Inhibition of NF-κB Sensitizes A431 Cells to Epidermal Growth Factor-induced Apoptosis, whereas Its Activation by Ectopic Expression of RelA Confers Resistance*

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Epidermal growth factor (EGF) is a well known mitogen, but it paradoxically induces apoptosis in cells that overexpress its receptor. We demonstrate for the first time that the EGF-induced apoptosis is accelerated if NF-κB is inactivated. To inactivate NF-κB, human epidermoid carcinoma cells (A431) that overexpress EGF receptor were stably transfected with an IκB-α double mutant construct. Under the NF-κB-inactivated condition, A431 cells were more sensitive to EGF with decreased cell viability and increased externalization of phosphatidylserine on the cell surface, DNA fragmentation, and activation of caspases (3 and 8 but not 9), typical features of apoptosis. These results were further supported by the potentiation of the growth inhibitory effects of EGF by chemical inhibitors of NF-κB (curcumin and sodium salicylate) and the protective role of RelA evidenced by the resistance of A431-RelA cells (stably transfected with RelA) to EGF-induced apoptosis. EGF treatment or ectopic expression of RelA in A431 cells induced DNA binding activity of NF-κB (p50 and RelA) and the expression of c-IAP1, a downstream target of NF-κB. A431-RelA cells exhibited spontaneous phosphorylation of Akt (a downstream target of phosphatidylinositol 3-kinase and regulator of NF-κB) and EGF treatment stimulated it further. Blocking this basal Akt phosphorylation with LY294002, an inhibitor of phosphatidylinositol 3-kinase, did not affect their viability but blocking of EGF-induced phosphorylation of Akt sensitized the otherwise resistant A431-RelA cells to EGF-mediated growth inhibition. Our results favor an anti-apoptotic role for NF-κB in the regulation of EGF-induced apoptosis.

Epidermal growth factor (EGF)[1] is a polypeptide (6-kDa) that belongs to the EGF family of ligands (heparin binding EGF, transforming growth factor-α, amphiregulin, β-cellulin, epiregulin, and neuregulins) binding to specific cell surface receptors (1, 2). Upon ligand binding, the epidermal growth factor receptor (EGFR) dimerizes, autophosphorylates itself, and recruits a cascade of signaling molecules before transmitting potent mitogenic signals in many cellular systems (1, 3). EGFR is overexpressed in a number of human malignancies including cancers of the lung, head and neck, brain, bladder, and breast (4). Furthermore, increased EGFR expression correlates with a poorer clinical outcome for patients with breast and ovarian cancers (5, 6). Whereas EGF is a potent mitogen, it paradoxically induces apoptosis in cells that overexpress EGFR such as A431 (7). Experimentally increasing the level of EGFR expression in epithelial, mesenchymal, or glial cells also leads to ligand-dependent apoptosis (8). Another ligand of the EGF family, heregulin (also known as neuregulin), is known to induce apoptosis in cells that overexpress ErbB2, the second member of the EGFR family (9). In addition, epiregulin also inhibited cell growth in EGFR-overexpressing cells (10). Induction of amphiregulin mRNA was observed in EGF-induced apoptosis (11) and interaction of EGF with pro-heparin-binding EGF leads to growth inhibition and apoptosis (12). Growth factors other than EGF such as platelet-derived growth factor and hepatocyte growth factor can also trigger cell cycle arrest and death in a variety of cells (13, 14) and in addition, EGF enhanced the apoptotic effect of platelet-derived growth factor (14). An EGF-related protein, Cripto-1, promotes apoptosis in HC-11 mouse mammary epithelial cells (15). Anoikis, activation of EGFR tyrosine kinase, Ras-MAP kinase signaling, and the elevation of Stat1 and p21 levels have been advocated as mechanisms driving EGF-induced apoptosis (11, 16–18) but, the actual mechanism appears to be more elusive and complicated.

Apoptosis or programmed cell death is a physiological process characterized by distinct morphological and biochemical features that include membrane blebbing, chromatin condensation, cytoplasmic shrinking, DNA fragmentation, and activation of different caspases (19). Typically two different pathways, extrinsic receptor-mediated and intrinsic mitochondria-mediated, leading to apoptosis have been identified (20, 21). Mostly cytokines of the tumor necrosis factor (TNF) superfamily induce apoptosis by interaction of the ligand with its death receptor, which sequentially recruits TNF receptor-associated death domain, Fas-associated death domain, caspase 8, and caspase 3, the last then cleaves various substrates leading to apoptosis. In contrast, the mitochondria-mediated pathway involves the release of cytochrome *c* from the mitochondria, and cytochrome *c* together with Apaf1 activates caspase 9, and the latter then activates caspase 3, resulting in apoptosis (20, 21). Tumor cells often evade apoptosis by expressing several anti-apoptotic proteins such as Bcl-2, down-regulation and mutation of pro-apoptotic genes and alterations of p53, PI-3K/Akt, or NF-κB

[1] The abbreviations used are: EGF, epidermal growth factor; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; IκB, inhibitor κB; IKK, IκB kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; PARP, poly-(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; PS, phosphatidylserine; TNF, tumor necrosis factor; IAP, inhibitor of apoptosis protein; PI-3K, phosphatidylinositol 3-kinase; AFC, 7-amino-4-trifluromethyl coumarin; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

pathways that give them survival advantage and thereby resist therapy-induced apoptosis (20).

NF-κB is a family of transcription factors activated by a diverse number of stimuli including EGF, cytokines, such as TNF-α and interleukin-1, UV irradiation, and lipopolysaccharides (22). EGF has been reported to activate NF-κB in smooth muscle cells, fibroblasts, and in several EGFR-overexpressing cell lines (23–25). Binding of IκB to NF-κB masks nuclear localization signals and prevents its translocation to the nucleus (26). Stimulation of cells with a diverse array of stimuli results in phosphorylation of IκB-α on serines 32 and 36 at its NH$_2$-terminal. This leads to the ubiquitination and degradation of IκB-α, allowing NF-κB to translocate to the nucleus and activate transcription (22, 26). Inhibition of NF-κB activity potentiates cell killing of human breast cancer and fibrosarcoma cell lines by TNF-α, ionizing radiation, and daunorubicin (27–29). NF-κB inhibition sensitized tumors in mice to chemotherapeutic compound CPT-11-mediated cell killing (30). NF-κB directly causes increased expression of proteins that contribute to the survival of tumor cells such as inhibitors of apoptotic proteins (IAPs) (31, 32). Results from our laboratory have shown earlier that ectopic expression of the RelA subunit of NF-κB into murine fibrosarcoma cells protects them from curcumin-induced apoptosis (33).

To understand whether NF-κB plays any role in EGF-induced apoptosis, we used A431 cells that overexpress EGFR and stably transfected them with a mutant IκB (known to inactivate NF-κB) or RelA (known to activate NF-κB). Using several parameters to assess apoptosis such as viability, externalization of phosphatidylserine (PS) on the cell surface, DNA fragmentation, and activation of caspases we report that A431 cells are more sensitive to EGF-induced apoptosis under NF-κB-inactivated conditions whereas its activation confers resistance.

EXPERIMENTAL PROCEDURES

Reagents, Chemicals, and Antibodies—EGF (isolated from male mouse submaxillary glands), Dulbecco's minimum essential medium, and fetal bovine serum were procured from Invitrogen. Curcumin, sodium salicylate, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate mixture, and a mouse monoclonal antibody to β-actin (A-5441) were purchased from Sigma. Fluorimetric substrates for caspase 3 (Ac-DEVD-AFC number 264157) and caspase 9 (Ac-LEHD-AFC number 218765) were obtained from Calbiochem. Rabbit polyclonal antibodies to p50 (sc-7178), RelA (sc-109), hemagglutinin (HA) (sc-7392), IκB-α (sc-271), and c-IAP1 (sc-7943) were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to caspase 8 (1C12), rabbit polyclonal PARP antibody (number 9542), and phospho-Akt pathway sampler kit (number 9916 containing antibodies to Akt and phospho-Akt, and LY294002) were purchased from Cell Signaling Technology (Beverly, MA), and the mouse monoclonal EGFR antibody (clone 111) raised against the extracellular domain of EGFR was a gift from Dr. Yosef Yarden, Weizmann Institute of Science, Israel.

Cell Lines and Culture—Human epidermoid carcinoma cell line A431 was obtained from the National Center for Cell Science, Pune, India. The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO$_2$ and 95% air.

Transient and Stable Transfections—A431-IκB-α cells were transiently transfected with *relA* in pMT2T vector (33, 34) using the calcium-phosphate transfection kit (Invitrogen) according to the manufacturer's protocol. Stable transfections in A431 cells with *relA* in pMT2T vector (co-transfected with pcDNA3) or the empty vector pcDNA3 or pcDNA3-IκB-α were carried out by the LipofectAMINE method (35). For the preparation of liposome solution, 20 μmol/ml of stock (prepared by mixing 6.6 μmol of dimethyl dioctadecyl ammonium bromide and 13.4 μmol of dioleyl-L-phosphatidylethanolamine in 1 ml of ethanol) was diluted into 1 nmol/μl in water. For transfection, the cells were seeded to attain 70% confluence in 35-mm Petri dishes. For each dish,

2 μg of DNA and 24 μl of liposome solution were mixed in 500 μl of Dulbecco's minimum essential medium free from serum and antibiotics, vortexed, and incubated at room temperature for 30 min. The liposome solution (1 ml) was layered over the cells previously rinsed with serum-free medium and left for 4 h in a CO$_2$ incubator and then the medium was replenished with 20% fetal bovine serum and reverted back to 10% fetal bovine serum after 24 h. After 72 h, cells were grown in selection medium (400 μg/ml G418) and clones formed were picked up and maintained separately with 100 μg/ml G418.

Western Blotting—Cells were lysed in whole cell lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, 0.1% Triton X 100, 1 mM DTT, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 4 mM sodium orthovanadate). Equal amounts of total protein for each sample were separated by SDS-PAGE and electrotransferred onto nitrocellulose filters, probed with the primary antibodies and appropriate peroxidase-conjugated secondary antibodies, and visualized with the enhanced chemiluminescence (ECL) method as per the manufacturer's protocol (Amersham Biosciences). For some experiments, alkaline phosphatase-conjugated secondary antibodies from Sigma were used and the bands were detected using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate.

Electrophoretic Mobility Shift Assay (EMSA)—Cells were washed with cold PBS and suspended in 150 μl of lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine). The cells were allowed to swell for 30 min, after which 4.5 μl of 10% Nonidet P-40 was added, vortexed, and centrifuged, and the pellet was suspended in 25 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine). The nuclear extract (8 μg of protein) collected after 30 min by centrifugation was used to perform EMSA by incubating it with 16 fmol of ^{32}P-end labeled 45-mer double stranded NF-κB oligonucleotide from the human immunodeficiency virus-1 long terminal repeat (5′-TTGTTACAAGGGACTTTCCGCTGG-GGACTTTCCAGGGAGGCGTGG-3′) in the presence of 1 μg/ml poly(dI-dC) in a binding buffer (25 mM HEPES (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, and 5% glycerol) for 30 min at 37 °C and the DNA-protein complex was resolved using a 6.6% native polyacrylamide gel. The gels were dried and the radioactive bands were visualized by phosphorimaging (Bio-Rad Personal FX).

MTT Assay—For MTT assay, 25 μl of MTT solution (5 mg/ml in PBS) was added to cells (untreated and treated) cultured in 96-well plates. Cells were incubated for 2 h and 0.1 ml of the extraction buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide) was added after removal of MTT with a PBS wash. After an overnight incubation at 37 °C, the optical densities at 570 nm were measured using a plate reader (Bio-Rad), with the extraction buffer as a blank. The relative cell viability in percentage was calculated as (A_{570} of treated samples/A_{570} of untreated samples) × 100.

[^{3}H]Thymidine Incorporation—Cells grown in 96-well plates were treated with or without the indicated concentrations of EGF and at the end of 18 h, [^{3}H]thymidine was added to each well (0.5 μCi/well) and the incubation was continued for a total period of 24 h. The culture medium was then removed, washed twice with PBS, and the proteins were precipitated with 5% trichloroacetic acid. The supernatant was removed and after washing with ethanol, the cells were solubilized with 0.2 N NaOH, and the radioactivity was counted using a liquid scintillation counter.

Annexin-PI Staining—The cells (10^4 cells/well) were seeded in 48-well plates and treated with or without EGF for 16 h. Then the cells were washed with PBS and treated with 1× assay buffer, annexin-fluorescein isothiocyanate and propidium iodide (PI) as per the protocol described in the annexin V apoptosis detection kit (sc-4252 AK) from Santa Cruz Biotechnology. After 10–20 min, the wells were washed with PBS and greenish apoptotic cells were viewed using a Nikon fluorescent microscope and photographed.

Comet Assay—Comet assay was carried out essentially as described (36). Briefly, the cells (treated with or without EGF) were pelleted and resuspended in 0.5% low melting point agarose at 37 °C and layered on a frosted microscope slide previously coated with a thin layer of 0.5% normal melting agarose and kept for 5 min at 4 °C. After solidification, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.5, 1% Triton X-100, and 10% Me$_2$SO) for 1 h at 4 °C. The slides were then electrophoresed for 20 to 30 min at 25 V. The slides after electrophoresis were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide (1 μg/ml) and observed under a Nikon fluorescent microscope.

Assays of Caspase 3 and Caspase 9—The enzymatic activities of caspase 3 or caspase 9 were assayed spectrofluorimetrically (37). Briefly, the whole cell lysate was incubated with 50 μM fluorimetric substrates of caspase 3 (Ac-DEVD-AFC) or caspase 9 (Ac-LEHD-AFC) in a total volume of 500 μl of reaction buffer (50 mM HEPES-KOH, pH 7.0, 10% glycerol, 0.1% CHAPS, 2 mM EDTA, 2 mM DTT) at 37 °C for 1 h. The released AFC was quantitated using a spectrofluorimeter (PerkinElmer LS-50 B) with the excitation and emission wavelengths of 380 and 460 nm, respectively. Values of relative fluorescence units released per mg of protein were calculated.

RESULTS

EGF Induces NF-κB DNA Binding Activity in A431-Neo but Not A431-IκB-α Cells—The human epidermoid carcinoma cell line, A431, overexpresses EGFRs (about 2×10^6 EGFRs/cell) and has been extensively used as a model system to study the effects of EGF on cell proliferation (7, 38–40). To study the role of NF-κB in EGF-induced apoptosis, we used A431 cells and stably transfected them with either the empty vector (pcDNA3) or pcDNA3-IκB-α, a double mutant construct in which both the serines (32 and 36) at the amino-terminal of IκB-α are mutated to alanine. Because the double mutant form of IκB-α lacks the crucial serine residues that need to be phosphorylated by NF-κB activators, it is popularly employed to strongly inhibit NF-κB (28). As the construct, pcDNA3-IκB-α, contains the hemagglutinin tag (HA tag), Western blotting of HA protein was used to ascertain the presence of the mutant IκB protein. As expected, all six clones of A431-IκB-α cells (selected by G418) showed the presence of HA protein whereas the A431-Neo cells transfected with control vector (pcDNA3) did not show its expression (Fig. 1*A*). Further experiments with IκB-transfected cells were done using only clone 6 of A431-IκB-α cells (showing very high HA expression) unless stated otherwise. Western blotting with a polyclonal IκB-α antibody confirmed that clone 6 had indeed a higher level of IκB-α expression compared with that in the parental as well as A431-Neo cells (Fig. 1*B*). For the Western blots, β-actin was used as a control and the results confirm equal loading of the samples (Fig. 1, *A* and *B*). To see whether transfection procedures affected the level of EGFR, Western blotting was carried out in parental, A431-Neo, and A431-IκB-α cells and the results confirmed that EGFR levels remained unaffected in these cells (Fig. 1*C*). Because the very purpose of stable transfection was to inactivate NF-κB, it was logical to check whether EGF could stimulate the NF-κB DNA binding activity in A431-Neo and A431-IκB-α cells (clone 6) by EMSA. Whereas 50 ng/ml EGF enhanced the NF-κB DNA binding activity in A431-Neo cells, even 100 ng/ml EGF could not induce it in A431-IκB-α cells and in the absence of EGF there were no active DNA-binding complexes of NF-κB in both the cells (Fig. 1*D*). To confirm whether the active complex contains the classical NF-κB partners, p50 and RelA, the nuclear extracts prepared from A431-Neo cells stimulated with 50 ng/ml EGF were incubated with antibodies to RelA or p50 and then EMSA was carried out. As shown in Fig. 1*E*, both antibodies shifted the active NF-κB complex (supershift) whereas incubation with excess of an unlabeled oligonucleotide containing the NF-κB binding site removed the active complex. Fig. 1*E* also shows that transient transfection of A431-IκB-α cells with *relA* restored the NF-κB active complex, and *relA* transfection was used by us earlier to constitutively activate NF-κB in L-929 cells (33). These results confirm that the A431-IκB-α cells express the mutant form of IκB-α that inactivated NF-κB and hence, EGF could not stimulate NF-κB DNA binding activity in these cells. The results also indicate that EGF can induce the NF-κB DNA binding activity in A431-Neo cells and the active NF-κB complex contains the heterodimers, p50 and RelA. In addition, the results show that RelA, being one of the heterodimeric partners of active NF-κB, favors the formation of
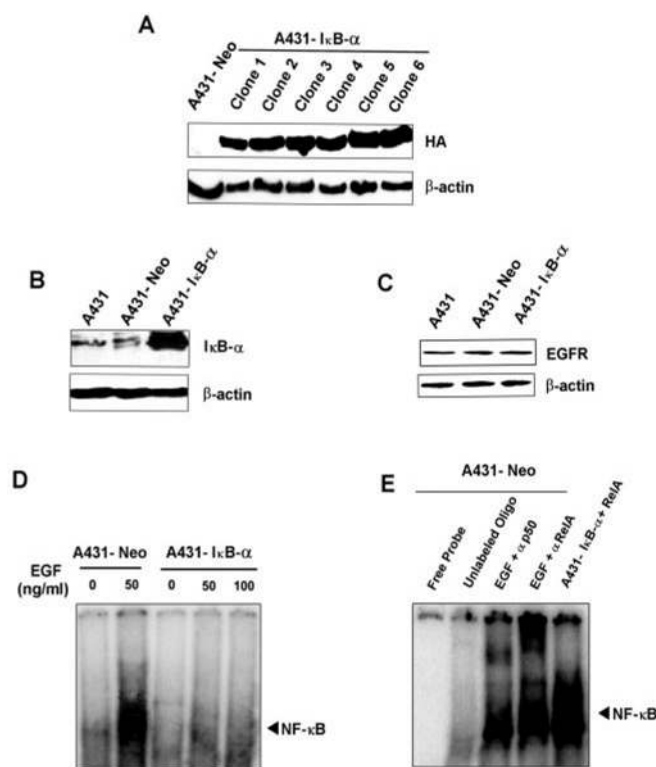

Fig. 1. **Western blotting for HA and IκB-α, and assessment of NF-κB DNA binding activity in transfected and untransfected A431 cells.** *A*, A431 cells were transfected with pcDNA3 vector or the pcDNA3-IκB-α construct using LipofectAMINE and the G418-resistant clones were selected as described under "Experimental Procedures." Cell lysates (40 μg of protein) from the vector-transfected A431-Neo cells and the different clones of A431-IκB-α cells were subjected to SDS-PAGE and immunoblotted with HA antibody or β-actin (control) by ECL as described under "Experimental Procedures." *B*, cell lysates from A431, A431-Neo, and the clone-6 of A431-IκB-α cells were subjected to Western blotting with an antibody to IκB-α or β-actin (control) as described above. *C*, similarly, the cell lysate from A431, A431-Neo, or A431-IκB-α cells was subjected to Western blotting with an antibody to EGFR or β-actin (control). *D*, A431-Neo cells or A431-IκB-α cells grown in 35-mm Petri dishes (1×10^6 cells/dish) were treated with EGF at the indicated concentrations at 37 °C for 1 h. Nuclear extracts were prepared and EMSA was done as described under "Experimental Procedures." *E*, A431-Neo cells were treated with 50 ng/ml EGF as described above and EMSA was done as before. The nuclear extracts from EGF-stimulated cells were also incubated with either RelA or p50 antibody or with 10 times excess of unlabeled oligo. One of the lanes had the nuclear extract prepared from A431-IκB-α cells transiently transfected with *relA* as described under "Experimental Procedures" and another lane contained the labeled oligo (free probe) without the addition of nuclear extract. All the experiments above were done at least two times with similar results. The *arrowheads* shown in *panels D* and *E* indicate the positions of the active DNA-binding complexes of NF-κB.

active NF-κB DNA binding complexes and has the potential to reverse the NF-κB-inactivating effect of IκB.

A431-IκB-α Cells Are More Sensitive to EGF-induced Cytotoxicity and DNA Fragmentation Than A431-Neo Cells—To study the effects of EGF on cell growth under conditions of NF-κB inactivation, A431-Neo and A431-IκB-α cells were exposed to various concentrations of EGF and the cell viability in percentage over untreated control was determined after 72 h by MTT assay. The MTT assay is a convenient screening assay for the measurement of cell death while it does not discriminate between apoptosis and necrosis. EGF treatment at 0.01 and 0.1 ng/ml had a stimulatory effect on A431-Neo cells with cell viabilities of 130 and 110%, respectively, if the viability of untreated control was taken as 100% at the end of 72 h (Fig. 2*A*). In contrast, EGF inhibited the A431-IκB-α cells with only 81 and 67% of cells being alive for the concentrations of 0.01
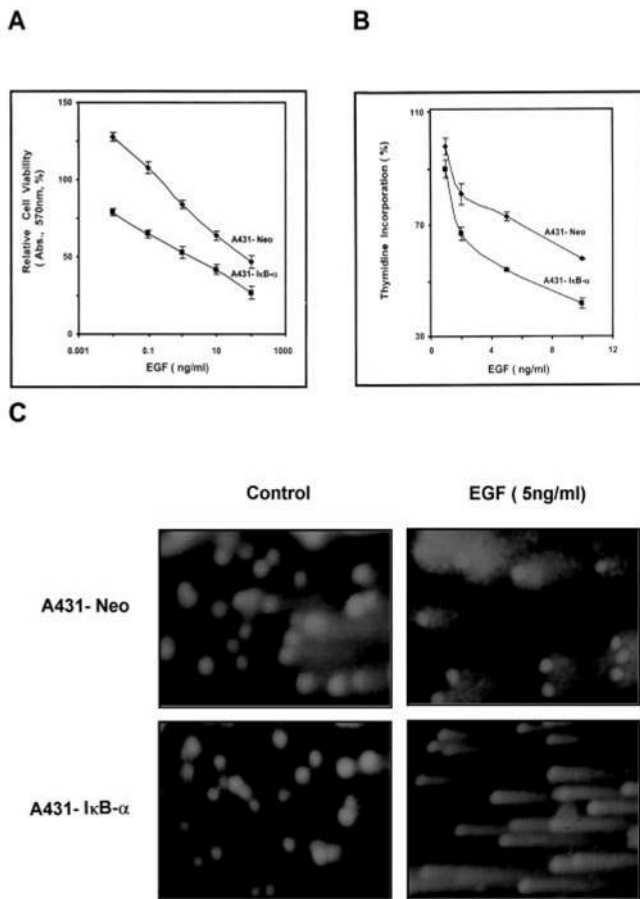
FIG. 2. **Inhibition of growth and DNA synthesis and induction of DNA fragmentation in A431-Neo and A431-IκB-α cells by EGF.** *A*, cells grown in 96-well plates (5 × 10³ cells/well) were incubated for 72 h either with medium or different concentrations of EGF as indicated and cell viability was assessed by the MTT assay as described under "Experimental Procedures." *B*, cells grown in 96-well plates (5 × 10³ cells/well) were incubated with different concentrations of EGF for 24 h, and then the thymidine incorporation as a measure of DNA synthesis was determined as described under "Experimental Procedures" and the results are expressed as percentage over the control (untreated cells). The above experiments were repeated two times with similar results and all determinations were made in triplicate and the *error bars* show the standard deviations. *C*, cells (1 × 10⁶) were seeded in 35-mm Petri dishes and incubated with 5 ng/ml EGF for 24 h. After incubation, cells were trypsinized, pelleted, and the comet assay was done as described under "Experimental Procedures." The experiment was repeated once more with similar results.

and 0.1 ng/ml, respectively, compared with the control (Fig. 2*A*). As can be seen in Fig. 2*A*, EGF at a concentration of 1 ng/ml and above was inhibitory to both A431-Neo and A431-IκB-α cells. Thus, even at concentrations that were stimulatory to A431-Neo cells, EGF inhibited the growth of A431-IκB-α cells (Fig. 2*A*) suggesting that A431-IκB-α cells are much more sensitive to EGF-induced cytotoxicity and these results were also confirmed in 2 other clones of A431-IκB-α (data not shown). We also examined the effect of EGF on DNA synthesis by the method of thymidine incorporation after exposing the cells to different concentrations of EGF (1–10 ng/ml) found to be inhibitory to both A431-Neo and A431-IκB-α cells by the MTT assay. EGF inhibited DNA synthesis in both A431-Neo and A431-IκB-α cells in a dose-dependent manner and again the A431-IκB-α cells were more sensitive to EGF-induced inhibition of DNA synthesis (Fig. 2*B*). DNA fragmentation is another hallmark of apoptosis and to detect this, we have used the single cell gel electrophoresis (Comet assay), a sensitive technique that allows detection of DNA strand breaks. DNA strand

breaks create fragments that when embedded in agarose gels migrate in an electric field. Cells with damaged DNA when stained with ethidium bromide appear like a comet and the length of the comet tail represents the extent of DNA damage. Fig. 2*C* clearly indicates that well formed comets are more in number in A431-IκB-α than A431-Neo cells when induced with 5 ng/ml EGF while the untreated cells did not exhibit the comet morphology in both the cells. These results suggest that A431-IκB-α cells with inactivated NF-κB are more susceptible to cell death induced by EGF compared with A431-Neo cells.

Relatively More A431-IκB-α Cells Undergo EGF-induced Externalization of Phosphatidylserine—To assess whether the cell death induced by EGF involves typical changes encountered during apoptosis, we first looked for changes in PS on the cell membrane. Under defined salt and calcium concentrations, annexin V is predisposed to bind PS that is externalized to the cell surface in the very early stages of apoptosis (41, 42). Hence, apoptotic cells were detected using annexin V labeled with fluorescein isothiocyanate and photographed with a camera-attached fluorescent microscope. Addition of PI helps to distinguish the early apoptotic cells from late apoptotic or necrotic cells because PI cannot enter the cells in the early stages of apoptosis when the membrane integrity is intact (41, 42). In A431-IκB-α cells 37 and 65% were annexin positive after treatment with 5 and 10 ng/ml EGF, respectively, whereas only 9 and 23% of the A431-Neo cells showed annexin positivity (greenish yellow) for the same corresponding EGF concentrations (Fig. 3). Untreated A431-Neo and A431-IκB-α cells either did not show annexin positivity or had a very minimum number of positively stained cells (Fig. 3). However, a small percentage of A431-Neo and A431-IκB-α cells also showed typical PI staining (yellowish red) suggesting the appearance of late apoptotic or necrotic cells with 10 ng/ml EGF treatment (Fig. 3). These results indicate that in comparison with A431-Neo cells, a relatively large number of A431-IκB-α cells exhibit externalization of PS, a typical feature of apoptosis upon treatment with EGF.

A431-IκB-α Cells Are More Sensitive to EGF-induced Apoptosis That Involves Cleavage of PARP, Activation of Caspases 3 and 8, but Not Caspase 9—In many systems caspase 8 and caspase 9 act as initiator caspases and caspase 3, the effector, signals for the final execution of the cells. Pro-caspase 8 was cleaved into its active fragments (p43/41 and p18) by 10 ng/ml EGF in A431-Neo cells while even 5 ng/ml EGF could easily do it in A431-IκB-α cells as visualized by Western blotting and the untreated cells did not show any of the cleavage products (Fig. 4*A*). Caspase 3 activity was determined spectrofluorimetrically using a substrate, Ac-DEVD-AFC, an acetylated synthetic tetrapeptide corresponding to the upstream amino acid sequence of the caspase 3 cleavage site in PARP, and the fluorphor AFC (7-amino-4-trifl64ldin). Whereas EGF-induced caspase 3 activity was 2.1-fold more than the untreated control in A431-IκB-α cells, it was only 1.2-fold more than the control in A431-Neo cells (Fig. 4*B*). Caspase 9 activity was also determined fluorimetrically and EGF treatment could not induce caspase 9 activity in A431-IκB-α and A431-Neo cells, whereas curcumin used as a positive control activated caspase 9 in both cells (Fig. 4*C*). We also examined the cleavage of a well characterized caspase 3 substrate, PARP, from its 116-kDa intact form into the 89-kDa fragment by Western blotting. When A431-IκB-α cells were treated with 5 or 10 ng/ml EGF, the 116-kDa form of PARP decreased and the 89-kDa form increased indicating that the full-length PARP was converted to an apoptotic fragment while the untreated cells showed only the uncleaved fragment (Fig. 4*D*). In A431-Neo cells, both control and 5 ng/ml EGF-treated cells did not exhibit the cleaved
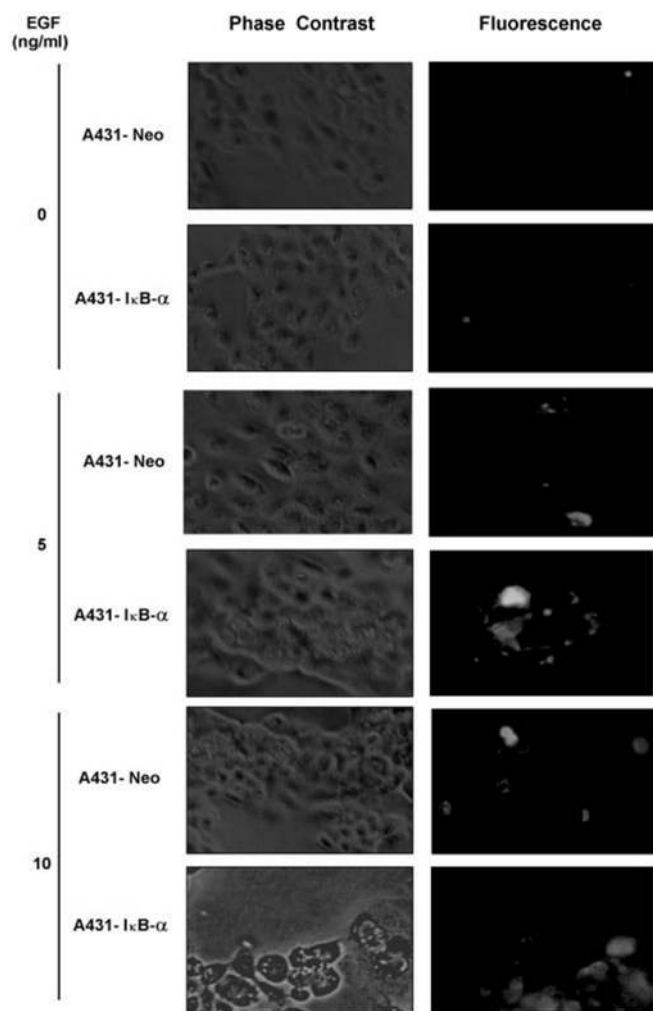
FIG. 3. **EGF-induced changes in annexin-PI staining.** Cells were incubated with or without the indicated concentrations of EGF for 16 h, and stained for annexin-PI positivity (not shown in color) as described under "Experimental Procedures." The same results were confirmed in another independent experiment.

FIG. 4. **Effects of EGF on the activities of caspases 8, 3, and 9, and PARP cleavage.** A, cells (1×10^6) were seeded in 35-mm Petri dishes and treated with or without EGF for 24 h. To detect the active caspase 8 fragments, the cell lysates were resolved on 12% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, probed with caspase 8 antibody (1:3000), and detected by the alkaline phosphatase method using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate. B, cells (1×10^6) were seeded in 35-mm Petri dishes and treated with or without EGF for 24 h. Fifty micrograms of total protein was incubated with 50 μM caspase 3 fluorimetric substrate in a total volume of 500 μl of the reaction buffer and the fluophor released was quantitated spectrofluorimetrically as described under "Experimental Procedures." C, cells (1×10^6) were seeded in 35-mm Petri dishes and treated with EGF or curcumin along with untreated control for 24 h. Fifty micrograms of total protein was incubated with 50 μM caspase 9 fluorimetric substrate and the activity was quantitated spectrofluorimetrically as above. The reproducibility of these experiments was ascertained by repeating them at least two times. D, cells (1×10^6) were seeded in 35-mm Petri dishes and treated with or without EGF for 24 h. To detect the cleavage of PARP, whole cell lysate (40 μg) was resolved on a 7.5% polyacrylamide gel, electrotransferred, probed with PARP antibody (1:3000), and detected by ECL reagent as described earlier. Similar results were obtained when the experiment was repeated.

product and even 10 ng/ml EGF could induce only a slight PARP cleavage (Fig. 4D). The above results confirm that the A431-IκB-α cells are more sensitive to EGF-induced apoptosis than A431-Neo cells by showing increased PARP cleavage and higher activation of caspases 3 and 8 but not 9 suggesting the operation of a caspase 8-mediated extrinsic pathway.

Chemical Inhibitors of NF-κB Enhance the Susceptibility of A431-Neo Cells whereas Activation of NFκB by RelA Reverses the Susceptibility of A431-IκB-α Cells to EGF-induced Cytotoxicity—To know whether chemical inhibitors of NF-κB would also enhance EGF-induced apoptosis, the A431-Neo cells were treated with known inhibitors of NF-κB such as sodium salicylate (50 μM) or curcumin (10 μM) for 2 h prior to EGF treatment. When compared with untreated cells, 77% A431-Neo cells were alive in wells treated with 5 ng/ml EGF for 24 h (Fig. 5A). If the viability of A431-Neo cells treated with 10 μM curcumin alone for 24 h was taken as 100, treatment with EGF after pretreatment with curcumin reduced it to 71% (Fig. 5A). Similarly, if 50 μM sodium salicylate pretreatment is compared with or without EGF the viability of A431-Neo cells was only 61% (Fig. 5A). If IκB-mediated inhibition of NF-κB can positively regulate EGF-induced apoptosis, then this effect is expected to be reversed by NF-κB. As expected, the transient transfection of *relA* into A431-IκB-α cells partly reversed the cytotoxic effect of EGF as measured by an MTT assay (Fig. 5B).
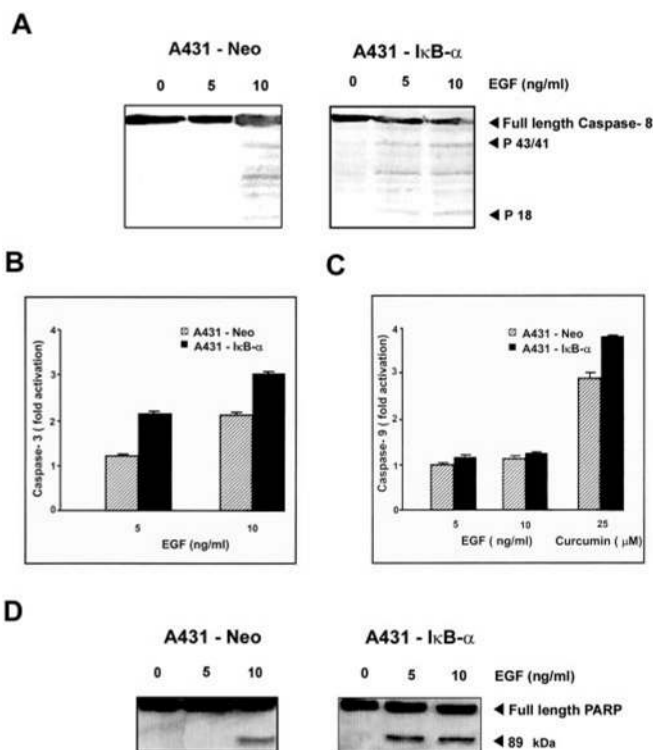
The higher expression of RelA in the transfected cells was confirmed by Western blotting and to ensure that proteins were loaded equally, β-actin controls were used (Fig. 5B, *inset*). These results suggest that similar to IκB, NF-κB inhibitors also have the potential to enhance EGF-induced cell death and NF-κB has a protective role suggested by the higher level of resistance of A431-IκB-α cells transiently transfected with *relA* to EGF-induced cytotoxicity.

RelA Protects A431 Cells from EGF-induced Apoptosis—Because RelA reversed the effect of IκB-α it became relevant to know whether, on its own, it can protect the parental A431 cells from EGF-induced apoptosis. To this end, we transfected the A431 cells stably with *relA* and confirmed the higher expression of RelA in A431-RelA cells (clone 1 and clone 2) compared with A431 and A431-Neo cells by Western blotting (Fig. 6A). When A431-Neo and A431-RelA cells (clone 1) were compared for their relative viability in the presence of varying concentrations of EGF by the MTT assay, A431-RelA cells were notably more resistant to EGF (Fig. 6B) and these results were also confirmed using clone 2 (data not shown)
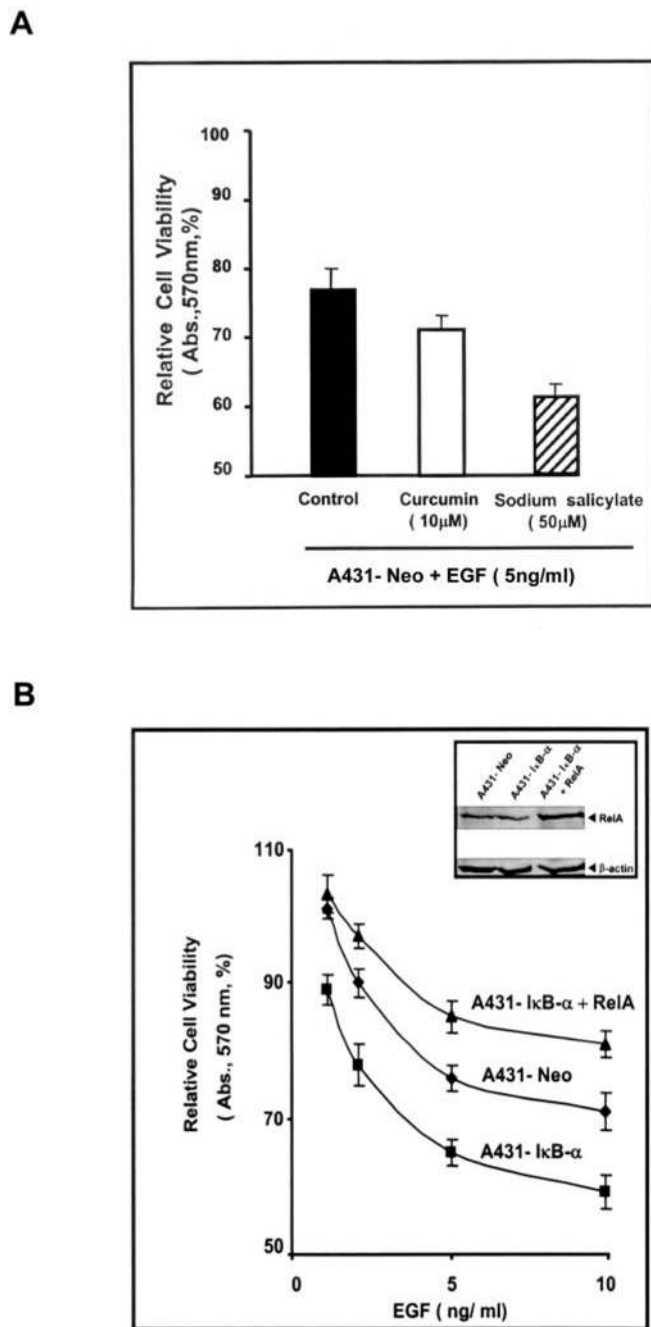
A

B

FIG. 5. **Influence of chemical inhibitors of NF-κB and *relA* transfection on EGF-mediated growth inhibition of A431-Neo and A431-IκB-α cells, respectively.** *A*, A431-Neo cells (5×10^3/well) were seeded into 96-well plates and pretreated with 10 μM curcumin, 50 μM sodium salicylate, or medium alone for 2 h. The cells were then treated with or without 5 ng/ml EGF for 24 h and the MTT assay was done as described earlier. Triplicate samples were used and the *error bars* indicate the standard deviations and the results were confirmed in another independent experiment. *B*, A431-IκB-α cells were transiently transfected with *relA* as described under Fig. 1*E*. A431-Neo and A431-IκB-α and the transfected cells (A431-IκB-α+RelA) were seeded into 96-well plates (5×10^3/well) and treated with the indicated concentrations of EGF for 24 h and the MTT assay was done as described earlier. Triplicate samples were used and the *error bars* indicate the standard deviations. The results were confirmed in another independent experiment. The *inset* shows Western blots of RelA and β-actin control in A431-Neo, A431-IκBα, and the transfected cells (*A431-IκB-α+RelA*).

but further experiments were done using clone 1 of A431-RelA cells. Similarly EGF-mediated inhibition of thymidine incorporation was relatively more in A431-Neo than A431-RelA cells confirming the protective role of RelA against
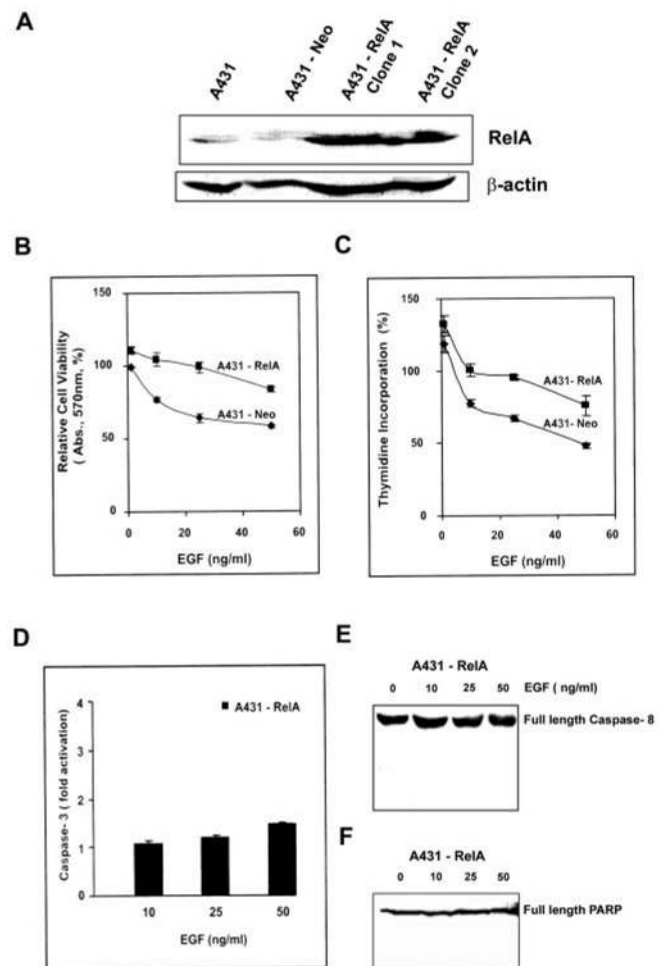

FIG. 6. **Western blotting for RelA and EGF-mediated changes in growth, DNA synthesis, caspase activation, and PARP cleavage.** *A*, A431 cells were cotransfected with pcDNA3 vector and *relA* PMT2T construct using LipofectAMINE and the G418-resistant clones were selected as described under "Experimental Procedures." Cell lysates (60 μg of protein) from the vector-transfected A431-Neo cells and different clones of A431-RelA cells were subjected to SDS-PAGE and immunoblotted with RelA or β-actin (control) antibody by the alkaline phosphatase method as described under "Experimental Procedures." *B*, cells were treated with or without the indicated concentrations of EGF for 24 h and the MTT assay was done under the conditions described for Fig. 2. Triplicate samples were used and the *error bars* indicate the standard deviations. The results were confirmed in another independent experiment. *C*, cells were treated with or without the indicated concentrations of EGF for 24 h and thymidine incorporation assays were done under the conditions described for Fig. 2. Triplicate samples were used and the *error bars* indicate the standard deviations. The results were confirmed in another independent experiment. *D*, A431-RelA cells were treated with or without the indicated concentrations of EGF for 24 h and caspase 8 activity was determined as described for Fig. 4. *E*, cells were treated with or without the indicated concentrations of EGF for 24 h and caspase 3 activity was determined as described for Fig. 4. *F*, cells were treated with or without the indicated concentrations of EGF for 24 h and PARP cleavage was determined as described for Fig. 4.

EGF-mediated cell death (Fig. 6*C*). In addition, varying EGF concentrations even up to 50 ng/ml could not induce caspase 8 (Fig. 6*D*) or caspase 3 (Fig. 6*E*) activities or PARP cleavage (Fig. 6*F*) in A431-RelA cells.

EGF Up-regulates the Expression of c-IAP1 in A431-Neo Cells and Its Basal Expression Is Higher in A431-RelA Cells—As IAP is considered to be one of the survival proteins induced by NF-κB, it was of interest to study the effect of EGF and RelA on IAP expression. Expression of c-IAP1 was observed by Western blotting in A431-Neo cells stimulated with 10 ng/ml EGF for
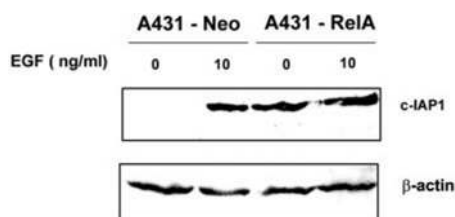
NF-κB Regulates EGF-induced Apoptosis

FIG. 7. **Effects of EGF on IAP expression in A431-Neo and A431-RelA cells.** Cells (1×10^6) were seeded in 35-mm Petri dishes and treated with or without EGF (10 ng/ml) for 24 h. To detect IAP expression, the cell lysates were resolved on 10% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, probed with c-IAP1 or β-actin (control) antibody, and detected by ECL as described under "Experimental Procedures." The results were confirmed in another independent experiment.

FIG. 8. **Effect of LY294002 on Akt phosphorylation and survival of A431-Neo and A431-RelA cells.** *A*, cells (1×10^6) grown in 35-mm Petri dishes were pretreated with LY294002 (20 μM) for 2 h and then treated with EGF (10 ng/ml) for 1 h and the cells that received no treatment served as control. Cell lysates were probed with phospho-Akt (Thr[308]) antibody (1:1000) by Western blotting by ECL as described above and the same blot was reprobed with an antibody (1:1000) to Akt. *B*, cells were pretreated with LY294002 (20 μM) for 2 h and then with EGF (10 ng/ml) and incubated for 24 h and the MTT assay was done as described under "Experimental Procedures." The above experiments were repeated with similar results.

24 h and A431-RelA cells had a higher basal level of c-IAP1 that was not further increased by EGF (Fig. 7). These results suggest that RelA up-regulates the expression of c-IAP1 and EGF up-regulates it presumably through the activation of NF-κB.

LY294002, an Inhibitor of PI-3K, Sensitizes A431-RelA Cells but Not A431-Neo Cells to EGF-induced Cytotoxicity—We also examined for the involvement of the PI-3K pathway known to activate Akt that enables survival of cells by the activation of NF-κB. In Western blot analysis using a phosphorylation-specific antibody to Akt, there was no detectable phosphorylation of Akt in A431-Neo cells in the presence or absence of EGF, whereas even the unstimulated A431-RelA cells exhibited phosphorylation of Akt that was further enhanced by EGF (Fig. 8*A*). An inhibitor of PI-3K, LY294002, inhibited EGF-induced phosphorylation of Akt in A431-RelA cells whereas the expression of total Akt protein remained unaffected by EGF treatment in A431-Neo and A431-RelA cells (Fig. 8*A*). EGF decreased the growth of A431-Neo cells whereas LY294002 alone did not affect their viability and addition of LY294002 together with EGF had no further effect on the growth of A431-Neo cells (Fig. 8*B*). In contrast, EGF did not inhibit the growth of A431-RelA cells but addition of LY294002 together with EGF decreased their viability (Fig. 8*B*). These results suggest that RelA could activate Akt and EGF could activate it further in A431-RelA cells and even though inhibition of PI-3K by LY294002 could not enhance EGF-induced cell death of A431-Neo cells, it did sensitize A431-RelA cells to EGF.

Taken together, the results of the present study suggest that EGF induces typical features of apoptosis in A431 cells such as the externalization of PS, DNA fragmentation, and activation of caspases (3 and 8 but not 9). These hallmarks of apoptosis induced by EGF were potentiated by NF-κB inhibition by IκB-α and RelA relieved the effects of IκB-α on EGF-mediated cytotoxicity. The chemical inhibitors of NF-κB also enhanced the growth inhibitory effects of EGF and these results together support an anti-apoptotic role for NF-κB. This is further supported by the protective role of RelA observed in A431-RelA cells against EGF-mediated apoptosis. A model for the regulation of EGF-induced apoptosis incorporating the known mechanisms and contributions from the present study is shown in Fig. 9.

DISCUSSION

Several studies have reported that high (nanomolar) concentrations of EGF caused growth inhibition and apoptosis of A431 cells while low (picomolar) concentrations of EGF promoted cell proliferation (7, 38, 40, 43, 44). Many hypotheses have been proposed to explain the mechanisms by which EGF inhibits cell proliferation, arrests cell cycle, and induces apoptosis. Increase in EGFR tyrosine kinase activity because of excessive ligand
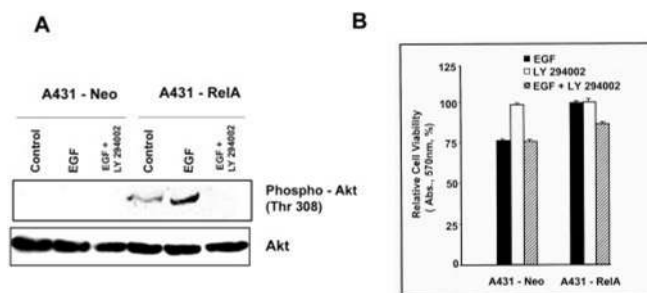
binding is said to result in growth inhibition and programmed cell death and the level of EGFR tyrosine kinase activity regulates the expression of p21 (17, 38). It has also been suggested that EGF arrests cell cycle progression through Stat1 activation and elevation of p21 that eventually inhibits cdk2 activity (43–45). However, Stat1 activation by EGF was not detected in a number of cancer cell lines with abnormal EGFR expression (18, 45). The dual effect of EGF on the proliferation of A431 cells is also attributed to differential activation of p42 MAP kinase at low and high concentrations of EGF (11, 46). Another hypothesis proposes that EGF-induced apoptosis is the result of a decline in cell adhesion (47), and detachment of cells because of anoikis is said to be responsible for the paradoxical anti-proliferative effects of EGF (16, 48). Our results do not support the view that EGF-induced apoptosis results from the loss of cell-substratum attachment and many other workers also have not observed EGF-induced anoikis of A431 or other EGFR-overexpressing cells (8, 11, 49, 50). The results of the present study suggest that EGF-induced apoptosis operates through the extrinsic pathway mediated through caspase 8 and not the mitochondrial pathway involving caspase 9. In contrast, EGF-induced apoptosis of MDA-MB-468 breast cancer cells that also overexpress EGFR has been reported to involve the mitochondrial pathway of caspase activation (16). Similar to our results with EGF, heregulin also enhanced the degradation of PARP but unlike EGF, heregulin activated caspase 9 without any significant activation of caspase 3 (51). Cripto-1 mediates the induction of caspase 3-like protease and down-regulates the expression of Bcl-XL (15). UCVA-1 cells, derived from human pancreas adenocarcinoma, have a high number of EGFRs but their growth is not inhibited by EGF, highlighting the complexity of the mechanisms involved in EGF-induced apoptosis (52). Thus, the regulation of EGF-induced apoptosis appears to be a complex process involving the interplay of several regulatory molecules.

In addition to the mechanisms by which the growth factors exhibit both stimulatory and inhibitory activity in a single cell, the final outcome is presumably influenced by a host of regulatory molecules other than the growth factors and their receptors (53, 54). It is thus clearly important to recognize that a potent mitogen like EGF also sends out apoptotic signals and identify conditions in which these signals are regulated. In the present study, we have hypothesized such a condition and demonstrated for the first time that NF-κB inhibition makes A431 cells more susceptible to EGF-induced apoptosis whereas RelA protects them against it. EGF stimulation in A431 cells
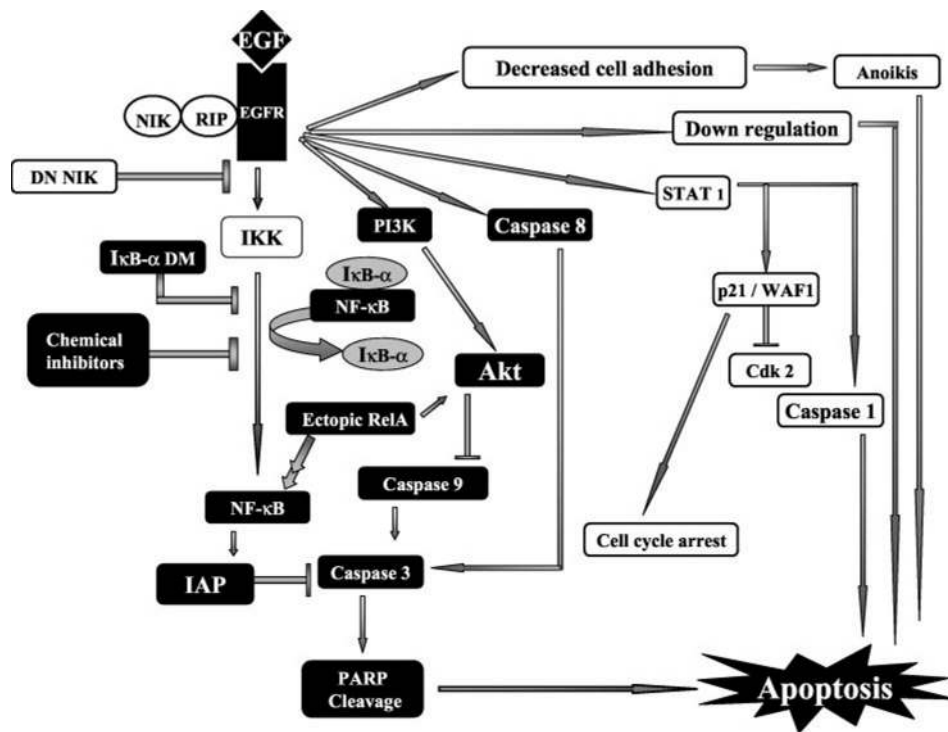
FIG. 9. **Proposed model for the regulatory steps involved in EGF-induced apoptosis.** EGF-induced activation of its receptor is said to decrease cell adhesion leading to anoikis and apoptosis. Down-regulation of EGFR is also postulated to induce apoptosis, and activation of Stat-1 has been reported to induce p21/WAF-1 or caspase 1 that may result in cell cycle arrest or apoptosis, respectively. In addition to these mechanisms that induce apoptosis, anti-apoptotic regulation also exists that includes activation of Akt or NF-κB. EGF-induced activation of EGFR is known to recruit NIK and RIP to activate IKK resulting in the activation of NF-κB that blocks apoptosis through IAPs. Ectopic expression of RelA activates Akt and also induces c-IAP1 and blocks apoptosis while the use of chemical inhibitors or IκB-α DM accelerates EGF-induced apoptosis.

enhances the degradation of IκB-α, but not IκB-β and proteasome inhibitors such as ALLN or MG132, block EGF-mediated NF-κB activation, indicating that EGF-induced NF-κB activation requires proteasome-dependent IκB degradation (25). Similar to the findings of the present study, EGF-induced DNA binding complex of NF-κB in A431 was found to be composed of p50/RelA heterodimers, but not c-Rel (25). In agreement with our results, a recent report has shown that both the protein kinase C inhibitor Go6976 (also inhibits NF-κB) and expression of dominant-negative NF-κB inhibitor kinase mutants caused apoptosis of EGF-stimulated mammary tumor cells whereas EGF alone did not induce apoptosis in these cells (55). In contrast to our results, it has been reported that infection of adenoviral vectors expressing IκB reversed the EGF-induced cell growth inhibition of A431 cells (56). Use of high concentrations of EGF (100 ng/ml), transient expression of IκB, and drawing conclusions only based upon cell counting (proportion of live and dead cells not shown) may account for this discrepancy. Furthermore, caspase activities or other apoptotic parameters were not measured in that study. Consistent with our results, blocking Ras (known to activate NF-κB) with dominant negative Ras mutants in EGFR overexpressing cells also potentiated EGF-induced apoptosis (8). EGF did not affect the levels of several proteins that regulate apoptotic pathways, including Bcl-2, Bcl-XL, Bax, and p53 in human ovarian cancer cells (57). Many workers have shown that Akt, a serine/threonine protein kinase and a downstream target of PI-3K, suppresses apoptosis by activating NF-κB (58–60) and accordingly blocking the EGF-induced phosphorylation of Akt sensitizes the otherwise resistant A431-RelA cells to EGF-mediated growth inhibition. However, the spontaneous phosphorylation of Akt in A431-RelA cells places Akt as a downstream target of NF-κB and it is interesting to note that blocking this basal phosphorylation with LY294002 does not affect their viability.

Consistent with our results, overexpression of RelA stimulated the phosphorylation of Akt in NIH 3T3 cells and in addition increased the expression of Akt mRNA and protein (61). Akt does not seem to be involved in EGF-induced NF-κB activation of MDA-MB-468 cells that also overexpress EGFR (62). The mechanism by which ectopic expression of RelA results in the phosphorylation of Akt is not known and further work is in progress to understand the contribution of Akt in EGF-induced apoptosis. Similar to the EGF-induced expression of c-IAP1 in the present study, TNF also stimulated its expression (63). Cbl-b inhibits EGF-induced apoptosis by enhancing ubiquitination and degradation of activated EGFR (64).

Our results support an anti-apoptotic role for NF-κB and suggest the possibility that EGF-induced apoptosis *in vivo* may be regulated by NF-κB. However, whether EGF-induced apoptosis occurs *in vivo* in tumor cells is not known. If EGF-induced apoptosis does not occur *in vivo*, it is still logical to think that anti-apoptotic factors such as NF-κB guard the tumor cells against responding to such apoptotic stimuli. Consistent with this speculation, NF-κB is constitutively activated in a number of tumors (22, 26). In addition, tumor cells are also known to express many anti-apoptotic proteins such as Bcl-2 and their role in EGF-induced apoptosis remains to be determined. Our identification of a role for NF-κB in EGF-induced apoptosis opens up new lines of investigation pertaining to EGF receptor-mediated signaling. Further studies on the role of NF-κB and its regulators in EGF-induced apoptosis are likely to be relevant for a better understanding of the biology of human tumors as well as EGF-induced normal growth.

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