The Journal of Biological Chemistry Vol. 279, No. 52, Issue of December 24, pp. 54610–54619, 2004
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Oncogenic and Anti-apoptotic Activity of NF-κB in Human Thyroid Carcinomas*

Received for publication, March 30, 2004, and in revised form, September 23, 2004
Published, JBC Papers in Press, October 8, 2004, DOI 10.1074/jbc.M403492200

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Thyroid cancer includes three types of carcinomas classified as differentiated thyroid carcinomas (DTC), medullary thyroid carcinomas, and undifferentiated carcinomas (UTC). DTC and medullary thyroid carcinomas generally have a good prognosis, but UTC are usually fatal. Consequently, there is a need for new effective therapeutic modalities to improve the survival of UTC patients. Here we show that NF-κB is activated in human thyroid neoplasms, particularly in undifferentiated carcinomas. Thyroid cell lines, reproducing *in vitro* the different thyroid neoplasias, also show basal NF-κB activity and resistance to drug-induced apoptosis, which correlates with the level of NF-κB activation. Activation of NF-κB in the DTC cell line NPA renders these cells resistant to drug-induced apoptosis. Stable expression of a super-repressor form of IκBα (IκBαM) in the UTC cell line FRO results in enhanced sensitivity to drug-induced apoptosis, to the loss of the ability of these cells to form colonies in soft agar, and to induce tumor growth in nude mice. In addition, we show that FRO cells display a very low JNK activity that is restored in FRO-IκBαM clones. Moreover, inhibition of JNK activity renders FRO-IκBαM clones resistant to apoptosis induced by chemotherapeutic agents. Our results indicate that NF-κB plays a pivotal role in thyroid carcinogenesis, being required for tumor growth and for resistance to drug-induced apoptosis, the latter function very likely through the inhibition of JNK activity. Furthermore, the strong constitutive NF-κB activity in human anaplastic thyroid carcinomas, besides representing a novel diagnostic tool, makes NF-κB a target for the development of novel therapeutic strategies.

Thyroid carcinomas are one of the most common neoplasias of the endocrine system (1). Four types of thyroid cancer comprise more than 98% of all thyroid tumors: papillary thyroid carcinoma, follicular thyroid carcinoma, both of which may be summarized as differentiated thyroid carcinoma, medullary thyroid carcinoma (MTC),[1] and undifferentiated anaplastic thyroid carcinoma (ATC) (2). Papillary thyroid carcinoma, follicular thyroid carcinoma, and ATC are derived from the thyroid follicular epithelial cells, whereas MTC is derived from the parafollicular C cells (3). Papillary thyroid carcinoma is the most common malignant thyroid neoplasm in countries with sufficient iodine diets and comprises up to 80% of all thyroid malignancies. Follicular thyroid carcinoma is more common in regions with insufficient iodine diets and represents ~10–20% of all thyroid malignancies. The overall 5–10 year survival rate of patients with papillary thyroid carcinoma is about 80–95%, whereas that of patients with follicular thyroid carcinoma is about 70–95% (3). The incidence of MTC is not well known because epidemiologic studies are rare. Generally, it is believed that MTC comprises about 5–10% of all thyroid malignancies (2–4). ATC is one of the most aggressive human malignancies, with a very poor prognosis. Although rare, accounting for up to 10% of clinically recognized thyroid cancers, the overall median survival is limited to months (2, 3, 5). Surgery, radiotherapy, and chemotherapy, based primarily on doxorubicin and cisplatin treatment, do not meaningfully improve survival of ATC patients (5, 6). Consequently, there is a need for new therapeutic tools for the treatment of these tumors.

The NF-κB family of transcription factors regulates the expression of a wide spectrum of genes involved in inflammation, immune response, cellular stress, cancer, and apoptosis (7–9). In most cell types, NF-κB is present in a latent form in the cytoplasm and bound to NF-κB inhibitory proteins collectively termed IκBs (10). A wide variety of extracellular signals, such as pro-inflammatory cytokines, bacterial and viral infections, oxidative stress, etc., initiate signaling cascade that culminates in the phosphorylation and subsequent degradation of IκBs, through a proteasome-dependent pathway (11, 12). Degradation of IκB frees NF-κB to enter the nucleus and to activate transcription of target genes (11). IκB phosphorylation is mediated by a large complex referred to as the IκB kinase complex (IKK complex), composed of two catalytic subunits, IKKα and IKKβ (13) as well as a regulatory protein, named NEMO/IKKγ (14, 15).

* This work was supported by grants from Associazione Italiana Ricerca sul Cancro, MIUR-FIRB RBNE0155LB, and Centro di Competenza GEAR. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MTC, medullary thyroid carcinoma; NF-κB, nuclear factor-κB; IKK, IκB kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; DTC, differentiated thyroid carcinomas; UTC, undifferentiated carcinomas; ATC, anaplastic thyroid carcinoma; EMSA, electromobility shift assay; Ab, antibody; PBS, phosphate-buffered saline; XIAP, X-linked inhibitor of apoptosis; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; GADD, growth arrest and DNA damage; TNF, tumor necrosis factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt.

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Numerous studies have indicated that NF-κB activation can block cell-death pathways (16). NF-κB-inducible anti-apoptotic factors include those that inhibit caspase function, such as cellular inhibitor of apoptosis (17), X-linked inhibitor of apoptosis (XIAP) (18), and Fas-associated death domain-like interleukin 1β-converting enzyme (19), those that inhibit NF-κB signaling after TNF receptor stimulation, such as TNF receptor-associated factors (20), those that preserve function of mitochondria, such as Bcl-XL (21), and those that block JNK function, such as XIAP and growth arrest and DNA damage (GADD)45β (22). Because it has been proposed that suppression of apoptosis is associated with oncogenic potential, and given the ability of NF-κB inhibitors to increase susceptibility to apoptosis in transformed cells (23), one role for NF-κB activation could be to suppress transformation-associated apoptosis. The NF-κB signal transduction pathway is misregulated in a variety of human cancers, especially ones of lymphoid origin, due either to genetic changes (such as chromosomal rearrangements, amplifications, and mutations) or to chronic activation of the pathway by epigenetic mechanisms (24). Basal activation of the NF-κB pathway can contribute to the oncogenic state in several ways: by driving proliferation (25), by enhancing cell survival (26), by promoting angiogenesis, or by metastasis (27). Because the development of a highly malignant tumor requires several changes in cellular metabolism, including ones affecting apoptosis, it is not surprising that the NF-κB signal transduction pathway, which influences various aspects of cellular physiology, has emerged as playing a major role in many human cancers.

c-Jun NH_2-terminal protein kinase (JNK), also known as stress-activated protein kinase (28), is a member of the mitogen-activated protein kinase superfamily (29), having three isoforms, JNK1, JNK2, and JNK3, with splicing variants (30).

Several lines of evidence suggest that JNK signaling is involved in stress-induced apoptosis via the mitochondrial pathway (30, 31), even though some studies have indicated that JNK may also contribute to cell survival (30). From different experimental evidence, it seems clear that the duration of JNK activation affects its role in apoptosis: *i.e.* prolonged but not transient JNK activation promotes TNF-α-induced apoptosis (32, 33). However, it has also been demonstrated that prolonged JNK activation alone may not be sufficient to induce apoptosis (33). Thus, the dual role of JNK in both apoptotic and antiapoptotic signaling pathways suggests that the function of JNK is complex and that the physiological response most likely reflects a balance between the ability of JNK to signal both apoptosis and cell survival. It has long been speculated that the role of the JNK pathway in apoptosis is affected by other signaling pathways. This has been recently demonstrated by two different groups (32, 33) in two independent ways. Both of them showed that NF-κB is able to inhibit TNF-α-induced apoptosis by negatively regulating activation of the JNK pathway. They also demonstrated that the negative regulation of JNK activity exerted by NF-κB is mediated by two genes, GADD45β (32) and XIAP (33), both of them under the transcriptional control of NF-κB.

Very little is known about the role that NF-κB plays in thyroid physiology. There is little evidence that NF-κB may be important in RET-mediated carcinogenesis and that may be involved in maintaining the transformed phenotype of thyroid cell lines (34, 35). However, the role that NF-κB plays "*in vivo*" in human thyroid neoplasia has not been fully investigated. Here we show that NF-κB is aberrantly expressed in primary human thyroid carcinomas, and we provide evidence that NF-κB is required for maintaining the transformed phenotype of UTC cells.

TABLE I

NF-κB nuclear localization in normal and pathological human thyroid tissues

The percentage of cells with nuclear staining for NF-κB was scored from 0 to 3: 0, no positive cells; 1+, <10% of positive cells; 2+, 11–50% of positive cells; 3+, 76–100% of positive cells.

Histological type of thyroid samples	No. positive cases/no. total cases analyzed by immunohistochemistry	NF-κB staining score
Normal thyroid	0/4	0
Papillary carcinoma	6/6	1+
Follicular carcinoma	5/5	2+
Anaplastic carcinoma	4/4	3+

EXPERIMENTAL PROCEDURES

Cell Lines—NPA is a cell line derived from human papillary thyroid carcinoma (36); FRO is a cell line derived from human anaplastic thyroid carcinoma (37); and WRO is a cell line derived from human follicular thyroid carcinoma (38). They were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma). FRO cells were transfected with the empty expression vector pcDNA3.1-FLAG (Invitrogen) (FRO Neo cells) or with the expression vector pcDNA3.1-FLAG vector encoding a mutant form (S32A/S36A) of IκBα (FRO IκBαM cells) (a kindly gift of Dr. G. Franzoso) (32). NPA cells were transfected with the empty expression vector pcDNA3.1-FLAG (Invitrogen) (NPA Neo cells) or with the expression vector pcDNA3.1-FLAG vector encoding human IKKβ (39) (NPA IKKβ cells). The stable transfected clones were selected and maintained

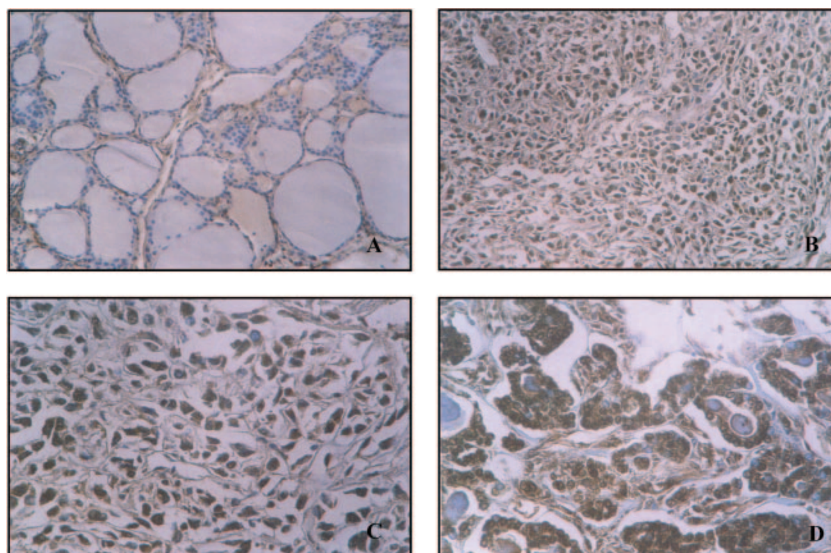


FIG. 1. **Immunohistochemical analysis of NF-κB activity in normal human thyroid and anaplastic human thyroid carcinomas.** Localization of RelA (p65) *in situ* was determined by immunohistochemistry in sections from normal thyroid tissue (*A*) and three different anaplastic thyroid carcinomas (*B–D*).

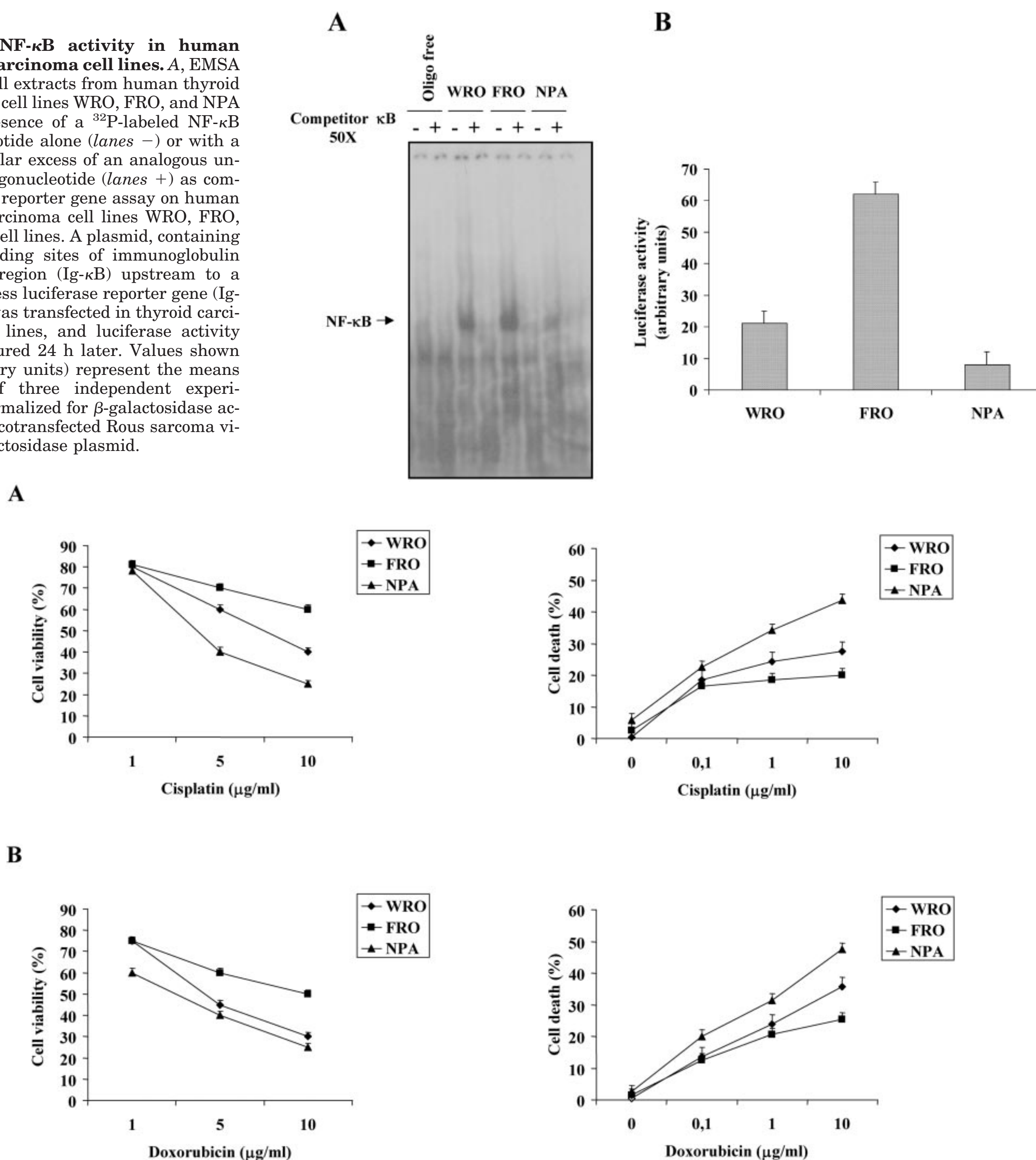


FIG. 2. **NF-κB activity in human thyroid carcinoma cell lines.** *A*, EMSA on total cell extracts from human thyroid carcinoma cell lines WRO, FRO, and NPA in the presence of a ^{32}P-labeled NF-κB oligonucleotide alone (*lanes* −) or with a 50-fold molar excess of an analogous unlabeled oligonucleotide (*lanes* +) as competitor. *B*, reporter gene assay on human thyroid carcinoma cell lines WRO, FRO, and NPA cell lines. A plasmid, containing NF-κB-binding sites of immunoglobulin promoter region (Ig-κB) upstream to a promoterless luciferase reporter gene (Ig-κB-Luc), was transfected in thyroid carcinoma cell lines, and luciferase activity was measured 24 h later. Values shown (in arbitrary units) represent the means (±S.D.) of three independent experiments, normalized for β-galactosidase activity of a cotransfected Rous sarcoma virus-β-galactosidase plasmid.

FIG. 3. **Cytotoxic effects of chemotherapeutic drugs on human thyroid carcinoma cell lines.** 1×10^3 cells/well were seeded in 96-well culture plates and incubated for 48 h at 37 °C with different concentrations of cisplatin (*A*) or doxorubicin (*B*). Cell survival was examined by using MTS and an electron coupling reagent (phenazine methosulfate), according to the manufacturer's instructions (Promega, Madison, WI). Cell death was assessed by staining of exposed phosphatidylserine on cell membranes with fluorescein isothiocyanate-conjugated annexin V (Pharmingen). Samples were analyzed by flow cytometry using a FACSCalibur (Beckman Instruments, Fullerton, CA), equipped with ModFit Software. Results were mean ± S.D. of at least three separate experiments.

in the presence of geneticin (200 μg/ml).

Immunohistochemical Analysis—Specimens from normal and pathological human thyroid were isolated, rinsed with PBS, fixed in 4% buffered neutral formalin, and embedded in paraffin. 5–6-μm-thick paraffin sections were then deparaffinized and placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining. Slides were then incubated overnight at 4 °C in a humidified chamber with antibody anti-p65 diluted 1:100 in PBS and subsequently incubated, first with biotinylated goat anti-rabbit IgG for 20 min (Vectostain ABC kits, Vector Laboratories) and then with pre-mixed reagent ABC (Vector Laboratories) for 20 min. The antibody anti-p65 used was a rabbit polyclonal antibody from Santa Cruz Biotechnology (SC 7151). The same results were obtained by using a mouse monoclonal anti-nuclear localization signal from Roche Applied Science. The immunostaining was performed by incubating slides in diaminobenzidine (Dako) solution containing 0.06 mM diaminobenzidine and 2 mM hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 min, and after chromogen development, slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent mounting medium (Permount).

Electromobility Shift Assays—To analyze NF-κB DNA binding activity, total cell extracts were prepared using a detergent lysis buffer (50 mM Tris (pH 7.4), 250 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, and Complete protease inhibitor mixture (Roche Applied Science)). Cells were harvested by centrifugation, washed once in cold PBS, and resuspended in detergent lysis

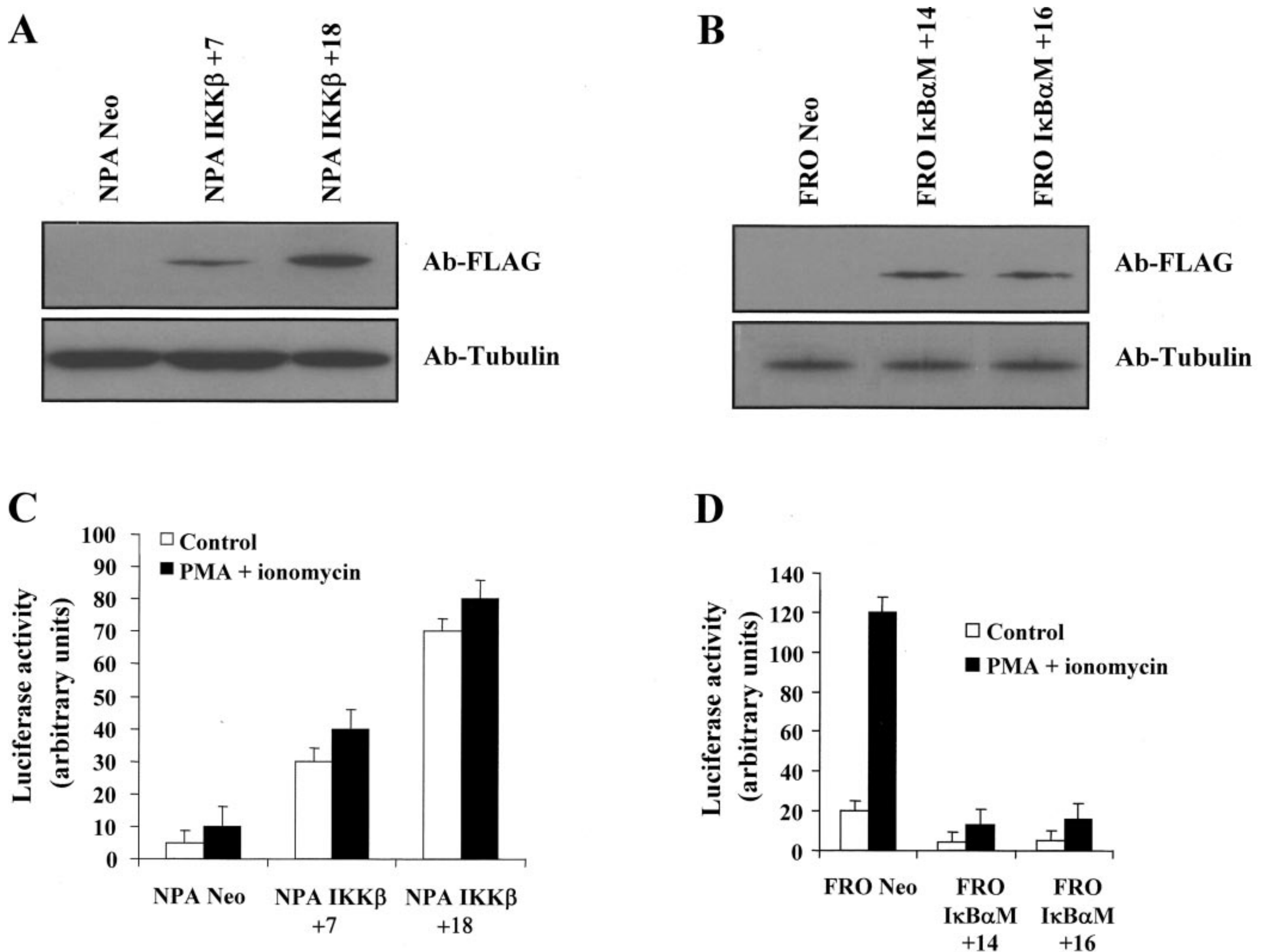


FIG. 4. **Characterization of NPA IKKβ and FRO IκBαM clones.** NPA cells were transfected with the empty vector pcDNA3.1-FLAG (Invitrogen) (NPA Neo cells) or with the pcDNA3.1-FLAG vector containing the full-length cDNA encoding human IKKβ (NPA IKKβ cells). FRO cells were transfected with the empty vector pcDNA3.1FLAG (Invitrogen) (FRO Neo cells) or with the pcDNA3.1FLAG vector containing the mutant form (S32A/S36A) of IκBα super-repressor (FRO IκBαM cells). The stable transfectants, selected and maintained in the presence of geneticin (200 μg/ml), were characterized for their ability to express FLAG-tagged IKKβ (*A*) or IκBαM (*B*) proteins by Western blot analysis with the Ab-FLAG (F 7425) polyclonal antibody (Sigma), and to respond to phorbol 12-myristate-13-acetate (*PMA*) plus ionomycin treatment (*C* and *D*) by luciferase assay.

buffer (30 μl/5 × 10^6 cells). The cell lysate was incubated on ice for 30 min and then centrifuged for 5 min at 10,000 × *g* at 4 °C. The protein content of the supernatant was determined, and equal amounts of protein (10 μg) were added to a reaction mixture containing 20 μg of bovine serum albumin, 2 μg of poly(dI-dC), 10 μl of binding buffer (20 mM HEPES (pH 7.9), 10 mM $MgCl_2$, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride), and 100,000 cpm of a ^{32}P-labeled oligonucleotide, containing specific binding sites for NF-κB, in a final volume of 20 μl. Samples were incubated at room temperature for 30 min and run on a 4% acrylamide gel.

To analyze AP-1 DNA binding activity, nuclear extracts were prepared as follows: cells were harvested by centrifugation, washed once in cold PBS, and resuspended in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM EGTA). The cells were then centrifuged for 5 min at 1,000 × *g* and resuspended in cold buffer C (20 mM HEPES (pH 7.9), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, and 25% glycerol). The cell resuspension was subjected to strong shaking for 30 min at 4 °C and then centrifuged for 15 min at 1,000 × *g*. The protein content of the supernatant was determined, and equal amounts of protein (10 μg) were added to a reaction mixture containing 20 μg of bovine serum albumin, 2 μg of poly(dI-dC), 10 μl of binding buffer (20 mM HEPES (pH 7.9), 10 mM $MgCl_2$, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride), and 100,000 cpm of wild-type or mutant ^{32}P-labeled AP-1 oligonucleotides, in a final volume of 20 μl. Samples were incubated at room temperature for 30 min and run on a 4% acrylamide gel.

Luciferase Assays—4 × 10^5 cells/well were seeded in a 6-well plate. After 12 h, cells were transfected with 0.5 μg of the Ig-κB-luciferase reporter gene plasmid. Cell extracts were prepared 24 h after transfection, and reporter gene activity was determined by the luciferase system (Promega). A pRSV-β-galactosidase vector (0.2 μg) was used to normalize for transfection efficiencies. Cells were stimulated with phorbol 12-myristate-13-acetate (400 ng/ml) plus ionomycin (2 μM) for 3 h before lysis.

In Vitro and in Vivo Tumorigenicity Assays—To analyze the ability of the various FRO clones to form colonies in soft agar, 1 × 10^4 cells were seeded in 60-mm dishes onto 0.3% Noble Agar (Difco) on top of a 0.6% bottom layer. Colonies larger than 50 cells were scored after 2 weeks incubation at 37 °C (40).

To analyze the ability of the various FRO clones to induce tumor growth in nude mice, 2 × 10^7 cells were injected subcutaneously on a flank of each 6-week-old nude mouse (Charles River Breeding Laboratories, Lecco, Italy). Thirty days later, mice were killed, and tumors were excised. Tumors weight was determined, and their diameters were measured with calipers. Tumors volumes were calculated by the formula: $a^2 \times b \times 0.4$, where a is the smallest diameter, and b is the diameter perpendicular to a. No mouse showed signs of wasting or other visible indications of toxicity. The mice were maintained at the Dipartimento di Biologia e Patologia Cellulare e Molecolare animal facility and housed in barrier facilities on a 12-h light-dark cycle with food and water available *ad libitum*. The animal experiments described here were conducted in accordance with accepted standards of animal care and in accordance with the Italian regulations for the welfare of ani-

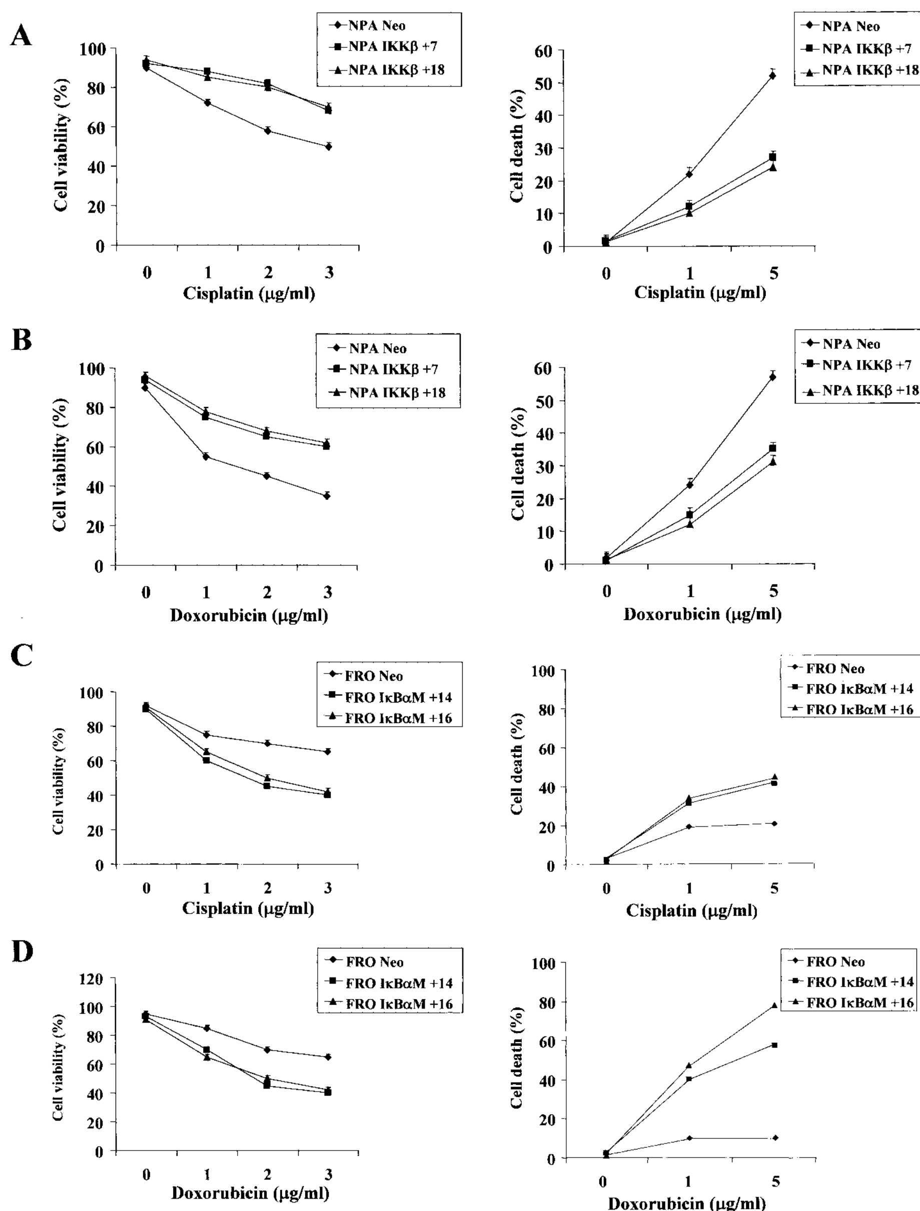


FIG. 5. **Cytotoxic effects of chemotherapeutic drugs on parental and transfected thyroid carcinoma cells.** 1×10^3 cells/well were seeded in 96-well culture plates and incubated for 48 h at 37 °C with different concentrations of cisplatin or doxorubicin. Cell survival was examined by using MTS and an electron coupling reagent (phenazine methosulfate), according to manufacturer's instructions (Promega, Madison, WI). Cell death was assessed by staining of exposed phosphatidylserine on cell membranes with fluorescein isothiocyanate-conjugated annexin V (Pharmingen). Samples were analyzed by flow cytometry using a FACSCalibur (Beckman Instruments, Fullerton, CA), equipped with ModFit Software. A representative experiment out of three is shown. *A* and *B*, NPA Neo cells and NPA IKKβ clones; *C* and *D*, FRO Neo cells and FRO IκBαM clones.

mals used in studies of experimental neoplasia, and the study was approved by our institutional committee on animal care.

Cytotoxic Treatments and Measurements of Apoptosis—1×10^3 cells/well were seeded in 96-well culture plates and incubated for 48 h at 37 °C with different concentrations of cisplatin or doxorubicin and, where indicated, with/without 10 μM JNK inhibitor SP600125 (BioMol Research Laboratories Inc., Plymouth Meeting, PA). Cell survival was examined by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), and an electron coupling reagent (phenazine methosulfate), according to the manufacturer's instructions (Promega, Madison, WI). Cell death was assessed by staining of exposed phosphatidylserine on cell membranes with fluorescein isothiocyanate-conjugated annexin V (Pharmingen) or by propidium iodide staining according to Nicoletti *et al.* (41). Samples were analyzed by flow cytometry using a FACSCalibur (Beckman Instruments, Fullerton, CA), equipped with ModFit Software. Results were mean ± S.D. of at least three separate experiments.

Western Blot Analysis—Subconfluent monolayers of parental and transfected FRO cells were washed three times with PBS and then lysed in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, and a mixture of protease inhibitors (Sigma). An aliquot of the cell lysates was used to evaluate the protein content by colorimetric assay (Bio-Rad). Total proteins from cell lysates (50 μg) were analyzed by 10% SDS-PAGE. After electroblotting of gels onto polyvinylidene difluoride sheets (Millipore, Bedford, MA), filters were blocked for 1 h at room temperature with 10% non-fat dry milk in TBST buffer (10 mM Tris-HCl (pH 8.0), 0.1% Tween 20, 150 mM NaCl). The filters were then incubated in the same buffer for 14–16 h at 4 °C with 1:2000 dilution of the Ab-FLAG (F 7425) polyclonal antibody (Sigma). After TBST washing, blots were incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase-conjugated antibodies (Amersham Biosciences) diluted 1:5000 in TBST buffer and then revealed by ECL (Amersham Biosciences).

A similar procedure was achieved to analyze phospho-JNK and total JNK levels on lysates from parental and transfected FRO cells after treatment with 10 μg/ml anisomycin (Sigma) at different time intervals. In this case, the monoclonal Ab-pJNK1/2 (9255) (Cell Signaling Technology Inc., Beverly, MA) recognizing phosphorylated JNK or the polyclonal Ab-JNK1/2 (9252) (Cell Signaling Technology Inc.) recognizing total JNK were used at 1:1000 dilution. After TBST washing, blots were incubated for 1 h at room temperature with secondary antibodies horseradish peroxidase-conjugated (Amersham Biosciences), diluted 1:5000 in TBST buffer, and then revealed by ECL (Amersham Biosciences).

[^{3}H]Thymidine DNA Incorporation—5×10^4 cells/well were seeded in 12-well culture plates and incubated for 4 h at 37 °C with 0.5 μCi/well of [^{3}H]thymidine (Amersham Biosciences). After three washings with cold PBS, cells were incubated for 10 min at 4 °C with 0.5 ml of 20% trichloroacetic acid. The trichloroacetic acid was removed, and cells were lysed with gentle shaking for 30 min at 37 °C in the presence of 1 N NaOH (0.5 ml/well). An aliquot of lysates (0.1 ml) was used to

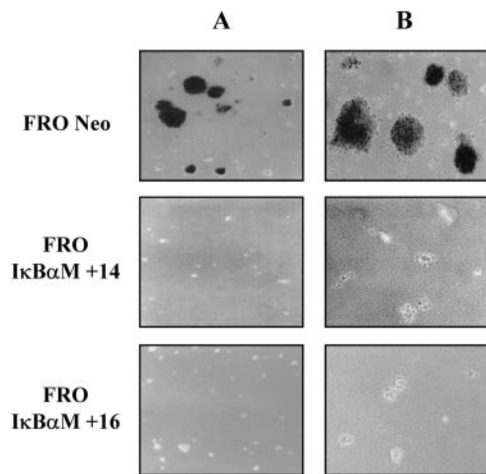


FIG. 6. ***In vitro* oncogenic potential of FRO Neo cells and FRO IκBαM clones.** 1×10^4 cells were seeded in 60-mm dishes onto 0.3% Noble Agar (Difco) on top of a 0.6% bottom layer. Colonies larger than 50 cells were scored after 2 weeks of incubation at 37 °C. FRO Neo cells = >50 colonies/plate; FRO IκBαM +14 cells = 0 colonies/plate; FRO IκBαM +16 = ≤5 colonies/plate. *A,* ×100 magnification. *B,* ×200 magnification.

TABLE II
In vivo tumor growth induced by FRO Neo cells and FRO IkBaM clones

2×10^7 cells were injected subcutaneously on a flank of each 6-week-old nude mouse (Charles River Breeding Laboratories, Lecco, Italy). Thirty days later, mice were killed, and tumors were excised. Tumor weight was determined, and their diameters were measured with calipers. Tumor volumes were calculated by the formula: $a2 \times b \times 0.4$, where a is the smallest diameter, and b is the diameter perpendicular to a.

Cell type	Tumor incidence	Tumor volume average	Tumor weight average
		cm³	*g*
Parental cells			
FRO Neo	6/6	1.48 ± 0.4	0.146 ± 0.04
Transfected cells			
FRO IκBαM +14	0/6		
FRO IκBαM +16	2/6	0.27 ± 0.06	0.029 ± 0.002

evaluate the protein content by colorimetric assay (Bio-Rad), whereas the remnant was analyzed at the β-counter (Beckman Instruments) after adding an equal volume (0.4 ml) of 1 N HCl to neutralize the samples. Results were the means ± S.D. of two separate experiments.

CFSE Cell Proliferation Assay—The analysis of cell proliferation was performed by labeling cells with CFSE (Molecular Probes, Eugene, OR) as originally described (42). Flow cytometry and data analysis were performed by using a two laser equipped FACSCalibur apparatus and the Cellquest analysis software (BD Biosciences), as described (43).

RESULTS

Basal NF-κB Activity in Human Thyroid Carcinomas—To determine the activation state of NF-κB in primary thyroid cancer tissues, human specimens from normal thyroid, papillary, follicular and anaplastic thyroid carcinomas were collected and stained with anti-p65 (RelA) antibodies. The results of these experiments are summarized in Table I. No nuclear staining for RelA was detected in normal thyroid follicular cells (Fig. 1*A*), whereas few nuclei from papillary carcinoma cells were positive for RelA. Follicular carcinoma cells showed ~50% of their nuclei stained for NF-κB, whereas anaplastic carcinoma cells showed almost 100% of their nuclei strongly positive for NF-κB (Fig. 1, *B–D,* and Table I). Most interestingly, the increased nuclear localization of NF-κB correlates with the increased malignant phenotype of thyroid carcinomas, suggesting that sustained activation of NF-κB confers an advantage for clonal selectivity. These results indicate that the nuclear localization of p65 is a characteristic of human anaplastic thyroid carcinomas and suggest a role of NF-κB in human thyroid carcinomas.

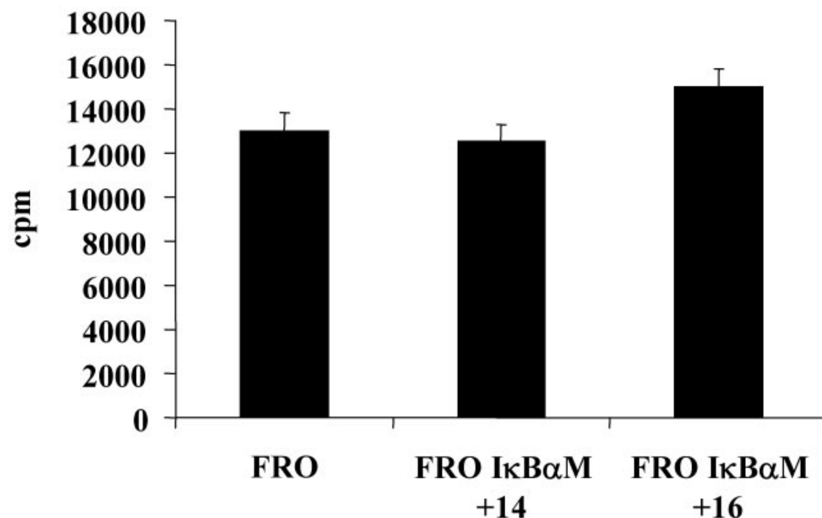
NF-κB Transcriptional Activity in Human Thyroid Transformed Cells—The high levels of basal NF-κB activity in human thyroid carcinomas prompted us to investigate the role of NF-κB in thyroid neoplastic transformation. To this aim, we used three different cell lines that resembled the features of thyroid tumors. These cell lines are as follows: WRO (38), derived from a human follicular thyroid carcinoma, FRO (37), derived from a human anaplastic thyroid carcinoma, and NPA (36), derived from a human papillary thyroid carcinoma.

In these cells we analyzed by EMSA the nuclear localization of NF-κB and, by reporter assay, its transcriptional activity. As shown in Fig. 2*A*, FRO cells showed the highest NF-κB DNA binding activity, whereas it was barely detectable in NPA cells (Fig. 2*A*). The specificity of the protein-DNA complex was confirmed by a competition assay with nonradioactive κB oligonucleotide. To demonstrate that the nuclear NF-κB was transcriptionally active, we performed reporter gene assays in all three cell lines. Consistent with EMSA experiments, FRO cells showed the highest NF-κB transcriptional activity, whereas NPA cells showed an almost undetectable activity (Fig. 2*B*). Taken together, these data indicate that NF-κB was transcriptionally activated in thyroid carcinoma cell lines, particularly in the human anaplastic thyroid cell line FRO.

NF-κB Activity Is Essential to Confer Resistance to Drug-induced Apoptosis in Thyroid Carcinoma Cell Lines—In order to determine a correlation between NF-κB activity and cell sensitivity to drug-induced apoptosis, we tested the ability of cisplatin and doxorubicin in promoting cell death in WRO, FRO, and NPA cells. As shown in Fig. 3, *A* and *B*, both drugs induced cell death in all three cell lines, at an extent that correlated with the levels of basal NF-κB activity. To test whether NF-κB activation protects neoplastic thyroid cells from apoptosis induced by chemotherapeutic drugs, we stably transfected NPA cells with an expression vector encoding IKKβ, to induce constitutive NF-κB activity, and FRO cells with a super-repressor form of IκBα (IκBαM), to suppress basal NF-κB activity. Two NPA clones, NPA IKKβ +7 and NPA IKKβ +18, as well as two FRO clones, FRO IκBαM +14 and FRO IκBαM +16, were used in our study. They expressed different levels of IKKβ (Fig. 4*A*) or IκBαM (Fig. 4*B*) and were differentially able to activate NF-κB (Fig. 4, *C* and *D*). In fact, although the constitutive IKKβ expression strongly increased basal NF-κB activity in NPA clones (Fig. 4*C*), the presence of IκBαM in FRO clones led to a decrease of both basal and phorbol 12-myristate-13-acetate/ionomycin-induced NF-κB activity (Fig. 4*D*).

Stably transfected and mock-transfected cells were then treated with increasing amounts of cisplatin (Fig. 5, *A* and *C*) or doxorubicin (Fig. 5, *B* and *D*) for 48 h, and the cell survival and cell death were measured. Although NPA Neo cells were sensitive to cell death induced by chemotherapeutic drugs, NPA IKKβ clones became resistant to apoptosis induced by either cisplatin (Fig. 5*A*) or doxorubicin (Fig. 5*B*). On the other hand, FRO Neo cells were still resistant to drug-induced cell death, whereas FRO IκBαM clones underwent apoptosis after treatment even at low dosage of either cisplatin (Fig. 5*C*) or doxorubicin (Fig. 5*D*). These results indicate that NF-κB activity is required and sufficient to confer resistance to drug-induced apoptosis of neoplastic thyroid cells.

Inhibition of FRO Cells Transforming Potential by NF-κB Inactivation—We next investigated if in addition to its role in protecting cells from apoptosis, NF-κB was also involved in other oncogenic properties of thyroid carcinoma cells. There-



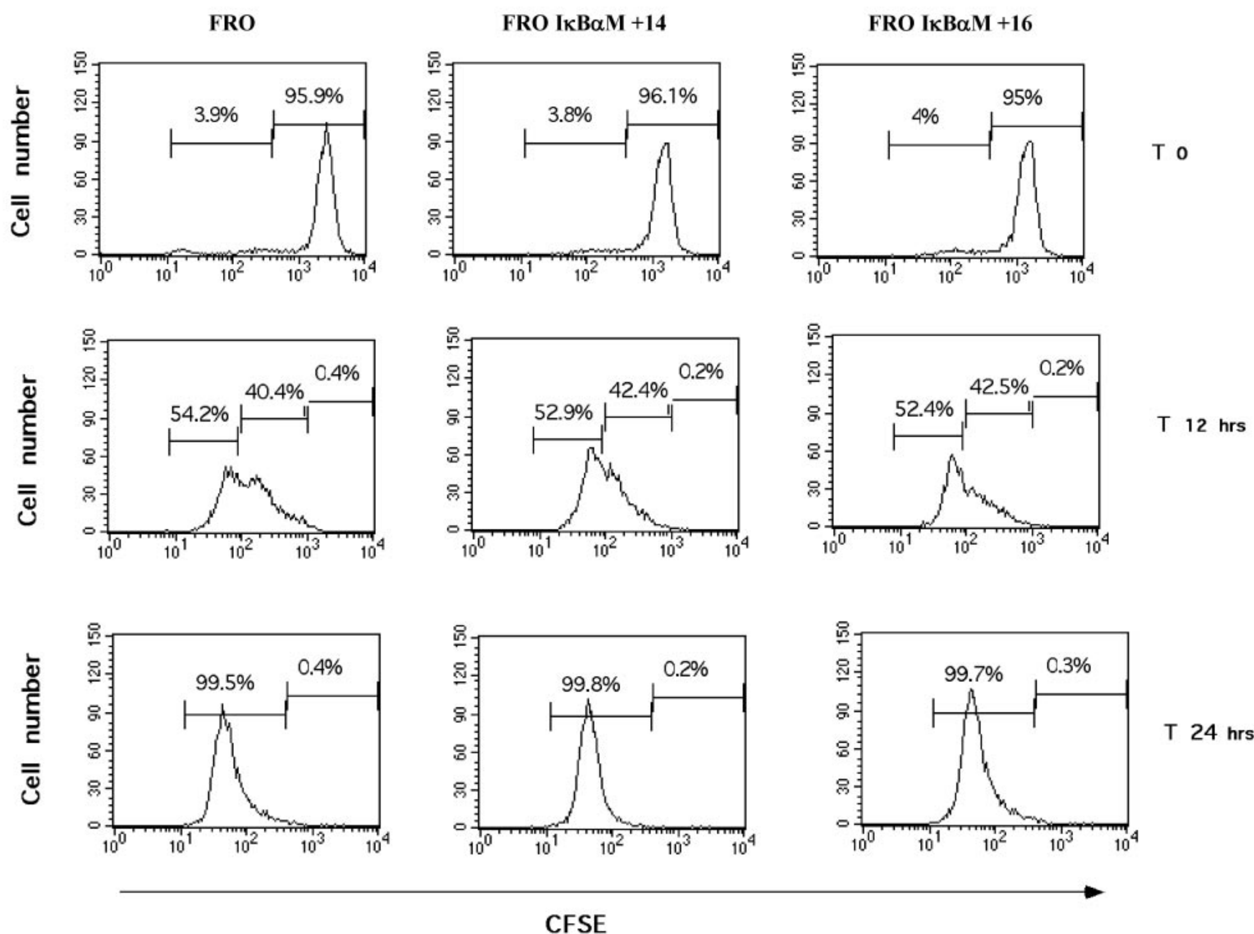


FIG. 7. **Analysis of cell proliferation rate in FRO Neo cells and FRO IκBαM clones.** *A*, 5×10^4 cells/well were seeded in 12-well culture plates and incubated for 4 h at 37 °C with 0.5 μCi/well of [^{3}H]thymidine (Amersham Biosciences). After three washings with cold PBS, cells were incubated for 10 min at 4 °C with 0.5 ml of 20% trichloroacetic acid. Trichloroacetic acid was then removed, and cells were lysed with gentle shaking for 30 min at 37 °C in the presence of 1 N NaOH (0.5 ml/well). An aliquot of lysates (0.1 ml) was used to evaluate the protein content by colorimetric assay (Bio-Rad), whereas the remnant was analyzed at the β-counter (Beckman Coulter) after adding an equal volume (0.4 ml) of 1 N HCl to neutralize the samples. Results were the mean ± S.D. of three separate experiments. *B*, FRO Neo cells and FRO IκBαM clones were collected and analyzed after 0, 12, and 24 h after CFSE labeling, as indicated. *x axes* indicate the CFSE fluorescence log intensity, whereas *y axes* refer to cell number count. Data are representative of one of two independent experiments.

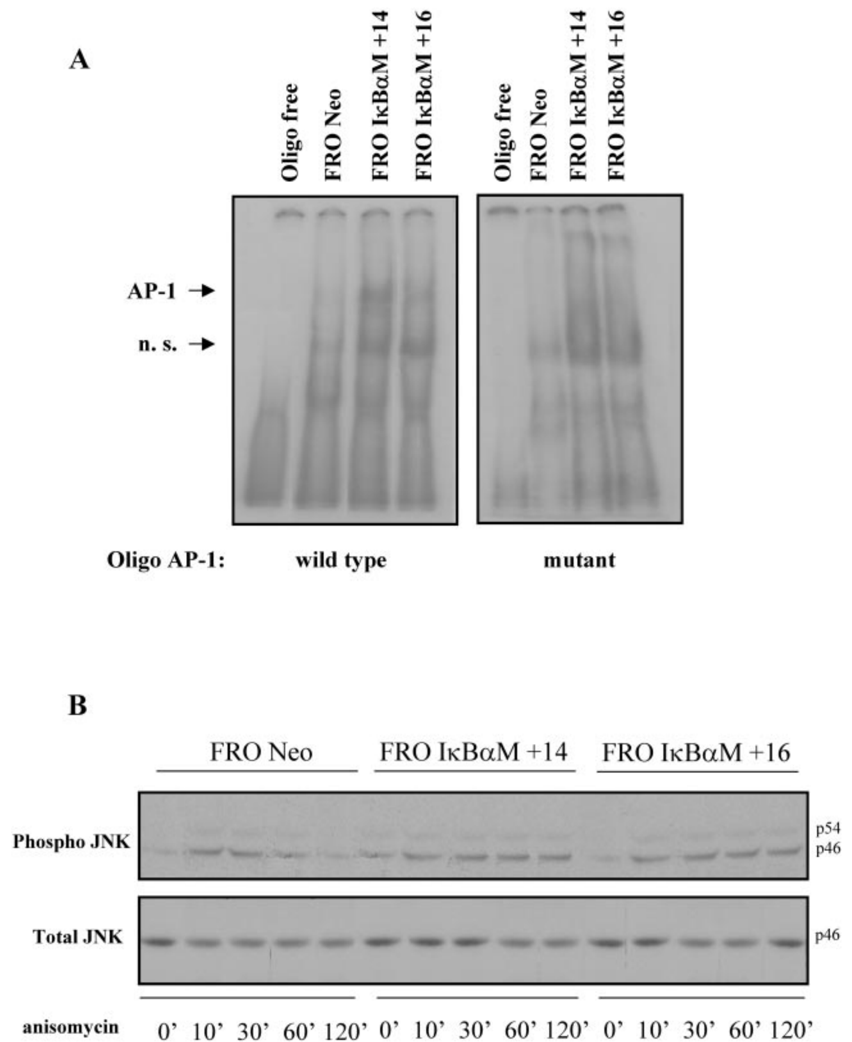


FIG. 8. **JNK activity in FRO Neo cells and FRO IκBαM clones.** *A*, EMSA on nuclear extracts from human thyroid carcinoma cell lines FRO Neo, FRO IκBαM +14, and +16 in the presence of a ^{32}P-labeled wild-type AP-1 oligonucleotide (*left panel*) or in the presence of a ^{32}P-labeled mutant AP-1 oligonucleotide (*right panel*). *n. s.* = nonspecific. *B*, 5×10^5 cells/well were seeded in 6-well culture plates and incubated for different time intervals at 37 °C with 10 μg/ml anisomycin (Sigma). Cell lysates were analyzed by Western blot assays with the monoclonal Ab-pJNK1/2 (9255) (Cell Signaling Technology Inc.) raised against phosphorylated JNK (*upper panel*) or with the polyclonal Ab-JNK1/2 (9252) (Cell Signaling Technology, Inc.) raised against total JNK (*lower panel*).

fore, we analyzed *in vitro* and *in vivo* the transforming potential of FRO Neo and FRO IκBαM cells. The *in vitro* assay was performed by analyzing the ability of transformed cells to form colonies on soft agar. As shown in Fig. 6, whereas parental FRO cells (FRO Neo) gave rise to numerous and large colonies in soft agar (*upper panels*), FRO IκBαM +14 and FRO IκBαM +16 clones lost this property (*middle* and *lower panels*). These results were also confirmed by *in vivo* assays. Table II shows that injection in nude mice of FRO Neo cells induced tumor formation in 6 out of 6 nude mice. Injection of FRO IκBαM +14 and FRO IκBαM +16 cells induced tumor formation in 0 out of 6 and 2 out of 6 mice, respectively. In addition, the two tumors developed from FRO IκBαM +16 cells were about 5-fold smaller than that formed after injection of parental cells (Table II). Thus, inhibition of NF-κB activity in FRO cells led to a strong decrease of their transforming potential, suggesting a role for this transcription factor in thyroid carcinogenesis.

The Inhibition of NF-κB Activity in FRO Cells Did Not Affect Their Proliferative State—The imbalance between proliferation and apoptosis is one of the critical cellular events that lead to oncogenesis. Because NF-κB controls transcription of genes involved in the regulation of apoptosis and cell proliferation, the inhibition of NF-κB activity in FRO cells could affect both proliferation and apoptosis. To test for this, we analyzed the proliferation rate of parental and transfected FRO cells by [^{3}H]thymidine DNA incorporation and by CFSE assay (Fig. 7). No significant differences in the rate of [^{3}H]thymidine DNA incorporation were appreciable between FRO Neo cells and FRO IκBαM clones (Fig. 7*A*), and a very similar doubling time was detected after 12 and 24 h of CFSE treatment (Fig. 7*B*), indicating that NF-κB activation is not required to control proliferation of FRO cells.

The Anti-apoptotic Activity of NF-κB Was Mediated by Down-regulation of JNK Activity—NF-κB is crucial to oncogenesis and to chemoresistance in cancer, controlling activation of pro-survival genes and down-regulation of pro-apoptotic genes (16). For example, it has been shown recently that NF-κB inhibits TNF-α-induced apoptosis by repressing the JNK pathway (32, 33). Therefore, we investigated if inhibition of NF-κB activity in FRO IκBαM clones affects JNK activation. To this aim, we first analyzed transcriptional activity of AP-1, a target of JNK activity, by EMSA on nuclear extracts from FRO Neo cells and FRO IκBαM clones (Fig. 8*A*). As shown in the Fig. 8, AP-1 DNA binding activity, almost undetectable in FRO Neo cells, was partially restored in FRO IκBαM clones (Fig. 8*A*, *left panel*). Next, we investigated JNK activity in FRO Neo cells and FRO IκBαM clones by analyzing its phosphorylation status following anisomycin stimulation (Fig. 8*B*, *upper panel*). As shown in Fig. 8, anisomycin induced JNK phosphorylation as early as 10 min of treatment in all cell lines, but, while the pJNK level in FRO Neo cells decreased with time, it remained sustained in FRO IκBαM clones. Anisomycin treatment had no effect on JNK expression, as assessed by Western blot analysis.

These data suggest that the basal NF-κB activation repressed JNK activity in FRO cells, and suggested that the

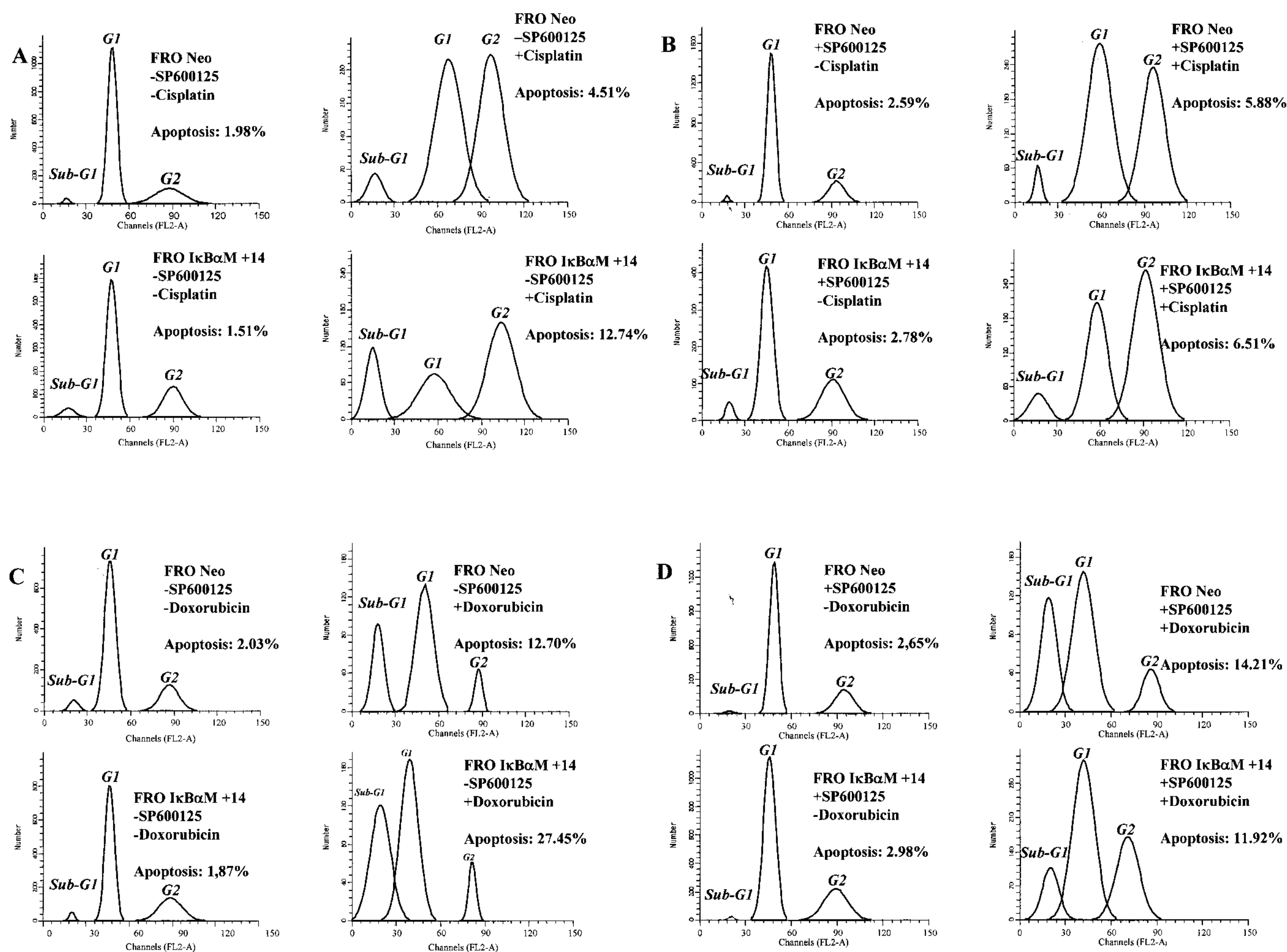


FIG. 9. **Inhibition of JNK activity restored cell death resistance in FRO IκBαM clones.** 1×10^5 cells/well were seeded in 6-well culture plates and incubated for 48 h at 37 °C with 5 μg/ml cisplatin (*A* and *B*) or doxorubicin (*C* and *D*) in the presence of 10 μM JNK inhibitor SP600125. Cell death was assessed by propidium iodide staining according to Nicoletti *et al.* (41). Samples were analyzed by flow cytometry using a FACSCalibur (Beckman Coulter, Fullerton, CA), equipped with ModFit Software. A representative experiment out of two is shown.

sensitivity of FRO IκBαM clones to apoptosis induced by chemotherapeutic agents could be due to restoration of JNK activity. To investigate this hypothesis, we analyzed the apoptosis rate in FRO Neo and FRO IκBαM +14 cells after treatment with 5 μg/ml cisplatin or doxorubicin in the presence of 10 μM SP600125, a specific inhibitor of JNK activity (44). Cell death was assessed by propidium iodide staining and was represented by the fraction of cells in sub-G_1 phase (Fig. 9). The inhibition of JNK activity by SP600125 rendered FRO IκBαM resistant to apoptosis induced by cisplatin (Fig. 9*B*) or doxorubicin (Fig. 9*D*). These results suggest that NF-κB inhibits chemotherapeutic drug-induced apoptosis of FRO cells by, at least partially, negatively regulating JNK signaling.

DISCUSSION

In the present paper, we show that the transcription factor NF-κB plays an important role in thyroid cancer. Its activity was constitutively elevated in primary human thyroid carcinomas and was correlated with malignant phenotype. In particular, anaplastic thyroid carcinoma cells displayed almost 100% of their nuclei positively stained for NF-κB. Activated NF-κB was also detected in an *in vitro* model of human thyroid cancer that resembles the *in vivo* differentiated and undifferentiated thyroid tumors. In these cell lines we demonstrate that persistent NF-κB activity was progressively detected in papillary thyroid carcinoma cells (NPA) to follicular carcinoma cells (WRO) until reaching the highest levels in anaplastic carcinoma cells (FRO), suggesting that sustained activation of NF-κB confers an advantage for clonal selectivity. An interesting question is how thyroid cancer cells acquired a basal NF-κB activity. By using Western blot analysis, we detected low levels of IκBα protein in FRO cells (data not shown). Given that transcription of the IκBα gene is strongly up-regulated by NF-κB, the low level of IκBα protein suggests a high turnover of the protein. One may speculate that thyroid cells may have acquired defects in components that regulate IκBα phosphorylation, ubiquitination, and degradation, thus resulting in basal NF-κB activity. Another possibility is that thyroid cells produce an autocrine factor that chronically activates the NF-κB pathway. The latter hypothesis is supported by the recent evidence that at least some differentiated thyroid carcinomas secrete growth factors and chemotactic factors potentially responsible for NF-κB up-regulation (45). In addition, it has been demonstrated recently that CXC chemokine receptor 4 is highly expressed in a human thyroid cell line (46) and that the ability of breast cancer cells to migrate and form metastasis depends on the NF-κB-mediated CXC chemokine receptor 4 expression (47).

NF-κB inhibition in FRO cells strongly enhanced the sensitivity of these cells to undergo apoptosis induced by cisplatin or doxorubicin treatment, and caused a dramatic decrease of their transforming potential. These *in vitro* functions of NF-κB were consistent with its role in tumor growth *in vivo*; FRO IκBαM

cells failed to form tumors in nude mice. The increased susceptibility to apoptosis of FRO IκBαM and the evidence that constitutive activation of the NF-κB pathway rendered NPA cells resistant to drug-induced apoptosis confirms and extends the idea that one of the roles played by NF-κB in neoplastic transformation is protection from apoptosis.

Even though escape from apoptosis may play an important role in the development of cancer, deregulation of this process, alone, may not fully explain the inability of FRO IκBαM to grow in soft agar and to form tumors in nude mice. Given that NF-κB controls expression of genes involved in different cell functions such as proliferation (cyclin D1 and *myc*), apoptosis, and drug resistance (MDR1), it is very possible that a combination of different factors are responsible for the loss of the transforming potential of these cells. However, stable transfected FRO IκBαM +14 and +16 did not show gross differences in proliferation rate compared with the mock-transfected counterpart, indicating that NF-κB is not playing a central role in controlling proliferation of neoplastic cells, at least in our experimental system.

Our data also suggest that the anti-apoptotic function of NF-κB was mediated by the inhibition of JNK signaling. In fact, JNK activity was restored in FRO IκBαM cells, where its duration was prolonged after treatment with anisomycin. In addition, incubation of FRO IκBαM cells with the specific JNK inhibitor SP600125 restored their resistance to chemotherapeutic drug-induced apoptosis. The role of JNK in programmed cell death is debated. Recently, it has been shown that NF-κB is able to inhibit TNF-α-induced apoptosis by negatively regulating activation of the JNK pathway (32, 33). This effect is mediated by two genes, GADD45β and XIAP, both of them under the transcriptional control of NF-κB. In our experimental system GADD45β expression paralleled the levels of NF-κB activity in thyroid carcinoma cells and decreased in FRO IκBαM clones (data not shown), suggesting that this gene might mediate the anti-apoptotic role of NF-κB in thyroid cancer. Taken together, the data presented in this paper clearly substantiate the fundamental role of NF-κB in thyroid oncogenesis and could open new perspectives for diagnosis and therapy of human ATC.

Acknowledgments—We thank G. Franzoso for providing the pCDNA3IκBαM expression vector and M. Santoro and D. Salvatore for critical reading of the manuscript.

REFERENCES

1. Figge, J. (1999) in *Thyroid Cancer: A Comprehensive Guide to Clinical Management* (Wartofsky, L., ed) pp. 515–535, Humana Press, Inc., Totowa, NJ
2. Sherman, S. I. (2003) *The Lancet* **361,** 501–511
3. Gimm, O. (2001) *Cancer Lett.* **163,** 143–156
4. Ball, D. W., Baylin, S. B., and de Bustros, A. C. (1996) in *The Thyroid* (Werner, S. C., and Ingbars, D. H., eds) pp. 1166–1184, 7th Ed., Lippincott-Raven, Philadelphia
5. McIver, B., Hay, I. D., Giuffrida, D. F., Dvorak, C. E., Grant, C. S., Thompson, G. B., van Heerden, J. A., and Goellner, J. R. (2001) *Surgery* **130,** 1028–1034
6. Tennvall, J., Lundell, G., Hallquist, A., Wahlberg, P., Wallin, G., and Tibblin, S. (1994) *Cancer* (*Phila.*) **74**, 1348–1354
7. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16,** 225–260
8. Tak, P. P., and Firestein, G. S. (2001) *J. Clin. Investig.* **107,** 7–11
9. Karin, M., and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* **18,** 621–663
10. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) *Genes Dev.* **9,** 2723–2735
11. Pahl, H. L. (1999) *Oncogene* **18,** 6853–6866
12. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995) *Science* **267,** 1485–1488
13. Karin, M. (1999) *Oncogene* **18,** 6867–6874
14. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) *Cell* **93,** 1231–1240
15. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) *Nature* **395,** 297–300
16. Baldwin, A. (2001) *J. Clin. Investig.* **107,** 241–246
17. Deveraux, Q. L., Roy, N., Stennicke, H. R., Van Arsdale, T., Zhou, Q., Srinivasula, S. M., Alnemri, E. S., Salvesen, G. S., and Reed, J. C. (1998) *EMBO J.* **17,** 2215–2223
18. Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. E., MacKenzie, A., and Korneluk, R. G. (1996) *Nature* **379,** 349–353
19. Muzio, M., Chinnayan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85,** 817–827
20. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) *Science* **281,** 1680–1683
21. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993) *Cell* **74,** 597–608
22. Takekawa, M., and Saito, H. (1998) *Cell* **95,** 521–530
23. Orlowski, R. Z., and Baldwin, A. S., Jr. (2002) *Trends Mol. Med.* **8,** 385–389
24. Gilmore, T., Gapuzan, M. E., Kalaitzidis, D., and Starczynowski, D. (2002) *Cancer Lett.* **181,** 1–9
25. Bargou, R. C., Emmerich, F., Krappmann, D., Bommert, K., Mapara, M. Y., Arnold, W., Royer, H. D., Grinstein, E., Greiner, A., Scheidereit, C., and Dörkin, B. (1997) *J. Clin. Investig.* **100,** 2961–2969
26. Duffey, D. C., Chen, Z., Dong, G., Ondrey, F. G., Wolf, J. S., Brown, K., Siebenlist, U., and Van Waes, C. (1999) *Cancer Res.* **59,** 3468–3474
27. Huang, S., Pettaway, C. A., Uehara, H., Bucana, C. D., and Fidler, I. J. (2001) *Oncogene* **20,** 4188–4197
28. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369,** 156–160
29. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev.* **7,** 2135–2148
30. Davis, R. J. (2000) *Cell* **103,** 239–252
31. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288,** 870–874
32. De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001) *Nature* **414,** 308–313
33. Tang, G., Minemoto, Y., Dibling, B., Purcell, N. H., Li, Z., Karin, M., and Lin, A. (2001) *Nature* **414,** 313–317
34. Pang, X. P., Hershman, J. M., Chung, M., and Pekary, A. E. (1989) *Endocrinology* **125,** 1783–1788
35. Visconti, R., Cerutti, J., Battista, S., Fedele, M., Trapasso, F., Zeki, K., Miano, M. P., de Nigris, F., Casalino, L., Curcio, F., Santoro, M., and Fusco, A. (1997) *Oncogene* **15,** 1987–1994
36. Ludwig, L., Kessler, H., Wagner, M., Hoang-vu, C., Dralle, H., Adler, G., Böhm, B. O., and Schmid, R. M. (2001) *Cancer Res.* **61,** 4526–4535
37. Fagin, J. A., Matsuo, K., Karmakar, A., Chen, D. L., Tang, S. H., and Koeffler, H. P. (1993) *J. Clin. Investig.* **91,** 179–184
38. Estour, B., Van Herle, A. J., Juillard, G. J., Totanes, T. L., Sparkes, R. S., Giuliano, A. E., and Klandorf, H. (1989) *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **57,** 167–174
39. Leonardi, A., Chariot, A., Claudio, E., Cunningham, K., and Siebenlist, U. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10494–10499
40. Macpherson, I., and Montagnier, I. (1964) *Virology* **23,** 291–294
41. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991) *J. Immunol. Methods* **139,** 271–279
42. Lyons, A. B. (1999) *Immunol. Cell Biol.* **77,** 516–518
43. Lanier, L. L., and Recktenweld, D. J. (1991) *Methods Companion Methods Enzymol.* **2,** 192–195
44. Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S. S., Manning, A. M., and Anderson, D. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 13681–13686
45. Russell, J. P., Shinohara, S., Melillo, R. M., Castellone, M. D., Santoro, M., and Rothstein, J. L. (2003) *Oncogene* **22,** 4569–4577
46. Hwang, J. H., Hwang, J. H., Chung, H. K., Kim, D. W., Hwang, E. S., Suh, J. M., Kim, H., You, K. H., Kwon, O. Y., Ro, H. K., Jo, D. Y., and Shong, M. (2003) *J. Clin. Endocrinol. Metab.* **88,** 408–416
47. Helbig, G., Christopherson, K. W., II, Bhat-Nakshatri, P., Kumar, S., Kishimoto, H., Miller, K. D., Broxmeyer, H. E., and Nakshatri, H. (2003) *J. Biol. Chem.* **278,** 21631–21638