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Antianoikis Effect of Nuclear Factor-κB through Up-regulated Expression of Osteoprotegerin, BCL-2, and IAP-1*

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Epithelial cells undergo a form of apoptosis termed anoikis when they lose extracellular attachments. We evaluated the role of transcription factor NF-κB in the regulation of anoikis susceptibility of intestinal epithelial cells. Culture of rat intestinal epithelial cells in suspension induced NF-κB activation, which blocked the anoikis of those cells, as assessed by internucleosomal DNA fragmentation and caspase-3 cleavage. Activation of NF-κB after the loss of extracellular attachments required focal adhesion kinase tyrosine 397 phosphorylation. This triggered a signaling cascade through phosphatidylinositol 3-kinase and AKT, to induce DNA binding of the RelA/p65 NF-κB polypeptide. NF-κB activated in this manner induced the up-regulated expression of a distinct program of genes that included osteoprotegerin, BCL-2, and IAP-1 (inhibitor of apoptosis protein-1). Chromatin immunoprecipitation experiments revealed that NF-κB directly regulated the promoters of these 3 genes. Knock-down of the expression of osteoprotegerin, BCL-2, or inhibitor of apoptosis protein-1 by RNA interference showed that these factors inhibit anoikis, and genetic reconstitution of their expression alone or in combination restored normal levels of anoikis to NF-κB-inactive intestinal epithelial cells. Together, these findings have identified the molecular components of a previously unrecognized antianoikis pathway in intestinal epithelial cells.

Contact with extracellular matrix provides survival signals to epithelial cells. When epithelial cells lose such contacts, a form of apoptosis results, which has been termed anoikis (1). Physiologically, anoikis is important in the regulation of cell number in the intestinal tract, where epithelial cells are shed from villus tips in the small intestine and from the intercrypt epithelium of the colon and die by anoikis (2, 3). Pathophysiologically, resistance to anoikis has been associated with metastatic spread of carcinoma cells (4, 5), which reflects the ability of malignantly transformed epithelial cells to evade apoptosis when deprived of extracellular matrix attachment during dissemination in lymph or blood. The molecular mechanisms that govern susceptibility to anoikis have not been fully characterized and could provide important insights into epithelial homeostasis and carcinogenesis.

It was previously established that the survival of cells cultured without extracellular attachments is promoted by PI 3-K[2] and AKT because inhibition of these kinases increased anoikis (6, 7). Moreover, the anchorage-independent survival of cancer cells that is induced by transforming oncogenes such as Ras and Src depends in part on PI 3-K and AKT (8–10). However, the molecular mechanisms by which cells activate PI 3-K and AKT after loss of extracellular attachments and through which this pathway blocks anoikis have not been completely characterized; nor have the molecular factors that ultimately control the susceptibility of cells to undergo anoikis been identified.

NF-κB constitutes a family of transcription factors defined by the presence of a Rel homology domain. When exposed to certain stresses, such as inflammation, infection, oxidative stress, or DNA double strand breaks, NF-κB dissociates from inhibitory IκB molecules, translocates from cytoplasm to nucleus, and binds to κB sequences in the promoter regions of specific target genes, up-regulating their expression (11). Prominent among the more than 150 genes known to be regulated by NF-κB proteins are a subset whose products inhibit apoptosis, including BCL-X_L, BCL-2, IAP-1 and -2, c-FLICE inhibitory protein, growth arrest and DNA damage associated protein 45β, and tumor necrosis factor (TNF) receptor-associated proteins 1 and 2 (12). NF-κB activation is strongly associated with resistance to apoptosis induced by TNF-α (13), ionizing radiation (14), and cancer chemotherapeutic drugs (15). An important role for NF-κB in carcinogenesis has been proposed, based on the oncogenicity of v-*rel* (16), the effect of NF-κB in promoting chemotherapy and radiotherapy resistance of cancer cells (17), and the functional requirement for NF-κB in tumorigenesis in the colon, breast, and liver (18–20). Because of the importance of NF-κB in apoptosis resistance and carcinogenesis, we suspected that this factor might regulate anoikis.

To address this possibility, we evaluated a functional role for NF-κB in the regulation of anoikis susceptibility, using a suspension culture model of intestinal epithelial cells. We found that following the loss of extracellular attachments in intestinal epithelial cells, FAK triggers a prosurvival signaling pathway through PI 3-K and AKT that activates NF-κB. Moreover, our results have identified a distinct program of genes up-regulated by NF-κB that inhibit anoikis, revealing this transcription factor to be a central regulator of intestinal epithelial cell survival following the loss of extracellular attachments.

EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise stated, reagents were obtained from Sigma. Wortmannin, LY294002, bisindolylmaleimide I, and MG132 were obtained from Calbiochem. In all experiments utilizing chemical inhibitors, control conditions contained an equal volume of vehicle (dimethyl sulfoxide or saline).

Cell Lines—Rat intestinal epithelial (RIE-1) cells (21) were maintained in Dulbecco's modified Eagle's medium (Invitrogen), containing

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[2] The abbreviations used are: PI 3-K, phosphatidylinositol 3-kinase; FAK, focal adhesion kinase; IKK, inhibitor of κB kinase; IκB, inhibitor of κB; OPG, osteoprotegerin; TNF, tumor necrosis factor; RIE-1, rat intestinal epithelial; poly-HEMA, polyhydroxyethylmethacrylate; BrdUrd, bromodeoxyuridine; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; TRAIL, TNF-related apoptosis-inducing ligand; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay.

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2 mM L-glutamine and 5% fetal bovine serum at 37 °C in 95% air, 5% CO_2. REV cells were prepared by transfecting RIE-1 cells with the empty vector (pRCβ-actin), and RSR cells were prepared by transfecting RIE cells with an expression vector for the NF-κB superrepressor (pRCβ-actin IκBα^{AA}), as previously described (22). RSR and REV cells were maintained in the same medium as RIE-1 cells supplemented with 0.4 mg/ml G418 (Invitrogen). For cell proliferation experiments, cells were seeded into 12-well plates in triplicate, and after varying durations of culture, cells were trypsinized and counted using a hemocytometer.

Suspension Culture—Cells were grown to confluence in 100-mm or 6-well tissue culture dishes. For culture in suspension, cells were detached from tissue culture plates using 0.25% trypsin, 0.38 g/liter EDTA, or 10 mM EDTA in calcium and magnesium-free Hanks' balanced salt solution (Invitrogen). Preliminary experiments revealed comparable results with these two detachment techniques but faster detachment using the trypsin/EDTA combination, so all subsequent experiments utilized this combination. Once detached, cells were cultured in complete medium in 30-mm Petri dishes that had been coated with polyhydroxyethylmethacrylate (poly-HEMA). Poly-HEMA-coated dishes were prepared by two applications of a 10 mg/ml solution of poly-HEMA in 95% ethanol onto Petri dishes. After drying, the dishes were washed three times with Dulbecco's phosphate-buffered saline before use. Cells were incubated in standard medium as described above during suspension culture. After various durations of suspension culture on poly-HEMA-coated dishes, cells were collected and reseeded onto tissue culture plates or immediately processed for analysis as described below.

Cell Lysates—Cells were collected, washed with ice-cold PBS, and incubated in ice-cold whole cell lysis buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton X-100, and 10% glycerol), including phosphatase inhibitors (40 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 0.9 mM sodium fluoride) and protease inhibitors (protease inhibitor mixture III, 5 μl/ml; Calbiochem) for 15 min at 4 °C. Samples were then centrifuged at 10,000 r.p.m. for 15 min at 4 °C, and supernatant was saved for use in Western blotting, electrophoretic mobility shift assays, and enzyme-linked immunosorbent assays. Protein concentration in the lysates were determined using the Bradford assay (Bio-Rad).

Bromodeoxyuridine (BrdUrd) Incorporation—BrdUrd (10 μM) was added to culture medium for 2 h. Cells were then fixed in methanol, treated with 2 N HCl for 15 min followed by 0.1 M borax for 10 min. Cells were permeabilized with 0.1% Nonidet P-40 and incubated in 1:60 diluted biotin-conjugated mouse anti-BrdUrd (Caltag/Invitrogen), followed by CY3-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA). Cells were counterstained with Hoechst 33342 and visualized using a Zeiss epifluorescence microscope fitted with appropriate filters. Digital photographs of random fields were taken using filters for both Hoechst and CY3, and BrdUrd incorporation was expressed as the percentage of total cells staining CY3-positive.

Electrophoretic Mobility Shift Assay—This was carried out as previously described (23). Supershift antibodies used were sc-372X (RelA/p65), sc-114X (p50), sc-70X (c-Rel), and sc-226 (RelB), all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and 05-361 (p50) from Upstate Biotechnology, Inc. (Lake Placid, NY).

Trans-Am Assay for NF-κB Activation—This method utilizes a 96-well plate format to quantify active NF-κB RelA/p65 in cell extracts and was performed according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, 5 μg of cellular extract was placed into wells of the 96-well plate, in which there is plate-bound oligonucleotide containing the NF-κB consensus site to which only activated (IκB-free) NF-κB can bind. NF-κB RelA/p65 bound to the immobilized oligonucleotide is detected using an anti-RelA/p65 antibody with a horseradish peroxidase-conjugated secondary and colorimetric detection at 450 nm (Molecular Devices, Menlo Park, CA).

Survival Experiments—Cells were trypsinized and counted using a hemocytometer, and 5×10^5 viable (exclusion of trypan blue) cells were resuspended in culture medium and transferred to poly-HEMA-coated dishes. After various durations of culture in suspension, cells were replated onto 6-well tissue culture plates and incubated overnight. The next day, cells were trypsinized and counted again to evaluate survival. The percentage of survival after each duration of suspension culture was determined by dividing the cell number surviving at that time point by the number surviving with immediate replating.

Cell Death Detection ELISA—This assay detects internucleosomal fragmented DNA, a feature of apoptotic cells, and is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones (24). For each condition, 10^5 cells were used, and the assay was performed according to the manufacturer's instructions (Roche Applied Science). Briefly, cells were grown in 6-well plates. After treatment or various time points of culture in suspension, cells were collected and lysed, and samples were incubated at room temperature for 30 min. Samples were transferred to anti-histone biotin-coated 96-well plates and incubated for 90 min. After three washes, anti-DNA peroxidase antibodies were added to each well and incubated for 90 min. After three washes, the peroxidase substrate was added to each well, and absorbance at 405 nm was read using a 96-well plate reader. Results were expressed as DNA fragmentation relative to attached or untreated cells.

Immunoblotting—Samples containing 30 μg of total protein were separated on 10% SDS-polyacrylamide gels (Bio-Rad) and then were transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA), after which membranes were blocked with a 5% (w/v) solution of nonfat milk powder in Tris-buffered saline/0.1% Tween 20 for 1 h at room temperature. Membranes were probed with antibodies specific for phosphoserine 32 IκBα, IκBα, AKT, phosphoserine 473 AKT, active caspase-3, IAP-1 (Cell Signaling Technology, Beverly, MA) FAK, phosphotyrosine 397 FAK (Upstate Biotechnology), BCL-2 (eBioscience, San Diego, CA), OPG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or β-actin (Oncogene Research Products, San Diego, CA). Detection was performed using horseradish peroxidase-conjugated donkey anti-rabbit antibody or sheep anti-mouse antibody (Amersham Biosciences), followed by enhanced chemiluminescence detection (Amersham Biosciences) and exposure to x-ray film (Biomax; Eastman Kodak Co.)

Plasmids—The following expression plasmids were used: a kinase-dead mutant of AKT, pCMV-6 HA-AKT (K179M), provided by Dr. S. Kaufmann (25); a dominant negative mutant of the p85 subunit of PI 3-K, pEF-BOSΔRI-Δp85, provided by Dr. G. Gores (26); a kinase-dead IKKβ, pRCβactin IKKβ K-A, or a dominant negative IKKβ, pRCβactin IKKβ AA, provided by Dr. M. Karin (27); a full-length BCL-2, pSFFV-BCL-2, provided by Dr. S. Kaufmann (28); a full-length c-IAP1, pcDNA3-myc-cIAP-1, provided by Dr. J Ashwell (29); or an OPG-Fc fusion construct (pSFFV-OPG-Fc) encoding the first 201 amino acids of human OPG fused to Fc. Transfections were performed by square wave electroporation, using a single pulse, 325 V, 10 ms (BTX, Holliston, MA). A total of 30 μg of plasmid DNA and 10^7 cells for each condition were used, as previously described (23). pcDNA3 (Invitrogen) lacking an insert was mixed with experimental plasmids to keep the total amount of transfected DNA constant. Preliminary experiments using a green

fluorescence protein expression plasmid indicated that ~60–90% of cells were transfected under these conditions.

Expression Profiling Array—Total cellular RNA was isolated from monolayered REV and RSR cells and after 4 h of detachment using Qiagen RNEasy kit (Qiagen Inc., Valencia, CA). The apoptosis GEArray Q series array was obtained from SuperArray Bioscience Corp. (Frederick, MD). Briefly, total RNA from the experimental samples was used as a template to generate cDNA probes using the GEAprimer mix as a reverse transcriptase primer. The cDNA probes, which represent abundance of mRNA population, were then denatured, and hybridization was conducted in GEHybridization solution to two nylon membranes spotted with gene-specific cDNA fragments. Membranes were then washed in 2× SSC, 1% SDS twice for 20 min each followed by 0.1× SSC, 0.5% SDS twice for 20 min each. Detection was performed using by CDP-star chemiluminescent solution and exposure to x-ray film.

RT-PCR Analysis—RNA was prepared from REV and RSR cells using Qiagen RNEasy kit (Qiagen Inc.). cDNA was prepared from the RNA using Superscript II (Invitrogen). PCR was carried out using the following conditions: 25 cycles of denaturation at 95 °C for 60 s, annealing at 56 °C for 60 s, and extension at 72 °C for 2 min (primer sequences available on request). One-tenth of the PCR product was analyzed by separation on a 2% agarose gel and stained with ethidium bromide.

Chromatin Immunoprecipitation (ChIP)—Cells were cross-linked with formaldehyde for 20 min at 25 °C, harvested in SDS-lysis buffer (Upstate Biotechnology), and sheared to fragment DNA to ~500 bp. Samples were then immunoprecipitated using RelA/p65 antibody (sc-372; Santa Cruz Biotechnology) or agarose beads alone at 4 °C overnight. Following immunoprecipitation, samples were washed and eluted using the Chromatin Immunoprecipitation system (Upstate Biotechnology) according to the manufacturer's instructions. Cross-links were removed at 65 °C for 4 h and immunoprecipitated DNA was purified using phenol/chloroform extraction (500 μl) and ethanol precipitation. PCR was utilized to detect RelA/p65 interaction with OPG, BCL-2, and IAP-1 promoters (primer sequences available on request).

RNA Interference—The 19-nucleotide small interfering RNA (siRNA) for BCL-2, OPG, and IAP-1 were designed by using Genebee software to predict probable RNA secondary structures, assesses target accessibility, and define RNA-targeting nucleic acids. Multiple sets of sequences were tested for their ability to selectively target BCL-2, OPG, or IAP-1, and the following sets were chosen: BCL-2, GAACTGGGGGAGGATTGTGTT and CACAATCCTCCCCCAGTTCTT; OPG, GCTTATAGTTAGGAATGTT and CATTCCTAACTATAAGCATT; IAP-1, CGGCCTGGATGACATGAGATT and TCTCATGTCATCCAGGCCGTT. Control siRNA was a scrambled sequence without any specific target that did not affect base-line or inducible levels of expression of BCL-2, OPG, IAP-1, or β-actin: CCGTCCTGCTCCAGCTAGATT and TCTAGCTGGAGCAGGACGGTT. Oligonucleotides were annealed with the complementary strand with dTdT overhangs. For the transfection, siRNA or control siRNA solution was added to Dulbecco's modified Eagle's medium containing Oligofectamine (Invitrogen) and allowed to incubate for 20 min at room temperature to create the transfection mixture. Transfection was done as per the manufacturer's guidelines for 4 h. After 4 h, medium was supplemented with RPMI 1640 and fetal bovine serum (to a final concentration of 10%). RNA was harvested after 72 h.

Statistical Analysis—Statistical analyses were performed using Student's *t* test or analysis of variance with *post hoc* Tukey tests, as appropriate. p values of <0.05 were accepted as statistically significant.

RESULTS

NF-κB Activation Promotes the Survival of RIE-1 Cells Cultured in Suspension—Initially, we evaluated the role of NF-κB in regulating anoikis, using a model system in which rat intestinal epithelial cells were detached from plastic tissue culture surfaces and cultured in suspension. Experiments were preformed with parental RIE-1 cells or with RIE-1-derived clones that we previously engineered to stably express the NF-κB superrepressor, a mutant nondegradable form of IκBα in which serines 32 and 36 are replaced with alanines (RSR cells) or a control empty vector (REV cells) (30). RSR cells cannot activate NF-κB in response to typical stimuli, such as interleukin-1 or TNF-α. We cultured two clones of RSR and two clones of REV cells in suspension on poly-HEMA-coated dishes for 1–6 h before reseeding on standard tissue culture plates. The next day, surviving cells that adhered to the tissue culture plates were counted and compared with the number of starting cells to determine percentage of survival. Across all of the time points of this experiment, control REV clones survived in greater numbers than did NF-κB-inactive RSR clones (Fig. 1*A*), differences that were statistically significant after 2 h. After 6 h of suspension culture, ~60% of control REV cells survived, compared with 30% RSR cells. To exclude the possibility that the differences in cell numbers between REV and RSR clones might be due to differences in rates of proliferation between the control and NF-κB-inactive cells, we assessed rates of growth and DNA synthesis. Parental RIE-1 cells, control REV-3 cells, and NF-κB-inactive RSR-6 cells grew at comparable rates (Fig. 1*B*). REV-3 and RSR-6 cells also had very similar BrdUrd uptake rates (Fig. 1*C*). Together these findings excluded the possibility that the greater numbers of surviving suspension-cultured REV cells compared with RSR cells was due to a proliferation advantage. Rather, the approximate 2-fold reduction in survival conferred by an inability to activate NF-κB suggested that NF-κB prevented apoptotic death of these cells. To test this idea, we quantified internucleosomal DNA fragmentation, a marker of apoptosis, in REV and RSR cells cultured both attached to tissue culture plates and in suspension. We observed a progressive induction of DNA fragmentation in both REV and RSR cells by suspension culture of 2 or 4 h (Fig. 1*D*). Notably, the amount of DNA fragmentation induced by suspension culture was ~3 times greater in RSR than in REV cells after 4 h. To support the results of DNA fragmentation analysis, we evaluated the activation of the death effector caspase-3 using Western blotting for cleaved caspase-3, a marker of its activation. Both REV and RSR cells cleaved caspase-3 when cultured in suspension but to a greater degree in the NF-κB-defective RSR cells (Fig. 1*E*). These findings indicate that NF-κB provides an antiapoptotic signal to intestinal epithelial cells that supports the survival of those cells when cultured without extracellular attachments.

The prosurvival role of NF-κB in suspension-cultured intestinal epithelial cells suggested that the loss of extracellular attachment activated this transcription factor. To test this possibility, we prepared whole cell protein lysates of attached and suspension-cultured RIE-1 cells and analyzed NF-κB activity. An electrophoretic mobility shift assay revealed that RIE-1 cells cultured in suspension activated NF-κB DNA binding 1 h after detachment, whereas the DNA binding activity of the unrelated transcription factor NF-Y was not affected (Fig. 2*A*). Similar results were obtained when RIE-1 cells were detached from tissue culture plates by incubation with trypsin (*left*) or with EDTA (*right*), indicating that NF-κB activation occurs independent of the method used to disrupt extracellular attachment of cells. Using antibodies against the five known mammalian NF-κB polypeptides, supershift analysis revealed that the detachment-induced NF-κB DNA binding complex contained RelA/p65 and p50 but not RelB, c-Rel, or p100/p52 (results not shown).

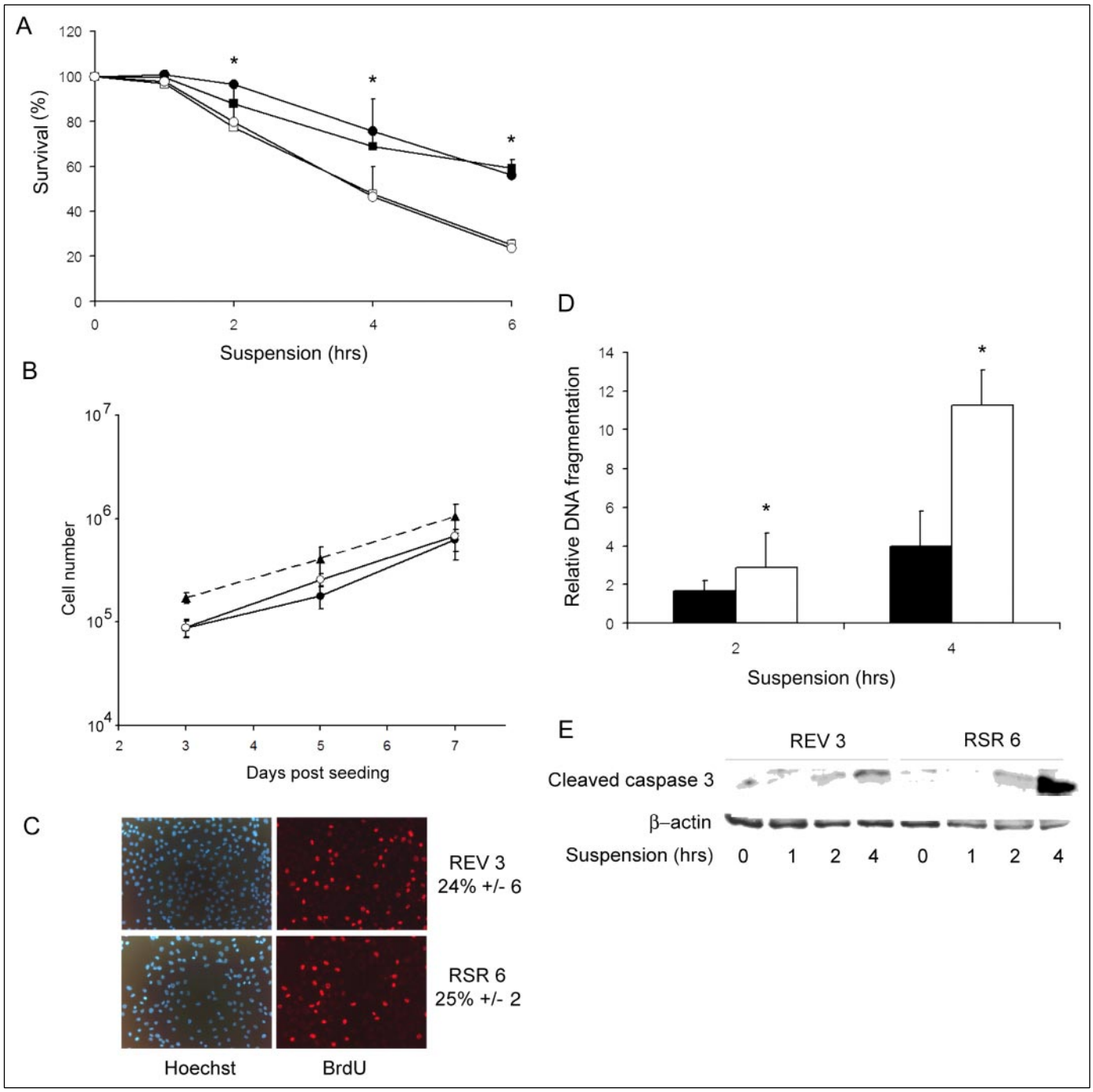


FIGURE 1. **NF-κB inhibits anoikis of intestinal epithelial cells.** *A*, two clones of control REV cells (*closed symbols*) and two clones of NF-κB-inactive RSR cells (*open symbols*) were trypsinized, counted, and cultured in suspension. After the indicated durations of suspension culture, the cells were reseeded onto tissue culture plates and cultured overnight. Surviving cells were counted, and results were expressed as mean ± S.E. percentage of survival. *, $p < 0.05$, both REV clones compared with both RSR clones. *B*, parental RIE-1 cells (*triangles*), REV-3 cells (*closed circles*), and RSR cells (*open circles*) were cultured for the indicated number of days, and cell counts were obtained on days 3, 5, and 7. *C*, 1 day after seeding, REV-3 and RSR-6 cells were pulsed with BrdUrd for 2 h before being fixed and stained for DNA (Hoechst) and BrdUrd incorporation. The *numbers* represent the average and S.E. percentage of cells labeled with BrdUrd. $p > 0.05$, REV-3 cells compared with RSR-6 cells. *D*, REV-3 cells (*closed bars*) and RSR-6 cells (*open bars*) were cultured in suspension. After the indicated durations of suspension culture, relative DNA fragmentation was determined by comparing internucleosomal DNA fragmentation in suspension-cultured cells with that in attached cells. Results were expressed as mean ± S.E. *, $p < 0.05$, REV-3 cells compared with RSR-6 cells. *E*, whole cell protein extracts were prepared from REV-3 and RSR-6 cells after the indicated durations of suspension culture. Western blotting using equal amounts of protein was performed using antibodies against cleaved caspase-3 and β-actin.

We used a quantitative ELISA-based assay specific for the RelA/p65 NF-κB polypeptide and found a time-dependent induction of RelA/p65 DNA binding activity in detached RIE-1 cells that was maximal after 1 h of suspension culture and declined after 2 and 4 h (Fig. 2*B*). Together, these findings indicate that RIE-1 cells activate an NF-κB RelA/p65-p50 heterodimer by suspension culture.

Activation of RelA/p65-p50 heterodimers by prototypic stimuli, such as TNF-α, is controlled by the "canonical" NF-κB pathway, involving IKKβ-dependent phosphorylation of IκBα (11). Using an antibody against serine 32-phosphorylated IκBα, by Western blot analysis we found that suspension culture of RIE-1 cells induced this phosphorylation, indicating activation of the IKK complex (Fig. 2*C*, *top*). When we

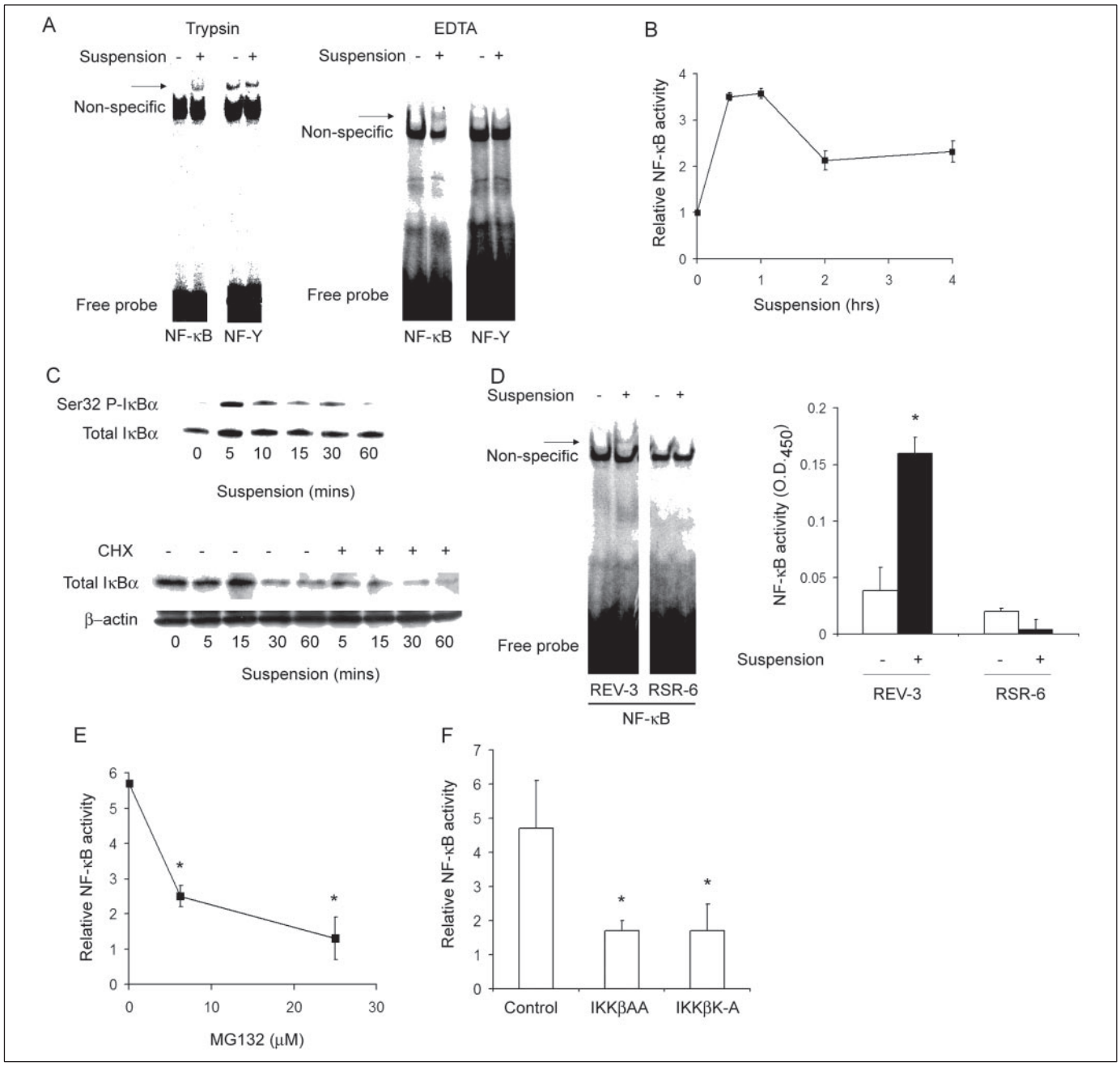


FIGURE 2. **Suspension culture activates NF-κB RelA/p65 through the canonical IKKβ-dependent pathway.** *A*, parental RIE-1 cells were cultured attached to tissue culture surfaces or in suspension for 1 h. Whole cell extracts were prepared and assessed for NF-κB and control NF-Y DNA binding activity using an electrophoretic mobility shift assay. The *arrow* indicates the specific shifted DNA binding. Cells were detached from tissue culture surfaces using trypsin (*left*) or EDTA (*right*). *B*, parental RIE-1 cells were cultured attached to tissue culture surfaces or in suspension for the indicated durations. Whole cell protein extracts were prepared and assessed for NF-κB RelA/p65 DNA binding using an ELISA-based assay. Results were expressed as mean ± S.E. NF-κB activity relative to attached cells. *C*, parental RIE-1 cells were cultured attached to tissue culture surfaces or in suspension for the indicated durations. Whole cell protein extracts were prepared, and equal amounts of protein were subjected to Western blotting using antibodies against phosphorylated serine 32 of IκBα or total IκBα (*top*). In other experiments, RIE-1 cells were incubated with or without cycloheximide (50 μg/ml) for 30 min before and for the indicated durations after detachment from tissue culture plates. Whole cell protein extracts were prepared, and equal amounts of protein were subjected to Western blotting using antibodies against total IκBα or β-actin (*bottom*). *D*, REV-3 and RSR-6 cells were cultured attached to tissue culture surfaces or in suspension for 1 h. Whole cell protein extracts were prepared and assessed for NF-κB RelA/p65 DNA binding using an electrophoretic mobility shift assay (*left*) and ELISA (*right*). ELISA results are expressed as mean ± S.E. absorbance units. *, $p < 0.05$, effect of suspension culture on NF-κB activity. *E*, parental RIE-1 cells were treated with the indicated concentrations of MG132. After 1 h of suspension culture, whole cell protein extracts were prepared and assessed for NF-κB RelA/p65 DNA binding using an ELISA-based assay. Results are expressed as mean ± S.E. NF-κB activity relative to attached cells. *, $p < 0.05$, effect of MG132 on NF-κB activity. *F*, parental RIE-1 cells were transfected with control plasmid or expression plasmids encoding the dominant negative mutant IKKβAA or IKKβK-A. Cells were detached, and after 1 h of suspension culture, whole cell protein extracts were prepared and assessed for NF-κB RelA/p65 DNA binding using an ELISA-based assay. Results are expressed as mean ± S.E. NF-κB activity relative to attached cells. *, $p < 0.05$, NF-κB activity compared with control.

examined total IκBα levels by Western blot, surprisingly, we did not observe a robust decline in protein abundance in suspension-cultured RIE-1 cells, since usually serine 32/36-phosphorylated IκBα is targeted to proteasomal degradation. To determine if the persistence of IκBα expression in suspension-cultured cells might be due to rapid resynthesis of IκBα, we compared protein levels of IκBα in suspension-cultured cells in the absence or presence of protein synthesis inhibitor cycloheximide (Fig. 2*C*, *bottom*). This analysis revealed that cycloheximide treat-

ment greatly reduced IκBα expression in suspension-cultured RIE-1 cells, which indicates that these cells rapidly resynthesize this protein after NF-κB activation. To verify the importance of IKKβ-triggered IκBα degradation in the NF-κB activation by this pathway, we compared NF-κB activity in control REV cells and mutant IκBα-expressing RSR cells. Expression of nondegradable mutant IκBα completely abolished suspension culture-induced NF-κB activation, as assessed by electrophoretic mobility shift assay (Fig. 2*D*, *left*) and by ELISA (Fig. 2*D*, *right*). Moreover, blockade of proteasome function with MG132 also dose-dependently inhibited NF-κB activity induced by culture in suspension, which supports the importance of proteasome activity in NF-κB activation (Fig. 2*E*). The role of IKKβ in NF-κB activation was further evaluated by transiently transfecting dominant negative mutant forms of this kinase into RIE-1 cells. Both a T loop serine 177/181 to alanine mutant and a kinase-dead K44A mutant plasmid of IKKβ greatly reduced NF-κB activity induced by culture in suspension (Fig. 2*F*). Together, these results support the importance of IKKβ-dependent IκBα phosphorylation and subsequent proteasomal degradation in the activation of NF-κB by suspension culture in intestinal epithelial cells.

FAK Tyrosine 397 Phosphorylation in Attached Cells Triggers an Antianoikis Pathway through PI 3-K, AKT, and NF-κB—We next sought to identify the mechanism of activation of the NF-κB pathway in RIE-1 cells induced by loss of extracellular attachment. We examined focal adhesions in an attempt to define early signaling events that occur after loss of extracellular attachments. Western blotting revealed that RIE-1 cells cultured on plastic expressed tyrosine 397-phosphorylated FAK (Fig. 3*A*). Within minutes of trypsinization, tyrosine 397 of FAK was dephosphorylated. We transfected RIE-1 cells with an expression plasmid encoding a tyrosine 397 to phenylalanine mutant (Y397F) that blocks downstream signaling dependent on this phosphotyrosine of FAK. This plasmid decreased NF-κB activation induced by loss of extracellular attachment, indicating that FAK signaling is required for activation of the NF-κB survival program (Fig. 3*B*). The fact that FAK tyrosine 397 phosphorylation is required for triggering the NF-κB pathway after loss of extracellular attachment yet decreases in abundance following detachment suggests that the pathway is triggered by FAK before dephosphorylation. To evaluate the functional importance of FAK in regulating anoikis, we again transfected the Y397F mutant into RIE-1 cells and cultured the cells in suspension. Cells transfected with the mutant FAK underwent more anoikis than control plasmid transfected cells or cells transfected with wild-type FAK (Fig. 3*C*). Together, these findings indicate that FAK functions as a molecular trigger that regulates the subsequent survival of cells after loss of extracellular attachment, linking adherence-dependent signaling through FAK to the activation of NF-κB.

Phosphorylation of FAK at tyrosine 397 creates a high affinity Src homology 2 binding site for several candidate adaptor and signaling molecules, including PI 3-K (31). To assess if PI 3-K might act downstream of FAK tyrosine 397 in limiting anoikis, we analyzed activation of protein kinase AKT, an effector of PI 3-K, by using Western blotting to detect phosphorylation of AKT at serine 473, a marker of its activation. Detachment of RIE-1 cells from tissue culture plates produced a rapid serine 473 phosphorylation of AKT, which was detectable as early as 5 min after detachment and persisted for at least 1 h (Fig. 4*A*). This phosphorylation could be decreased by tyrosine 397 → alanine mutant FAK (Fig. 4*B*). Moreover, wortmannin and LY294002, both chemical inhibitors of PI 3-K, dose-dependently decreased AKT serine 473 phosphorylation induced by suspension culture (Fig. 4*C*). We next evaluated potential signaling from PI 3-K and AKT to NF-κB. In suspension-cultured RIE-1 cells, LY294002 treatment reduced NF-κB activity by

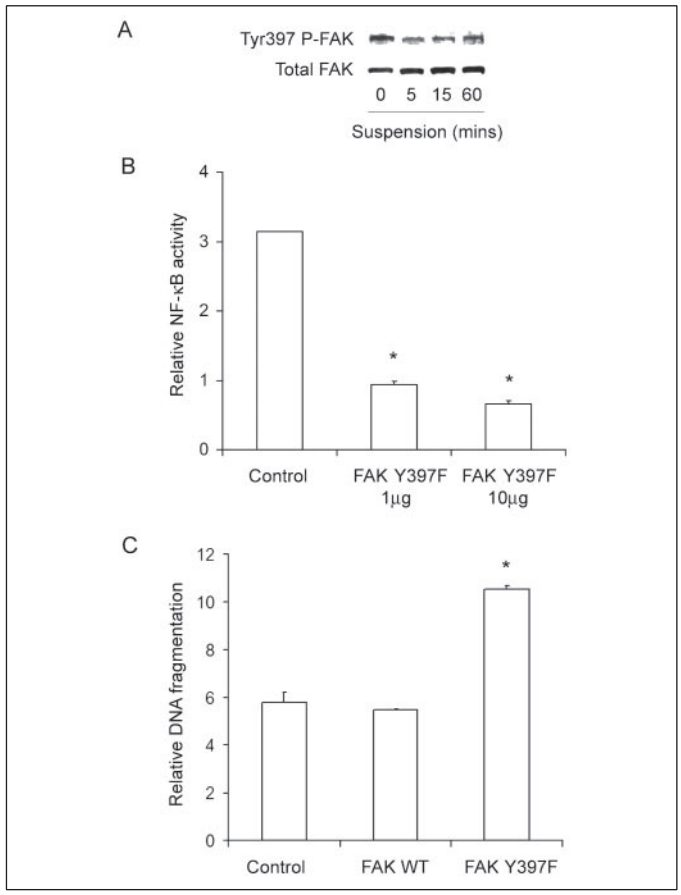


FIGURE 3. **FAK tyrosine 397 controls NF-κB activity and anoikis.** *A*, parental RIE-1 cells were cultured attached to tissue culture surfaces or in suspension for the indicated durations. Whole cell protein extracts were prepared, and equal amounts of protein were subjected to Western blotting using antibodies against phosphorylated tyrosine 397 of FAK or total FAK. *B*, parental RIE-1 cells were transfected with control plasmid or the indicated amounts of expression plasmid encoding the dominant negative mutant FAK Y397F. Cells were detached, and after 1 h of suspension culture, whole cell protein extracts were prepared and assessed for NF-κB RelA/p65 DNA binding using an ELISA-based assay. Results were expressed as mean ± S.E. NF-κB activity relative to attached cells. *, $p < 0.05$, NF-κB activity compared with control. *C*, parental RIE-1 cells were transfected with control plasmid or with expression plasmids encoding wild type (*WT*) FAK or the dominant negative mutant FAK Y397F. After 4 h of suspension culture, relative DNA fragmentation was determined by comparing internucleosomal DNA fragmentation in suspension-cultured cells with that in attached cells. Results were expressed as mean ± S.E. *, $p < 0.05$, compared with control.

about 50%, whereas wortmannin reduced NF-κB activity by ~70% (Fig. 4*D*). In contrast, neither LY294002 nor wortmannin significantly affected interleukin-1-induced NF-κB activation in RIE-1 cells (results not shown), indicating selectivity of PI 3-K for suspension-induced NF-κB activation. Results of NF-κB activity obtained with pharmacological inhibitors were confirmed by transient expression of a dominant-negative PI 3-K and AKT mutants, which also dose-dependently inhibited suspension-induced NF-κB activity (Fig. 4*E*). The protein kinase C family, especially the atypical members, are downstream effectors of PI 3-K that have also been implicated in NF-κB activation (32). However, bisindolylmaleimide I, a broad specificity inhibitor of protein kinase C family members, did not affect NF-κB activation induced by detachment (results not shown), arguing against a role for this protein kinase family in antianoikis signaling.

The functional importance of PI 3-K in the FAK-triggered antianoikis pathway in RIE-1 cells was established by an approximate 2-fold increase in DNA fragmentation in the presence of wortmannin or LY294002 (Fig. 4*F*). Transient expression of dominant negative mutants

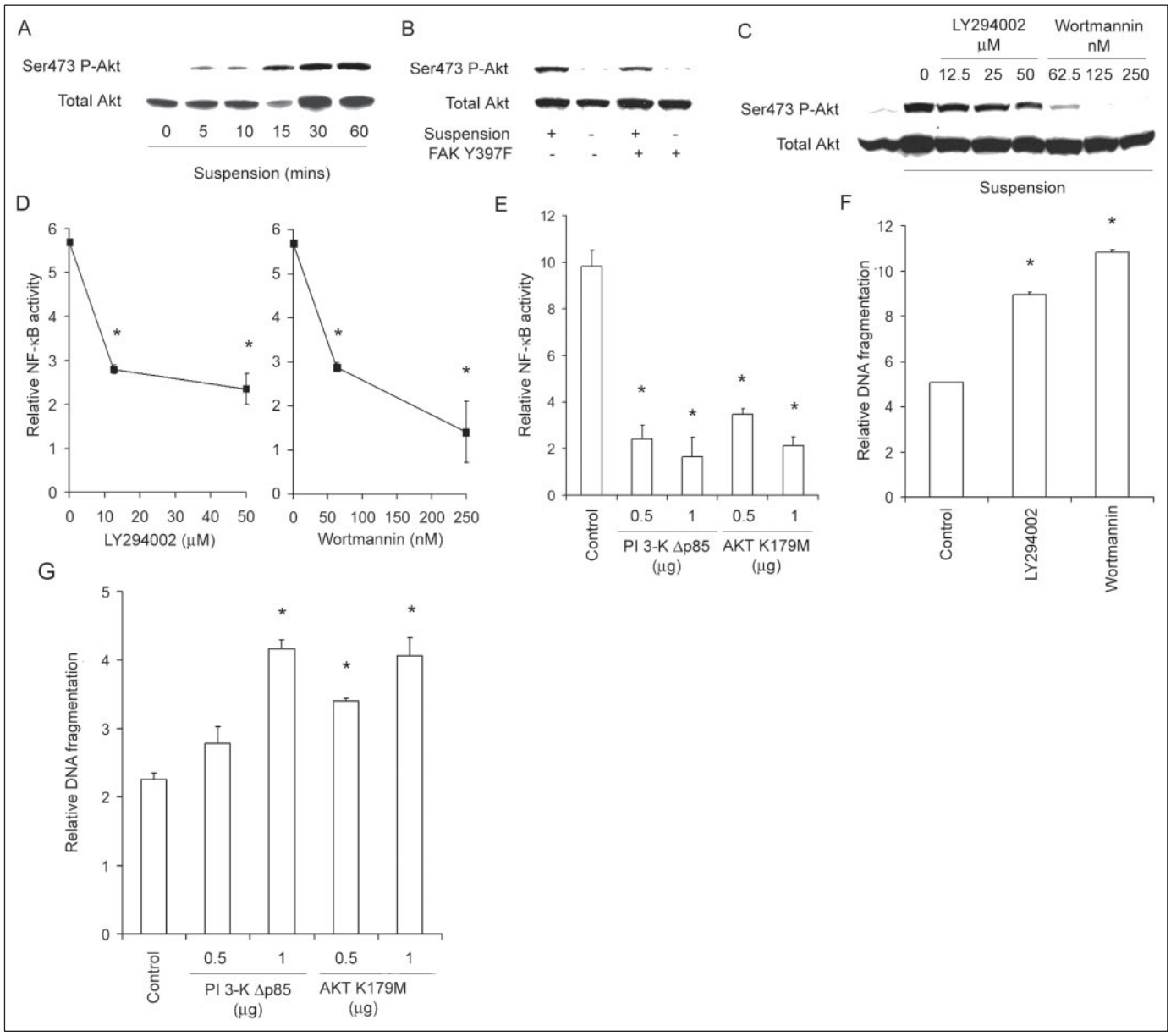


FIGURE 4. **PI 3-K and AKT transmit a FAK-derived survival signal to NF-κB in suspension-cultured cells.** *A*, parental RIE-1 cells were cultured attached to tissue culture surfaces or in suspension for the indicated durations. Whole cell protein extracts were prepared, and equal amounts of protein were subjected to Western blotting using antibodies against phosphorylated serine 473 of AKT or total AKT. *B*, parental RIE-1 cells were transfected with control plasmid (−) or with an expression plasmid encoding the dominant negative mutant FAK Y397F (+). Cells were detached, and after 30 min of suspension culture, whole cell protein extracts were prepared, and equal amounts of protein were subjected to Western blotting using antibodies against phosphorylated serine 473 of AKT or total AKT. *C*, parental RIE-1 cells were treated with the indicated concentrations of LY294002 or wortmannin. After 30 min of suspension culture, whole cell protein extracts were prepared, and equal amounts of protein were subjected to Western blotting using antibodies against phosphorylated serine 473 of AKT or total AKT. *D*, parental RIE-1 cells were treated with the indicated concentrations of LY294002 or wortmannin. After 1 h of suspension culture, whole cell protein extracts were prepared and assessed for NF-κB RelA/p65 DNA binding using ELISA. Results are expressed as mean ± S.E. NF-κB activity relative to attached cells. *, $p < 0.05$, effect of LY294002 or wortmannin on NF-κB activity. *E*, parental RIE-1 cells were transfected with control plasmid or the indicated amounts of expression plasmid encoding the dominant negative mutants PI 3-K Δp85 or AKT K179M. Cells were detached, and after 1 h of suspension culture, whole cell protein extracts were prepared and assessed for NF-κB RelA/p65 DNA binding using ELISA. Results are expressed as mean ± S.E. NF-κB activity relative to attached cells. *, $p < 0.05$, NF-κB activity compared with control. *F*, parental RIE-1 cells were treated with LY294002 (50 μM) or wortmannin (250 nM). After 4 h of suspension culture, relative DNA fragmentation was determined by comparing internucleosomal DNA fragmentation in suspension-cultured cells with that in attached cells. Results were expressed as mean ± S.E. *, $p < 0.05$ compared with control. *G*, parental RIE-1 cells were transfected with control plasmid or the indicated amounts of expression plasmid encoding the dominant negative mutants PI 3-K Δp85 or AKT K179M. After 4 h of suspension culture, relative DNA fragmentation was determined by comparing internucleosomal DNA fragmentation in suspension-cultured cells with that in attached cells. Results were expressed as mean ± S.E. *, $p < 0.05$ compared with control.

of PI 3-K or AKT also increased anoikis (Fig. 4*G*), further confirming the functional importance of these signaling intermediates in the transmission of survival signals in detached intestinal epithelial cells. Together, the results of these experiments identify FAK, PI 3-K, and AKT as molecular components of the signaling pathway to activation of anoikis inhibitor NF-κB.

BCL-2, OPG, and IAP-1 Contribute to the Antianoikis Activity of NF-κB—Next, we wished to identify target genes of NF-κB that were up-regulated by loss of extracellular attachment that might function as effectors of NF-κB. We used a focused expression profiling approach to assess the mRNA expression levels of 96 apoptosis-related genes. Compared with attached cells, 4 h of suspension culture induced up-regu-

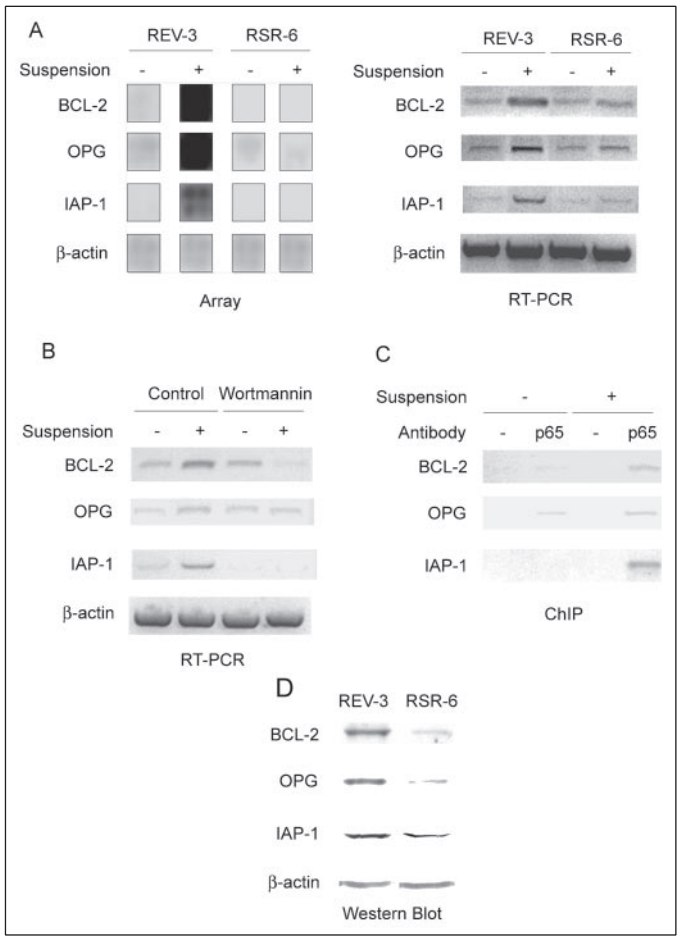


FIGURE 5. **Suspension culture up-regulates expression of BCL-2, OPG, and IAP-1 through NF-κB.** *A*, REV-3 cells and RSR-6 cells were cultured adherent to tissue culture plastic (−) or in suspension for 4 h (+). RNA was prepared and pooled from three independent experiments and used to assess relative expression of 96 apoptosis-related genes using a focused array (*left*). Shown are the array positions corresponding to BCL-2, OPG, IAP-1, and β-actin. In independent experiments, REV-3 and RSR-6 cells were treated identically, and the mRNA expression of BCL-2, OPG, IAP-1, and β-actin were determined by RT-PCR (*right*). *B*, parental RIE-1 cells were left untreated or were treated with wortmannin 250 nM. Cells were cultured adherent to tissue culture plastic (−) or in suspension for 4 h (+). RNA was prepared and the mRNA expression of BCL-2, OPG, IAP-1, and β-actin were determined by RT-PCR. *C*, parental RIE-1 cells were cultured attached to tissue culture plastic (−) or in suspension for 1 h (+). Sheared DNA was immunoprecipitated using anti-RelA/65 antibody or no antibody (−). The binding of RelA/p65 to the promoters of BCL-2, OPG and IAP-1 was assessed using ChIP with promoter-specific PCR. *D*, whole cell protein extracts were prepared from REV-3 and RSR-6 cells after 4 h of suspension culture and were subjected to Western blotting for BCL-2, OPG, IAP-1, and β-actin.

lated expression of BCL-2, OPG, and IAP-1 mRNA in control REV cells (Fig. 5*A*). However, none of these genes were up-regulated in RSR cells, which indicates NF-κB dependence. The results of array-based expression analysis were validated using RT-PCR on RNA extracted from REV and RSR cells from independent experiments. We confirmed up-regulated expression of BCL-2, OPG, and IAP-1 mRNA in REV but not in RSR cells 4 h after detachment (Fig. 5*B*). Since PI 3-K regulated anoikis and NF-κB activation by loss of extracellular attachment, we reasoned that the expression of this set of NF-κB target genes might depend on PI 3-K. We tested this notion by measuring the mRNA expression of these targets by RT-PCR from attached and suspension-cultured RIE-1 cells treated with PI 3-K inhibitor wortmannin. Results show that wortmannin decreased expression of BCL-2, OPG, and IAP-1 induced by culture in suspension (Fig. 5*C*). This confirms the functional linkage of the PI 3-K/AKT system with the downstream mediator NF-κB in the up-regulated expression of this set of suspension culture-induced genes.

BCL-2 and IAP-1 have been previously identified as NF-κB target genes based on comparisons of their expression in cells with or without functional NF-κB, usually under conditions of cytokine stimulation (33, 34). However, OPG has not been extensively characterized with respect to NF-κB dependence of expression. When we examined the DNA sequences of the 5′ regulatory regions of these genes, we found candidate NF-κB binding sites in them all. *In vivo* binding of the NF-κB RelA/p65 polypeptide to these promoters was then evaluated using ChIP with promoter-specific PCR. Some RelA/p65 binding was detected on the OPG promoter in attached parental RIE-1 cells (Fig. 5*D*). After 1 h of culture in suspension, RelA/p65 was recruited to the promoter regions of all of these genes. Western blotting experiments of REV and RSR cell extracts revealed that under suspension culture conditions, NF-κB promoted the expression of BCL-2, OPG, and IAP-1 at the protein level, confirming and extending the previous observations at the promoter and mRNA levels (Fig. 5*D*). Taken together, these data establish the importance of NF-κB in the direct suspension culture-induced regulation of BCL-2, OPG, and IAP-1.

Next, we wished to assess the functional importance of BCL-2, OPG, and IAP-1 in regulating anoikis of intestinal epithelial cells. We designed siRNA oligonucleotides directed against these three targets and used them to transfect RIE-1 cells. 72 h after transfection, the cells were placed in suspension culture for 4 h, and we compared the mRNA expression levels of BCL-2, OPG, and IAP-1. Although control siRNA did not reduce expression of any of the targets, we did observe a decrease in the suspension culture-induced up-regulated expression of BCL-2, OPG, and IAP-1 by their respective siRNAs (Fig. 6*A*). Having developed an effective strategy for decreasing expression of these three putative regulators of anoikis, we measured the effect of the small interfering RNAs on anoikis. Compared with the control oligonucleotides, relative levels of anoikis increased by about 2-fold in the presence of any of the siRNAs targeting BCL-2, OPG, or IAP-1 (Fig. 6*B*).

The increase in relative levels of anoikis of intestinal epithelial cells caused by knockdown of BCL-2, OPG, or IAP-1 strongly suggested that their induced expression by NF-κB might mediate the antianoikis effect of this transcription factor. To explore this possibility further, we genetically reconstituted the expression of these genes either alone or in combination to control REV and NF-κB-inactive RSR cells and subsequently quantified anoikis in those cells. Compared with control DNA-transfected cells, transfection of BCL-2, OPG, or IAP-1 plasmids had little effect on anoikis in REV cells (Fig. 6*C*). In contrast, transfection of NF-κB-inactive RSR cells with BCL-2, OPG, or IAP-1 plasmids reduced anoikis by about 50%, to a level similar to that observed in the control REV cells. Thus, restoring the expression of these three NF-κB target genes rescues the anoikis susceptibility induced by a loss of NF-κB activity.

Together, these findings further highlight the importance of NF-κB in suppressing anoikis and show that BCL-2, OPG, or IAP-1 function as NF-κB-regulated molecular effectors that inhibit anoikis in intestinal epithelial cells.

DISCUSSION

These experiments were initiated to characterize NF-κB as a putative mediator of the antianoikis effects of PI 3-K and AKT. We found that NF-κB was activated after loss of extracellular attachments and served to inhibit anoikis in intestinal epithelial cells. The activation of NF-κB after loss of extracellular attachments required FAK, which triggered a signaling cascade through PI 3-K and AKT that induced the canonical IKKβ-dependent pathway of NF-κB activation. NF-κB, activated in this manner, up-regulated the expression of a subset of target genes, which

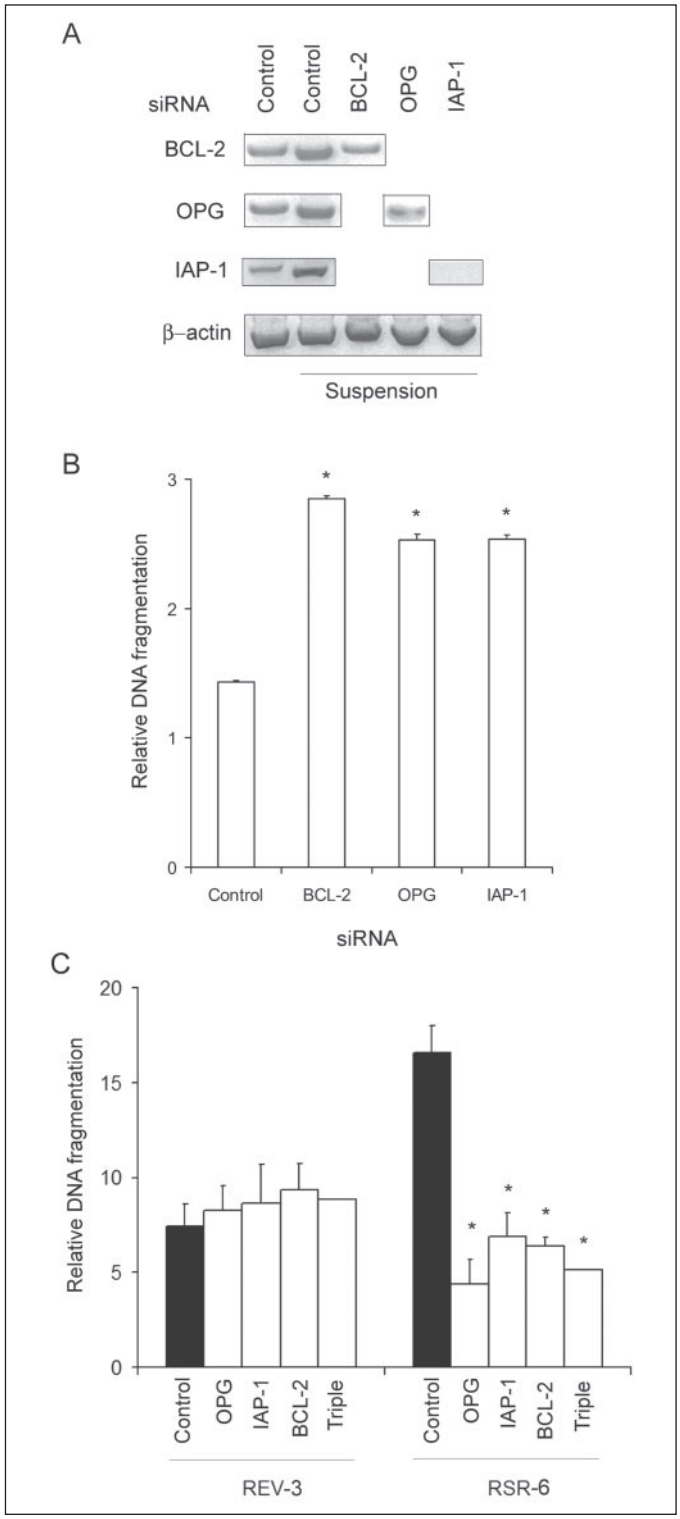


FIGURE 6. **OPG, IAP-1, and BCL-2 expression mitigate the anoikis susceptibility of RSR-6 cells.** *A*, RIE-1 cells were transfected with siRNA targeting BCL-2, OPG, or IAP-1 or with a control siRNA. 72 h after transfection, cells were detached and cultured in suspension for 4 h. Total RNA was extracted and subjected to reverse transcription PCR to assess relative expression of BCL-2, OPG, IAP-1, and β-actin. *B*, RIE-1 cells were transfected with control, BCL-2, OPG, or IAP-1 siRNA. 72 h after transfection, cells were detached and cultured in suspension. After 4 h of suspension culture, relative DNA fragmentation was determined by comparing internucleosomal DNA fragmentation in suspension-cultured cells with that in attached cells. Results were expressed as mean ± S.E. *, $p < 0.05$, compared with control. *C*, REV-3 and RSR-6 cells were transfected with control plasmid or with expression plasmids encoding OPG, IAP-1, BCL-2, or all three plasmids (*Triple*). After 4 h of suspension culture, relative DNA fragmentation was determined by comparing internucleosomal DNA fragmentation in suspension-cultured cells with that in attached cells. Results were expressed as mean ± S.E. *, $p < 0.05$ compared with control.

included BCL-2, OPG, and IAP-1. Inhibition of the up-regulated expression of these genes increased anoikis, whereas restoration of their expression reversed the anoikis susceptibility of NF-κB-inactive cells. This confirms their functional importance as downstream effectors of NF-κB-mediated anoikis inhibition. Therefore, these findings have filled three important knowledge gaps concerning anoikis regulation in intestinal epithelial cells: FAK is revealed as a molecular trigger for an antianoikis pathway; NF-κB acts downstream of PI 3-K and AKT to inhibit anoikis; and OPG, BCL-2, and IAP-1 function as downstream molecular effectors of this survival pathway.

Attachment-dependent epithelial cell survival requires phosphorylation of FAK tyrosine 397, which is stimulated by the interaction of plasma membrane integrins with their extracellular ligands. When cells lose extracellular attachments, FAK tyrosine 397 is dephosphorylated (35), a finding we confirmed in RIE-1 intestinal epithelial cells. The functional importance of FAK in transmitting a survival signal from integrins was previously demonstrated by the ability of constitutively active FAK to prevent anoikis (36, 37), but downstream effectors of FAK had not been identified. Our results show the importance of FAK in the induction of an NF-κB-mediated antianoikis pathway through PI 3-K and AKT, since blockade of FAK signaling both inhibited AKT and NF-κB activation and increased anoikis. FAK tyrosine 397 phosphorylation seemed to trigger activation of the antianoikis pathway very rapidly following detachment, since the abundance of this phosphotyrosine decreased within minutes of suspension culture yet was required for the activation of the pathway. Therefore, FAK acts as a link that couples adherence-dependent survival to the induction of a signaling pathway that controls the survival of cells once adherence has been lost.

We found that PI 3-K is a mediator of the FAK-triggered antianoikis signal. Previously, it was recognized that PI 3-K could be stimulated by growth factors acting through receptor tyrosine kinases (38) or by expression of transforming forms of proto-oncogenes, such as Ras or Src (8, 10, 39). When so activated in epithelial cells cultured in suspension, PI 3-K was in part required for the antianoikis functions of those factors. Consistent with a previous report in colon cancer cells (7), we have found that, even in the absence of stimulation of RIE-1 cells with growth factors or oncogenes, the release of these cells from extracellular attachments alone results in robust activation of PI 3-K, as assessed by phosphorylation of AKT, a downstream target. A recent paper reported that Src family kinases are transiently activated after detachment of intestinal epithelial cells, which activated PI 3-K (6). Our observation that the activation of PI 3-K and AKT after detachment depended in part on phosphorylation of FAK tyrosine 397 is consistent with a role for Src, since these two kinases functionally co-associate through interaction at this site (40).

The role of the PI 3-K/AKT pathway in NF-κB activation is controversial. Whereas some reports have suggested that these kinases are required for IKKβ-dependent NF-κB activation by inflammatory stimuli, such as TNF-α (41), others have found no involvement of AKT in this pathway (42). Our own results show no effect of PI 3-K inhibitors on interleukin-1-induced NF-κB activation in RIE-1 cells, which argues against a role for this kinase in cytokine-induced NF-κB activation in intestinal epithelial cells. However, we did observe a role for PI 3-K and AKT in NF-κB activation induced by suspension culture of RIE-1 cells, judged both by the PI 3-K-dependent serine 473 phosphorylation of AKT and the inhibition of NF-κB by pharmacologic and genetic blockers of PI 3-K and AKT. These results, which reveal the NF-κB RelA/p65-p50 heterodimer as a downstream target of the PI 3-K/AKT survival pathway in suspension-cultured epithelial cells, are consistent with a number of prior reports that identified NF-κB as a target of PI 3-K/AKT

after stimulation by Gram-negative bacteria (43), oncogenic Ras (44), and growth factor receptor tyrosine kinases (45). A number of mechanisms of anti-apoptosis have been attributed to AKT. These include phosphorylation and subsequent inactivation of proapoptotic molecules caspase-9 (46) and Bad (47) and up-regulated expression of anti-apoptotic BCL-2 (48). Our experiments with NF-κB-inactive RSR cells clearly show the important antianoikis and prosurvival effects of this transcription factor. Moreover, the important roles for PI 3-K and AKT in signaling suspension culture-induced NF-κB activation and blocking anoikis of intestinal epithelial cells point to the activation of NF-κB as a novel mechanism of anoikis inhibition by AKT.

Two important pathways of NF-κB activation have been described. The canonical pathway involves IKKβ-dependent IκBα phosphorylation and subsequent proteasomal degradation to control RelA/p65-p50 heterodimer activity, whereas an alternative pathway induces p100 processing through IKKα to control p52-RelB activity (11, 49). When RIE-1 cells were released from extracellular attachments and cultured in suspension, we observed serine 32 phosphorylation of IκBα within 5 min, indicating rapid activation of the IKK complex. Western analysis of total IκBα abundance in RIE-1 cells after detachment failed to show the almost complete reduction in levels that typically accompanies IKKβ activation by prototypic NF-κB stimuli. It had previously been noted in a variety of intestinal epithelial cell lines that some stimuli that lead to IKKβ-dependent NF-κB activation do not induce robust IκBα degradation (50). This suggests that such cells might rapidly resynthesize IκBα, itself an NF-κB target gene (51). This notion was supported by our experimental observation that cycloheximide, a protein synthesis inhibitor, caused a marked reduction in IκBα expression in suspension-cultured RIE-1 cells. The potent inhibition of NF-κB activation by expression of nondegradable superrepressor IκBα, by proteasome inhibitor MG132, and by dominant negative IKKβ mutant plasmids nevertheless indicate the involvement of the canonical IKKβ-dependent pathway to NF-κB activation in suspension-cultured RIE-1 cells.

Well characterized NF-κB-activating stimuli include proinflammatory cytokines such as TNF-α, microbial cell wall products such as lipopolysaccharide and flagellin, and DNA double strand breaks induced by ionizing radiation or DNA-damaging drugs. In intestinal epithelial cells, the loss of extracellular attachment can now be added to the list of NF-κB-activating stimuli. Interestingly, a prior study showed that reattachment of suspension-cultured human colon cancer HT-29 cells to tissue culture surfaces resulted in NF-κB activation, which promoted the survival of those cells (52). In that study, NF-κB activity in HT29 cells was accompanied by greater metastasis in immunodeficient mice. A similar prometastasis role for NF-κB has also been made a syngeneic Balb/c mouse model of endotoxemia and colon cancer metastasis (53). One other prior study had shown that NF-κB, when stimulated in intestinal epithelial cells by trefoil factors, inhibited anoikis of those cells (54). However, the mechanism of NF-κB activation by trefoil factors and the mechanism by which NF-κB mediated the antianoikis effect of trefoil factors were not elucidated. In our study, we have delineated the trigger for NF-κB activation following loss of extracellular attachment and have also identified the specific molecular effectors of NF-κB that result in the blockade of anoikis. The functional importance of our results relates to the fact that this distinct prosurvival mechanism may contribute to the promotion of colon cancer metastasis by NF-κB.

The antiapoptosis functions of NF-κB have been ascribed to the up-regulated expression of several target genes (12). Our results have identified BCL-2, OPG, and IAP-1 as genes that are up-regulated by NF-κB in intestinal epithelial cells after detachment. Interestingly, the program of genes activated in an NF-κB-dependent manner in suspension-cultured cells was distinct from that previously reported to be activated by different apoptosis inducers. For example, we observed no significant up-regulation of TNF receptor associated factor-1 or -2, BCL-XL, or X-IAP, all previously reported to be up-regulated by NF-κB after treatment of cells with death receptor ligands or DNA-damaging agents (12, 17). We identified NF-κB binding sites in the promoters of these three genes, and ChIP analysis revealed the binding of the p65/RelA subunit to those sites. In the case of BCL-2 and IAP-1, this confirms the role of NF-κB as a direct regulator of their expression (33, 34). In the case of OPG, a prior publication had noted lower expression levels in endothelial cells expressing the NF-κB superrepressor (55). Our results expand this finding to intestinal epithelial cells, and the ChIP experiments conclusively show for the first time direct regulation of the OPG promoter by NF-κB. Importantly, using an underexpression/overexpression experimental paradigm, we could demonstrate the functional importance of BCL-2, OPG, and IAP-1 in the inhibition of anoikis in intestinal epithelial cells.

Some prior studies have indicated an antiapoptotic function for OPG wherein OPG acts as a decoy receptor for TNF-related apoptosis-inducing ligand (TRAIL), which in cancer cells induces a death receptor pathway of apoptosis through the DR5 receptor (56, 57). The functional antianoikis effect of OPG that was shown by restoration of OPG expression in NF-κB-inactive cells suggests that TRAIL might be involved in the induction of anoikis. Alternatively, OPG might function to block anoikis in a TRAIL-independent manner, as has also been noted in growth factor withdrawal-induced apoptosis of endothelial cells (55). TRAIL is expressed on natural killer and natural killer T cells in the liver, where it functions to limit colon cancer metastasis (58). The up-regulated expression of OPG by NF-κB after detachment raises the possibility that colon cancer cells might use OPG expression as a decoy to mitigate apoptosis by TRAIL during the establishment of liver metastases. This clinically important possibility will be evaluated in future experiments.

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