cytoskeleton (15). Gα_{12/13} are potent oncopgenes in some cell types (16, 17), and may also stimulate apoptosis. In transiently transfected COS cells, Gα_{12} and Gα_{13} induced apoptosis through activation of MEKK1 and Ask1 in a Bcl-2-dependent manner (18), and in a human adenocarcinoma cell line, a mutant Gα_{13} stimulated apoptosis through JNK (19). However, there is very little known about the role of Gα_{12} in regulating apoptosis in non-transfected cells, and few studies have investigated G protein regulation of apoptosis in epithelial cells. Recently, we identified an interaction of Gα_{12} with the regulatory subunit of the serine/threonine phosphatase protein phosphatase 2A (PP2A) and demonstrated that Gα_{12} stimulated PP2A activity in vitro and in MDCK cells (20, 21). PP2A is implicated in many of the same cellular processes that have been described for Gα_{13} (reviewed in Ref. 22). PP2A regulates apoptosis by maintaining dephosphorylated Bcl-2 (23) as well as regulating several other members of the Bcl-2 family. Identification of the Gα_{12}-PP2A interaction prompted us to investigate whether these signaling proteins were linked to apoptosis in epithelial cells. Using a well characterized MDCK cell culture model of inducible expression of Gα_{12}, we report that Gα_{12} is a potent activator of apoptosis in epithelial cells and identify critical regulation of Bcl-2 protein levels by Gα_{12}, PP2A, and JNK. Furthermore, we demonstrate for the first time that activation of endogenous Gα_{12}-coupled signaling pathways stimulates apoptosis in epithelial cells and recapitulates the signaling pathways identified in the inducible Gα_{12}-MDCK cell model.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Rabbit polyclonal anti-Gα_{12}, anti-JNK1, anti-ERK, anti-NF-κB, anti-IκBα, and goat anti-PP2A catalytic unit were from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-pThr^{185}/pTyr^{185}-JNK, anti-pThr^{202}/pTyr^{204}-ERK, and anti-Bcl-xL were from Cell Signaling Technology (Danvers, MA), mouse anti-Bcl-2 was from BD Biosciences (San Jose, CA), and rabbit anti-phospho-c-Jun was from Biovision (Mountain View, CA). Rabbit polyclonal anti-β-actin was from Sigma. Fosstrecin and okadaic acid were from Calbiochem, SP600125 was from A.G. Scientific (San Diego, CA), and lactacystin was from Sigma. Human α-thrombin was from Enzyme Research Laboratories (South Bend, IN). Plasticware and culture slides were from BD Falcon (Lincoln Park, NJ).

**Cell Culture**—Establishment, characterization, and culture conditions for Tet-Off MDCK-II cell lines (Clontech) with inducible wild type Gα_{12} and constitutively activated QLα_{12} expression were previously described (13). Briefly, cells were maintained in Dulbecco’s modified Eagle’s medium (Cellgro, Herndon, VA) containing 5% Tet system-approved fetal bovine serum (Clontech), 100 μg/ml G418 (Cellgro), 50 IU/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). 100 μg/ml hygromycin (Roche Applied Science), and 40 ng/ml doxycycline (dox) (Sigma). Non-transfected Tet-Off MDCK-II cell lines were maintained in the same culture medium without hygromycin. Human embryonic kidney (HEK293) 293 cell lines (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

**Flow Cytometry—Gα_{12}** and QLα_{12}-expressing MDCK cells were grown to confluence and cultured ± dox for 72 h. Adherent cells were collected by trypsinization and pooled with floating cells (1–2 × 10^{6}) by low speed centrifugation, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol in PBS at 4 °C for 30 min. Subsequently, cells were incubated with 1 μg/ml RNase A in PBS for 30 min at room temperature, after which cells were stained in 50 μg/ml propidium iodide (Invitrogen) in PBS for 30 min at room temperature and analyzed by flow cytometry in the propidium iodide/PE Texas Red channel. 10,000 cells were analyzed per experiment.

**DNA Fragmentation Assay—Gα_{12}** and QLα_{12}-expressing MDCK cells were cultured ± dox for 72 h. Adherent cells were collected by trypsinization and pooled with floating cells by low speed centrifugation, washed with PBS, and lysed with genomic DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K). Extracts were incubated for 24 h at 50 °C and subsequently for 1 h at 37 °C with 1 μg/ml RNase A (MP Biomedical, Solon, OH). DNA was extracted with an equal volume of phenol/chloroform (1:1) and precipitated at −70 °C for 24 h with 3 volume equivalents of absolute ethanol. DNA pellets were resuspended in 20 μl of 10 mM Tris (pH 7.8), 1 mM EDTA buffer and analyzed by electrophoresis on a 0.5% agarose gel run at 50 V for 4 h. Images were obtained using a Kodak DC290 Zoom Digital Camera (Eastman Kodak).

**Caspase 9 Activity—Gα_{12}** or QLα_{12}-expressing MDCK cells were cultured ± dox for 72 h. Cells were lysed and assessed for caspase-9 activation using LEHD-pNA as a substrate, as per the manufacturer’s instructions (Caspase 9 Colorimetric Assay Kit, Chemicon/Millipore, Billerica, MA).

**Cell Proliferation Assay—Gα_{12}** or QLα_{12}-expressing MDCK cells were cultured ± dox for 72 h in a 96-well plate. Subsequently, cells were incubated with the tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenoxy)-2H-5-tetrazolio]-1,3-benzene disulfonate (Biovision, Mountain View, CA) in Electroluting Solution (Biovision) substrate for 2 h. The plate was read at 450 nm to quantify formazan produced, which correlates directly to mitochondrial dehydrogenase activity.

**Kinexus Kinetworks™ Phospho-site Phosphorylation Screen**—1 × 10^{7} QLα_{12}-expressing MDCK cells were grown to confluence, cultured ± dox for 72 h. To obtain whole cell lysates, monolayers were washed with ice-cold PBS, scraped in lysis buffer (100 mM NaCl, 2 mM EDTA, 10 mM HEPES, pH 7.5, 1 mM Na3VO4, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, and proteases inhibitors (Roche)), sonicated gently, and subjected to low speed centrifugation. Supernatants were collected by trypsinization, and protein concentration. 750 μl of 1 mg/ml was analyzed for phosphorylation of target proteins (Kinexus Bioinformatics Corp., Vancouver, B.C., Canada).

**c-Jun N-terminal Kinase Activity—Gα_{12}** or QLα_{12}-expressing MDCK cells were cultured ± dox for 72 h. Floating cells and adherent cells were collected by trypsinization for 5 min and...
low speed centrifugation, washed with PBS, and lysed with INK Extraction Buffer (Biovision). Lysates were normalized for protein concentration and analyzed according to the manufacturer’s instructions (KinaseSTAR JNK Activity Screening Kit, Biovision). Briefly, lysates were incubated with GST-cjun on glutathione-Sepharose beads to pull down total JNK, which was resuspended with 200 μM ATP and incubated at 30 °C for 30 min. Samples were eluted in Laemmli sample buffer and analyzed by SDS-PAGE and Western blot for phospho-cjun.

**RNA Isolation and Semiquantitative RT-PCR Analysis—Gα12- or QLα12-expressing MDCK cells were cultured ± dox for 72 h and lysed with TRIzol (Invitrogen). Total RNA was purified according to the manufacturer’s protocol and quantified. 5 μg of total RNA was reverse transcribed using the Transcript Reverse Transcriptase Kit (Roche). Equal aliquots of cDNA (5 μl) were subsequently amplified for Bcl-2 and β-actin. The oligonucleotide primer sequences used for Bcl-2 and β-actin (Operon, Huntsville, AL) were as follows: Bcl-2: sense, 5'-ATG-GCGCACGCTGGGC-3'; antisense, 5'-TCACATTAGGCAGATATGCAC-3'; β-actin: sense, 5'-ATGGACGATGAATGCGGCG-3'; antisense, 5'-CTAGAAGCATTTGGCGGCG-3'. After 35 cycles, the amplified fragments were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining using a Kodak DC290 Zoom Digital Camera (Kodak).

**Immunoblot Analysis—**Gα12- or QLα12-expressing MDCK cells were cultured ± dox for 72 h. Lysates were prepared as described above and analyzed by SDS-PAGE and Western blot with the following primary antibodies: Gα12 (1:1000), Bcl-2 (1:500), JNK1 (1:500), pThr183/pTyr185-JNK (1:500), ERK1 (1:500), Bcl-xL (1:500), IκBα (1:500), NF-κB (1:500), or β-actin (1:10,000). After washing and incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 60 min at room temperature, signal was detected with SuperSignal West Pico horseradish peroxidase substrate system (Pierce) and autoradiography (Biomax MR, Kodak).

**GST-TPR Pull Down Assay—**The GST-TPR construct was kindly provided by Dr. M. Negishi, Kyoto University, Kyoto Japan. GST-TPR and GST were expressed in Escherichia coli and purified from bacterial lysates as described previously (24). Cells were lysed in Lysis Buffer (100 mM NaCl, 2 mM EDTA, 10 mM HEPES, pH 7.5, 1 mM Na3VO4, 25 mM NaF, 1 mM phenyl-
methylsulfonyl fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Roche)) and normalized for protein concentration. 1 μg of GST or GST-TPR coupled to glutathione-agarose beads (Amersham Biosciences) was added to 800 μg of total protein and rocked overnight at 4°C. Beads were pelleted with low speed centrifugation and washed three times with PBS, 0.1% Triton X-100, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE and Western blot.

siRNA Knockdowns—PP2A catalytic subunit was selectively knocked down using SMARTpool pooled siRNAs (Upstate, Lake Placid, NY) that contains four different siRNAs (catalog number D-001206-13-01). A nonspecific control pool (catalog number D-001206-13-01) was used for the negative control and also contained four different siRNAs. HEK293 cells were transfected with siRNA (200 nM) using Lipofectamine (Invitrogen) following the manufacturer’s instructions. After overnight incubation, fresh media was added to the cells, and the cells were cultured an additional 24 h. Lysates were prepared and analyzed by Western blot with goat anti-PP2A catalytic subunit antibody at 1:2000. For thrombin stimulation, cells were serum starved overnight prior to treatment with 2 units/ml INK phosphorylation and Bcl-2 expression were examined by Western blot at 8 h, and apoptosis was quantified after 24 h.

Quantification and Statistics—Western blots and agarose gels were scanned using an Epson 1640 desktop scanner and band intensity quantified using NIH Image (Wayne Rasband) after subtracting background and determining linear range. Statistics were done in GraphPad Prism (San Diego, CA). Significance was determined by using the two-tailed t test.

RESULTS

Expression of QLα12 in MDCK-II Cells Induces Apoptosis—Several factors including the disruption of cell-matrix interactions and dome formation associated with confluence have been shown to trigger apoptosis in MDCK cells (25, 26). The pathways associated with these phenotypes are not known. The study of Ga12 has been limited by low levels of expression in cells, and by its novel protein characteristics that make it difficult to express in vitro and in cell culture models. We have established and extensively characterized the inducible expression of wild type Ga12 and constitutively active QLα12 using the Tet-off MDCK cell culture model (12, 13). The removal of dox for 72 h induces Ga12 expression (Fig. 1A), and the re-addition of dox suppresses Ga12 protein expression to background levels within 48 h (not shown, but in Refs. 12 and 13). We have previously demonstrated that expression of QLα12, but not wild type Ga12 leads to disruption of junctional complexes and inhibits dome formation (12). To determine whether Ga12 regulated apoptosis in MDCK cells, Ga12- and QLα12-MDCK cells were cultured ± dox for 72 h, followed by overnight serum starvation. Apoptosis was determined by propidium iodide flow cytometry (Fig. 1B), DNA laddering (Fig. 1C and D), and caspase 9 activity (Fig. 1E). In all assays, QLα12-MDCK (-dox) cells showed a marked increase in apoptosis compared with the other conditions. In flow cytometry analysis (Fig. 1B), there was a 4–5-fold increase in the percentage of apoptotic cells with QLα12 expression when compared with + dox and with Ga12-MDCK cells ± dox. Moreover, there was a parallel reduction in the percentage of cells in the G0/G1 and mitosis phases of the cell cycle. Fig. 1C shows the DNA laddering in Ga12- and QLα12-MDCK cells ± dox. Total intact genomic DNA was reduced to less than 50% in the QLα12 expressing cells (-dox) (quantified in Fig. 1D), whereas there was little effect in Ga12-MDCK cells ± dox. However, compared with Ga12-MDCK cells, there was a small reduction in genomic DNA of QLα12-MDCK cells in + dox conditions (about 10–15%). This may reflect incomplete suppression of the tetracycline responsive element leading to a smaller amount of QLα12 expression (although QLα12 protein is not detectable by Western in + dox, Fig. 1A). Finally, to confirm the increase in apoptosis and to determine the role of the intrinsic pathway, we assessed the activity of caspase 9. Consistent with the flow cytometry results and DNA laddering, there was a marked increase in caspase 9 activity in the QLα12 expressing cells that was not seen in other conditions (Fig. 1E). This suggests that QLα12 activates the intrinsic apoptotic pathway, and this is distinct from apoptosis induced by dome formation and confluence, which is associated with distal activation of caspase 8 but not caspase 9 (25).

The Effect of Ga12 Expression on Proliferation—The results from flow cytometry (Fig. 1B) revealed little change in the fraction of cells in the synthesis stage suggesting that proliferation may not be increased under these conditions. To address the effects of Ga12 on proliferation in the inducible MDCK cell model, proliferation was quantified by measuring mitochondrial dehydrogenase activity (Fig. 1F), a well established correlate of cell proliferation (27). Proliferation was measured in confluent and subconfluent monolayers. When Ga12- and QLα12-MDCK cells were plated at confluence (identical to conditions for apoptosis assays) and switched to ± dox for 72 h, there were no significant differences in proliferation (Fig. 1F). However, when Ga12-MDCK cells were plated at subconfluent density (~50%) and switched into ± dox after plating, there was increased proliferation in Ga12- and QLα12-MDCK cells.
**Gα₁₂ Stimulates Apoptosis**

(-dox) at 72 h (Fig. 1G). Fig. 1G also shows that at 24 and 48 h proliferation rates were minimally stimulated in Gα₁₂ and QLα₁₂-MDCK cells (± dox) compared with Tet-off control cells. These results indicate that Gα₁₂ stimulates proliferation in MDCK cells when cultured under specific conditions.

**QLα₁₂ Expression Results in Reduced Bcl-2 Expression**—We next examined the effects of Gα₁₂ expression on the protein levels of the two major anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-xL. Fig. 2 (A and B) shows that inducing QLα₁₂ (−dox) leads to nearly complete loss of Bcl-2 protein, in comparison with + dox and with Gα₁₂-MDCK cells (± dox). In contrast, there was no appreciable difference in the levels of Bcl-xL protein for any of the conditions (Fig. 2A). The absence of any effect of Gα₁₂ expression on Bcl-2 and Bcl-xL protein levels is consistent with the lack of an effect in Gα₁₂-MDCK cells on apoptosis. To establish whether the QLα₁₂-stimulated loss of Bcl-2 was mediated by changes in gene expression, semi-quantitative RT-PCR was performed for each condition. Fig. 2 (C and D) reveals no significant differences in Bcl-2 transcript in the QLα₁₂-MDCK cells in −dox when compared with the other conditions.

**Expression of QLα₁₂ Activates JNK1**—To screen for candidate signaling molecules activated in QLα₁₂-MDCK cells in −dox, a phosphoprotein screen comparing lysates from QLα₁₂-MDCK cells ± dox was performed (Kinexus Kinetworks™). QLα₁₂ expression (−dox) specifically increased the phosphorylation of JNK1 and completely inhibited the phosphorylation of ERK1, without significantly affecting other MAPK family members, JNK2, ERK2, or p38 (Fig. 3A). To confirm the increased JNK1 activity detected in the phosphoprotein screen, JNK1 activity was determined by phosho-JNK1 Western blot (Fig. 3, B and C) and by direct measurement of JNK activity (Fig. 3, D and E). Fig. 3 shows that inducing QLα₁₂ protein expression (−dox) led to a nearly 15-fold induction of JNK1 activity as determined by p-JNK1 immunoreactivity, a value similar to the effect seen in the phosphoprotein screen. QLα₁₂ expression did not significantly affect total JNK1 levels. Additionally, Fig. 3B shows that ERK1 phosphorylation is inhibited by overexpression of both Gα₁₂ and QLα₁₂, whereas ERK2 phosphorylation remains unchanged. JNK activity was also directly measured in these cell lines during a time course of Gα₁₂ protein induction (Fig. 3D). As previously described, Gα₁₂ is induced at low levels by 24 h (not apparent in Gα₁₂ Western blot, Fig. 3D), and is steadily expressed by 72 h. There was a small amount of baseline JNK activity in both Gα₁₂- and QLα₁₂-MDCK cells in + dox (no Gα₁₂ expression, Fig. 3D). Within 24 h of Gα₁₂ expression, there was a significant increase in JNK activity in QLα₁₂-MDCK cells that remained elevated through 72 h (Fig. 3, D and E). There was a small but consistent increase in JNK activity in Gα₁₂-MDCK cells at 72 h in −dox (Fig. 3D). Taken together, QLα₁₂ significantly stimulates JNK activity in this MDCK cell model.

**Activation of JNK1 Leads to Proteosomal Degradation of Bcl-2**—Phosphorylation of Bcl-2 inactivates its anti-apoptotic properties and leads to its degradation (23). To determine
whether JNK1 activation in QLα12-MDCK cells was important for loss of Bcl-2 expression. Bcl-2 protein levels were analyzed in Go12- and QLα12-MDCK cells in the presence and absence of the highly specific JNK inhibitor SP600125 (IC50 = 100 nM) (28). JNK1 directly phosphorylates Bcl-2 in response to microtubule damage (29), and we hypothesized that Go12-stimulated JNK activity may lead to loss of Bcl-2 expression. Fig. 4 shows that there was no effect of JNK inhibition on Bcl-2 expression in Go12-MDCK cells (+ dox), and in QLα12-MDCK cells + dox. However, the QLα12-induced loss of Bcl-2 (− dox) was nearly completely inhibited in cells treated with the JNK inhibitor. Furthermore, inhibiting Bcl-2 degradation through the proteasome pathway with lactacystin also nearly completely inhibited the QLα12-stimulated loss of Bcl-2. Taken together, these findings support the notion that Go12 stimulates apoptosis by JNK activation and Bcl-2 phosphorylation leading to Bcl-2 degradation through the proteasome pathway.

QLα12 induces the expression of IκBα through JNK1 and Bcl-2—NF-κB inhibits apoptosis through numerous pathways and is directly regulated by its inhibitor, IκBα (30). NF-κB participates in a cyclic regulatory pathway with Bcl-2. NF-κB stimulates Bcl-2 transcription (31, 32), and Bcl-2 sustains NF-κB activation by enhancing the degradation of IκBα (summarized in Fig. 10). To determine whether NF-κB signaling plays a role in Go12-stimulated apoptosis, we examined NF-κB and IκBα expression in Go12- and QLα12-MDCK cells (+ dox; Fig. 5). In QLα12-MDCK cells (− dox), there was significant induction of IκBα protein levels in comparison with Go12-MDCK (+ dox) and QLα12-MDCK (+ dox), whereas the expression of NF-κB was relatively constant (Fig. 5A). To establish whether the QLα12-stimulated induction of IκBα was mediated through JNK1, Go12- and QLα12-MDCK cells + dox were incubated with the JNK inhibitor SP600125. Fig. 5B shows that there was no detectable IκBα in QLα12-MDCK cells in + dox, whereas the QLα12-MDCK cells in − dox demonstrated a large increase in IκBα expression that was nearly completely inhibited with SP600125. As expected, inhibiting the proteasome with lactacystin had subtle effects on IκBα expression and this likely contributed to Go12-mediated apoptosis.

JNK1 Activation in QLα12-MDCK Cells Is Mediated by PP2A—PP2A is a major cellular serine/threonine phosphatase and a known regulator of apoptosis (33). We recently reported that...
**Gα₁₂ Stimulates Apoptosis**

**A**  

| Vehicle | Okadaic Acid | Fostriecin | pJNK1 | β-actin |
|---------|-------------|-----------|------|--------|
| Gα₁₂   | -           | +         | +    | +      |
| QLα₁₂  | +           | -         | -    | -      |

**B**  

| Vehicle | Okadaic Acid | Fostriecin | p-c-Jun | β-actin |
|---------|-------------|-----------|--------|--------|
| Gα₁₂   | -           | +         | +      | +      |
| QLα₁₂  | +           | -         | -      | -      |

**C**  

| Vehicle | Okadaic Acid | Fostriecin | Bcl-2 | β-actin |
|---------|-------------|-----------|------|--------|
| Gα₁₂   | -           | +         | +    | +      |
| QLα₁₂  | +           | -         | -    | -      |

**D**  

| Vehicle | Okadaic Acid | Fostriecin | Bcl-2 |
|---------|-------------|-----------|-------|
| Tet-Off |             |           |       |
| MDCK    |             |           |       |

**FIGURE 6.** QLα₁₂-stimulated JNK1 activation is blocked by PP2A inhibitors fostriecin and okadaic acid. A, JNK phosphorylation. Western blots for pThr<sup>183</sup>/pTyr<sup>185</sup>-JNK1, total JNK1, and β-actin were performed on lysates from Gα₁₂- and QLα₁₂-MDCK cells (+ dox for 72 h) incubated with vehicle, fostriecin (200 nM; IC<sub>50</sub> = 1.5–5.5 nM), or okadaic acid (50 nM; IC<sub>50</sub> = 0.1–0.3 nM). The blot was stripped and reprobed for β-actin. B, JNK activity. Total JNK was pulled-down using GST-cJun and incubated in vitro with 200 μM ATP to assess kinase activity as measured by GST-cJun phosphorylation. C, JNK activity quantification. Relative JNK activity was quantified using the β-actin loading control using NIH Image. Results are the mean ± S.E. of three independent experiments. D, Tet-off MDCK-II (non-transfected) cells were incubated with PP2A inhibitors okadaic acid or fostriecin for 72 h, after which Western blots for Bcl-2 expression were performed on cell lysates. The blots were stripped and reprobed for β-actin. *: significance at p < 0.05; **: significance at p < 0.05 when compared with *.

Gα₁₂ binds to the α subunit of PP2A and that QLα₁₂ stimulates PP2A activity in QLα₁₂-MDCK cells (21). To assess the potential role of PP2A in this pathway, Gα₁₂- and QLα₁₂-MDCK cells (+ dox) were cultured in the presence of PP2A inhibitors fostriecin (IC<sub>50</sub> = 1.5–5.5 nM) or okadaic acid (IC<sub>50</sub> = 0.1–0.3 nM) (34). Fig. 6A shows the QLα₁₂-induced (+ dox) increase in p-JNK1 was completely inhibited by fostriecin and okadaic acid, and the inhibitors had no demonstrable effect on total JNK1 levels in Gα₁₂- or QLα₁₂-MDCK cells ± dox. This finding was confirmed by determining JNK activity in each condition (Fig. 6B). There was a small but consistent increase in JNK activity in Gα₁₂-MDCK cells (+ dox) similar to what was seen in other experiments (Fig. 4, D and E). As expected, QLα₁₂-MDCK cells (+ dox) demonstrated a large increase in JNK activity that was nearly completely inhibited to baseline levels with okadaic acid or fostriecin (Fig. 6, B and C). To determine whether inhibiting PP2A in MDCK-II cells leads to loss of Bcl-2 expression, Tet-off MDCK cells were cultured for 72 h in the presence of PP2A inhibitors, okadaic acid and fostriecin. As reported in other cell types, there was complete loss of Bcl-2 expression, and increased apoptosis (not shown) with PP2A inhibition (Fig. 6D), and identical findings were seen in Gα₁₂- and QLα₁₂-MDCK cells ± dox (results not shown). This suggests that potent inhibition of PP2A with okadaic acid and fostriecin cannot be overcome by Gα₁₂ stimulation of PP2A, and other signaling pathways are likely to lead to Bcl-2 phosphorylation and degradation in the presence of PP2A inhibition.

**Thrombin Stimulates Endogenous Gα₁₂ Activation and Apoptosis in MDCK-II and HEK293 Cells through JNK—** To determine the physiological relevance of Gα₁₂-coupled signaling in inducing apoptosis, we examined the effects of thrombin stimulation of endogenous Gα₁₂ on apoptosis in two non-transfected cell lines. MDCK-II and HEK293 cells were stimulated with α-thrombin, a known agonist for Gα₁₂-coupled protease-activated receptor family of membrane receptors (35). Fig. 7A shows the results of the flow cytometry analysis for MDCK-II cells and HEK293 cells serum starved overnight and then stimulated with thrombin or vehicle for 24 h. Similar to the effects of QLα₁₂ expression in MDCK cells (Fig. 1D), there was a significant increase in the percentage of apoptotic cells with thrombin stimulation. In addition, the increase in apoptosis was associated with decreases in G<sub>0</sub> and mitotic stages, mirroring the observations in the QLα₁₂ MDCK cells (− dox). The flow cytometry analysis was repeated in the presence of thrombin and the JNK inhibitor SP600125, and the thrombin-stimulated increase in apoptosis was completely inhibited in MDCK cells, and partially (but significantly) inhibited in HEK293 cells. To confirm that thrombin stimulation of apoptosis was associated with Gα₁₂ activation, GST-TPR pull-downs were performed on cell lysates obtained from MDCK-II and HEK293 cells stimulated with thrombin at specific time points. The TPR domain of protein phosphatase 5 interacts with active (GTP-ligated) conformations of Gα₁₂ and Gα<sub>13</sub> (36, 37). This assay was first validated by comparing GST pull-downs of GST and GST-TPR on MDCK cell lysates prepared from Gα₁₂- and QLα₁₂-MDCK cells cultured in ± dox (Fig. 7B). Western blots of pull-downs with GST alone did not detect Gα₁₂ in any condition, and there was no detectable Gα₁₂ seen in GST-TPR pull-downs from Gα₁₂-MDCK cells ± dox. However, there was easily detectable Gα₁₂ from QLα₁₂-MDCK cells in − dox that was not apparent in GST-TPR pull-downs from QLα₁₂-MDCK cells + dox (Fig. 7B). Next, we utilized this assay to confirm Gα₁₂ activation in thrombin-treated cells, and Fig. 7C shows that Gα₁₂ was robustly activated within 20 min of stimulation, and Gα₁₂ remained activated through 24 h. These findings reveal that thrombin stimulates apoptosis in a JNK-dependent manner and activates endogenous Gα₁₂ over 24 h.
Thrombin Stimulates Apoptosis in MDCK and HEK293 Cells through Similar Pathways—To determine whether the same apoptosis pathway(s) identified in QLα12-MDCK cells were activated in non-transfected cells, a time course of thrombin effects on Bcl-2, phospho-JNK1, pSer70-Bcl-2, IκBα, and NF-κB were determined at specific time points in MDCK-II and HEK293 cells (Fig. 8). Analogous to the QLα12-regulated JNK1 activation and loss of Bcl-2, thrombin had a similar effect in both cell types. Bcl-2 phosphorylation increased within the first 4 h and Bcl-2 protein levels steadily declined in both cell types to less than 25% at 24 h. JNK1 was phosphorylated within 20 min, and peaked at about 1 h without affecting JNK levels. JNK1 phosphorylation preceded Bcl-2 phosphorylation in both cell types. Similarly, IκBα expression was induced at the early time points and increased over the subsequent 24 h although there was little effect on NF-κB levels. Although the time course of thrombin-stimulated activation of JNK and loss of Bcl-2 differed slightly in MDCK and HEK293 cells, the overall findings were remarkably similar. To confirm JNK activation and regulation of Bcl-2 expression, thrombin-stimulated MDCK and HEK293 cells were analyzed at 24 h ± JNK inhibition with SP600125 (Fig. 8). The degradation of Bcl-2 was prevented in both cell types in the presence of the JNK inhibitor (Fig. 8). When normalized to total Bcl-2 levels, the phospho-Bcl-2 levels were increased in both cell types with thrombin stimulation (consistent with targeting to degradation), and this was blocked in the presence of JNK inhibition with SP600125 (Fig. 8). The time course of activation for these pathways correlates with the timing of Gα12 activation, but we cannot exclude the possibility that thrombin also activates additional pathways that contribute to the stimulation of apoptosis in these cells.

Thrombin/Gα12-regulated Apoptosis Requires PP2A—To establish that thrombin-stimulated Gα12 leads to loss of Bcl-2 and JNK activation in a PP2A-dependent manner, HEK293 cells were characterized in the presence and absence of silenced PP2A. The catalytic subunit was silenced, and Fig. 9A shows a 70% reduction in catalytic subunit expression by Western blot. In the absence of thrombin, silencing PP2A or the use of control oligonucleotides did not affect apoptosis (Fig. 9B). However, as expected with thrombin stimulation, there was a significant increase in apoptosis that was not affected by control oligonucleotides or the transfection procedure (Fig. 9C). In cells with silenced PP2A catalytic subunit, there was partial rescue of the increased apoptosis. This partial reduction in thrombin-stimulated apoptosis in PP2A-silenced HEK293 cells was significant, similar to the effect seen with JNK inhibition (Fig. 7A, and expected in an experiment where 30–50% of the cells were silenced). To confirm that this pathway regulates JNK and Bcl-2 expression, HEK293 cells ± silenced PP2A were analyzed by Western blot. Fig. 9D shows that thrombin-stimulated HEK293 cells with silenced PP2A do not exhibit increased phospho-JNK or loss of Bcl-2 expression as seen in the non-silenced thrombin-stimulated conditions.

DISCUSSION

Cell proliferation and apoptosis are tightly regulated in the normal state, and disturbances in either process lead to major pathophysiologic changes resulting in numerous diseases. G proteins regulate both proliferation and apoptosis, but the mechanisms maintaining the appropriate balance are not well defined. Furthermore, the specific mechanisms regulating proliferation and apoptosis depend upon the stimulus and cell type. The findings of this study identify an important role for Gα12 in regulating epithelial cell apoptosis. To our knowledge, this is the first definitive demonstration that Gα12 regulates apoptosis and that this pathway can be activated through endogenous signaling pathways. Furthermore, these studies reveal that Gα12 regulates apoptosis through well established pathways that include Bcl-2 degradation, signaling through Jun kinases, and up-regulation of the NF-κB inhibitor, IκBα. In addition, these findings support our previous finding that Gα12 regulates PP2A, and reveal both Gα12-dependent and -independent regulation of apoptosis through PP2A. These observations are summarized in Fig. 10.

The studies on G protein regulation of apoptosis in specialized cells implicate several pathways depending upon the cell type. Go protein overexpression in neuronal cells increased sensitivity to apoptotic stimuli that was independent of cAMP and PKA activity (9). In renal tubular epithelium, tumor necrosis factor α-induced apoptosis that was associated with activation of NF-κB through a pertussis toxin-sensitive G protein pathway (4). In the heart, muscarinic receptor activation of Gαq pathways protected cells from apoptosis (7), through increased Akt activity, but in HeLa cells, Gαi stimulated apoptosis by decreasing Akt activation utilizing a Rho-dependent pathway.
In several hormone-dependent cancers, gonadotropin-releasing hormone antagonists inhibited apoptosis through direct effects on \( \alpha_{12} \)/H9251i pathways (6). \( \alpha_{12} \) and \( \alpha_{13} \) have been implicated in many cellular processes. Whereas several studies have implicated \( \alpha_{12} \) in proliferation and neoplasia (38, 39), recent studies suggest that \( \alpha_{12} \) does not stimulate proliferation in epithelial tumors, but promotes cell migration. Expression of \( \alpha_{13} \) was elevated in breast and prostate cancer, and thrombin stimulation resulted in increased cell invasion but not proliferation (40, 41). However, the effects of up-regulated \( \alpha_{12} \) levels on apoptosis were not addressed in these studies. Interestingly, we also found no significant difference in proliferation in overexpressing \( \alpha_{12} \) or QLQ12-MDCK when cultured at confluence, but did detect increased proliferation after 72 h in non-confluent monolayers. These findings suggest that the mitogenic potential of increased \( \alpha_{12} \) expression or activation of \( \alpha_{12} \) depends upon the cell type and may be influenced by cellular confluence. Remarkably, there have been few studies on apoptosis regulated by the \( \alpha_{12}/\alpha_{13} \) family. There is only one report on the role of \( \alpha_{12} \) in apoptosis, and this was in a transient transfection system (18). Additionally, \( \alpha_{13} \) stimulated apoptosis in Rat 1A cells through JNK (19), an important MAPK regulating apoptosis in other signaling pathways.

Based upon these observations, we utilized previously characterized MDCK cells with inducible \( \alpha_{12} \) and QLQ12-MDCK to ask whether \( \alpha_{12} \) regulates apoptosis in the MDCK cell model epithelia. The inducible expression of QLQ12 led to increased apoptosis as assessed by flow cytometry, DNA laddering, and caspase 9 activity. Unlike proliferation that was stimulated by overexpressing \( \alpha_{12} \) (at 72 h), there was no significant effect of \( \alpha_{12} \) overexpression on apoptosis.

The mechanism of \( \alpha_{12} \)-stimulated apoptosis in MDCK cells involves at least two well established apoptosis signaling pathways. At the center of these pathways is the ratio of pro-apoptotic and anti-apoptotic Bcl family of proteins. The loss of the anti-apoptotic protein Bcl-2 permits the homodimerization of the pro-
apoptotic protein Bax and the stimulation of apoptosis. For example, osteoblast and osteoclast apoptosis has been associated with an increase in Bax/Bcl-2 ratio (42), and apoptosis of renal proximal tubular epithelial cells due to local hydrogen peroxide generation is also associated with this shift in Bax/Bcl-2 ratio (43). Likewise, the mechanism of Gα12-stimulated apoptosis presented in our studies involves degradation of Bcl-2 that is mediated by JNK stimulation. JNK has been implicated in several Gα12-initiated signaling cascades, and the JNK family regulate several apoptosis pathways (44). JNK localizes at the mitochondrial membrane, and activated JNK1 has been shown to directly phosphorylate Bcl-2 in response to microtubule damage (29). The decrease in the percentage of QL12 expressing MDCK cells in the mitotic phase but not the synthesis phase (Fig. 1B) is also consistent with JNK phosphorylation of Bcl-2 at the G2/M checkpoint (45). It is possible that Gα12 activation of JNK1 specifically causes the targeting of Bcl-2 to degradation at this checkpoint, causing these cells to undergo apoptosis rather than continue into mitosis.

Selective activation of JNK1 but not JNK2 was associated with renal fibrosis and tubular cell apoptosis (46), phenotypes linked to polycystic kidney disease (47). Furthermore, polycystin-1, the major protein mutated in polycystic kidney disease may cause cystogenesis, and consistent with this possibility, overexpression of Bcl-2 in MDCK cells prevented cyst formation associated with anoikis-induced apoptosis (53). In vivo, the JNK-specific inhibitor SP600125 blocked Bcl-2 phosphorylation and inhibited apoptosis associated with brain ischemia (54).

In QLα12 expressing MDCK cells, apoptosis is clearly stimulated. However, the in vivo relevance of these observations needed to be established in non-transfected cells. Therefore, we
utilized MDCK-II and HEK293 cells to validate the observations from the Ga12-MDCK cell culture model. Thrombin activates Ga12-coupled protease-activated receptors (35) and has been implicated in numerous disease phenotypes, including ischemia-reperfusion injury (54). Thrombin significantly stimulated apoptosis in both MDCK-II and HEK293 cells. To confirm that thrombin activated endogenous Ga12, we first validated the GST-TPR pull-down assay-activated Ga12 (described in Refs. 36 and 37). Utilizing this novel assay, we confirmed that thrombin activated endogenous Ga12 throughout the 24 h of thrombin exposure. In addition, thrombin activated JNK and led to loss of Bcl-2 in MDCK-II and HEK293 cells, similar to thrombin exposure. In addition, thrombin activated JNK and apoptosis is not surprising (see Fig. 10). Although Ga12 has been previously shown to activate JNK through numerous signaling pathways, including nigral dopaminergic neurodegeneration (55), a pathology of Parkinsonism. However, our studies were not designed to distinguish between thrombin-stimulated Ga12 and other potential pathways important to apoptosis.

Like G proteins, PP2A has many cellular functions. Ga12 binds PP2A and stimulates phosphatase activity (20), and the binding domains were recently identified (21). The current results extend these observations and reveal regulation of apoptosis through PP2A using at least 2 different pathways: Ga12-dependent activation of JNK and a Ga12-independent pathway. The answer to the question of why Ga12-stimulated PP2A activity does not offset JNK activation and directly inhibit Bcl-2 degradation may reside in the relative potency of these pathways. The stoichiometry, localization of signaling components, and specific stimuli may affect which pathway predominates. The differing effects on apoptosis when PP2A is partially silenced versus chemically inhibited is likely to be explained by such a mechanism. The partial silencing of PP2A prevents Ga12-dependent effects, but does not alter direct effects of PP2A on Bcl-2 expression. This suggests that the available pool of functional PP2A contributes to determining which pathways are functional. In addition, PP2A is highly abundant, whereas the relative expression of Ga12 is much lower. Therefore, the finding of Ga12-dependent and -independent effects of PP2A on apoptosis is not surprising (see Fig. 10). Although Ga12 has been previously shown to activate JNK through numerous other signaling pathways, including small G proteins (56), Src (57), and various MAPK kinases (58), this is the first report linking PP2A to JNK activation by Ga12. In intestinal epithelial cells, PP2A stimulated apoptosis through a JNK-dependent mechanism that required ERK1 inactivation (59). The mechanism of PP2A activation of JNK in our studies was not explicitly examined, but we also observed loss of ERK1 phosphorylation (Fig. 3, A and B). This leads us to speculate that ERK1 inhibition may be released by Ga12-PP2A activation and, this contributes to JNK activation. The role of PP2A and JNK stimulating apoptosis in intestinal epithelia in combination with our findings in MDCK cells suggests that Ga12-coupled signaling pathways are a general mechanism regulating apoptosis in epithelia. Additional studies will be necessary to definitively answer this question, but the coupling of Ga12 to numerous receptors regulating proliferation and apoptosis make this an intriguing possibility (and potential therapeutic target).

Another major mechanism regulating apoptosis involves NF-κB. NF-κB prevents apoptosis by regulating transcription of pro-apoptotic genes (reviewed in Ref. 60). G proteins also regulate apoptosis through NF-κB. For example, Gaq stimulates NF-κB activity through the phosphatidylinositol 3-kinase pathway (61), and inhibits NF-κB activity by preventing IκBα degradation (62). In addition, Gα12 stimulates NF-κB activity through reactive oxygen species and Ca2+-mediated signaling pathways (63). Recently, Gα12-mediated activation of JNK was reported to induce proteasomal degradation of IκBα, leading to the activation of NF-κB in the regulation of cyclooxygenase transcription. However, this study did not examine apoptosis (64). Here, we reveal novel suppression of NF-κB activity by Ga12 through the targeted up-regulation of IκBα expression. In the absence of changes in Bcl-2 gene expression, it is likely that Bcl-2 is upstream of the NF-κB pathway in this model (see Fig. 10). Our finding of enhanced Bcl-2 degradation is also consistent with the up-regulation of IκBα, although the relative role of the NF-κB pathway in Ga12-stimulated apoptosis remains to be elucidated.

In conclusion, these studies reveal that Ga12 stimulates apoptosis in epithelial cells, and the mechanisms are nearly identical with inducible expression of QLα12, and with thrombin activation of endogenous Ga12. Activation of Ga12 leads to PP2A-mediated JNK1 activation, proteasomal degradation of Bcl-2, and stimulation of apoptosis (see Fig. 10). This identifies novel roles for PP2A and JNK1 in Ga12 signaling and specifically in the regulation of apoptosis. Furthermore, Ga12-stimulated JNK activity also fundamentally regulates NF-κB by stimulating expression of its inhibitor IκBα. The numerous reports of apoptosis regulation through thrombin and identification of pathways that utilize JNK, PP2A, and Bcl-2 suggest a general mechanism with proximal regulation by Ga12. Future studies will target Ga12-mediated apoptosis in vivo to develop therapies for specific diseases where these signaling pathways have gone awry.

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