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Circumvention of Nuclear Factor κB-Induced Chemoresistance by Cytoplasmic-Targeted Anthracyclines

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ABSTRACT

Nuclear factor κB (NF-κB) has been implicated in inducible chemoresistance against anthracyclines. In an effort to improve the cytotoxicity of anthracyclines while reducing their cardiotoxic effects, we have developed a novel class of extranuclear-localizing 14-*O*-acylanthracyclines that bind to the phorbol ester/diacylglycerol-binding C1b domain of conventional and novel protein kinase C (PKC) isoforms, thereby promoting an apoptotic response. Because PKCs have been shown to be involved in NF-κB activation, in this report, we determined the mechanism of NF-κB activation by *N*-benzyladriamycin-14-valerate (AD 198) and *N*-benzyladriamycin-14-pivalate (AD 445), two novel 14-*O*-acylanthracylines. We show that the induction of NF-κB activity in response to drug treatment relies on the activation of PKC-δ and NF-κB–activating kinase (NAK), independent of ataxia telengectasia mutated and p53 activities. In turn, NAK activates the IKK complex through phosphorylation of the IKK-2 subunit. We find that neither NF-κB activation nor ectopic expression of Bcl-X_L confers protection from AD 198-induced cell killing. Overall, our data indicate that activation of novel PKC isoforms by cytoplasmic-targeted 14-*O*-acylanthracyclines promotes an apoptotic response independent of DNA damage, which is unimpeded by inducible activation of NF-κB.

Anthracyclines, as exemplified by doxorubicin (DOX), comprise a class of antineoplastic agents widely used for the treatment of cancer because of their broad spectrum of activity. Although they are reported to produce many cellular effects, it is widely believed that their principal mechanism of cytotoxicity involves the production of DNA damage through intercalating with DNA, as well as by inhibiting topoisomerase II (topo II), thereby causing DNA double-strand breaks (DSBs) and promoting apoptosis (Burden and Osheroff, 1998). The development of cellular resistance mechanisms in response to DOX-induced apoptosis include P-glycoprotein and Bcl-2 overexpression, altered topo II activity, and loss of p53 function (Lothstein et al., 2001). In addition, recent studies have illustrated a novel mechanism of induced chemoresistance to topoisomerase-interactive agents derived from ATM-mediated induction of NF-κB in response to DSBs (Baldwin, 2001; Shiloh and Kastan, 2001).

NF-κB is a dimeric transcription factor that is expressed ubiquitously. Classic NF-κB is a heterodimer of p50 (NF-κB1) and p65 (RelA) subunits sequestered in the cytoplasm through interaction with inhibitory κB (IκB) proteins, of which IκB-α is the most well characterized (Barkett and Gilmore, 1999). In response to genotoxic stress, IκB-α is phosphorylated at serine residues 32 and 36 by the IKK complex, which includes two catalytic subunits, IKK-1 (IKK-α) and IKK-2 (IKK-β), and a regulatory subunit, IKK-γ.

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ABBREVIATIONS: DOX, doxorubicin; NF-κB, nuclear factor κB; IκB, inhibitor κB; PKC, protein kinase C; ATM, ataxia telengectasia mutated; NAK, nuclear factor κB-activating kinase; IKK, inhibitor κB kinase; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; URE, upstream regulatory region; Oct-1, octamer-1; A-T, ataxia telengectasia mutated; AD 198, *N*-benzyladriamycin-14-valerate; AD 445, *N*-benzyladriamycin-14-pivalate; AD 288, *N*-benzyladriamycin; topo II, topoisomerase II; DSB, double-strand break; HEK, human embryonic kidney; FBS, fetal bovine serum; TNF, tumor necrosis factor; siRNA, small interfering RNA; wt, wild type; dn, dominant negative; OD, optical density; PMSF, phenylmethylsulfonyl fluoride; PMA/I, 50 ng/ml PMA, phorbol 12-myristate 13-acetate/2 μg/ml ionomycin; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leuleucinal; NAK K/M, dominant-negative mutant of nuclear factor κB-activating kinase; IKK-1 K→M, dominant-negative form of inhibitor κB kinase-1; IKK-2 K→M, dominant-negative form of inhibitor κB kinase-2; IB, immunoblotting.

This event is required for subsequent ubiquitination and degradation of IκB-α through the proteasome pathway (Ghosh and Karin, 2002). After IκB-α degradation, NF-κB translocates to the nucleus in which it activates multiple genes, including antiapoptotic genes (Barkett and Gilmore, 1999), thereby promoting resistance to anticancer chemotherapy (Baldwin, 2001). In this regard, inhibition of NF-κB activation using either degradation-resistant IκB-α mutants or proteasome-targeted inhibitors has been shown to ameliorate the response to cancer chemotherapy (Baldwin, 2001).

In an effort to improve the cytotoxicity of anthracyclines while reducing their cardiotoxic effects, we are developing a novel class of extranuclear-localizing 14-*O*-acylanthracyclines that, despite being structurally related to DOX, display markedly distinct mechanisms of action (Israel et al., 1987; Lothstein et al., 2000). Thus, AD 198 and AD 445 rapidly localize in the cytoplasm, bind directly to the phorbol ester/diacylglycerol (DAG)-binding C1b domains of conventional and novel protein kinase C (PKC) isoforms (Roaten et al., 2001), and thereby directly promote an apoptotic response (Barrett et al., 2002). Consequently, inhibition of AD 198-mediated PKC-δ activation using the pharmacological inhibitor rottlerin results in protection from AD 198-induced apoptosis of 32D cells (Barrett et al., 2002). We have also shown that cell-killing by AD 198 and AD 445, but not DOX, is unaffected by cellular resistance mediated by overexpression of the P-glycoprotein and multidrug resistance protein multidrug transporters, as well as resistance caused by reduced topo II activity and the overexpression of Bcl-2 (Lothstein et al., 2001; Barrett et al., 2002). Given the antiapoptotic properties of NF-κB in response to antineoplastic agents, and the ability of AD 198 to directly stimulate PKC activity, in this report, we have examined whether treatment with AD 198 and AD 445 would activate NF-κB through PKCs and whether this activation would elicit an antiapoptotic response. Previously, PKC has been implicated in the regulation of NF-κB activity in response to the microtubule interactive agents vinca alkaloids and taxanes (Das and White, 1997), but the specific PKC isoform mediating this effect was not identified.

Here, we show that PKC-δ, but not -α or -ϵ, promotes NF-κB activation, presumably through sequential activation of an NAK to IKK signaling pathway in response to 14-*O*-acylanthracycline drug treatment. Importantly, we find that the inability of NF-κB to confer protection from 14-*O*-acylanthracycline–induced cell death is caused by circumvention of the antiapoptotic properties of the putative NF-κB downstream target Bcl-X_L. Overall, our data characterize a novel mechanism of inducible activation of NF-κB by extranuclear-targeted acylanthracyclines but one that does not confer protection from their novel cytotoxic effects.

Materials and Methods

Cell Culture and Treatment Conditions. Murine embryo fibroblasts (10)1 (Harvey and Levine, 1991) and 3T3 RelA and 3T3 RelA −/− (Beg et al., 1995) were grown in high-glucose Dulbecco's modified Eagle's medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) with 10% FBS (Invitrogen, Carlsbad, CA). HEK 293 cells were grown in RPMI 1640 medium (Cellgro; Mediatech, Herndon, VA) with 10% FBS. Simian virus 40-transformed human fibroblast lines from a healthy A-T heterozygote (GM00637) and a patient with A-T (GM09607) were obtained from Coriell Cell Repositories (Camden, NJ) and grown at low passage in minimum essential medium plus Earle's balanced salt (Sigma, St. Louis, MO) with 15% FBS. Porcine renal epithelial cells LLC-PK_1 expressing a Tet-off inducible PKC-α or PKC-δ were a kind gift from James Mullin (Lankenau Medical Research Center, Wynnewood, PA) (Rosson et al., 1997; Mullin et al., 1998). Murine IL-3–dependent myeloid 32D.3 cells expressing high levels of human Bcl-X_L were a generous gift of John Cleveland (St. Jude Children's Research Hospital, Memphis, TN). Murine 263 cells were derived from a hepatocellular carcinoma of a transforming growth factor-α/c-*myc* bitransgenic mouse and were cultivated in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum.

Where indicated, cells were treated with either AD 198, AD 288 (Lothstein et al., 1992b), or DOX (Sigma) dissolved in DMSO. As a control, cells were incubated with carrier DMSO solution or 20 ng/ml TNF-α (Sigma). For treatment, cells were also incubated for the indicated periods of time with 100 to 900 nM calphostin, 200 μM calpeptin (both from Calbiochem, San Diego, CA), or 50 ng/ml phorbol ester plus 2 μg/ml ionomycin (Sigma). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assays were conducted as specified in the manufacturer's instructions (Promega, Madison, WI).

Transfection Conditions. For transient transfection, HEK 293 cells were plated at low confluence in 96-well plates and transfected in triplicate with a solution of DNA and LipofectAMINE reagent according to the manufacturer's instructions (Invitrogen). Twenty-four hours after transfection, cells were treated with 5 μM AD 198, 5 μM AD 445, or DMSO carrier solution for 16 h. Cells were harvested after treatment according to the manufacturer's protocol in the Dual-Luciferase Reporter assay system (Promega), and lysates were analyzed with a Labsystems Luminoskan 96-well plate luminometer (Thermolab System, Needham Heights, MA). Firefly luciferase activity was normalized for Renilla luciferase activity, and results were expressed as a ratio (fold induction) of normalized luciferase activities in treated cells versus vehicle-treated cells.

Plasmids, siRNA, and Adenoviruses. The kinase dead mutant NAK (K→M) was a kind gift from Makoto Nakanishi (Nagoya City University Medical School, Nagoya, Japan) (Tojima et al., 2000). The GST-IKK-2 plasmid was a generous gift from Thomas Wirth (Wurzburg University, Wurzburg, Germany) (Baumann et al., 2000). The vector expressing the dominant-negative (dn) mutant IKK-2 (IKK SS/AA) has been described previously (Mercurio et al., 1997).

The siRNA specific for PKC-ϵ was purchased from Xeragon, Qiagen (Valencia, CA) and was lipofected into (10)1 cells according to the manufacturer's specifications. The double-stranded RNA sequence was synthesized from the following DNA target sequence in PKC-ϵ: 5′-AATGGCCTTCTTAAGATCAAA-3′. Upon Basic Local Alignment Search, this sequence did not display significant homology with other known genes (data not shown).

The adenoviral vectors expressing wild-type (wt) IKK-1, wt IKK-2, or dominant-negative forms of IKK-1 (IKK-1 K→M) or IKK-2 (IKK-2 K→M) have been described previously (Arsura et al., 2003). Virus stocks were amplified to high titer (Quantum Biotechnologies, Montreal, Canada). The concentration of viral particles was determined as described previously (Arsura et al., 2003).

EMSA. Nuclear extracts were prepared as described previously (Arsura et al., 2000). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The sequences of the URE-κB– and Octamer-1 (Oct-1)–containing oligonucleotides are as follows: URE-κB, 5′-AAGTCCGGGTTTTC-CCCAACC-3′; and Oct-1, 5′-TGTCGAATGCAAATCACTAGAA-3′. EMSA was performed as described previously (Arsura et al., 2000) using approximately 2 ng of labeled oligonucleotide (20,000 dpm) and 5 μg of nuclear extracts, and complexes were resolved in 4.5% polyacrylamide gels. Optical density (OD) was measured using NIH Image 1.63 Software.

Immunoblot Analysis and Kinase Assays. For isolation of whole-cell extracts, cells were resuspended at 4°C in extraction

buffer (40 mM Tris, pH 8, 500 mM NaCl, 6 mM EDTA, 6 mM EGTA, 10 mM glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 1 mM dithiothreitol, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.5% NP-40), sonicated briefly, and incubated on ice for 30 min. Extracts were then cleared by centrifugation at 40,000 rpm for 30 min at 4°C. Samples (20–40 μg) were subjected to electrophoresis on a 10% SDS-polyacrylamide gel electrophoresis, and immunoblotting was performed as described previously (Arsura et al., 1996). The antibody preparations for IκB-α (sc-371), actin (sc-1615), and hemagglutinin-A (sc-805), were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody specific for PKC-ε was obtained from BD Biosciences Transduction Laboratories (Lexington, KY). The anti-Flag (F4042) antibody was purchased from Sigma. The anti-Rabbit IgG (12–370) was purchased from Upstate Biotechnology (Lake Placid, NY). The antibody against the phosphorylated form of IκB-α at serines 32 and 36 was purchased from Cell Signaling Technology Inc. (Beverly, MA). The antibody preparation specific for phospho-IKK1/IKK-2 was obtained from Cell Signaling Technology Inc.

Subcellular Fractionation. For isolation of the total protein fraction, AD 198-treated cells were sonicated in lysis buffer (25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 500 μM PMSF, and 1 μg/ml pepstatin A). Aliquots of these samples were subjected to centrifugation at 52,000 rpm for 60 min at 4°C to separate the cytosolic fraction from the pelleted membrane and detergent-insoluble fractions. The pellet was resuspended and sonicated in lysis buffer plus 1% Triton X-100 and centrifuged at 20,000 rpm for 15 min at 4°C to separate the membrane fraction from the detergent-insoluble fraction. This latter fraction was resuspended in 2× SDS-polyacrylamide gel electrophoresis sample buffer and boiled to dissolve the pellet. Samples were then subjected to immunoblotting analysis as described above.

NAK Kinase Assay. For the kinase assay, 293 cells were transfected with Flag-tagged NAK or pcDNA control vector. Cells were treated with the indicated doses of AD 198 or 50 ng/ml phorbol 12-myristate 13-acetate/2 μg/ml ionomycin (PMA/I) for the indicated time points the day after the transfection. Cells were lysed in extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween-20, 0.2% NP-40, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 2 μg/ml pepstatin A, and 5 μg/ml leupeptin), and immunoprecipitations were performed with approximately 500 μg of cell extracts. Cell extracts were precleared with Protein A/G beads (Oncogene Research Products, San Diego, CA) for 1 h followed by incubation for 2 h with 8 μg of polyclonal anti-Flag antibody. The protein antibody complexes were then captured by the addition of Protein A/G beads for 1 h. The immunoprecipitated proteins were immediately subjected to kinase assays using GST-IKK-2 as substrate essentially as described previously (Arsura et al., 2000).

PKC-ε Kinase Assay. Untreated or treated cells were lysed by sonication in cell buffer plus 10 μg/ml phenanthroline. Samples were centrifuged at 40,000 rpm at 4°C for 30 min. The supernatant was retained and used for immunoprecipitation with a monoclonal PKC-ε antibody (BD Biosciences). The PKC-ε–enriched immunoprecipitates (250 μg) were subsequently subjected to kinase assays carried out for 10 min at 30°C in a reaction buffer containing 20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 20 μg/ml leupeptin, 0.1 mM ATP, and 1 μCi P^{32}-γATP in the presence of 5 μg of a PKC-ε specific peptide substrate purchased from Calbiochem.

Results

14-*O*-Acylanthracyclines Activate NF-κB in Multiple Cell Lines Independent of p53 and ATM Activities. To assess NF-κB DNA binding activity in response to acylanthracycline drug treatment, we performed EMSA analyses from nuclear extracts of various cell lines treated with AD 198 or AD 445 for the indicated time points. As shown in Fig. 1A, AD 198 treatment of MCF-7 breast cancer cells resulted in the induction of NF-κB DNA binding activity at the 3- and 4-h time points. Similarly, AD 198 treatment enhanced NF-κB DNA binding activity in the human prostate cancer cell line LNCaP (Fig. 1B) and in the Snu387 and SK-Hep-1 human hepatocellular carcinoma cells (Fig. 1C). Furthermore, treatment of 3T3 cells with 5 μM AD 445 induced NF-κB DNA binding activity to an extent comparable with that seen upon treatment with AD 198 (Fig. 1D). Thus, acylanthracycline congeners promote NF-κB activation in multiple human epithelial carcinomas.

Because we have shown previously that AD 198 and AD 445 localize almost exclusively in the cytoplasm (Lothstein et al., 2001), we asked whether nuclear events associated with DNA DSBs would be dispensable for NF-κB activation by AD 198 and AD 445 treatment. Indeed, treatment of p53 null 10(1) cells with AD 198 or AD 445 led to induction of NF-κB DNA binding activity (Fig. 2A) with kinetics similar to those observed in cells expressing a functional p53 gene (Fig. 1) (data not shown). Thus, p53 activity, which has been shown previously to promote activation of NF-κB through mitogen-activated protein kinase signaling (Ryan et al., 2000), is not required for NF-κB activation by 14-*O*-acylanthracyclines. Furthermore, the observation that both the antioxidant pyrrolidinedithiocarbamate and the protein synthesis inhibitor cycloheximide could not inhibit NF-κB activation by AD 198 strongly indicates that the induction of NF-κB by 14-*O*-acylanthracycline–activated PKCs is not caused by autocrine secretion of a cytokine or by the induction of oxidative stress after drug treatment.

Next, we sought to assess the degree of AD 198- and AD 445-mediated activation of NF-κB in cells lacking functional

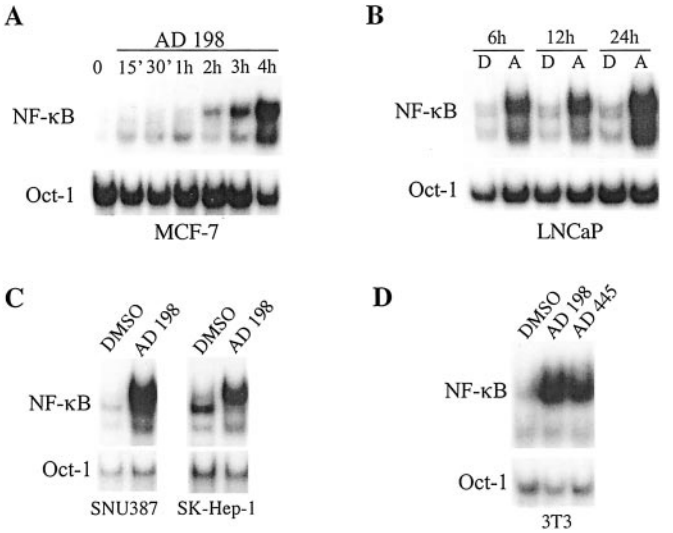

Fig. 1. 14-*O*-Acylanthracyclines activate NF-κB DNA binding activity in various epithelial carcinoma cell lines. Human breast cancer MCF-7 (A) and human prostate cancer LNCaP cells (B) were treated with 5 μM AD 198 or DMSO vehicle for the indicated times. C, human hepatocellular carcinoma SNU387 and SK-Hep-1 cell lines were treated with 5 μM AD 198 for 5 h. D, NIH 3T3 cells were treated with 5 μM AD 198 or 5 μM AD 445 for 6 h. Nuclear extracts (5 μg) were subjected to EMSAs using the upstream (URE) NF-κB element from the c-*myc* gene as probe. Equal protein loading was assessed using the Oct-1 oligonucleotide.

ATM, which is essential for NF-κB activation in response to DNA DSBs (Piret et al., 1999; Li et al., 2001; Panta et al., 2004a). As expected, treatment of wild-type GM00637 fibroblasts with 5 μM DOX, AD 198, or AD 445 for 6 h promoted activation of NF-κB DNA binding activity (Fig. 2B). As we have shown previously (Panta et al., 2004a), DOX-treated A-T GM09607 fibroblasts failed to display NF-κB activation (Fig. 2B). In contrast, treatment of A-T cells with AD 198 or AD 445 resulted in efficient activation of NF-κB (Fig. 2B), although the levels of NF-κB activation in response to AD 198 seemed slightly reduced. Thus, ATM is dispensable for the activation of NF-κB by extranuclear-localizing 14-*O*-acylanthracyclines.

The IKK Complex Is Required for NF-κB Activation by AD 198 and AD 445. Because the canonical pathway of NF-κB activation involves phosphorylation and degradation of IκB-α, we sought to determine the effects of AD 198 treatment on IκB-α phosphorylation. As shown in Fig. 3A, we detected phosphorylation of IκB-α at serines 32 and 36 in (10)1 cells treated for 1 h with AD 198. Furthermore, treatment of (10)1 cells with the proteasome inhibitors MG132 or lactacystin abolished the AD 198-elicited increase in NF-κB DNA binding activity (data not shown), indicating that IκB-α phosphorylation and degradation is required for NF-κB activation in response to AD 198 treatment.

To assess the role of the IKK complex during AD 198-mediated activation of NF-κB, we treated HEK 293 cells with AD 198 and assessed the levels of activation of the IKK complex using an antibody specific for the phosphorylated forms of IKK-1 and IKK-2 raised against serine 180 and 181, respectively, within their activation loop. We detected a significant enhancement of IKK phosphorylation levels after 1 h of AD 198 treatment that continued to increase up to the 3-h time point (Fig. 3B), consistent with the kinetics of IκB-α phosphorylation and NF-κB activation.

To determine the functionality of IKK complex activation, we infected (10)1 cells with 10 plaque-forming units of adenoviral constructs expressing wt or dn forms of IKK-1 (IKK-1 K→M) or IKK-2 (IKK-2 K→M) (Mercurio et al., 1997). In whole-cell extracts of these cells, we observed similar levels of expression of the various wild-type and mutant ectopic IKKs (data not shown). After 24 h of infection, cells were treated for 5 h with 5 μM AD 198 (Fig. 3C). As judged by EMSA, expression of IKK-2 K→M blocked the induction of NF-κB compared with adenoviral vectors containing green fluorescent protein-infected cells or cells expressing wt IKK-1 or wt IKK-2 (Fig. 3C). In contrast, levels of NF-κB DNA binding activity in response to AD 198 treatment were not significantly affected by ectopic expression of IKK-1 K→M, which suggests that AD 198-mediated induction of NF-κB relies predominantly on the IKK-2 subunit. In good agreement with previous reports (Mercurio et al., 1997; Li et al.,

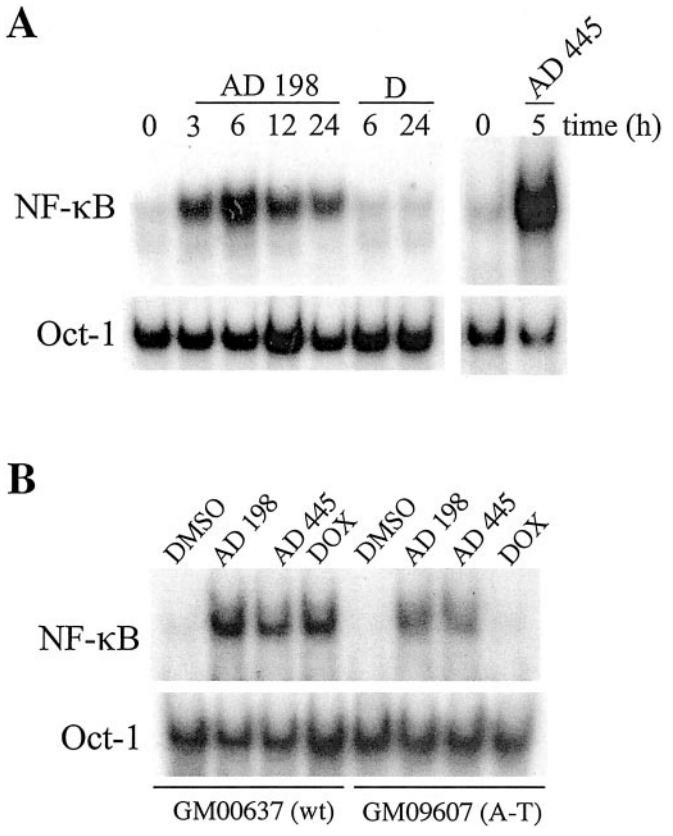

Fig. 2. p53 and ATM are not required for acylanthracycline-mediated activation of NF-κB. A, p53 null (10)1 murine embryo fibroblasts were treated for the indicated times with 5 μM AD 198, 5 μM AD 445, or the equivalent DMSO (D) vehicle volume. To measure the levels of NF-κB DNA binding activity, nuclear extracts (5 μg) were subjected to EMSA as described for Fig. 1. B, wild-type (GM00637) and A-T (GM09607) fibroblasts were treated for 5 h with 5 μM AD 198, AD 445, or DOX. NF-κB DNA binding activity was determined by EMSA as described previously.

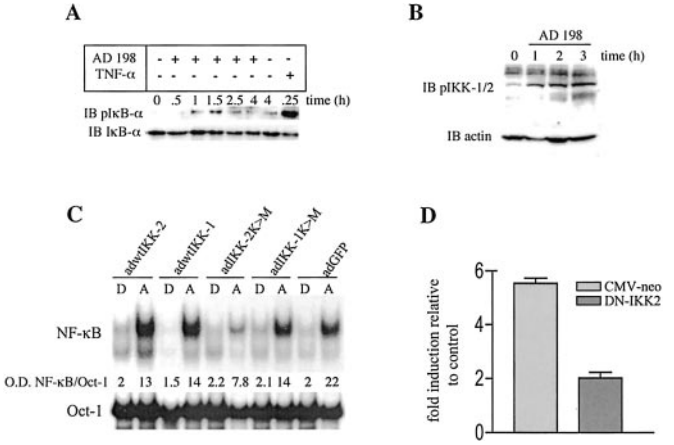

Fig. 3. AD 198 elicits NF-κB activation through the IKK complex. A, IκB-α is phosphorylated in response to AD 198 treatment. (10)1 murine embryoblasts were treated with 200 μM calpeptin for 1 h, followed by treatment with DMSO carrier solution, 5 μM AD 198, or 20 ng/ml TNF-α for the indicated times. Whole-cell extracts (30 μg) were subjected to immunoblotting (IB) using an antibody that recognizes IκB-α phosphorylated at serines 32 and 36 (top). The same blot was also hybridized with an antibody against IκB-α (bottom). B, HEK 293 cells were treated for the indicated times with 5 μM AD 198. Whole-cell extracts (30 μg) were subjected to IB using an antibody that recognizes IKK1/2 phosphorylated at serines180 and 181, respectively (top). The same blot was also hybridized with an antibody against actin (bottom). C, IKK-2 is required for NF-κB activation by AD 198. (10)1 cells were infected with adenoviral constructs directing the expression of wt or dn IKK-1 or IKK-2 and green fluorescent protein for 24 h. Infected cells were treated for 5 h with 5 μM AD 198 (A) or DMSO vehicle (D). D, HEK 293 cells were plated in duplicate in p35 plates and transfected by lipofection with 2 μg of kb-luciferase construct and 50 ng of pRL-SV40 in the presence of 1 μg of a pCMV-neo parental vector or a vector directing expression of a dominant-negative IKK-2 mutant (DN-IKK2). Twenty-four hours after transfection, cells were treated with either DMSO or 5 μM AD 198 for 16 h. Luciferase assays were performed using the Dual-Luciferase Reporter Kit (Promega) and a Labsystems luminometer (Thermolab System). Values are expressed as the fold induction of luciferase activity relative to control. The figure represents the means and standard deviation of three independent experiments, each carried out in duplicate.

1999; Arsura et al., 2003), IKK-2 K→M but not IKK-1 K>M affected NF-κB activation in response to TNF-α treatment (data not shown).

Similarly, ectopic expression of the IKK-2 SS/AA dominant-negative mutant that had been shown previously to block the IKK complex activation by TNF-α, and Ras (Mercurio et al., 1999; Arsura et al., 2000) inhibited AD 198-mediated induction of κB luciferase activity compared with vector-transfected cells (Fig. 3D). Thus, activation of the IKK complex is required for induction of NF-κB by AD 198.

PKC-δ Mediates the Activation of NF-κB by AD 198. Because PKCs have been implicated in NF-κB activation in response to phorbol esters (Tojima et al., 2000), and given the ability of AD 198 to interact with the C1b-DAG/phorbol ester binding domain of novel and conventional PKC isoforms (Roaten et al., 2001), we sought to determine whether AD 198 treatment promoted activation of NF-κB through PKCs. To determine the involvement of PKCs in NF-κB activation by AD 198, we treated cells with the broad PKC inhibitor calphostin C and examined NF-κB DNA binding activity after AD 198 treatment. As shown in Fig. 4A, treatment with calphostin C resulted in a dose-dependent inhibition of NF-κB DNA binding activity. In contrast, calphostin C did not prevent NF-κB activation by the catalytic inhibitor of topo II AD 288.

Because we have shown that AD 198 and AD 445 treatment can activate PKC-δ (Barrett et al., 2002), we assessed whether this other novel PKC isoform would mediate NF-κB activation. Indeed, rottlerin, a specific PKC-δ inhibitor, inhibited approximately 50% of the activation of NF-κB by AD 198 but not by PMA/I (Fig. 4B). Furthermore, rottlerin treatment attenuated the increase in IKK complex phosphorylation levels after AD 198 but not after PMA/I treatment (Fig. 4C).

To further explore the role of PKC-δ during AD 198-mediated activation of NF-κB, we measured NF-κB DNA binding activity in AD-198 treated porcine renal epithelial LLC-PK_1 cells that express PKC-δ in a tetracycline Tet-off system (Mullin et al., 1998). As predicted by our previous data, Tet-mediated repression of PKC-δ protein levels resulted in a significant decrease of NF-κB activation after AD 198 treatment but not after PMA/I treatment (Fig. 4D). The lack of inhibition of PMA/I-mediated NF-κB activation after repression of PKC-δ could be the consequence of a compensatory mechanism by other signaling pathways elicited by ionomycin treatment. In contrast, tetracycline treatment of LLC-PK_1 cells that express PKC-α in a tetracycline Tet-off system (Rosson et al., 1997) did not affect levels of NF-κB activity in response to AD 198 treatment (Fig. 4E). Thus, PKC-δ, but not -α, is involved in NF-κB activation by 14-*O*-acylanthracyclines. In addition, PKC-δ is not required for PMA/I-mediated activation of NF-κB in this cell type.

Because PKC-ε has been shown to mediate NF-κB activation by phorbol esters (Tojima et al., 2000), we asked whether PKC-ε would also play a role during NF-κB activation by AD 198. First, we assessed PKC-ε translocation to the membrane fraction, a hallmark of PKC-ε activation (Koide et al., 1992). After 30 min of AD 198 treatment, we observed a significant increase of PKC-ε in the membrane fraction of AD 198-treated (10)1 cells that started to decrease at the 3-h time point (Fig. 5A). This decrease was probably caused by the accumulation of PKC-ε in the detergent-insoluble fraction. Thus, the accumulation of PKC-ε in the membrane-associated protein fraction after AD 198 treatment is the result of translocation to the membrane. Consistent with the translocation data, PKC-ε–mediated phosphorylation of a substrate peptide was up-regulated in response to AD 198 treatment (Fig. 5B) with kinetics consistent with those seen in the translocation assay. Thus, AD 198 drug treatment induces PKC-ε kinase activity.

To assess whether PKC-ε was involved in the activation of NF-κB in response to AD 198 treatment, we used RNA interference to disrupt PKC-ε gene expression and measured the levels of NF-κB activation in response to AD 198 treatment. Surprisingly, silencing the expression of PKC-ε (Fig. 5C, bottom) did not affect the activation of NF-κB after AD 198 treatment of (10)1 cells (Fig. 5C, top). Thus, PKC-δ, but not -α or -ε, mediates NF-κB activation in response to 14-*O*-acylanthracycline treatment in these cell types.

NAK Mediates IKK Complex Activation in Response to AD 198 Stimulation. Because treatment with phorbol esters has been shown to induce NF-κB activity through the NF-κB activating kinase (NAK) (Tojima et al., 2000), we sought to determine whether NAK is activated in response to AD 198 treatment. For this purpose, whole-cell extracts of untreated or AD 198-treated HEK 293 cells that had been transfected with a vector directing the expression of Flag-tagged NAK or empty-backbone pcDNA vector were immunoprecipitated with an anti-Flag antibody and subjected to kinase assays using GST-IKK-2 as substrate. As reported previously (Tojima et al., 2000), overexpression of NAK resulted in high levels of basal phosphorylation (Fig. 6A), presumably caused by autophosphorylation. Importantly, NAK phosphorylation was further enhanced after AD 198 or PMA/I treatment (Fig. 6A). Furthermore, we observed a 3-fold increase of NAK-mediated phosphorylation of GST-IKK2 in response to AD 198 treatment, with kinetics consistent with those of NAK phosphorylation (Fig. 6A). To determine whether NAK acts downstream of PKC-δ, we assessed the levels of NAK phosphorylation by AD 198 in the presence or absence of the PKC-δ inhibitor rottlerin. For this purpose, Flag-NAK immunoprecipitants from HEK 293 cells that had been treated with 5 μM AD 198 for 1 h in the absence or presence of 10 μM rottlerin were subjected to kinase assay. To reduce the background levels of NAK phosphorylation in untreated cells, we performed a 10-min kinase reaction in the presence of nonradioactive ATP, which was followed by a 10-min kinase reaction in the presence of P^{32}-γATP. As shown in Fig. 6B, we observed a strong induction of NAK phosphorylation levels after AD 198 treatment that was reversed by pretreatment with rottlerin. Thus, PKC-δ kinase activity is required for the induction of NAK phosphorylation levels after AD 198 stimulation. Consistent with the idea that NAK is an upstream regulator of NF-κB transcriptional activity in response AD 198 stimulation, cotransfection of a dominant-negative mutant of NAK (NAK K/M) that has previously been shown to inhibit PMA-driven activation of NF-κB (Tojima et al., 2000) reduced the induction of κB-luciferase activity in response to AD 198 or PMA/I treatment (Fig. 6C). Furthermore, ectopic expression of NAK K/M, as well as pretreatment with rottlerin, inhibited the induction of IKK1/2 phosphorylation levels after AD 198 treatment (Fig. 6D). Thus, NAK acts downstream of PKC-δ and medi-

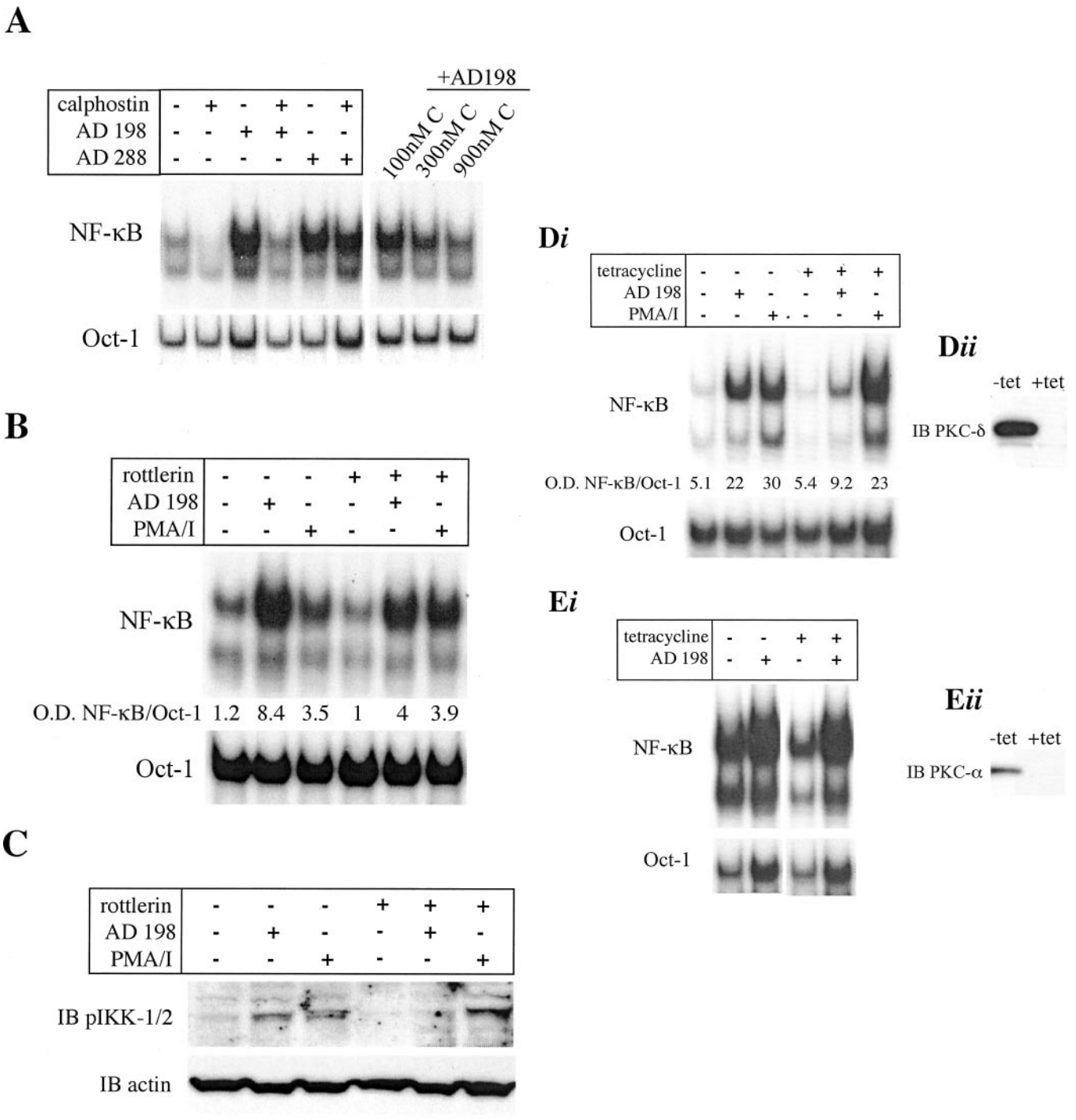

Fig. 4. PKC-δ is a mediator of NF-κB activation in response to AD 198. A, (10)1 cells were treated for 5 h with DMSO, 5 μM AD 198, or 5 μM AD 288, or for 1 h with PMA/I, in the absence or presence of 600 nM (unless otherwise indicated) calphostin (C) pretreatment for 1 h. Nuclear extracts (5 μg) were subjected to EMSA as described in the legend to Fig. 1. B, (10)1 cells were treated with DMSO only or 10 μM rottlerin, a specific PKC-δ inhibitor, for 2 h followed by treatment with DMSO or 5 μM AD 198 for 5 h or PMA/I for 1 h. Nuclear extracts were isolated, and EMSA was performed as described in the legend to Fig. 1. C, HEK 293 cells were treated for 3 h with 5 μM AD 198 or for 1 h with PMA/I in the absence or presence of 10 μM rottlerin. Whole-cell extracts (50 μg) were subjected to IB using an antibody that recognizes IKK1/2 phosphorylated at serines 180 and 181, respectively (top). The same blot was also hybridized with an antibody against actin (bottom). D*i*, porcine renal epithelial LLC-PK$_1$ cells that express PKC-δ in a tetracycline Tet-off system were cultured in the presence or absence of 1 μg/ml tetracycline. Subsequently, cells were treated for 3 h with either 5 μM AD 198 or DMSO vehicle, or for 1 h with PMA/I. Nuclear extracts (5 μg) were subjected to EMSA as described above. D*ii*, whole-cell extracts were obtained from PKC-δ–LLC-PK$_1$ cells cultured in the presence and absence of tetracycline and subjected to IB using an antibody against PKC-δ. E*i*, LLC-PK$_1$ cells that express PKC-α in a Tet-off system were cultured in the presence or absence of 1 μg/ml tetracycline. Subsequently, cells were treated for 3 h with 5 μM AD 198 or DMSO. Nuclear extracts (5 μg) were subjected to EMSA as described above. E*ii*, whole-cell extracts were obtained from PKC-α–LLC-PK$_1$ cells cultured for 24 h in the presence and absence of tetracycline and subjected to IB using an antibody for PKC-α.

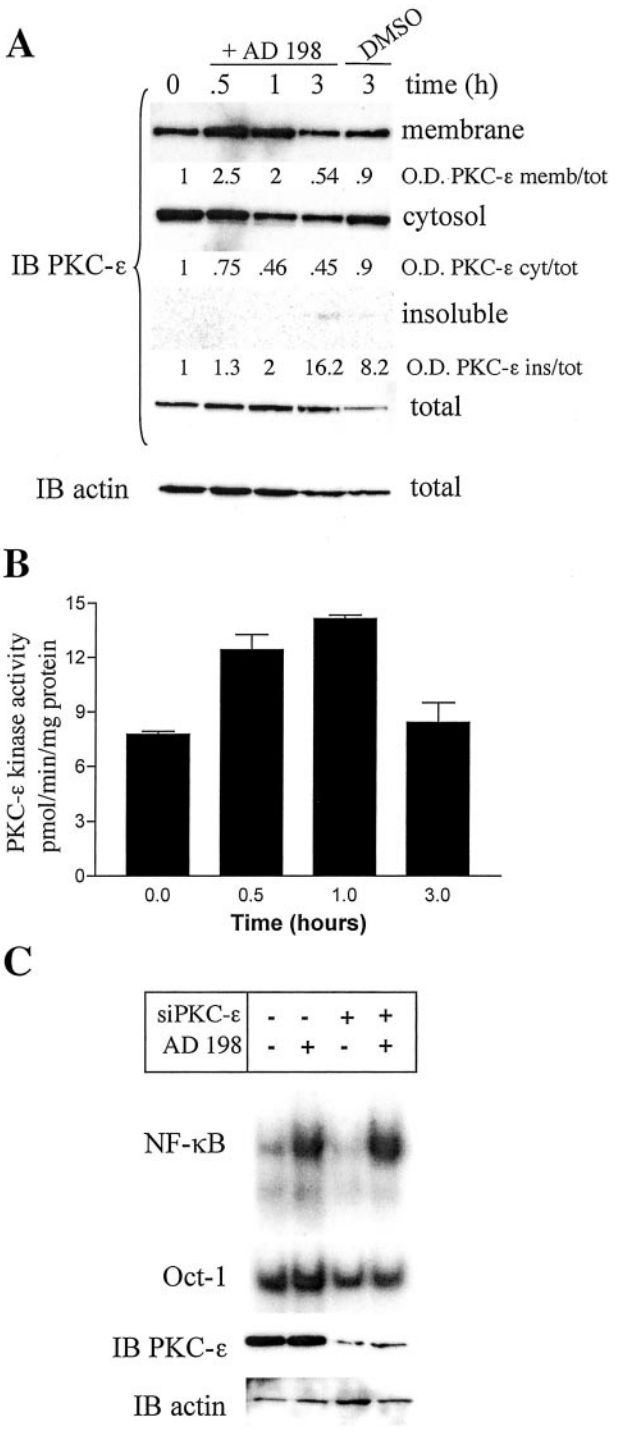

Fig. 5. PKC-ε is not involved in NF-κB activation in response to AD 198 treatment. A, (10)1 cells were treated with 5 μM AD 198 or DMSO for the indicated times. After subcellular fractionation as described under *Materials and Methods*, cytosolic, membrane, insoluble, and total protein fractions were subjected to IB for PKC-ε or for actin. Bands were quantified by densitometric analysis and were expressed as the ratio of the OD of PKC-ε in each fraction to that of total PKC-ε. B, (10)1 cells were treated with 5 μM AD 198 for the indicated times. Whole-cell extracts were isolated, and immunoprecipitation of PKC-ε was carried out. The PKC-ε–enriched immunoprecipitates were subjected to kinase assays in triplicate using a PKC-ε–specific peptide substrate. This graph represents two independent experiments. Error bars represent standard deviations. C, (10)1 cells were lipofected with PKC-ε siRNA (siPKC-ε). Twenty-four hours after transfection, cells were treated with 5 μM AD 198 or DMSO for 5 h. Nuclear extracts and whole-cell extracts were subjected to EMSA (top two) or IB using antibodies against PKC-ε or actin (bottom two).

ates the induction of NF-κB DNA binding and transcriptional activities by AD 198 through activation of the IKK complex.

NF-κB and Bcl-X_L Do Not Protect from AD 198-Induced Cell Death. Because we found a more pronounced rate of cell death in DOX-treated RelA null cells compared with that of wild-type 3T3 cells that were similarly treated (Panta et al., 2004a), we asked whether inducible activation of NF-κB by AD 198 would result in enhanced cell survival. Surprisingly, AD 198 treatment killed wild-type 3T3 cells to the same extent of RelA-deficient cells (Fig. 7A). Furthermore, adenoviral-mediated expression of the dominant-negative IKK-2 K→M mutant did not enhance AD 198- or AD 445-mediated cell killing of murine hepatocellular carcinoma cells or (10)1 embryoblasts (Fig. 7B) (data not shown). Thus, RelA-mediated cell survival, and presumably that of other Rel family members whose regulation relies on the activation of the IKK complex, is circumvented by the cytotoxic action of extranuclear-targeted 14-*O*-acylanthracyclines. In addition, inhibition of NF-κB by dn IKK-2 did not enhance the percentage of AD 198-treated HEK 293 cells arrested in the G_2/M phase compared with control cells (data not shown). Therefore, inhibition of NF-κB is unable to rescue AD 198-treated cells from cell-cycle arrest.

Because inducible activation of NF-κB in response to genotoxic stress has been shown to promote chemoresistance through transactivation of multiple antiapoptotic genes (Barkett and Gilmore, 1999), we asked whether the induction of cell death by AD 198 would be affected by constitutive expression of the NF-κB antiapoptotic target gene Bcl-X_L. To address this issue, we compared the extent of AD 198-mediated cell death of wild-type 32D.3 myeloid cells to that of 32D.3 cells stably expressing ectopic Bcl-X_L. As a positive control, we examined the extent of cell death by the topo II poison DOX and the topo II catalytic inhibitor AD 288 in the same cell line. Stable expression of Bcl-X_L resulted in complete protection of 32.D.3 cells from either DOX or AD 288 treatment (Fig. 7C). In contrast, ectopic expression of Bcl-X_L did not rescue 32.D.3 cells from AD 198-induced apoptosis (Fig. 7C). Thus, AD 198-mediated cytotoxicity can circumvent both NF-κB– and Bcl-X_L–induced chemoresistance.

Discussion

In this report, we have identified a novel signaling pathway of cytoplasmic-localizing 14-*O*-acylanthracyclines that involves activation of PKC-δ, NAK, and IKK complex kinase activities resulting in the induction of NF-κB. We report that the cytotoxic effects of AD 198 and AD 445 circumvent both NF-κB and Bcl-X_L–mediated chemoresistance. Thus, we propose a model in which direct activation of PKC-δ in response to treatment with extranuclear-localizing anthracyclines, such as AD 198 and AD 445, promotes an apoptotic response that is independent of DNA damage and is unimpeded by the inducible activation of NF-κB.

Previously, PKCs have been implicated in vinca alkaloid- and taxane-mediated activation of NF-κB (Das and White, 1997). However, the authors of this study did not address the mechanism of PKC activation nor did they determine the specific PKC isoform(s) mediating this effect. Our study clearly identifies PKC-δ as being involved in inducible activation of NF-κB by the novel cytotoxic agents. This finding is entirely reasonable given that PKC-δ belongs to the subfam-

ily of novel PKCs, which are activated upon DAG binding to their C1b domain (Toker, 1998), the molecular site of which has been identified as a target for AD 445 and AD 198 by molecular modeling studies (Roaten et al., 2001). Drug binding promotes PKC translocation to the membrane, which then results in downstream phosphorylation of specific substrates (Roaten et al., 2002).

Interestingly, we show that PKC-ϵ, which was previously shown to mediate the induction of NF-κB by phorbol ester (Tojima et al., 2000), does not seem to be involved in NF-κB activation by AD 198. Furthermore, we did not address the possibility that the atypical PKC isoforms ζ and λ (Lallena et al., 1999) might also be involved in NF-κB activation in response to drug treatment because they display only one C1b binding domain, which cannot be accessed by phorbol esters or DAG (Storz and Toker, 2003). However, from our data, we cannot rule out the possibility that other PKC isoforms, such as PKC-θ and protein kinase D, which can be activated by phorbol ester and have been implicated in the activation of NF-κB (Genot et al., 1995; Rozengurt et al., 1995; Storz and Toker 2003), might also participate in NF-κB induction by 14-*O*-acylanthracyclines in a cell-type specific manner. Furthermore, we show that AD 198 and PMA/I activate NF-κB through different pathways. The observation that PKC-δ is dispensable for NF-κB activation by PMA/I could be the consequence of a compensatory mechanism by other calcium-dependent signaling pathways elicited by ionomycin treatment such as calcineurin, calpain, and calmodulins, which have been implicated in the regulation of NF-κB activity (Ghosh and Karin, 2002). Interestingly, we show that both PMA/I and AD 198 elicited pathways that seem to converge on the downstream common mediator NAK (TBK/T2K), which has been shown previously to be required for phorbol ester-mediated activation of NF-κB (Tojima et al., 2000).

The function of this recently identified kinase during NF-κB activation remains controversial (Peters and Maniatis, 2001). Previously, NAK has been shown to form a ternary complex with TRAF2 and TANK that in turn induces NF-κB through activation of the IKK complex (Pomerantz and Baltimore, 1999). However, knockout studies have indicated that NAK promotes NF-κB transcriptional activity instead of inducing nuclear translocation through IκB-α degradation (Bonnard et al., 2000). Consistent with the phorbol esters and platelet-derived growth factor signaling pathways (Tojima et al., 2000), we show that after AD 198 treatment, NAK phosphorylates the IKK-2 subunit, which then presumably promotes NF-κB activation through IκB-α degradation. Furthermore, we did not detect enhanced Gal4-mediated transcription in AD 198-treated cells that had been transfected with an expression vector containing the transcrip-

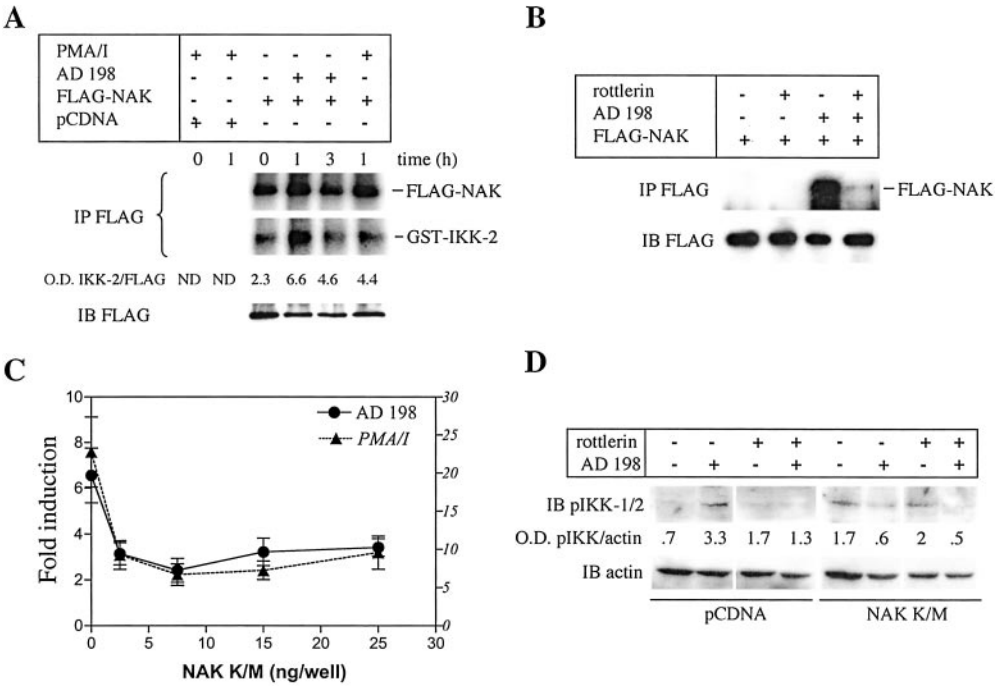

Fig. 6. NAK phosphorylates the IKK-2 subunit and promotes NF-κB transcriptional activation after acylanthracycline drug treatment. A,HEK 293 cells were transfected by lipofection with vectors directing the expression of Flag-tagged wild-type NAK (FLAG-NAK) or pcDNA empty vector. After 24 h, transfected cells were treated for the indicated times with 5 μM AD 198 or PMA/I. After immunoprecipitation (IP) with an antibody against Flag, kinase assay was performed using GST-IKK-2 as substrate (middle). The immunoprecipitants were subjected to IB with the M5-FLAG antibody (bottom). B, HEK 293 cells were transfected by lipofection as described above with a vector directing the expression of FLAG-NAK. After 24 h, transfected cells were treated for 1 h with 5 μM AD 198 in the absence or presence of 10 μM rottlerin. After IP with an antibody for Flag, kinase assay was performed to assess levels of NAK phosphorylation. Equal aliquots of the immunoprecipitants were subjected to IB as above. C, HEK 293 cells were plated in triplicate in 96-well plates and transfected by lipofection with 100 ng of kb-luciferase construct plus 2.5 ng pRL-SV40 in the presence of the indicated amount of a vector directing the expression of NAK K/M. After 16 h of treatment with 5 μM AD 198 or PMA/I, luciferase activity was determined as described in the legend to Fig. 3. Values are expressed as fold induction of luciferase activity of drug-treated cells relative to vehicle-treated cells. The values expressed on the left *y*-axis correspond to AD 198-treated cells. The values on the right *y*-axis represent PMA/I-treated cells. Means and standard deviations represent at least three independent experiments, each carried out in triplicate. D, HEK 293 cells were transfected by lipofection with vectors directing the expression of FLAG-NAK or pcDNA empty vector. After 24 h, transfected cells were treated for 1 h with 5 μM AD 198 in the absence or presence of 10 μM rottlerin. Whole-cell extracts (50 μg) were subjected to IB using an antibody that recognizes IKK1/2 phosphorylated at serines 180 and 181, respectively (top). The same blot was also hybridized with an antibody for actin (bottom). Bands were quantified by densitometric analysis and were expressed as the ratio of the OD of phosphorylated IKK1/2 to that of actin.

tional activation domain of RelA (TA1) fused to the DNA binding domain of Gal4 (data not shown). Thus, we conclude that in our cell system, NAK activates NF-κB through the activation of the IKK complex rather than by enhancing the transcriptional activity of RelA.

AD 198 and AD 445 are novel compounds that are functionally distinct from DNA DSB-inducing anthracyclines such as DOX and daunorubicin. They very rapidly accumulate in the cytoplasm, in which they bind to and activate conventional and novel PKCs (Roaten et al., 2001), thereby triggering an apoptotic response (Barrett et al., 2002). Previously, we have shown that 14-*O*-acylanthracyclines were able to circumvent multiple mechanisms of drug resistance, including the loss of p53 expression (G. Panta and M. Arsura, unpublished results), reduced topo II activity, and increased expression of P-glycoprotein or multidrug resistance protein (Lothstein et al., 1992a, 1994). Additionally, we found that 14-*O*-acylanthracyclines circumvented Bcl-X_L–, or Bcl-2–mediated cell survival (this report and Barrett et al., 2002, respectively). This latter effect is consistent with the ability of AD 198 and AD 445 to induce cell death through PKC-δ–induced mitochondrial depolarization and cytochrome *c* release independent of both the activation of the permeability transition pore and Ca^{2+} influx (Leonard Lothstein, personal communication). Here, we report that in addition to these other well-established mechanisms of cellular chemoresistance, the cytotoxic effect of 14-*O*-acylanthracyclines is unimpeded by inducible activation of NF-κB. This unexpected finding differs from what we have observed after DNA damage inflicted by the classic anthracycline DOX. In that instance, inhibition of ATM-mediated activation of NF-κB potentiated cell killing by DOX (Panta et al., 2004b). Thus, it is tempting to speculate that NF-κB–mediated cell survival can be circumvented by compounds such as 14-*O*-acylanthracy-

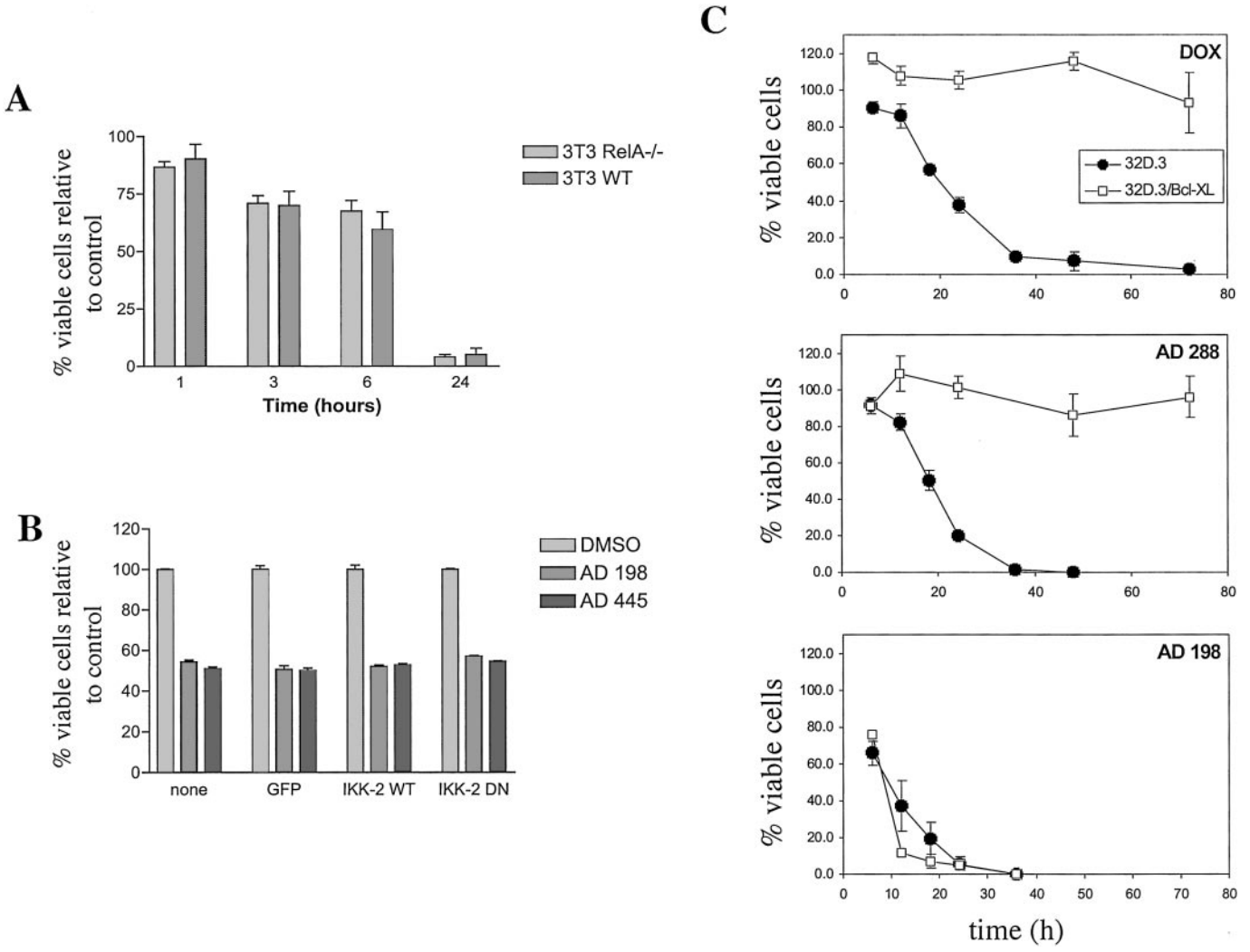

Fig. 7. AD 198- or AD 445-induced NF-κB activation has no significant effect on cell survival. A, cultures of wild-type and RelA null NIH 3T3 cells were incubated in medium containing 5 μM AD 198 or DMSO carrier solution for the indicated times. Cell viability was monitored by conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to its formazan product. Results are expressed as percentage of survival relative to DMSO-treated cells, which was set to 100%. Means and standard deviations represent at least three independent experiments, each carried out in triplicate. B, murine hepatocellular carcinoma 263 cells were either uninfected or infected with adenoviruses directing the expression of either green fluorescent protein, wt IKK-2, or dn IKK-2 and subsequently treated with DMSO vehicle, 0.25 μM AD 445, or 0.25 μM AD 198 for 24 h. Results are expressed as above. C, 32D.3 and 32D.3/Bcl-X_L cells were cultured at a density of 5×10^5 cells/ml and exposed to 5 μM of the indicated drug for 1 h at 37°C. Cells were resuspended in fresh, drug-free medium and incubated at 37°C for up to 72 h. At the times indicated, aliquots of cells were withdrawn and stained with trypan blue. Viable cells were scored based on the exclusion of stain and gross morphological appearance. Each datum point represents the mean (±S.E.) of at least three independent determinations, each consisting of 300 to 500 cells per count.

clines that function independently of the expression, localization, and/or phosphorylation of anti-apoptotic Bcl-2 family members. Overall, our findings underscore the potential clinical relevance of extranuclear-targeted anthracyclines to current chemotherapeutic protocols and provide further impetus for their continued preclinical development.

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