MOLECULAR AND CELLULAR BIOLOGY, Feb. 2003, p. 1061–1074 Vol. 23, No. 3
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.3.1061–1074.2003

Inhibition of IκB Kinase by a New Class of Retinoid-Related Anticancer Agents That Induce Apoptosis

Yolanda Bayon,[1] Maria A. Ortiz,[1] Francisco J. Lopez-Hernandez,[1] Feng Gao,[1] Michael Karin,[2] Magnus Pfahl,[1,3] and F. Javier Piedrafita[1]*

Sidney Kimmel Cancer Center,[1] Maxia Pharmaceuticals,[3] and Department of Pharmacology, University of California–San Diego School of Medicine,[2] San Diego, California

Received 1 May 2002/Returned for modification 11 June 2002/Accepted 29 October 2002

The transcription factor NF-κB is overexpressed or constitutively activated in many cancer cells, where it induces expression of antiapoptotic genes correlating with resistance to anticancer therapies. Small molecules that inhibit the NF-κB signaling pathway could therefore be used to induce apoptosis in NF-κB-overexpressing tumors and potentially serve as anticancer agents. We found that retinoid antagonist MX781 inhibited the activation of NF-κB-dependent transcriptional activity in different tumor cell lines. MX781 was able to completely inhibit tumor necrosis factor alpha-mediated activation of IκB kinase (IKK), the upstream regulator of NF-κB. Inhibition of IKK activity resulted from direct binding of MX781 to the kinase, as demonstrated by in vitro inhibition studies. Two other molecules, MX3350-1 and CD2325, which are retinoic acid receptor gamma-selective agonists, were capable of inhibiting IKK in vitro, although they exerted variable inhibition of IKK and NF-κB activities in intact cells in a cell type-specific manner. However, *N*-(4-hydroxyphenyl)-retinamide, another apoptosis-inducing retinoid, and retinoic acid as well as other nonapoptotic retinoids did not inhibit IKK. Inhibition of IKK by the retinoid-related compounds and other small molecules correlated with reduced cell proliferation and increased apoptosis. Reduced cell viability was also observed after overexpression of an IKKβ kinase-dead mutant or the IκBα superrepressor. The induction of apoptosis by the retinoid-related molecules that inhibited IKK was dependent on caspase activity but independent of the retinoid receptors. Thus, the presence of an excess of retinoic acid or a retinoid antagonist did not prevent the inhibition of IKK activation by MX781 and CD2325, indicating a retinoid receptor-independent mechanism of action.

Aberrant expression of NF-κB has been associated with oncogenesis and carcinogenesis (35, 46), and constitutive high levels of NF-κB activity have been detected in tumor cells. Particularly high activity was found in breast cancer cells, and this activity increased in more advanced tumors (51, 62), although other studies have shown that in most human breast cancer cells, NF-κB is activated independently of the hormonal status (14). NF-κB is also activated in human melanoma, gastric carcinoma, colon carcinoma, and pancreatic carcinoma cells (3, 22, 41, 59, 74, 77).

NF-κB activation in cancer cells correlates with tumor resistance to induction of apoptosis by tumor necrosis factor (TNF) or irradiation, which is likely due to the activation of NF-κB-responsive genes that control survival pathways. For instance, NF-κB induces expression of TRAF-1 and TRAF-2, as well as inhibitor-of-apoptosis proteins 1 and 2 (IAP-1 and -2) and X-linked IAP (13, 63, 72), which inhibit caspase activity and prevent apoptosis (15). Upregulation of the caspase 8 inhibitor cFLIP by NF-κB also prevents apoptosis induced by Fas ligand (47). Furthermore, the Bcl-2 homolog Bfl-1/A1 was found to be induced by inflammatory cytokines and is a direct target of NF-κB (24, 70, 81). Consistent with this, inhibition of NF-κB by means of chemical inhibitors or microinjection of IκB protein or an anti-c-Rel antibody was shown to induce apoptosis in B cells (75), while NF-κB expression prevented TNF-α-induced apoptosis (7, 42, 68, 71).

More recently, it has been shown that inhibition of NF-κB enhances the antitumor activity of TNF-α and a camptothecin analog in nude mice (69). In addition to upregulating the expression of antiapoptotic genes, NF-κB also activates target genes involved in cell proliferation (cyclin D1) (25), angiogenesis (interleukin-8, vascular endothelial growth factor) (32, 36), and metastasis (matrix metalloproteinases) (11, 30, 31, 73). Therefore, compounds that block NF-κB activation pathways could serve as novel anticancer agents and, as recently suggested, might be particularly useful for the treatment of estrogen receptor-negative breast cancer (9, 10).

NF-κB is activated in response to inflammation, viral and bacterial infection, and various stress stimuli. In addition to apoptosis, NF-κB also participates in cellular transformation and oncogenesis. Activation of NF-κB usually requires phosphorylation of IκB at two specific serine residues at the N terminus of IκBα (Ser32/36) or IκBβ (Ser19/23), which triggers the polyubiquitination and subsequent proteasome-dependent degradation of the inhibitor (12, 16). This leaves free NF-κB that translocates into the nucleus and binds DNA to activate transcription of responsive genes (reviewed in reference 4). Cytokine-responsive phosphorylation of IκBα and IκBβ is achieved by the IκB kinase (IKK) (17), which is a complex formed by at least three different protein kinases, IKKα, IKKβ, and IKKγ. Both IKKα and IKKβ are catalytic subunits, which

* Corresponding author. Mailing address: 10835 Altman Row, San Diego, CA 92121. Phone: (858) 410-4188. Fax: (858) 450-3251. E-mail: jpiedrafita@skcc.org.

are brought together through interactions with the regulatory subunit IKKγ (reviewed in references 34 and 80).

Genetic studies have demonstrated that IKKβ is essential for cytokine-dependent activation of the IKK complex, while IKKα is required for the proliferation and differentiation of epidermal keratinocytes and skeletal patterning (29, 38–40). IKK therefore represents a major regulator of the NF-κB pathway, and because of the central role of NF-κB activity in important cellular functions, inhibitors of IKK show promise for the treatment of different human diseases.

The anticancer activity of natural retinoids has been connected to their ability to induce differentiation and/or to their ability to inhibit cell proliferation by antagonizing the transcription factor AP-1 in a retinoid receptor-dependent manner (20). In the search for more potent compounds, synthetic retinoid-related molecules (RRMs) that induce apoptosis in a variety of cancer cell lines in vitro and in vivo were discovered (21, 43, 53, 56, 61, 67). Most of the apoptotic RRMs are analogs of CD437, a retinoic acid receptor γ (RARγ)-selective agonist (8) that induces apoptosis by targeting the mitochondrial pathway (45). We found that this type of novel RRM causes a strong and sustained activation of c-Jun N-terminal kinase (JNK) and p38 kinase in Jurkat cells, leading to the release of cytochrome *c* into the cytosol and subsequent activation of caspases (54). This indicated that molecules with retinoid activity could also target nonretinoid signaling pathways. Another type of apoptotic RRM structurally not related to CD437 is the retinoid antagonist MX781, which showed significant anti-breast cancer activity in vitro and in vivo (21).

Here we investigated the effect of the antagonist MX781 and RARγ-selective RRMs on the NF-κB survival pathway. We observed that MX781 inhibited TNF-α-induced activation of NF-κB DNA binding and transcriptional activities in various cancer cells in which it induced apoptosis. We found that the inhibition of NF-κB activity was mediated by direct inhibition of IKK and that MX781 reversibly bound IKK and blocked kinase activity in vitro. In addition to the antagonist, several RARγ-selective RRMs that induced apoptosis also inhibited IKK and NF-κB activity in vitro but were overall weaker inhibitors in intact cells and exerted only partial effects in certain cancer cell lines. In contrast, retinoids that did not induce apoptosis also had no effect on IKK activity. Other inhibitors of IKK not related to retinoids prevented cell proliferation and induced apoptosis in cancer cells. Moreover, nonpharmacological inhibition of NF-κB activity achieved by overexpression of a dominant negative mutant of IKKβ or a nonphosphorylatable form of IκBα significantly reduced cell viability, demonstrating that interference with the IKK/NF-κB pathway may be sufficient to activate the apoptotic process.

Synthetic peptides that inhibit caspase activity prevented the induction of apoptosis by selective RRMs, suggesting a caspase-dependent mechanism. However, the induction of caspase activity and the inhibition of IKK by the apoptotic RRMs were not affected by the presence of an excess of all-*trans*-retinoic acid (RA) or a retinoid antagonist, which indicates a RAR-independent mechanism of action. Thus, our data point to new backbone structures that could be useful for the development of novel IKK inhibitors as anticancer drugs.

MATERIALS AND METHODS

Cell lines, reagents, and retinoids. Prostatic carcinoma cell lines (DU145 and PC3) were grown in RPMI with 10% fetal bovine serum. HeLa (cervical cancer) and A549 (non-small cell lung carcinoma) cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Glutamine, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) were added to the media.

TNF-α was purchased from R&D. RA and 15-deoxy-$\Delta^{12\text{-}14}$-prostaglandin J_2 (15dPGJ$_2$) were obtained from Biomol. Other chemicals were purchased from Sigma. Synthetic RRMs were obtained from Maxia Pharmaceuticals Inc.; 10 mM stock solutions were made in dimethyl sulfoxide and properly diluted in culture medium prior to use. Treatment with retinoids was performed in the presence of 0.5% fetal bovine serum, keeping the concentration of dimethyl sulfoxide under 0.2% (vol/vol). Prior to RRM exposure, cells were grown overnight (16 to 20 h) in medium containing 0.5% fetal bovine serum. Z-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) was purchased from Enzyme System Products. Cells were incubated for 1 h with 20 μM Z-VAD-FMK prior to RRM treatment. Fresh Z-VAD-FMK was added to the culture medium every 12 to 16 h during prolonged experiments.

Electrophoretic mobility shift assay. Nuclear extracts were prepared as previously described (18). Double-stranded oligonucleotides containing a consensus NF-κB (GGGGACTTTCCC) or Oct-1 (ATGCAAAT) binding site were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and 5 to 10 μg of protein extract was mixed with the labeled DNA (≈100,000 cpm) in the presence of 1 μg of poly(dI-dC) in 20 μl of binding buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 10% glycerol). After 30 min at 4°C (NF-κB) or room temperature (Oct-1), protein-DNA complexes were separated in a 5% polyacrylamide gel under nondenaturing conditions. Gels were subsequently dried and analyzed by autoradiography.

Transfections and luciferase assays. Typically, 5×10^4 HeLa or PC3 cells were seeded in 24-well plates the day before transfection. Cells were cotransfected with 100 ng of NF-κB–luciferase reporter and 10 ng of NF-κB-inducing kinase or constitutively active MEKK1 (MEKK1Δ70) expression vectors following a calcium phosphate-DNA precipitation procedure for 16 h (HeLa) or SuperFect transfection reagent for 2 h (PC3). Control cells were cotransfected with empty vector (pcDNA3). Carrier DNA (pBluescript) was added up to 1 μg/well. After transfection, cells were washed with phosphate-buffered saline and treated with retinoids or vehicle in Dulbecco's modified Eagle's medium supplemented with 0.5% charcoal-treated fetal bovine serum for 6 h, when cell extracts were prepared and assayed for firefly luciferase activity. Alternatively, cells were transfected with the NF-κB–luciferase reporter and carrier DNA, washed after transfection, and incubated with retinoids for 2 h prior to stimulation with 200 ng of tetradecanoyl phorbol acetate per ml for an additional 4 h. Protein concentration was measured to normalize the luciferase activity.

Kinase activity. Total cell extracts (150 μg) were incubated with 0.5 μg of anti-IKKα antibody (PharminGen) in lysis buffer (50 mM Tris [pH 8], 1 mM EDTA, 0.1% NP-40, 250 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 2 mM phenylmethylsulfonyl fluoride, 2 μg of leupeptin per ml) for 30 min at 4°C and then isolated with protein A-Sepharose beads (Amersham Pharmacia Biotech) after 3 h of incubation. The immunoprecipitates were washed three times with lysis buffer and once with kinase buffer (20 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM Na_3VO_4). The washed beads were incubated with 20 μl of kinase buffer containing 2 μg of glutathione *S*-transferase (GST)-IκBα(1-54), 20 μM ATP, 2 mM dithiothreitol, and 5 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The reactions were stopped by addition of 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and proteins were resolved by SDS–15% PAGE. The gels were dried and autoradiographed. Quantification of the radioactive bands was performed with a Storm phosphorimager (Molecular Dynamics). JNK, p38, and CDK2 activities were measured with immune complex kinase assays slightly modified from those described in the literature with whole-cell extracts and GST-c-Jun, GST-ATF2, and histone H1 as the substrate, respectively (52, 65).

Western blots. A total of 50 μg of total protein extract was separated by SDS-PAGE and transferred onto Immobilon-P (Millipore) by standard procedures. Endogenous IKKα and IκBα were detected with anti-IKKα (PharminGen) or anti-IκBα (Santa Cruz Biotechnology) used at a 1:1,000 dilution in Blotto (5% nonfat milk in Tris-buffered saline containing 0.5% Tween 20). Ectopic expression of the different IKK constructs transfected was detected with antihemagglutinin (HA; Covance) diluted 1:1,000 in Blotto. To examine the activation of caspases, cytosol extracts were prepared in CE buffer (25 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7], 25 mM KCl, 5 mM EGTA, 1 mM dithiothreitol, 10 μM cytochalasin B, 0.5% NP-40, and a mixture

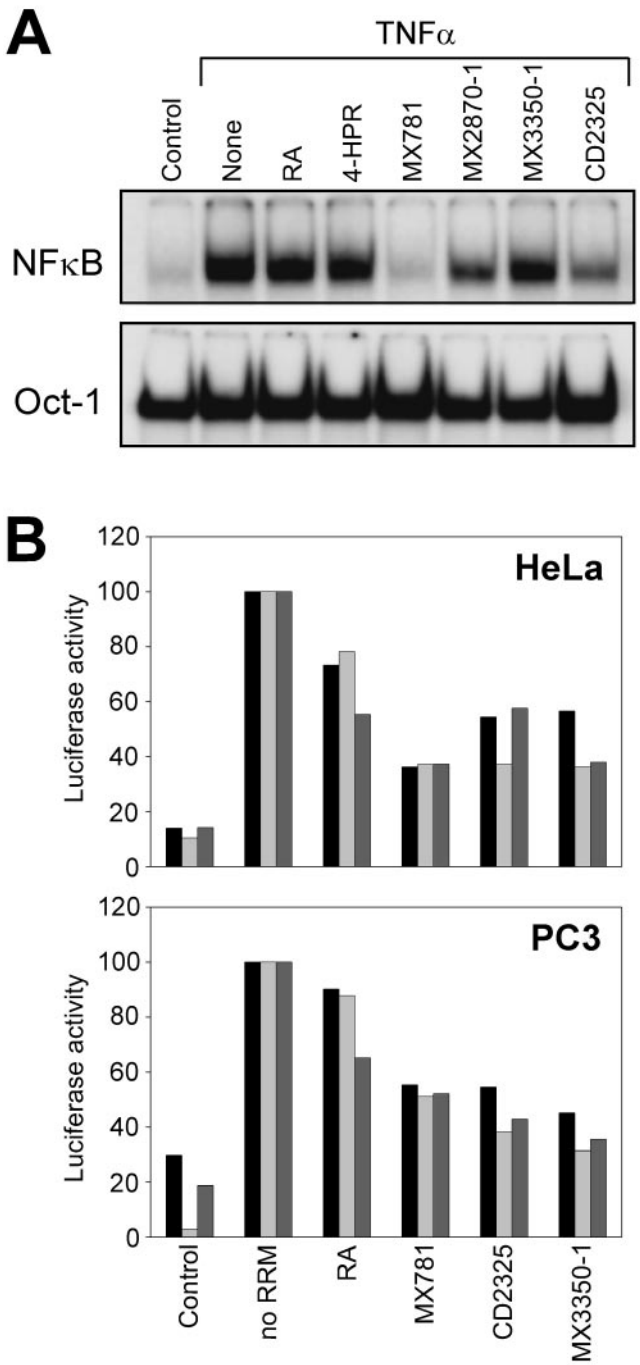

FIG. 1. Selective RRMs inhibit the induction of NF-κB activity by TNF-α. (A) Inhibition of NF-κB DNA binding activity. DU145 cells (2×10^6 in 60-mm dishes) were grown for 16 h in medium containing 0.5% fetal bovine serum prior to exposure to solvent (none) or a 6 μM concentration of the indicated RRMs for 4 h. Cells were then stimulated with 20 ng of TNF-α per ml for 10 min. Control cells were not stimulated with TNF. Nuclear extracts were prepared and examined for NF-κB and Oct-1 DNA binding activities. Data from one representative experiment are shown. (B) Inhibition of NF-κB transcriptional activity by retinoids. HeLa or PC3 cells were cotransfected with 100 ng of NF-κB–luciferase reporter together with 10 ng of NF-κB-inducing kinase (dark gray), MEKK1Δ70 (light gray), or empty vector (control). After transfection, cells were incubated with 1 μM RA, MX781, CD2325, or MX3350-1 for 6 h in medium containing 0.5% charcoal-treated fetal bovine serum. Alternatively, cells were incubated for 2 h with the indicated RRMs and then stimulated with tetradecanoyl phorbol acetate (black columns) or not (control) for 4 h. Cells were harvested and analyzed for luciferase activity and protein concentration. The percentage of luciferase activity relative to that in cells stimulated in the absence of RRMs (no RRM) is indicated. The experiment was performed at least two times in triplicate, and the results of one representative experiment are shown.

of protease inhibitors consisting of 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, and 1 μg of aprotinin per ml). From 25 to 50 μg of protein extract was analyzed by immunoblot with antibodies that recognize active caspases 3 and 9 (Cell Signaling Technologies) following the manufacturer's recommendations. Incubation with secondary antibodies, horseradish peroxidase-coupled donkey anti-rabbit or sheep anti-mouse immunoglobulin, was subsequently carried out for 1 h at room temperature. Proteins were detected by chemiluminescence with the ECL kit (Amersham Pharmacia).

Cell viability and apoptosis assays. PC3 and A549 cells were seeded in 96-well plates (3,000 cells per well) and allowed to attach. Cells were treated with retinoids or other inhibitors in medium containing 0.5% fetal bovine serum for the indicated periods of time, when cell proliferation was measured with a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) as the substrate. The induction of DNA fragmentation was determined with a cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche) following the manufacturer's instructions. A total of 10,000 cells in 96-well plates were treated in medium containing 0.5% fetal bovine serum with the compounds for 24 h and then lysed, and 20 μl of lysate was used for the ELISA. The induction of apoptosis was calculated with untreated cells as a control. Individual cell death was analyzed by labeling fragmented DNA with terminal deoxynucleotide transferase with a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) labeling assay in situ cell death detection kit (fluorescein or tetramethylrhodamine red) (Roche) and subsequent flow cytometry analysis. Labeled cells were further cytospun onto glass slides and examined by fluorescence microscopy.

Caspase activity was measured with 10 μg of cytosol extract prepared in CE buffer diluted in a total volume of 100 μl of caspase buffer (50 mM HEPES [pH 7.4], 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 0.1% CHAPS [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate], and 10% sucrose), which contained 100 μM acetyl-Asp-Glu-Val-Asp-7-amino-trifluoromethyl coumarin (Ac-DEVD-AFC; Enzyme Systems Products). The release of AFC was measured every 3 min as emission at 510 nm upon excitation at 390 nm with a Victor2 microplate reader (Perkin Elmer). Caspase activity (picomoles of AFC per milligram per minute) was calculated from the initial slope. A standard curve with known concentrations of free AFC (Enzyme Systems Products) was performed to calculate the amount of cleaved AFC.

To detect apoptosis in transfected cells, PC3 and A549 cells (3×10^5 per well) were cotransfected in six-well plates with 1 μg of β-galactosidase expression vector and 4 μg of IKKβ K44M, IκBα S32/36A, or empty vector with Superfect (Qiagen) (PC3 cells) or Lipofectin (Invitrogen) (A549 cells) following the manufacturer's instructions. At 24 h after transfection, cells were stained basically as described before (48). Briefly, cells were washed with phosphate saline buffer and stained with a 0.4% (wt/vol) solution of trypan blue in phosphate saline buffer for 10 min. After fixation, cells were stained with Red-Gal (Research Organics) until a pink color of the desired intensity was developed. Apoptotic transfected cells appeared rounder with dark pink-purple coloration due to the double staining with Red-Gal and trypan blue. At least eight different fields containing a total of 400 to 600 transfected cells were counted to quantitate the percentage of apoptotic cells.

RESULTS

MX781 inhibits activation of NF-κB. When analyzing the effect of a series of synthetic retinoid derivatives on the DNA binding activity of various transcription factors, we found that certain compounds that induced apoptosis also inhibited the binding of NF-κB to DNA without affecting the levels of expression of NF-κB proteins (data not shown). NF-κB activity is strongly induced upon stimulation with cytokines such as TNF-α. We therefore investigated the effect of several of these

RRMs on the DNA binding activity of NF-κB induced by TNF-α.

Strong NF-κB DNA binding activity was observed in nuclear extracts prepared from DU145 cells stimulated with 20 ng of TNF-α per ml (Fig. 1A). This increased NF-κB activity was completely inhibited when cells were incubated with 6 μM MX781 prior to TNF-α stimulation. The effect of this retinoid antagonist was dependent on the concentration and the time of incubation, and only those concentrations that were effective in the induction of apoptosis inhibited the DNA binding activity of NF-κB (data not shown). Interestingly, RARγ-selective RRMs such as MX2870-1 had a marginal effect on TNF-mediated NF-κB-DNA binding activity, although they were potent inducers of apoptosis in different cancer cell lines (54, 56) (data not shown). Another apoptotic retinoid, *N*-(4-hydroxyphenyl)-retinamide (4-HPR), as well as the natural RAR agonist RA had no significant effects on NF-κB DNA binding activity. The RRMs, however, did not affect the DNA binding activity of the POU family member Oct-1, indicating specific inhibition of NF-κB binding activity (Fig. 1A). Similar results were observed in other cancer cell lines, including PC3, LNCaP, Jurkat, and HeLa cells (not shown).

We next examined the inhibition of NF-κB mediated transcriptional activity by selected RRMs in HeLa and PC3 cells. The antagonist strongly inhibited (by 40 to 60%) the induction of NF-κB activity by tetradecanoyl phorbol acetate, MEKK1Δ70, and NF-κB-inducing kinase in both cell lines (Fig. 1B). The RARγ-selective compounds CD2325 and MX3350-1 also inhibited luciferase activity induced by all stimuli, while RA exerted a weaker but still significant effect (10 to 40% inhibition). This inhibition of NF-κB transcriptional activity by RA, MX3350-1, and CD2325 contrasted with the small effect observed on NF-κB DNA binding activity (see Fig. 1A) and could possibly be caused by negative cross talk mediated by liganded RARs, as previously observed with retinoid X receptors (49). The inhibition of TNF-α-mediated NF-κB transcriptional activation by MX781 further correlated with decreased expression of IAP-2 mRNA levels in a time- and RRM concentration-dependent manner, with no effect on other TNF-α-induced genes (TRAF-1 and A1). In contrast, the RARγ-selective compounds and RA showed no inhibition of the TNF-α-upregulated genes (data not shown).

MX781 inhibits TNF-mediated activation of IKK. Since IKK is an upstream activator of NF-κB, we examined if the observed inhibition of NF-κB activation was due to inhibition of IKK. IKK activity was stimulated by TNF-α in DU145 cells after exposure for various periods of time to increasing concentrations of MX781. At 6 μM, MX781 significantly inhibited TNF-mediated activation of IKK when cells were incubated for 2 h with the compound (Fig. 2A). Activation of IKK correlated with degradation of IκBα, which was prevented by increasing the time of incubation with MX781. IKKα protein levels were, however, not affected, indicating interference with IKK activity or activation. The effect of MX781 on TNF-α-induced IKK activity and IκBα degradation was dependent on the concentration of the compound (Fig. 2B). At least 6 μM MX781 was necessary to completely inhibit IKK, which correlated with the optimal concentration required to inhibit NF-κB DNA binding activity (data not shown). A very similar time- and dose-dependent response was observed in the other cancer cell lines investigated, including PC3 prostate carcinoma cells (data not shown).

The effect of other apoptosis-inducing RRMs on TNF-induced IKK activity was next investigated. IKK activity was stimulated with TNF-α in DU145 cells after incubation with several retinoids, including the antagonist MX781 and four RARγ-selective RRMs (MX2870-1, MX3350-1, CD2325, and 4-HPR) at concentrations known to induce apoptosis (not shown). Interestingly, only MX781 completely inhibited TNF-induced IKK activity (Fig. 2C). Although MX2870-1, MX3350-1, and CD2325 were strong inducers of apoptosis in DU145 cells, they elicited only a partial inhibition of IKK activation, which correlated with a partial inhibition of TNF-α-mediated activation of NF-κB DNA binding activity (see Fig. 1A). In contrast, IKK activity was not inhibited at all after incubation with RA or 4-HPR, which is another apoptosis-inducing retinoid. While the RARγ-selective RRMs elicited a partial effect on DU145 cells, they exerted a complete inhibition of TNF-α-mediated activation of IKK in other cell lines, including PC3 and U937 cells (see below and data not shown).

The induction of p38 mitogen-activated protein kinase and caspase activities has been shown to participate in the inhibition of NF-κB activity in certain cell models (1, 57). Since both MX781 and the RARγ-selective RRMs lead to strong activation of p38 mitogen-activated protein kinase and caspase activities in Jurkat cells (54) as well as in other cancer cell lines (see below), we examined the effect of caspase inhibitors (Z-DEVD-FMK and Z-VAD-FMK) and p38 kinase inhibitors (SB203580 and PD169316) on RRM-mediated inhibition of IKK and NF-κB activities. Our results indicated that the inhibition of TNF-α-mediated IKK activation was independent of caspase and p38 kinase activities, since it was not prevented by any of these inhibitors (data not shown).

Inhibition of exogenous IKK activity. To determine whether the RRMs analyzed here can inhibit the activity of overexpressed IKK and to examine if there is any selectivity towards a particular form of the kinase, we transfected cells with expression vectors containing epitope-tagged IKKα or IKKβ. In contrast to aspirin, which inhibits IKKβ but not IKKα (78), CD2325 and the antagonist MX781 were capable of inhibiting both IKK isoforms with similar efficacy in PC3 cells (Fig. 3) as well as in DU145 cells (not shown). These RRMs were also able to inhibit a constitutively active IKKβ kinase (S177E/S181E) (Fig. 3), indicating that MX781 and CD2325 might function directly at the level of IKK without interfering with any upstream steps that lead to the phosphorylation and activation of IKKs.

$15dPGJ_2$ and arsenite have been shown to covalently bind to a critical cysteine residue (C179) located within the activation loop, blocking kinase activity in an irreversible manner (33, 58). Since RRMs could potentially form covalent bonds to sulfhydryl groups through their carboxyl group, we examined if the inhibition of IKK activity mediated by these compounds was dependent on the Cys-179 residue. A C179A mutant of IKKβ was transfected into PC3 cells, and its activation by TNF-α was analyzed in the absence and in the presence of selective RRMs. Figure 3 shows that both antagonists MX781 and CD2325 inhibited the activation of the mutant kinase as well as the wild type, indicating that these compounds do not

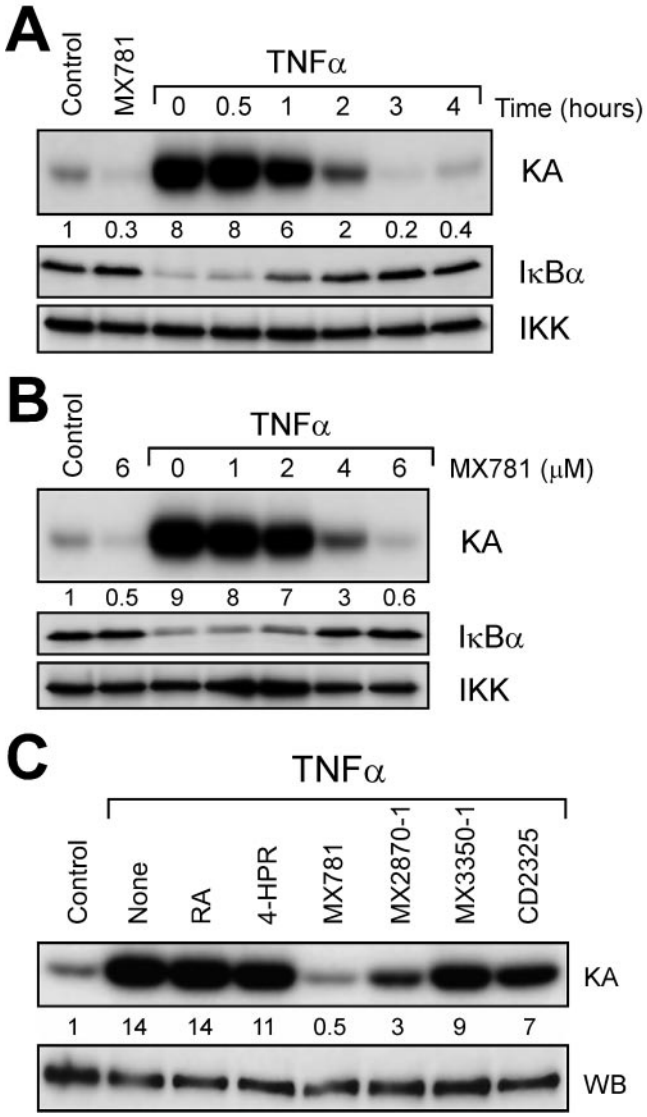

FIG. 2. MX781 inhibits TNF-α-induced IKK activity. (A) DU145 cells were incubated with 6 μM MX781 for the indicated periods of time prior to stimulation with TNF-α (20 ng/ml for 10 min). IKK activity was subsequently measured by immune complex kinase assay (KA), and the levels of IKKα and IκBα proteins were analyzed by Western blot (WB). The fold induction with respect to untreated (control) cells is indicated under the KA panel. (B) DU145 cells were incubated in the presence of 0.5% fetal bovine serum with the indicated concentrations of MX781 for 4 h prior to TNF-α stimulation for an additional 10 min where indicated. Cell extracts were obtained and analyzed as above. (C) Inhibition of TNF-α-mediated IKK activation by RRMs. DU145 cells were incubated with the indicated RRMs at 6 μM or with solvent (none) for 4 h and then stimulated with 20 ng of TNF-α per ml for 10 min. Control cells were left unstimulated. Whole-cell extracts were prepared and assayed for IKK activity (KA) and IKKα protein levels (WB). The fold induction of IKK activity is shown under the KA panel.

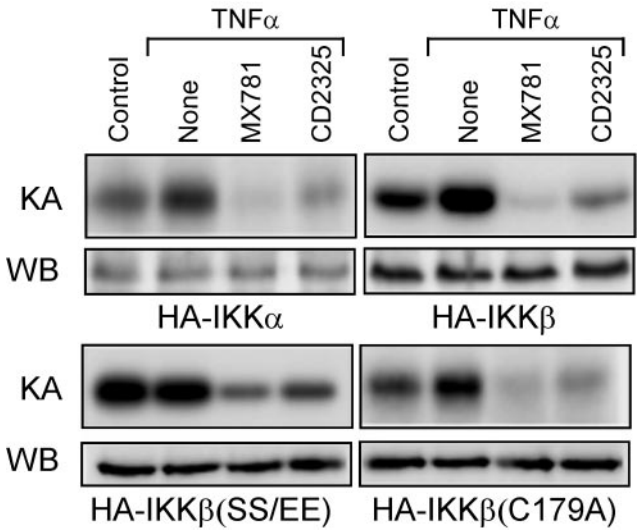

FIG. 3. Retinoid-related molecules inhibit the activity of overexpressed IKKs. PC3 cells were transiently transfected with expression vectors bearing HA-tagged IKKα, IKKβ, IKKβ (SS/EE), and IKKβ (C179A), as indicated. After 16 h, cells were incubated with 6 μM MX781, CD2325, or solvent (none) for 4 h prior to stimulation with 20 ng of TNF-α per ml for 10 min. Kinase activity (KA) was measured in HA immunoprecipitates with GST-IκBα(1-54) as the substrate. Control cells were not treated with TNF-α. Expression levels of the different transfected IKKs were measured by immunoblot (WB) with anti-HA antibody.

inhibit IKK through binding to Cys-179. Furthermore, 2,3-dimercaptopropane-1-sulfonic acid did not prevent RRM-mediated inhibition of IKK while interfering with arsenite action (data not shown), suggesting that interaction with a Cys residue is not responsible for RRM-mediated inhibition of IKK.

MX781 and CD2325 inhibit IKK in vitro. The data presented above suggested that some of the compounds investigated here could function directly on IKK. We therefore examined the effects in vitro of RRMs on active IKK purified by immunoprecipitation. Epitope-tagged IKKα and IKKβ were isolated from HeLa cells stimulated with 20 ng of TNF-α per ml and incubated in vitro with different RRMs. Figure 4A shows that the antagonist MX781 effectively inhibited IKK activity. Interestingly, the RARγ-selective compound CD2325 completely inhibited both IKKα and IKKβ activities, while other related molecules (MX2870-1 and MX3350-1) exerted a partial but considerable inhibitory effect. This contrasted with the results obtained in intact cells, in which these RARγ-selective RRMs elicited a comparable partial activity (see Fig. 2C and 5A below). In contrast, 4-HPR had no significant effect on IKK activity in vitro, confirming our findings in intact cells.

Both MX781 and CD2325 inhibited IKK in a concentration-dependent manner, and lower concentrations of CD2325 resulted in significant inhibition of IKK activity, with a 50% inhibitory concentration of approximately 15 μM. Higher concentrations of MX781 were required to effectively inhibit IKK in vitro, reflecting differences in the chemical structure of the two RRMs (Fig. 4B). The inhibition of IKK activity in vitro by these two RRMs was specific for IKK, since no substantial inhibition of JNK, p38 mitogen-activated protein kinase, or CDK2 activities was observed (Fig. 4C).

We investigated further whether inhibition of IKK activity was due to reversible or covalent binding. Immunopurified IKK was incubated in vitro with MX781, CD2325, or 15dPGJ$_2$, and the IKK-containing beads were washed or not three times with kinase buffer prior to measuring the remaining kinase

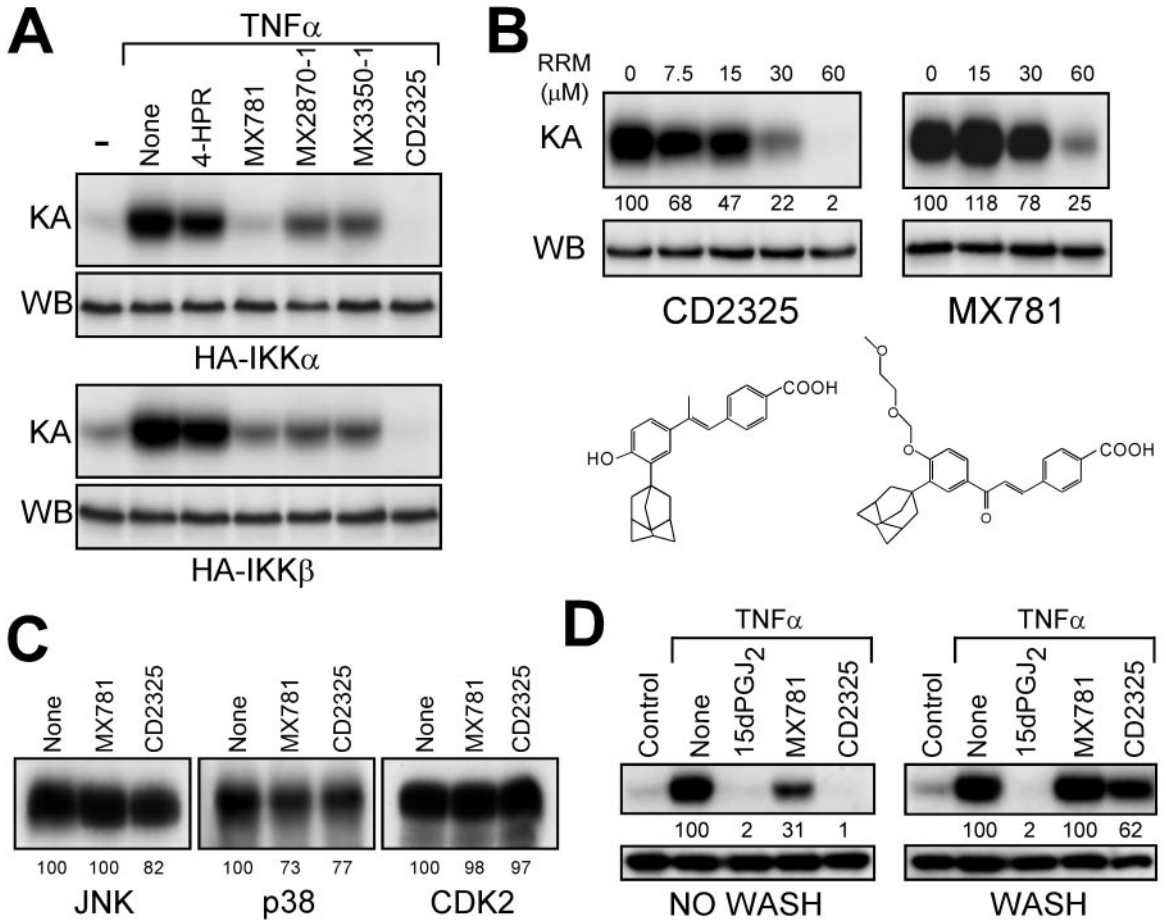

FIG. 4. Inhibition of IKK activity in vitro. (A) Retinoid-related molecules inhibit activated IKK isolated from cancer cells. HeLa cells were transfected with HA-IKKα or HA-IKKβ expression vectors and, at 16 h posttransfection, were stimulated with TNF-α for 10 min, after which the cells were lysed and HA-IKKα and HA-IKKβ were immunoprecipitated with an anti-HA antibody. IKKα- and IKKβ-containing Sepharose beads were incubated with the indicated compounds at 60 μM for 60 min on ice and then subjected to a kinase assay (KA) with GST-IκBα(1-54) as the substrate. Levels of IKKα and IKKβ were analyzed by Western blot (WB) with an anti-HA antibody. (B) Endogenous IKK complex was immunoprecipitated with an anti-IKKα antibody from HeLa (left) or DU145 (right) cells that were stimulated with 20 ng of TNF-α per ml for 10 min. Isolated IKK was incubated in vitro with solvent (lanes 0) or the indicated concentration of CD2325 or MX781, and kinase activity was subsequently measured. The percentage of kinase activity remaining after incubation with the RRM is indicated under the KA panel. The chemical structures of the RRMs are shown below for comparison. (C) Jurkat cells were irradiated with UV at 100 J/m^2 and incubated for 30 min to activate JNK. To induce p38 activity, cells were treated with 0.5 M sorbitol for 20 min, and nonstimulated cells were used to isolate CDK2. Kinases were immunoprecipitated with appropriate antibodies, and kinase-containing Sepharose beads were incubated with solvent (none) or the RRMs at 60 μM for 60 min on ice and subsequently used in a kinase assay with the corresponding substrate. The percentage of kinase activity remaining is indicated below. (D) Inhibition of IKK activity by RRMs is reversible. HeLa cells were stimulated or not (control) with TNF-α for 10 min, and endogenous IKK complex was isolated by immunoprecipitation with anti-IKKα antibody. Isolated IKK was then incubated in vitro in kinase buffer with the indicated RRMs or 15dPGJ$_2$ at 60 μM in duplicate tubes. One set of immunoprecipitates was washed three times with kinase buffer (wash, right), while the second set was kept on ice (no wash, left). The remaining IKK activity was subsequently measured (KA) and quantitated with a phosphorimager. The IKK protein levels were normalized by immunoblot (WB).

activity. Figure 4D shows that the RRM-mediated inhibitory effect was reversed after the washing steps, indicating that these compounds interact with IKK in a noncovalent manner. In contrast, the inhibition of IKK by 15dPGJ$_2$ was not reversed, confirming the covalent nature of this interaction (58). Confirming previous observations with immunoprecipitated endogenous IKK, MX781 elicited a partial inhibition in vitro (70 to 75%), while CD2325 was a more potent inhibitor when used at the same concentrations. This result contrasted with the observations in intact cells, in which the antagonist MX781 was a more potent inhibitor of IKK in all the cell lines analyzed.

Inhibition of IKK correlates with increased apoptosis. To confirm that the inhibition of IKK is relevant for the anticancer activity of MX781, we investigated the effect on cell proliferation and apoptosis of several related RRMs and other nonretinoid structures that inhibit IKK in PC3 and A549 cells. Cells were also treated with RA, MX3350-1, or CD2325 for comparison. First, the effect on TNF-α-mediated IKK activation was analyzed. As previously observed, MX781 effectively inhibited IKK activation by TNF-α in both cancer cell lines. MX3350-1 and CD2325 inhibited the induction of IKK activity to basal levels in PC3 cells but not in A549 cells, although the remaining IKK activity was similar in both cell lines (note that

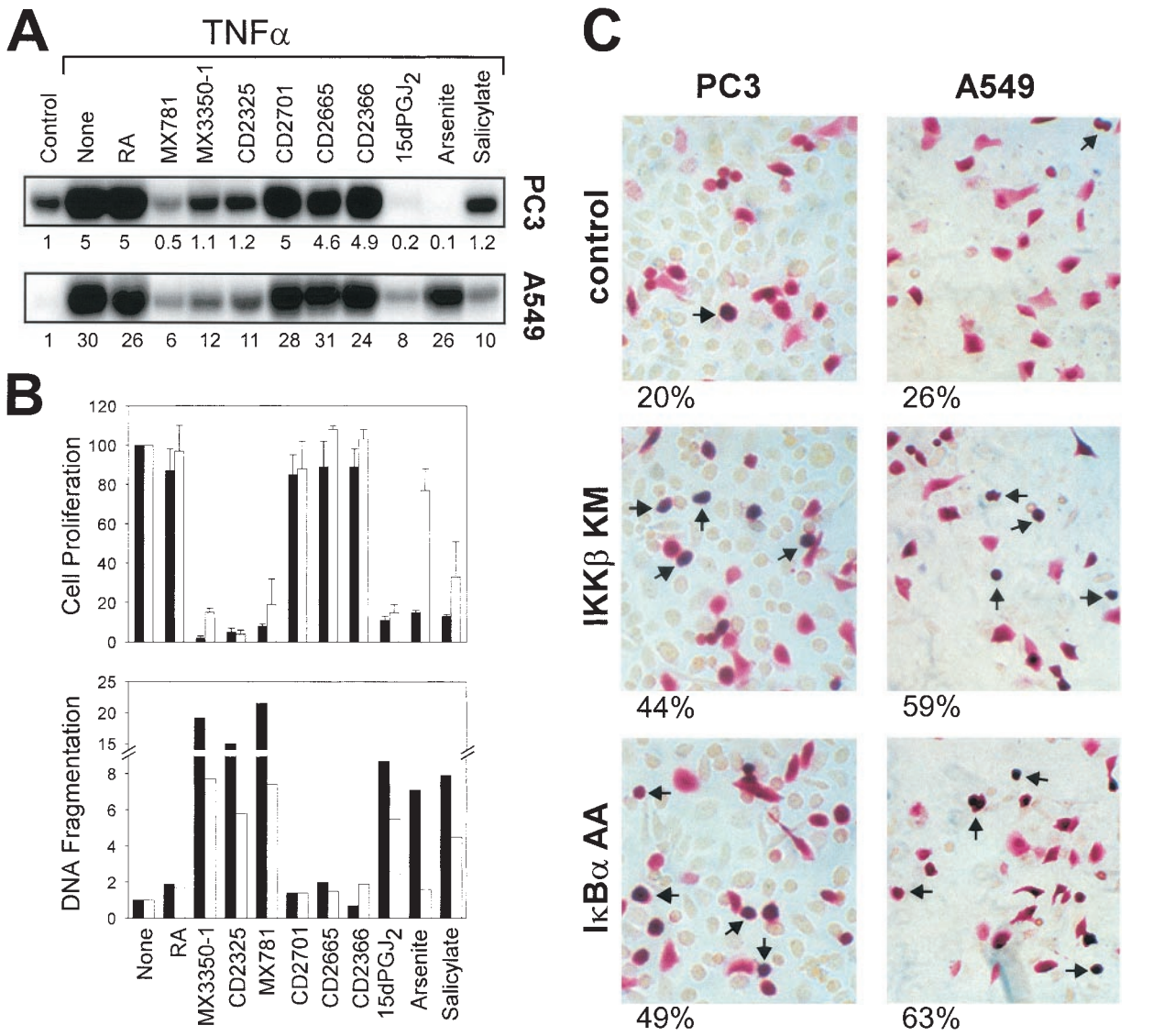

FIG. 5. Inhibition of IKK by small molecules correlates with the induction of cell death. (A) Effect of selective RRMs and other small molecules on IKK activation. PC3 and A549 cells were incubated with 6 μM RA or the indicated RRMs, 15 μM 15dPGJ2, 100 μM sodium arsenite, or 20 mM salicylate for 4 h prior to stimulation with 20 ng of TNF-α per ml for 10 min. Subsequently, whole-cell extracts were prepared and assayed for IKK activity with an immune complex kinase assay. Kinase activity was quantitated by phosphorimager, and the fold induction with respect to nonstimulated cells (control) is indicated. (B) Inhibition of cell growth and induction of apoptosis by IKK inhibitors. PC3 (black columns) and A549 (white columns) cells were incubated with the same concentrations of the indicated molecules as described above in medium containing 0.5% fetal bovine serum. Top: 24 h (PC3) or 72 h (A549) after treatment, cell proliferation was measured with an MTT assay. Untreated cells (none) served as the control (100%). The experiment was performed twice in triplicate, and the average ± standard deviation is shown. Bottom: PC3 (black columns) and A549 (white columns) cells were incubated with the indicated compounds as above for 24 h, when cell extracts were obtained and the amount of DNA fragmentation in the cytosol was quantitated by ELISA. The fold induction of apoptosis relative to untreated cells is indicated. The experiment was performed twice in duplicate, and data from one representative experiment are shown. (C) Inhibition of the IKK/NF-κB pathway reduces cell viability. PC3 and A549 cells were transiently transfected with empty vector (control) or mutants of IKKβ (K44M, KM) and IκBα (S32/36A, AA) together with a β-galactosidase expression vector. At 24 h after transfection, cells were double-stained with trypan blue and Red-Gal. Transfected cells (positive for β-galactosidase) and apoptotic cells (indicated by arrows) were counted in at least eight different fields (over 400 transfected cells), and the percentage of transfected apoptotic cells is indicated below each panel. The experiment was repeated at least four times with very similar results, and data from one representative experiment are shown.

IKK was constitutively activated in PC3 cells but not in A549 cells) (Fig. 5A). Other RRMs, CD2701, CD2665, and CD2366, as well as RA showed no effect on the activation of IKK in either PC3 or A549 cells. On the other hand, $15dPGJ_2$ and salicylate inhibited IKK activation in both cell lines, while arsenite was very effective in PC3 but not in A549 cells.

The effect of these molecules on cell growth was investigated after 24 h (PC3) or 72 h (A549) of incubation in the presence

of the IKK inhibitors. Only the antagonist MX781 and the RARγ-selective RRMs MX3350-1 and CD2325, but not the other retinoids, exerted a substantial inhibition of cell proliferation (Fig. 5B). Similarly, the other IKK inhibitors exerted a strong antiproliferative activity that correlated with their effect on IKK. While $15dPGJ_2$ and salicylate inhibited the growth of both PC3 and A549 cells, arsenite was only effective in PC3 cells, not in A549 cells.

We next investigated if the inhibition of cell proliferation correlated with induction of apoptosis. The appearance of histone-associated DNA fragments in the cytosol was quantitated in PC3 and A549 cells treated for 24 h with RRMs or the IKK inhibitors. Only those compounds that inhibited IKK and cell growth elicited a strong induction of DNA fragmentation as a measure of cell death in either PC3 or A549 cells (Fig. 5B). RA and the selective RRMs CD2701, CD2366, and CD2665 had no effect on DNA fragmentation, while MX3350-1, CD2325, and MX781 as well as 15dPGJ2 and salicylate elicited a strong induction of apoptosis in both cell lines. As expected, arsenite induced apoptosis in PC3 cells (7-fold) but not in A549 cells (1.6-fold), which correlated with its ability to inhibit IKK and cell proliferation only in the prostate carcinoma cell line. The lower apoptosis induction observed in A549 cells compared to PC3 cells with the RRMs correlates with the higher resistance to the killing activity of the molecules (note that 3 days of treatment were required in A549 cells to observe antiproliferative activity similar to that in PC3 cells, which were treated for only 24 h).

Alternatively, a nonpharmacological approach was carried out to investigate the effect of IKK/NF-κB inhibition in apoptosis signaling. NF-κB activity was inhibited specifically by overexpression of a dominant negative mutant of IKK (IKKβ K44M) or a nonphosphorylatable form of IκBα (IκBα S32/36A). Analysis of apoptosis in β-galactosidase-positive transfected cells indicated that transient expression of either of these mutants elicited a strong induction of cell death in both PC3 and A549 cells (Fig. 5C), as evidenced by changes in cell morphology (smaller and rounder cells) together with a purple coloration that appeared as a consequence of the double staining with Red-Gal (transfected cells) and trypan blue (nonviable cells).

Induction of apoptosis by selective RRMs is dependent on caspase activity. We have demonstrated that the RARγ-selective RRMs induced apoptosis in Jurkat cells in a caspase-dependent manner (54, 56). We further characterized the induction of apoptosis by the novel RRMs MX3350-1 and CD2325 as well as the antagonist MX781 in PC3 and A549 cells. The effect of different concentrations of RRM on the induction of caspase activity was first investigated in PC3 cells. The induction of DEVDase activity was significantly enhanced in the presence of increasing amounts of the RRMs (Fig. 6A). As expected, incubation with RA had no effect on caspase activity. The generation of DEVDase activity was completely abolished when cells were incubated in the presence of 20 μM Z-VAD-FMK, a specific paninhibitor of caspases. A concentration of at least 4 μM of the antagonist MX781 was necessary to induce DEVDase activity, which correlated with the concentration required to inhibiting IKK (see Fig. 2B and data not shown) and suggested a link between IKK inhibition and induction of apoptosis by this compound. In contrast, lower concentrations of MX3350-1 and CD2325 (0.6 μM) were sufficient to induce substantial DEVDase activity, which did not compare with the concentration of RRM necessary to inhibit IKK (not shown).

The stimulation of DEVDase activity by MX3350-1, CD2325, and MX781 could be further associated with a significant increase in the amount of cytosolic histone-associated DNA fragments (Fig. 6B) and DNA fragmentation, as determined by TUNEL labeling (Fig. 6C), which were completely prevented in the presence of the pancaspase inhibitor Z-VAD-FMK. Moreover, active caspases 3 and 9 were detected in protein extracts prepared from PC3 cells treated with the RRMs in the absence of Z-VAD-FMK, but not in extracts obtained from RA-treated cells (Fig. 6D). Incubation with Z-VAD-FMK abolished the cleavage of caspase 9 and the generation of the mature caspase 3 p17 subunit, but not the accumulation of the intermediary fragment of caspase 3, p19.

The induction of apoptosis in A549 cells was analyzed next. As expected, the selective RRMs but not RA induced a significant increase of cytosolic DEVDase-like activity, which was completely abolished by Z-VAD-FMK (Fig. 7A). As observed in PC3 cells, MX781, MX3350-1, and CD2325 induced activation of caspases 3 and 9 (Fig. 7B). Although the amount of active caspase 9 was similar in cells treated with the three RRMs, the amount of mature caspase 3 (p17 fragment) was substantially lower in MX781-treated cells, which correlated with reduced DEVDase activity and DNA fragmentation (Fig. 7A and data not shown). This could reflect a delayed activation of the apoptotic program by the retinoid antagonist. In support of this observation, we found that prolonged periods of time were necessary for the antagonist to induce levels of apoptosis similar to those obtained with MX3350-1 in A549 cells, as determined by TUNEL (Fig. 7C and data not shown). MX3350-1 and CD2325 induced apoptosis in 24.5% ± 1% and 26% ± 10% of cells, respectively, after 48 h of incubation. In the presence of Z-VAD-FMK, cell death was reduced to 10.4% and 2.5%, respectively, while only 0.3% of the control cells were stained positive. However, only 18% apoptotic cells were observed after 72 h of exposure to 6 μM MX781 (not shown).

Inhibition of IKK and induction of apoptosis are not mediated by retinoid receptors. Retinoids bind and activate the nuclear retinoid receptors (RARs and retinoid X receptors) to produce a biological response. Although retinoid-mediated cell differentiation is dependent on RARs, the role of the receptors in the induction of apoptosis by the RARγ-selective molecules is controversial. The induction of apoptosis by CD437 has been suggested to proceed independently of the RARs (28, 61, 66), although RARs could modulate CD437-induced apoptosis in ovarian carcinoma cells (27). Therefore, we decided to examine whether the RARs are involved in the apoptosis induced by the RRMS investigated here.

PC3 cells were incubated with CD2325 or MX781 in the absence or presence of a threefold molar excess of RA or the retinoid antagonist CD2366, which showed no anti-IKK or antiproliferative activity (see Fig. 5A and B). The induction of DEVDase activity by the apoptotic RRMs was not affected by saturation of the RARs with the natural ligand RA or the RAR antagonist CD2366 (Fig. 8A), suggesting that RRM-

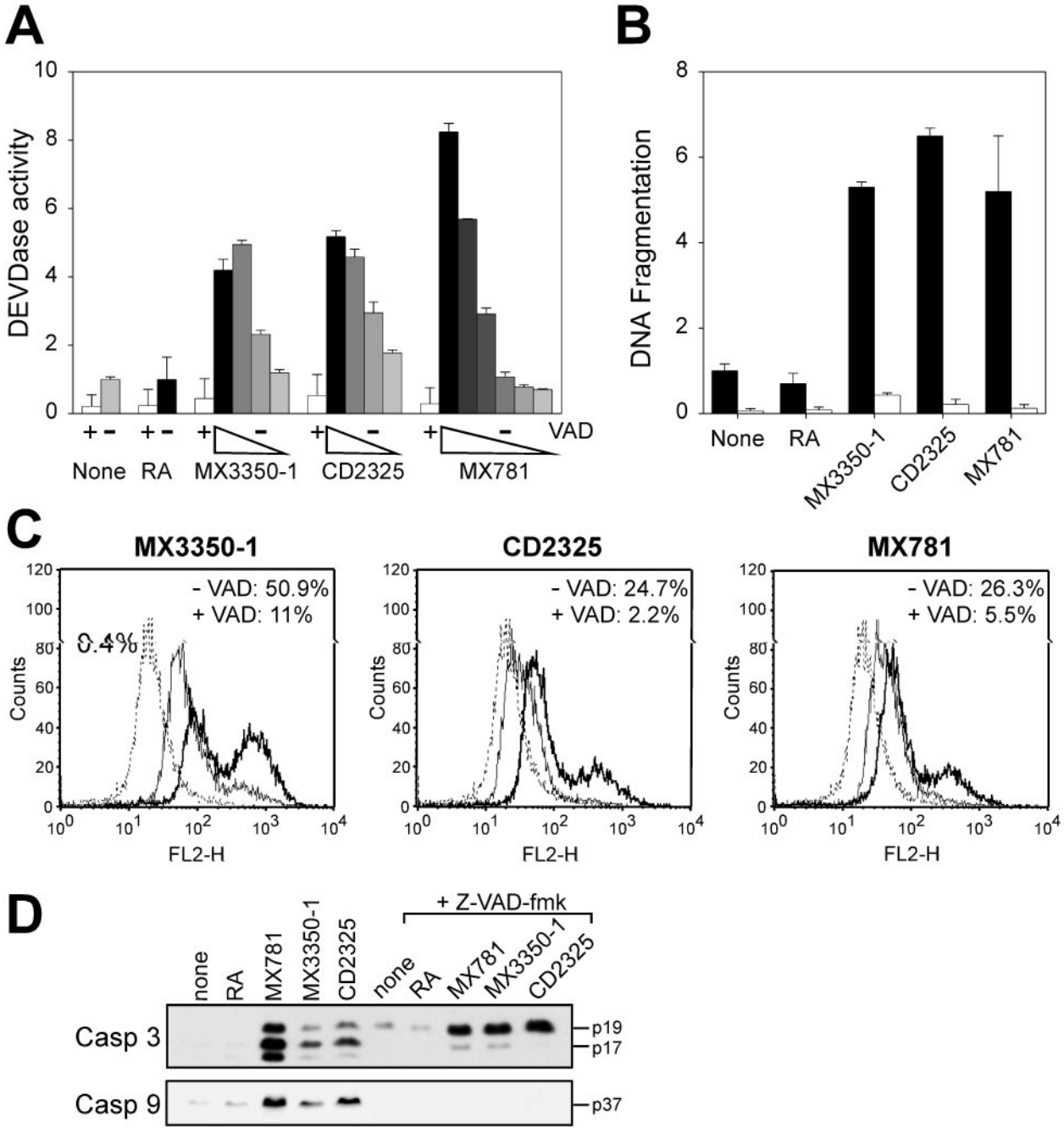

FIG. 6. Selective RRMs induce apoptosis in PC3 cells. (A) Induction of DEVDase activity is dependent on RRM concentration. PC3 cells were incubated with the following concentrations of RRMs: 0.3, 0.6, 2, or 6 μM MX3350-1 or CD2325; 1, 2, 3, 4, 5, or 6 μM MX781; or 6 μM RA. After 16 h, cytosol extracts were prepared and assayed for DEVDase activity. Control cells were incubated in the presence of an equivalent volume of solvent. Cells incubated with the different retinoids at 6 μM (black columns) were also incubated with 20 μM Z-VAD-FMK (white columns) for 1 h prior to retinoid treatment. The fold induction of two independent experiments performed in triplicate is shown. (B) Induction of DNA fragmentation by RRMs. PC3 cells were incubated (white columns) or not (black columns) with Z-VAD-FMK, and subsequently the indicated RRMs were added at 6 μM. At 16 h (MX3350-1 and CD2325) or 24 h (RA and MX781) after retinoid treatment, cell extracts were prepared and assayed for DNA fragmentation (expressed as fold induction) with a cell death detection ELISA. (C) A total of 10^6 cells were treated with the indicated RRMs at 6 μM in the absence (thick line) or in the presence (thin line) of 20 μM Z-VAD-FMK. Control cells (dotted line) were left untreated. At 36 h after RRM exposure, cells were harvested, labeled with a fluorescein TUNEL kit, and analyzed by flow cytometry. The percentage of apoptotic cells is indicated within each box. The experiment was performed at least five times with similar outcomes, and a representative result is shown. (D) Activation of caspases 3 and 9 by RRMs is inhibited in the presence of Z-VAD-FMK. PC3 cells were incubated or not with 20 μM Z-VAD-FMK and then treated for 16 h with the retinoids as indicated at 6 μM. Cytosol extracts were prepared and analyzed by immunoblot with antibodies recognizing active caspases 3 and 9.

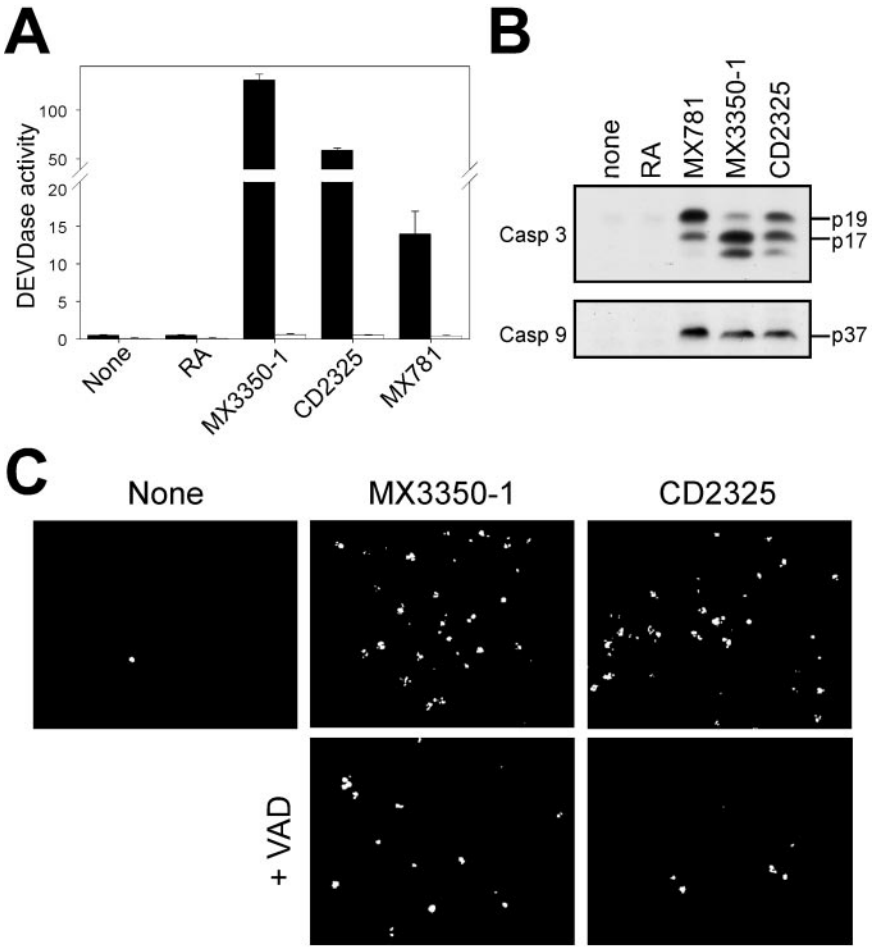

FIG. 7. Induction of apoptosis in A549 cells is dependent on caspase activity. (A) A549 cells (10^6 cells in 60-mm dishes) were treated with the indicated retinoids at 6 μM for 36 h in the absence (black columns) or in the presence (white columns) of Z-VAD-FMK. Cytosol extracts were prepared and assayed for DEVDase activity with a fluorometric assay. The activity (arbitrary units) of a representative experiment performed in triplicate is shown. (B) The extracts obtained in A were analyzed by Western blot with antibodies that recognize active caspases 3 and 9. (C) Induction of DNA fragmentation is inhibited by caspase inhibitors. A549 cells were incubated with 6 μM MX3350-1 or CD2325 for 48 h in the absence or in the presence of Z-VAD-FMK. Control cells were grown in the absence of retinoid. Cells were harvested and labeled with a tetramethylrhodamine-red TUNEL assay, analyzed by flow cytometry for quantification, and visualized by fluorescence microscopy. Representative fields containing similar numbers of cells under light microscopy are shown. The experiment was performed at least three times with similar results.

induced apoptosis is independent of RARs in PC3 cells. Although our data obtained in vitro supported a direct interaction of MX781 and CD2325 with IKK, independently of RARs, we cannot rule out the possibility that RARs could mediate IKK inhibition in intact cells. We therefore analyzed the effect of RA and CD2366 on RRM-mediated inhibition of IKK in PC3 cells.

Cells were incubated with RA or the antagonist CD2366 for 1 h prior to MX781/CD2325 treatment, and TNF-α-stimulated IKK activity was determined in whole-cell extracts with an immune complex kinase assay. Figure 8B shows that both MX781 and CD2325 completely inhibited the stimulation of IKK activity by TNF-α and that this inhibition was not reversed by incubation with RA or CD2366. These results suggest that inhibition of IKK in intact cells was not mediated by the RARs and support a direct retinoid-kinase interaction, as observed in vitro.

DISCUSSION

High levels of constitutive NF-κB activity have been shown to correlate in certain cancer cell lines with hyperactivated IKK (77) and increased expression levels of antiapoptotic genes (5). As a consequence, survival pathways are generally favored in tumor cells, making them more resistant to the induction of apoptosis by TNF, irradiation, or conventional chemotherapeutic drugs. Therefore, inhibition of NF-κB activity has been considered an attractive strategy to increase the sensitivity of cancer cells to chemotherapeutic agents.

One effective way to inhibit NF-κB activity is to target IKK, the kinase responsible for NF-κB activation (34, 80). Small molecules that inhibit IKK activity have indeed been described, including aspirin, prostaglandins containing cyclopentanone rings (e.g., $15dPGJ_2$), arsenite, and resveratrol (26, 33, 58, 78). Some of these molecules have been shown to induce

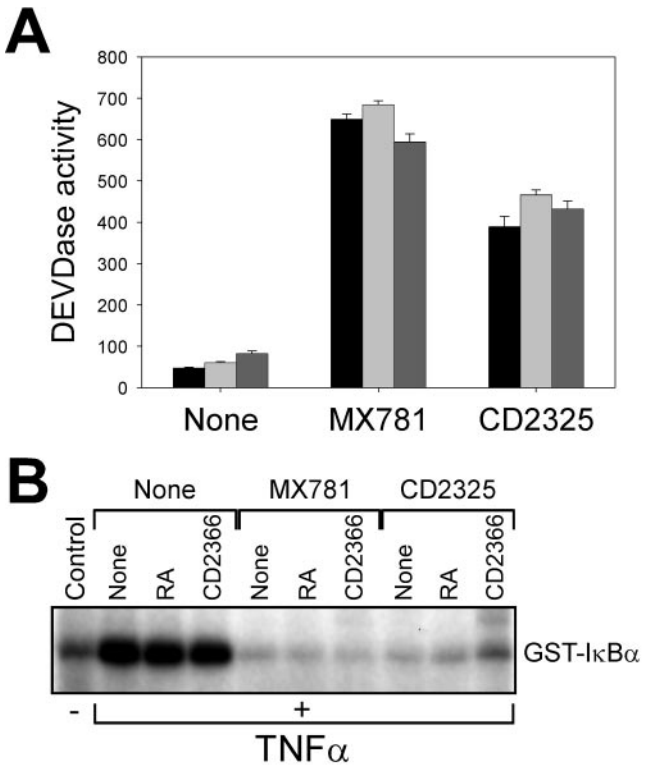

FIG. 8. Retinoid-related molecules function independently of RARs. (A) Induction of caspase activity is independent of RARs. PC3 cells were incubated with 12 μM RA (light gray) or CD2366 (dark gray) for 1 h prior to exposure to 4 μM MX781 or CD2325 for 24 h. Cells were harvested and lysed, and DEVDase activity (arbitrary units) was quantitated. One representative experiment done in triplicate is shown. (B) Inhibition of IKK is not mediated by RARs. PC3 cells were incubated for 1 h with 12 μM RA or CD2366 and then treated with 6 μM MX781 or CD2325 for an additional 4 h prior to stimulation with 20 ng of TNF-α per ml for 10 min. Whole-cell extracts were prepared and analyzed for IKK activity with an immune complex kinase assay. One representative experiment is shown.

apoptosis in cancer cells (26, 76). However, compounds that inhibit IKK with very high affinities have not been reported.

Here we describe the inhibition of IKK activity by a novel class of retinoid-related compounds or RRMs that show strong anticancer activity. In contrast to aspirin, which selectively inhibits IKKβ but not IKKα (78), the inhibitors described here inhibit the activity of both IKKα and IKKβ. Similar to aspirin but different from $15dPGJ_2$ and arsenite, binding of the RRMs to IKK is reversible and does not require the Cys-179 residue located on the kinase domain, to which $15dPGJ_2$ and arsenite bind covalently (33, 58). These molecules could provide a new scaffold for noncovalent inhibitors of IKK.

We present evidence from cell-free assays with immunopurified IKK complex that the inhibition of IKK activity by certain RRMs is mediated by direct binding of the molecule to the kinase, representing a novel retinoid action that is independent of the nuclear retinoid receptors. This is further supported by experiments in cellular systems, in which saturation of RARs with an excess of RA or a retinoid antagonist does not revert the inhibition of IKK elicited by the apoptotic RRMs. Therefore, RAR activity is dispensable for the inhibition of IKK as well as for the induction of apoptosis by these selective RRMs. We could hypothesize that pure IKK inhibitors with no RAR transactivating activity would lack the RAR-associated side effects that are characteristic of natural and most nonselective retinoids and could represent novel anticancer compounds with higher therapeutic potential as stand-alone therapies or in combination with other anticancer therapies.

Abolition of IKK activity causes a reduction of NF-κB transcriptional activity, as demonstrated by transient-transfection experiments. MX781 elicits a significant inhibition of NF-κB-driven luciferase activity in HeLa and PC3 cells stimulated with tetradecanoyl phorbol acetate or upon cotransfection with NF-κB-inducing kinase or MEKK1Δ70, which are known to phosphorylate and activate IKKs (37, 44), correlating with a strong inhibition of IKK and NF-κB DNA binding activities. Similarly, the RARγ-selective RRMs MX3350-1 and CD2325 inhibited NF-κB transcriptional activity while exerting little effect on IKK or NF-κB DNA binding activities. RA also inhibited NF-κB transcriptional activity but not IKK.

The inhibition observed with RA and the synthetic agonists MX3350-1 and CD2325 mirrors the effect of steroids and retinoids in B lymphoma cells (19), which could probably be due to negative interference exerted by agonist-bound RARs through direct NF-κB–RAR interactions similar to those described with retinoid X, glucocorticoid, and estrogen receptors (49, 60, 64). In addition, RARs bound by agonist compounds (RA, MX3350-1, and CD2325) but not antagonists (MX781) could inhibit NF-κB activity through squelching of the coactivators SRC-1, CBP, and p300 (23, 50, 55). In support of these interpretations, we found that low concentrations of RA and MX3350-1 but not the antagonist MX781 efficiently inhibited NF-κB transcriptional activity only when RARα was cotransfected (M. A. Ortiz and F. J. Piedrafita, unpublished observations). Whether or not these RRMs induce RAR–NF-κB interactions or how they affect the interactions between the receptors and coactivators/corepressors is currently under investigation. The most evident consequence of the inhibition of IKK/NF-κB by MX781 is the abolition of TNF-α-mediated induction of IAP-2 mRNA, a potent inhibitor of caspase activity (15). MX781 does not inhibit the induction of other NF-κB-responsive genes such as TRAF-1 and A1, indicating selective inhibition of a subset of NF-κB-induced survival genes.

In contrast to studies in the acute promyelocytic leukemia cell line NB4, in which RA induced NF-κB activity and subsequent upregulation of TRAF-1, -2, and IAP-1 and -2 expression (2), we found that only the RARγ-selective RRMs, not RA, are capable of inducing TRAF and IAP expression in DU145 cells, which was cell type specific and independent of TNF-α costimulation (Y. Bayon and F. J. Piedrafita, unpublished observations). How these RRMs elicit this effect needs further investigation.

Two types of synthetic RRMs have been found to potently induce apoptosis in various cancer cell lines in vitro. One is represented by the antagonist MX781, which was shown to be effective against solid tumors derived from breast cancer cells (21). The second class of apoptotic RRMs is best represented by MX3350-1, a RARγ-selective compound that is chemically related to MX2870-1, CD437, and CD2325. These later RRMs induce apoptosis in Jurkat cells in a tran-

scription-independent and caspase-dependent manner (54, 56) and are more powerful than the antagonist in inducing apoptosis. However, although they are strong inhibitors of IKK in vitro, in particular CD2325, they exert only a partial or cell type-selective inhibition of IKK activation in intact cells. This contrasts with the total inhibition of IKK by MX781 in all the cancer cell lines examined.

The discrepancy between a cell-free assay and the results obtained in cellular systems could be due to different reasons. The lack of total IKK inhibition in intact cells by the RARγ-selective RRMs could be attributed to chemical instability under cell culture conditions or intracellular metabolism. On the other hand, MX781 could be transformed into a more active compound within the cell. It is also possible that the selective RRMs bind to additional cellular targets, and therefore their activity in vivo cannot be expected to entirely compare to their in vitro inhibition of IKK activity. In any case, the concentration of MX781 necessary to induce apoptosis correlates with the effective concentration required to inhibit IKK, while the RARγ-selective RRMs are capable of inducing apoptosis at lower concentrations at which they exert no effect on IKK activity.

Taken together, these data suggest that interference with NF-κB-mediated survival signals might be one major pathway leading to the induction of apoptosis by MX781. In contrast, CD2325- and MX3350-1-mediated cell death appears to rely on the activation or inhibition of other signaling cascades, such as the activation of JNK and p38 mitogen-activated protein kinases (54). The inhibition of IKK activity seems to be of lesser relevance for the proapoptotic activity of such compounds in certain cell types in which they elicit partial inhibition of IKK but strong induction of apoptosis. Interestingly, not all RRMs that induce apoptosis are capable of inhibiting IKK, consistent with the assumption that they can target other pathways for the induction of cell death. However, all RRMs as well as other structurally unrelated molecules that inhibit IKK also elicit a strong antiproliferative activity and induce caspase-dependent apoptosis in prostate carcinoma cells (PC3) and non-small cell lung carcinoma cells (A549). In further support of a causal role of IKK/NF-κB inhibition in the induction of apoptosis, we observed that overexpression of IKKβ or IκBα mutants that block NF-κB activity is sufficient to induce cell death in PC3 and A549 cells.

Since NF-κB transcription factors are involved in several other disorders, in particular inflammatory and autoimmune diseases (6), inhibition of NF-κB activity by targeting IKK predicts the potential use of such inhibitors for treatment of additional disorders. Recently, the IKK inhibitor aspirin has been reported to reverse insulin resistance, and this effect is related to the inhibition of IKK (79). Molecules based on the structures reported here that inhibit IKK could therefore also serve as potential antidiabetic drugs. Taken together, we have identified here new retinoid-related structures that exhibit anti-IKK activities. These structures can serve as important leads for a new series of IKKα and IKKβ inhibitors that have applications in several major diseases.

ACKNOWLEDGMENTS

Research support was provided by grants from the NIH (CA 75033 to F.J.P., CA 55681 to M.P., and AI 43477 to M.K.) and the California Cancer Research Program (00-00778V-20253 to F.J.P. and 99-00529V-10249 to M.K.). Y.B. was partially supported by a postdoctoral fellowship from the Basque Government of Spain.

We thank Renata Hasim for technical assistance, Mireille Delhase for the IKK expression vectors, and Galderma Research Inc. for providing retinoids. We also thank Marion Sauter and Shamim Sheikh for secretarial assistance.

REFERENCES

1. **Alpert, D., P. Schwenger, J. Han, and J. Vilcek.** 1999. Cell stress and MKK6b-mediated p38 mitogen-activated protein kinase activation inhibit tumor necrosis factor-induced IκB phosphorylation and NF-κB activation. J. Biol. Chem. **274:**22176–22183.
2. **Altucci, L., A. Rossin, W. Raffelsberger, A. Reitmair, C. Chomienne, and H. Gronemeyer.** 2001. Retinoic acid-induced apoptosis in leukemia cells is mediated by paracrine action of tumor-selective death ligand TRAIL. Nat. Med. **7:**680–686.
3. **Arlt, A., J. Vorndamm, S. Muerkoster, H. Yu, W. E. Schmidt, U. R. Folsch, and H. Schafer.** 2002. Autocrine production of interleukin 1 beta confers constitutive nuclear factor κB activity and chemoresistance in pancreatic carcinoma cell lines. Cancer Res. **62:**910–916.
4. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
5. **Barkett, M., and T. D. Gilmore.** 1999. Control of apoptosis by Rel/NF-κB transcription factors. Oncogene **18:**6910–6924.
6. **Barnes, P. J., and M. Karin.** 1997. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. **336:**1066–1071.
7. **Beg, A. A., and D. Baltimore.** 1999. An essential role for NF-κB in preventing TNF-α-induced cell death. Science **274:**782–784.
8. **Bernard, A., J.-M. Bernardon, C. Delescluse, B. Martin, M.-C. Lenoir, J. maignan, B. Charpentier, W. R. Pilgrim, U. Reichert, and B. Shroot.** 1992. Identification of synthetic retinoids with selectivity for human nuclear RAR γ. Biochem. Biophys. Res. Commun. **186:**977–983.
9. **Biswas, D. K., A. P. Cruz, E. Gansberger, and A. B. Pardee.** 2000. Epidermal growth factor-induced nuclear factor κB activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. Proc. Natl. Acad. Sci. USA **97:**8542–8547.
10. **Biswas, D. K., S. C. Dai, A. Cruz, B. Weiser, E. Graner, and A. B. Pardee.** 2001. The nuclear factor κB (NF-κB): a potential therapeutic target for estrogen receptor negative breast cancers. Proc. Natl. Acad. Sci. USA **98:** 10386–10391.
11. **Bond, M., A. J. Chase, A. H. Baker, and A. C. Newby.** 2001. Inhibition of transcription factor NF-κB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. Cardiovasc. Res. **50:**556–565.
12. **Chen, Z., J. Hagler, V. J. Palombella, F. Melandri, D. Scherer, D. Ballard, and T. Maniatis.** 1995. Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Genes Dev. **9:**1586–1597.
13. **Chu, Z. L., T. A. McKinsey, L. Liu, J. J. Gentry, M. H. Malim, and D. W. Ballard.** 1997. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proc. Natl. Acad. Sci. USA **94:**10057–10062.
14. **Cogswell, P. C., D. C. Guttridge, W. K. Funkhouser, and A. S. Baldwin, Jr.** 2000. Selective activation of NF-κB subunits in human breast cancer: potential roles for NF-κB2/p52 and for Bcl-3. Oncogene **19:**1123–1131.
15. **Deveraux, Q. L., and J. C. Reed.** 1999. IAP family proteins—suppressors of apoptosis. Genes Dev. **13:**239–252.
16. **DiDonato, J., F. Mercurio, C. Rosette, J. Wu-Li, H. Suyang, S. Ghosh, and M. Karin.** 1996. Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. Mol. Cell. Biol. **16:**1295–1304.
17. **DiDonato, J. A., M. Hayakawa, D. M. Rothwarf, E. Zandi, and M. Karin.** 1997. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature **388:**548–554.
18. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
19. **Donjerkovic, D., C. M. Mueller, and D. W. Scott.** 2000. Steroid- and retinoid-mediated growth arrest and apoptosis in WEHI-231 cells: role of NF-κB, c-Myc and CKI p27(Kip1). Eur. J. Immunol. **30:**1154–1161.
20. **Fanjul, A., P. D. Hobbs, L. Jong, J. F. Cameron, E. Harlev, G. Graupner, X.-P. Lu, M. I. Dawson, and M. Pfahl.** 1994. A novel class of retinoids with selective anti-AP-1 activity exhibit anti-proliferative activity. Nature **372:** 107–111.
21. **Fanjul, A. N., F. J. Piedrafita, H. Al-Shamma, and M. Pfahl.** 1998. Apoptosis induction and potent antiestrogen receptor-negative breast cancer activity in vivo by a retinoid antagonist. Cancer Res. **58:**4607–4610.
22. **Feinman, R., K. O. Clarke, and L. E. Harrison.** 2002. Phenylbutyrate-induced apoptosis is associated with inactivation of NF-κB in HT-29 colon cancer cells. Cancer Chemother. Pharmacol. **49:**27–34.
23. **Gerritsen, M. E., A. J. Williams, A. S. Neish, S. Moore, Y. Shi, and T. Collins.** 1997. CREB-binding protein/p300 are transcriptional coactivators of p65. Proc. Natl. Acad. Sci. USA **94:**2927–2932.

24. **Grumont, R. J., I. J. Rourke, and S. Gerondakis.** 1999. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes Dev. **13:**400–411.
25. **Guttridge, D. C., C. Albanese, J. Y. Reuther, R. G. Pestell, and A. S. Baldwin, Jr.** 1999. NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol. Cell. Biol. **19:**5785–5799.
26. **Holmes-McNary, M., and A. S. Baldwin, Jr.** 2000. Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the IκB kinase. Cancer Res. **60:**3477–3483.
27. **Holmes, W. F., M. I. Dawson, R. D. Soprano, and K. J. Soprano.** 2000. Induction of apoptosis in ovarian carcinoma cells by AHPN/CD437 is mediated by RARs. J. Cell. Physiol. **185:**61–67.
28. **Hsu, C. A., A. K. Rishi, X. Su-Li, T. M. Gerald, M. I. Dawson, C. Schiffer, U. Reichert, B. Shroot, G. C. Poirer, and J. A. Fontana.** 1997. Retinoid induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway. Blood **89:**4470–4479.
29. **Hu, Y., V. Baud, T. Oga, K. I. Kim, K. Yoshida, and M. Karin.** 2001. IKKα controls formation of the epidermis independently of NF-κB. Nature **410:** 710–714.
30. **Huang, S., A. DeGuzman, C. D. Bucana, and I. J. Fidler.** 2000. Nuclear factor-κB activity correlates with growth, angiogenesis, and metastasis of human melanoma cells in nude mice. Clin. Cancer Res. **6:**2573–2581.
31. **Huang, S., C. A. Pettaway, H. Uehara, C. D. Bucana, and I. J. Fidler.** 2001. Blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Oncogene **20:**4188–4197.
32. **Huang, S., J. B. Robinson, A. DeGuzman, C. D. Bucana, and I. J. Fidler.** 2000. Blockade of nuclear factor-κB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer Res. **60:**5334–5339.
33. **Kapahi, P., T. Takahashi, G. Natoli, S. R. Adams, Y. Chen, R. Y. Tsien, and M. Karin.** 2000. Inhibition of NF-κB activation by arsenite through reaction with a critical cysteine in the activation loop of IκB kinase. J. Biol. Chem. **275:**36062–36066.
34. **Karin, M.** 1999. The beginning of the end: IκB kinase (IKK) and NF-κB activation. J. Biol. Chem. **274:**27339–27342.
35. **Karin, M., Y. Cao, F. R. Greten, and Z. W. Li.** 2002. NF-κB in cancer: from innocent bystander to major culprit. Nat. Rev. Cancer **2:**301–310.
36. **Koch, A. E., P. J. Polverini, S. L. Kunkel, L. A. Harlow, L. A. DiPietro, V. M. Elner, S. G. Elner, and R. M. Strieter.** 1992. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science **258:**1798–1801.
37. **Lee, F. S., R. T. Peters, L. C. Dang, and T. Maniatis.** 1998. MEKK1 activates both IκB kinase alpha and IκB kinase beta. Proc. Natl. Acad. Sci. USA **95:**9319–9324.
38. **Li, Q., Q. Lu, J. Y. Hwang, D. Buscher, K. F. Lee, J. C. Izpisua-Belmonte, and I. M. Verma.** 1999. IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev. **13:**1322–1328.
39. **Li, Q., D. Van Antwerp, F. Mercurio, K. F. Lee, and I. M. Verma.** 1999. Severe liver degeneration in mice lacking the IκB kinase 2 gene. Science **284:**321–325.
40. **Li, Z. W., W. Chu, Y. Hu, M. Delhase, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin.** 1999. The IKKβ subunit of IκB kinase (IKK) is essential for nuclear factor κB activation and prevention of apoptosis. J. Exp. Med. **189:**1839–1845.
41. **Lind, D. S., S. N. Hochwald, J. Malaty, S. Rekkas, P. Hebig, G. Mishra, L. L. Moldawer, E. M. Copeland III, and S. Mackay.** 2001. Nuclear factor-κB is upregulated in colorectal cancer. Surgery **130:**363–369.
42. **Liu, Z. G., H. Hsu, D. V. Goeddel, and M. Karin.** 1996. Dissection of TNF-receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell **87:**565–576.
43. **Lu, X. P., A. N. Fanjul, N. Picard, M. Pfahl, D. Rungta, K. Nared-Hood, B. Carter, F. J. Piedrafita, S. Tang, E. Fabbrizio, and M. Pfahl.** 1997. Novel RRMs as apoptosis inducers and effective inhibitors of human lung cancer cells in vivo. Nat. Med. **3:**686–690.
44. **Malinin, N. L., M. P. Boldin, A. V. Kovalenko, and D. Wallach.** 1997. MAP3K-related kinase involved in NF-κB induction by TNF-, CD95 and interleukin-1. Nature **385:**540–544.
45. **Marchetti, P., N. Zamzami, B. Joseph, S. Schraen-Maschke, C. Mereau-Richard, P. Costantini, D. Metivier, S. A. Susin, G. Kroemer, and P. Formstecher.** 1999. The novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. Cancer Res. **59:**6257–6266.
46. **Mayo, M. W., and A. S. Baldwin.** 2000. The transcription factor NF-κB: control of oncogenesis and cancer therapy resistance. Biochim. Biophys. Acta **1470:**M55–M62.
47. **Micheau, O., S. Lens, O. Gaide, K. Alevizopoulos, and J. Tschopp.** 2001. NF-κB signals induce the expression of c-FLIP. Mol. Cell. Biol. **21:**5299–5305.
48. **Miura, M., H. Zhu, R. Rotello, E. A. Hartwieg, and J. Yuan.** 1993. Induction of apoptosis in fibroblasts by interleukin-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. Cell **75:**653–660.
49. **Na, S. Y., B. Y. Kang, S. W. Chung, S. J. Han, X. Ma, G. Trinchieri, S. Y. Im, J. W. Lee, and T. S. Kim.** 1999. Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NF-κB. J. Biol. Chem. **274:**7674–7680.
50. **Na, S. Y., S. K. Lee, S. J. Han, H. S. Choi, S. Y. Im, and J. W. Lee.** 1998. Steroid receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor κB-mediated transactivations. J. Biol. Chem. **273:**10831–10834.
51. **Nakshatri, H., P. Bhat-Nakshatri, D. A. Martin, R. J. J. Goulet, and G. W. J. Sledge.** 1997. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol. **17:**3629–3639.
52. **Olson, N. E., J. Kozlowski, and M. A. Reidy.** 2000. Proliferation of intimal smooth muscle cells. Attenuation of basic fibroblast growth factor 2-stimulated proliferation is associated with increased expression of cell cycle inhibitors. J. Biol. Chem. **275:**11270–11277.
53. **Oridate, N., M. Higuchi, S. Suzuki, B. Shroot, W. K. Hong, and R. Lotan.** 1997. Rapid induction of apoptosis in human C33A cervical carcinoma cells by the synthetic retinoid 6-[3-(1-adamantyl)hydroxyphenyl]-2-naphtalene carboxylic acid (CD437). Int. J. Cancer **70:**484–487.
54. **Ortiz, M. A., F. J. Lopez-Hernandez, Y. Bayon, M. Pfahl, and F. J. Piedrafita.** 2001. Retinoid-related molecules induce cytochrome *c* release and apoptosis through activation of c-Jun NH_2-terminal kinase/p38 mitogen-activated protein kinases. Cancer Res. **61:**8504–8512.
55. **Perkins, N. D., L. K. Felzien, J. C. Betts, K. Leung, D. H. Beach, and G. J. Nabel.** 1997. Regulation of NF-κB by cyclin-dependent kinases associated with the p300 coactivator. Science **275:**523–527.
56. **Piedrafita, F. J., and M. Pfahl.** 1997. Retinoid-induced apoptosis and Sp1 cleavage occur independently of transcription and require caspase activation. Mol. Cell. Biol. **17:**6348–6358.
57. **Reuther, J. Y., and A. S. J. Baldwin.** 1999. Apoptosis promotes a caspase-induced amino-terminal truncation of IκBα that functions as a stable inhibitor of NF-κB. J. Biol. Chem. **274:**20664–20670.
58. **Rossi, A., P. Kapahi, G. Natoli, T. Takahashi, Y. Chen, M. Karin, and M. G. Santoro.** 2000. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature **403:**103–108.
59. **Sasaki, N., T. Morisaki, K. Hashizume, T. Yao, M. Tsuneyoshi, H. Noshiro, K. Nakamura, T. Yamanaka, A. Uchiyama, M. Tanaka, and M. Katano.** 2001. Nuclear factor-κB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. Clin. Cancer Res. **7:**4136–4142.
60. **Scheinman, R. I., A. Gualberto, C. M. Jewell, J. A. Cidlowski, and A. S. Baldwin, Jr.** 1995. Characterization of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. Mol. Cell. Biol. **15:**943–953.
61. **Shao, Z.-M., M. I. Dawson, X. S. Li, A. K. Rishi, M. S. Sheikh, Q.-X. Han, V. Ordonez, B. Shroot, and J. A. Fontana.** 1995. p53 independent G_0/G_1 arrest and apoptosis induced by a novel retinoid in human breast cancer cells. Oncogene **11:**493–504.
62. **Sovak, M. A., R. E. Bellas, D. W. Kim, G. J. Zanieski, A. E. Rogers, and A. M. Traish.** 1997. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Investig. **100:**2952–2960.
63. **Stehlik, C., R. de Martin, I. Kumabashiri, J. A. Schmid, B. R. Binder, and J. Lipp.** 1998. Nuclear factor (NF-)-κB-regulated X-chromosome-linked *iap* gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. J. Exp. Med. **188:**211–216.
64. **Stein, B., and M. X. Yang.** 1995. Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-κB and C/EBP beta. Mol. Cell. Biol. **15:**4971–4979.
65. **Sudo, T., and M. Karin.** 2000. Assays for JNK and p38 mitogen-activated protein kinases. Methods Enzymol. **322:**388–392.
66. **Sun, S. Y., P. Yue, R. A. Chandraratna, Y. Tesfaigzi, W. K. Hong, and R. Lotan.** 2000. Dual mechanisms of action of the retinoid CD437: nuclear RAR-mediated suppression of squamous differentiation and receptor-independent induction of apoptosis in UMSCC22B human head and neck squamous cell carcinoma cells. Mol. Pharmacol. **58:**508–514.
67. **Sun, S. Y., P. Yue, and R. Lotan.** 2000. Implication of multiple mechanisms in apoptosis induced by the synthetic retinoid CD437 in human prostate carcinoma cells. Oncogene **19:**4513–4522.
68. **Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma.** 1996. Suppression of TNF-alpha-induced apoptosis by NF-κB. Science **274:** 787–789.
69. **Wang, C. Y., J. C. J. Cusack, R. Liu, and A. S. J. Baldwin.** 1999. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-κB. Nat. Med. **5:**412–417.
70. **Wang, C. Y., D. C. Guttridge, M. W. Mayo, and A. S. Baldwin.** 1999. NF-κB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Moll. Cell. Biol. **19:**5923–5929.
71. **Wang, C. Y., M. W. Mayo, and A. S. J. Baldwin.** 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science **274:**784–787.
72. **Wang, C. Y., M. W. Mayo, R. G. Korneluk, D. V. Goeddel, and A. S. J. Baldwin.** 1998. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science **281:**1680–1683.

73. **Wang, W., J. L. Abbruzzese, D. B. Evans, and P. J. Chiao.** 1999. Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated RelA. Oncogene **18:**4554–4563.
74. **Wang, W., J. L. Abbruzzese, D. B. Evans, L. Larry, K. R. Cleary, and P. J. Chiao.** 1999. The nuclear factor-κB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin. Cancer Res. **5:**119–127.
75. **Wu, M., H. Lee, R. E. Bellas, S. L. Schauer, M. Arsura, D. Katz, M. J. FitzGerald, T. L. Rothstein, D. H. Sherr, and G. E. Sonenshein.** 1996. Inhibition of NF-κB/Rel induces apoptosis of murine B cells. EMBO J. **15:**4682–4690.
76. **Yamamoto, Y., M. J. Yin, K. M. Lin, and R. B. Gaynor.** 1999. Sulindac inhibits activation of the NF-κB pathway. J. Biol. Chem. **274:**27307–27314.
77. **Yang, J., and A. Richmond.** 2001. Constitutive IκB kinase activity correlates with nuclear factor-κB activation in human melanoma cells. Cancer Res. **61:**4901–4909.
78. **Yin, M. J., Y. Yamamoto, and R. B. Gaynor.** 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-beta. Nature **396:**77–80.
79. **Yuan, M., N. Konstantopoulos, J. Lee, L. Hansen, Z. W. Li, M. Karin, and S. E. Shoelson.** 2001. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of IKKβ. Science **293:**1673–1677.
80. **Zandi, E., and M. Karin.** 1999. Bridging the gap: composition, regulation, and physiological function of the IκB kinase complex. Mol. Cell. Biol. **19:** 4547–4551.
81. **Zong, W. X., L. C. Edelstein, C. Chen, J. Bash, and C. Gelinas.** 1999. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNF-α-induced apoptosis. Genes Dev. **13:**382–387.