Cisplatin Mimics ARF Tumor Suppressor Regulation of RelA (p65) Nuclear Factor-κB Transactivation

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Abstract

The RelA (p65) nuclear factor-κB (NF-κB) subunit can contribute towards tumor cell survival through inducing the expression of a variety of antiapoptotic genes. However, the NF-κB response can show great diversity and is not always antiapoptotic. Here, we find that cisplatin, a DNA cross-linking agent and commonly used anticancer compound, does not affect RelA nuclear translocation but modulates its transcriptional activity. Similar to other genotoxic agents, such as daunorubicin and UV light, cisplatin treatment in the U-2 OS osteosarcoma cell line represses RelA activity and inhibits expression of the NF-κB antiapoptotic target gene *Bcl-x$_L$*. The mechanism through which cisplatin achieves these effects is different to daunorubicin and UV light but shows great similarity to the RelA regulatory pathway induced by the ARF tumor suppressor: cisplatin regulation of RelA requires ATR/Chk1 activity, represses Bcl-x$_L$ but not XIAP expression, and results in phosphorylation of RelA at Thr^{505}. In contrast to these results, another chemotherapeutic drug etoposide activates NF-κB and induces expression of these target genes. Thus, within a single tumor cell line, there is great heterogeneity in the NF-κB response to different, commonly used chemotherapeutic drugs. These observations suggest that it might be possible to minimize the ability of RelA to inhibit cancer therapy by diagnostically predicting the type of chemotherapeutic drug most compatible with NF-κB functionality in a tumor cell type. Moreover, our data indicate that at least with respect to RelA, cisplatin functions as an ARF mimic. Other drugs capable of mimicking this aspect of ARF function might therefore have therapeutic potential. (Cancer Res 2006; 66(2): 929-35)

Introduction

Aberrantly active nuclear factor-κB (NF-κB) is associated with diseases of an inflammatory origin, such as rheumatoid arthritis and inflammatory bowel disease, as well as cancer (1–4). Moreover, NF-κB provides the link between chronic inflammation and tumor development, thought to cause up to 20% of human cancers (5–7). These effects result not only from NF-κB's ability to control the expression of genes required for inflammation and the immune response but also from its function as an important regulator of apoptosis and cellular proliferation (8, 9). NF-κB, and specifically the RelA (p65) subunit, is frequently considered an antiapoptotic protein due to the liver apoptosis and subsequent embryonic lethality found with *rela* null mice, resulting from increased sensitivity to tumor necrosis factor-α (TNF-α)–induced cell death (10). NF-κB has now been found to promote resistance to apoptosis by a wide variety of agents, which in addition to natural molecules, such as TNF, include a number of chemotherapeutic drugs and ionizing radiation (9). Therefore, in addition to a role in tumorigenesis, NF-κB is also thought to reduce the efficiency of cancer therapy (11). For these reasons, drugs targeting the NF-κB pathway are being actively investigated and developed as both anticancer and anti-inflammatory agents (12–14).

The antiapoptotic function of NF-κB results principally from its ability to transcriptionally induce the expression of antiapoptotic genes, such as *cIAP1, cIAP2, XIAP, Blf/A1*, and *Bcl-x$_L$* (9). However, in common with many other transcriptional regulators, NF-κB subunits do not always target the same genes regardless of activating stimuli. For example, RelA is not always antiapoptotic, and in some circumstances, the RelA subunit can induce the expression of proapoptotic target genes, such as *Fas, FasL*, and death receptors *DR4* and *DR5* (9). Moreover, we have recently shown that under some circumstances, RelA can repress the expression of antiapoptotic genes. We observed that atypical inducers of NF-κB activity, such as the chemotherapeutic agents daunorubicin and doxorubicin as well as UV-C light, resulted in RelA-dependent repression of Bcl-x$_L$, XIAP, and A20 gene expression (15). Daunorubicin and doxorubicin are structurally related topoisomerase II inhibitors and DNA interchelators, whereas UV-C treatment induces pyrimidine dimer cross-linking resulting in single-strand DNA breaks. These stimuli all induced RelA DNA binding, and chromatin immunoprecipitation analysis showed that RelA was promoter associated under these conditions. In contrast to those activators of RelA associated with increased expression of these genes, these atypical inducers resulted in the association of RelA with histone deacetylases (HDAC), which function as repressors of gene expression. Therefore, whether NF-κB prevents or facilitates apoptosis is stimulus, cell type, and context dependent (9).

In another work from our laboratory, we have discovered that the ARF tumor suppressor, although not an inducer of NF-κB DNA binding, modulates RelA transcriptional activity. Indeed, similar to the results described above, we find that ARF expression results in repression of the antiapoptotic gene *Bcl-x$_L$* by RelA through induced association with HDAC1 (16). Furthermore, ARF sensitizes cells to TNF-induced cell death (16). However, the effect of ARF on RelA was in other ways distinct to that of daunorubicin/doxorubicin and UV-C. Significantly, ARF-induced repression of NF-κB transcriptional activity requires the evolutionarily conserved Thr^{505} residue of RelA and the ATR/Chk1 kinase pathway (16, 17). We found that ARF modulates ATR/Chk1 activity, and that Chk1 phosphorylates Thr^{505} (17). In contrast,

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repression of transcription by RelA in response to daunorubicin/doxorubicin and UV-C does not require Thr[505] or ATR/Chk1, despite the fact that these DNA-damaging agents also activate Chk1 (15, 16).

These findings led us to hypothesize that there may be multiple classes of "repressor" RelA, with distinct properties (18). Furthermore, they indicated that the function of NF-κB in cancer, and RelA in particular, might be more complex than merely functioning as a tumor promoter (19). Indeed, NF-κB might function more analogously to a tumor suppressor under some circumstances, particularly in the early stages of tumorigenesis before the function of the ARF/p53 pathway is lost (19). Our data also suggested that the response of NF-κB to distinct cancer therapies might also vary, depending on the cell type and context. Although in the cell types we examined, daunorubicin/doxorubicin treatment resulted in a form of RelA that repressed transcription, we also observed that another topoisomerase II inhibitor (etoposide) induced the opposing form of NF-κB, at least in a reporter gene assay (15). Therefore, DNA damage per se does not result in the transcriptional repressor form of RelA. Rather, these observations, together with the results from our ARF studies, indicated that there is great potential for heterogeneity in the NF-κB response to cell stimuli. Given the role of NF-κB in tumorigenesis and apoptosis, such differing responses could profoundly affect the effectiveness of cancer therapies, particularly if NF-κB inhibitors come into common clinical usage. With this in mind, we have begun to extend our studies to investigate the precise mechanisms through which other chemotherapeutic agents affect NF-κB–dependent transcription. In this report, we have investigated the effects of cisplatin on RelA function. Cisplatin is a DNA cross-linking agent used in the treatment of solid tumors of testes, ovaries, and the head and neck (20). It is also used in the treatment of osteosarcoma (21). Cisplatin generates DNA adducts but unlike daunorubicin, doxorubicin, or etoposide does not inhibit topoisomerase II. Here, we show that similar to daunorubicin, cisplatin induces repression of NF-κB–dependent reporter gene and endogenous $Bcl\text{-}x_L$ expression. Surprisingly, this repression is distinct to that seen with daunorubicin and UV-C. Rather, cisplatin mimics the effects we had previously seen with ARF. In particular, cisplatin modulation of RelA is ATR/Chk1 dependent and requires phosphorylation of RelA at Thr[505].

Materials and Methods

Cells. U-2 OS osteosarcoma cells were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom) and were not grown beyond passage 35. NARF2 cells are a derivative of U-2 OS cells, which contain a stably integrated, isopropyl-L-thio-B-D-galactopyranoside (IPTG) inducible ARF expression plasmid and have been described previously (16, 22). HeLa 57A cells, which contain a chromosomally integrated NF-κB luciferase reporter plasmid, were grown and analyzed as previously described (23).

Where indicated, endogenous NF-κB activity was induced by 10 ng/mL TNF-α (Sigma, Gillingham, United Kingdom), 1 μmol/L daunorubicin (Affiniti, Exeter, United Kingdom), 4 μg/mL cisplatin (Sigma), or 40 J/m^2 UV-C (254 nm) using a Stratalinker (Stratagene, Amsterdam, the Netherlands). Endogenous Chk1 activity was inhibited by prestimulation with 1 μmol/L Gö6976 (Calbiochem, Nottingham, United Kingdom).

Antibodies. All antibodies have been described previously (15, 17), except the new goat anti-RelA phospho-T505 antibody. This antibody used the same peptide immunogen as described previously (17) and was raised by Pacific Immunology Corp. (Ramona, CA) as part of a collaboration with Active Motif. This antibody was purified using both phospho-peptide and non–phospho-peptide affinity chromatography. Peptide ELISA confirmed that the resulting purified antibody specifically recognizes the phosphorylated T505 epitope.

Other experimental procedures. All reporter plasmid luciferase assays, chromatin immunoprecipitation assays, semiquantitative PCR, immunofluorescence microscopy, electrophoretic mobility shift assay (EMSA), Western blots, and immunoprecipitations were done as described previously (15–17). All antibodies, plasmids, PCR primers, and small interfering RNAs (siRNA) have been described and characterized before (15–17). Luciferase assays were done according to the manufacturer's instructions (Promega, Southampton, United Kingdom), and results were normalized for protein concentration with all experiments done a minimum of three times before calculating means and SD as shown in the figures.

Results

Cisplatin represses NF-κB–dependent transcription. Treatment of human osteosarcoma U-2 OS cells with daunorubicin or doxorubicin results in active repression of transcription by the RelA subunit of NF-κB (15). Here, we extend these observations to another chemotherapeutic drug, cisplatin, which, although a DNA-damaging agent, is chemically and mechanistically distinct to these other compounds. It was a surprise, therefore, when treatment of U-2 OS cells with cisplatin for 8 hours repressed the activity of an NF-κB–responsive luciferase reporter plasmid to a magnitude similar to that seen with daunorubicin (Fig. 1*A*). In contrast, and as expected, TNF strongly induced NF-κB activity. These effects were dependent upon the κB elements present in this plasmid, suggesting a direct effect on NF-κB (Fig. 1*A*). Cisplatin treatment also repressed the activity of a chromosomally integrated, 3× κB luciferase reporter plasmid in HeLa 57A cells (Fig. 1*B*), which we have previously shown to be strongly activated by TNF (23).

Cisplatin represses $\mathbf{Bcl\text{-}x_L}$ but not XIAP expression. Previously, we had identified *XIAP* and $Bcl\text{-}x_L$ as key antiapoptotic genes induced by TNF and other typical stimuli but down-regulated by NF-κB in response to daunorubicin and UV light (15). Therefore, we next investigated whether cisplatin treatment also affected the expression of these genes. Significantly, after 4 hours of treatment, $Bcl\text{-}x_L$ mRNA became down-regulated in response to cisplatin, whereas *XIAP* was not (Fig. 1*C*). We have shown previously, using a RelA siRNA, that expression of $Bcl\text{-}x_L$ in U-2 OS cells is RelA dependent, in both unstimulated and stimulated cells (15, 16). We also observed similar specific down-regulation of $Bcl\text{-}x_L$ but not *XIAP* when investigating the effects of ARF on RelA function in NARF2 cells, a derivative of U-2 OS cells containing a stably integrated IPTG-inducible ARF expression plasmid (Fig. 1*D*). As previously, both UV-C and daunorubicin treatment repressed both *XIAP* and $Bcl\text{-}x_L$ expression (Fig. 1*D*). Interestingly, another DNA-damaging agent and topoisomerase II inhibitor, etoposide strongly induced both $Bcl\text{-}x_L$ and *XIAP* expression (Fig. 1*C*), in a similar manner to that seen with TNF (15). Therefore, DNA damage per se does not result in repression of these genes. Rather, the nature of the stimulus determines the effect seen; furthermore, effects on $Bcl\text{-}x_L$ and *XIAP* can be mechanistically distinct.

Cisplatin stimulation does not induce NF-κB DNA binding or nuclear translocation. Analysis of NF-κB DNA binding by EMSA revealed that cisplatin did not induce binding to the consensus HIV κB site over an 8-hour time course of stimulation in U-2 OS cells (Fig. 2*A*). In contrast, the other DNA-damaging agents daunorubicin and etoposide both robustly induced NF-κB DNA-binding in the same cells (Fig. 2*A*). Furthermore, immunofluorescence analysis confirmed that cisplatin, unlike UV-C–induced DNA damage, does not induce RelA nuclear localization;

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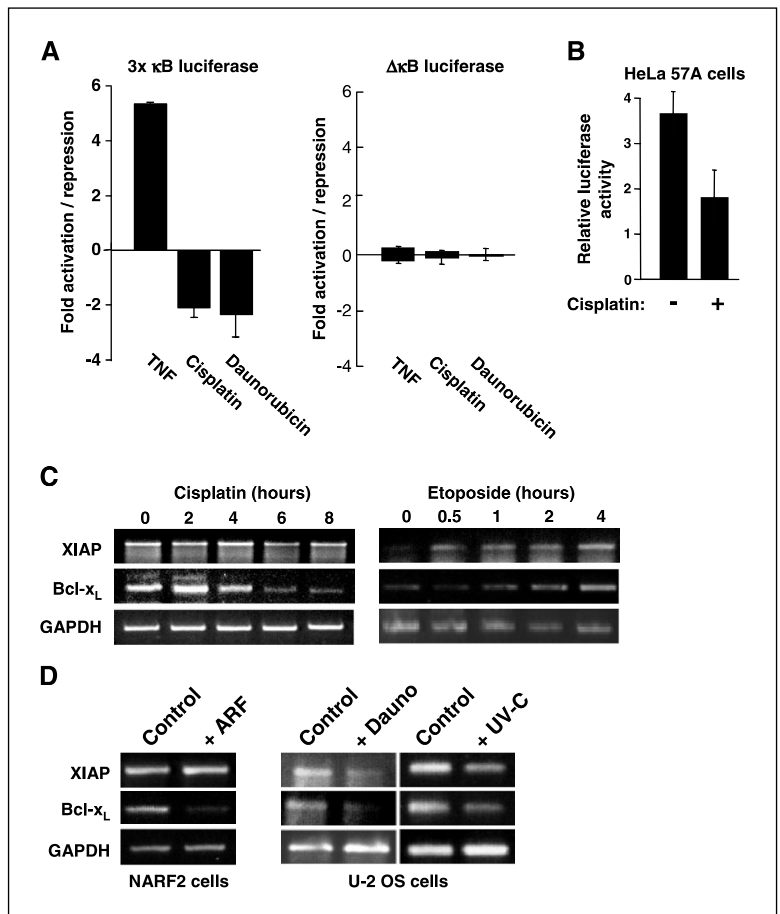

Figure 1. Cisplatin represses NF-κB–dependent transcription. *A,* TNF activates, whereas cisplatin and daunorubicin repress NF-κB reporter plasmid activity. U-2 OS cells were transfected with 2 μg of 3× κB concanavalin A luciferase NF-κB reporter plasmid or a control lacking κB sites as indicated. Cells were either unstimulated or stimulated for 8 hours with 10 ng/mL TNF, 4 μg/mL cisplatin, or 1 μmol/L daunorubicin, 36 hours after transfection. Fold activation or repression relative to levels seen in untreated controls. Normalized such that no change in luciferase activity has a value of 0. *B,* cisplatin represses NF-κB activity in HeLa cells. HeLa 57A cells, which contain a chromosomally integrated 3× κB reporter plasmid, were treated as in (*A*). *C-D,* cisplatin inhibits expression of *Bcl-x$_L$* but not *XIAP* mRNA. RNA was prepared from U-2 OS cells treated with either 4 μg/mL cisplatin or 15 μmol/L etoposide for the indicated times (*C*), 1 μmol/L daunorubicin, or 40 J/m^2 UV-C for 0 or 6 hours (*D, right*) or the U-2 OS cell derivative NARF2, where ARF was induced by 1 mmol/L IPTG for 0 or 24 hours (*D, left*). Semiquantitative PCR analysis was done using 5 ng total RNA, with primers specific to human *XIAP, Bcl-x$_L$,* or a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control.

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therefore, the failure to observe stimulation of DNA binding by EMSA does not reflect more subtle effects on NF-κB's affinity for its binding site (Fig. 2*B*). This effect was again reminiscent of the effects we had previously seen with ARF. Here, ARF does not induce NF-κB DNA binding but rather modulates the activity of the basal or induced NF-κB already present in the nucleus (16). This effect of ARF was confirmed by immunofluorescence analysis (Fig. 2*B*). We had previously observed that ARF was capable of repressing the transcriptional activity of TNF-induced NF-κB but did not affect TNF-induced NF-κB DNA binding (16). To determine whether cisplatin could exert a similar dominant effect on TNF-induced NF-κB, U-2 OS cells were treated with both stimuli. This experiment revealed that cisplatin does inhibit the transcriptional activity of TNF-induced NF-κB, although not completely (Fig. 2*C*). Moreover, cisplatin does not inhibit TNF-induced NF-κB DNA binding. In fact, a slight enhancement was typically observed (Fig. 2*D*). Supershift analysis also revealed that both the basal and induced levels of NF-κB in U-2 OS cells consisted primarily of RelA containing complexes (Fig. 2*D*). It should be noted that in U-2 OS cells, the ARF promoter is methylated; thus, the cells are functionally ARF null (24). In addition, we have confirmed that cisplatin treatment does not induce ARF expression in these cells (data not shown).

Consistent with the EMSA data (Fig. 2*A*), chromatin immunoprecipitation analysis showed that cisplatin treatment did not affect RelA occupancy of the *Bcl-x$_L$* promoter (Fig. 3). However, an increase in HDAC1 recruitment together with a concomitant decrease in histone H3 acetylation was observed (Fig. 3), providing a possible explanation for cisplatin-induced repression of *Bcl-xL* expression.

Cisplatin uses the same pathway as ARF to repress transcription through the RelA transactivation domain. We had previously observed that the effect of ARF, daunorubicin, and UV-C all occurred, at least in part, through regulation of the RelA transactivation domain (15–17). There are important differences, however, with ARF repression being dependent upon phosphorylation of Thr^{505} (16, 17). Mutation of this residue does not affect repression by daunorubicin and UV-C (15). Given the similarities between the effects of cisplatin and ARF on NF-κB described above, we decided to further investigate the mechanism through which cisplatin regulates NF-κB transactivation. Significantly, cisplatin treatment repressed, in a Thr^{505}-dependent manner, the activity of a Gal4 fusion with the RelA transactivation domain (amino acids 428-551) Gal4 RelA (TAD; Fig. 4*A*). We have also recently reported that ARF repression of RelA, but not that seen with daunorubicin or UV-C, is dependent upon the ATR/Chk1 checkpoint kinase pathway (17). It was therefore of great interest to observe that overexpression of a kinase-dead, dominant-negative ATR plasmid prevented cisplatin- but not UV-C–induced repression of an NF-κB–responsive luciferase reporter plasmid (Fig. 4*B*). Furthermore, siRNAs to ATR and Chk1 but not ATM inhibited cisplatin-induced repression of *Bcl-x$_L$* (Fig. 4*C*). Together, these results showed that similar to ARF, cisplatin uses an ATR/Chk1–dependent pathway for repression by RelA. Cisplatin is a

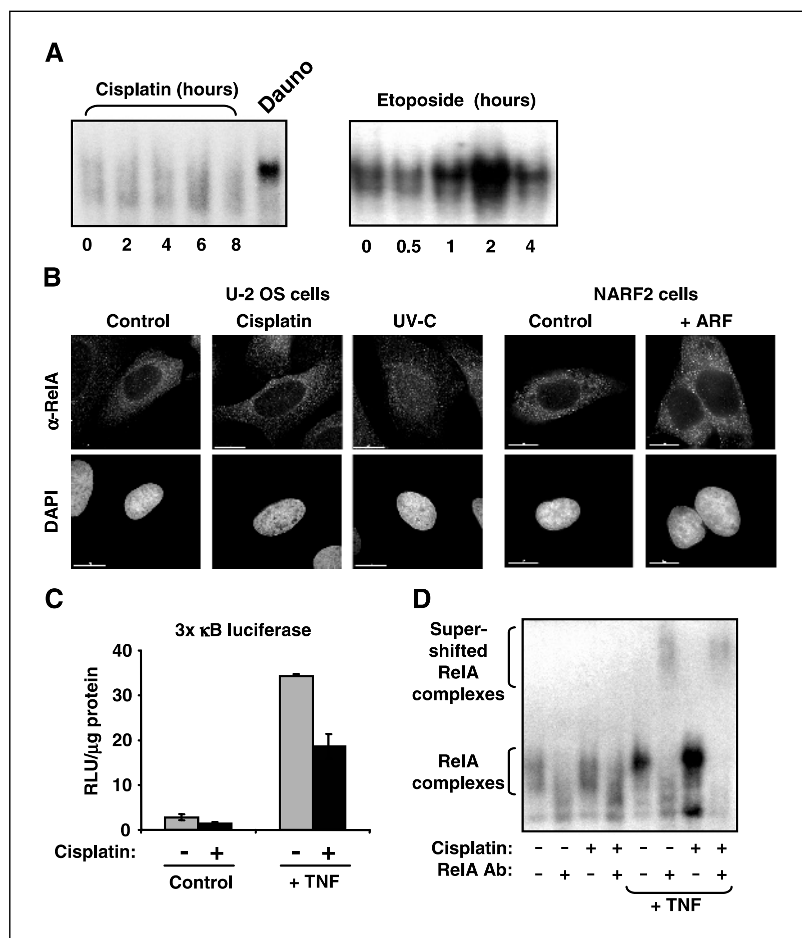

Figure 2. Cisplatin and ARF do not induce NF-κB DNA binding or RelA nuclear translocation. *A,* EMSA analysis using nuclear protein extracts from U-2 OS cells treated with either 4 μg/mL cisplatin (*left*) or 15 μmol/L etoposide (*right*) for the indicated times. A lane using 1 μmol/L daunorubicin-treated cells (3 hours) is also shown. EMSA analysis was done using a ^{32}P-labeled consensus Ig/HIV κB probe. *B,* immunofluorescence analysis of RelA nuclear localization following cisplatin treatment. U-2 OS were plated onto coverslips and fixed after 4 μg/mL cisplatin (4 hours) or 40 J/m^2 UV-C (4 hours) treatment. Cells were stained with rabbit anti-RelA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 4′,6-diamidino-2-phenylindole (*DAPI*) to reveal DNA. In addition, NARF2 cells were treated with 1 mmol/L IPTG for 0 or 24 hours to induce ARF and similarly stained for RelA. Cells were analyzed, and images were acquired using a DeltaVision microscope. *C,* cisplatin treatment represses TNF-induced NF-κB activity. U-2 OS cells were transfected as in Fig. 1*A*, except that some transfected cells were also treated with TNF (8 hours) and/or cisplatin (6 hours). *D,* cisplatin does not inhibit TNF-induced NF-κB DNA binding. EMSA analysis was done as in (*A*), except that cells were treated with 10 ng/mL TNF for 30 minutes or cisplatin for 4 hours either alone or in combination. Where indicated, supershift analysis was done with a specific anti-RelA antibody.

known activator of ATR activity (25). Consistent with this, cisplatin treatment induced phosphorylation of Chk1 kinase at Ser^{317} and Ser^{345}, both target sites for ATR (26), confirming activation of this pathway (Fig. 4*D*).

Cisplatin induces Thr^{505} phosphorylation of RelA. We have previously shown that a consequence of ARF-induced activation of the ATR/Chk1 pathway is the phosphorylation of RelA at Thr^{505} by Chk1 (refs. 16, 17; also Fig. 5*A*). This does not occur with other inducers of Chk1 activity, such as daunorubicin and UV-C, however, suggesting that specific scaffold proteins determine substrate specificity. Because there were a number of parallels between ARF and cisplatin effects on NF-κB, we next investigated whether cisplatin is also an inducer of RelA Thr^{505} phosphorylation. Using two distinct phosphospecific anti-Thr^{505} RelA antibodies raised in rabbit and in goat, we observed that the relatively low level of basal RelA in U-2 OS cells became significantly Thr^{505} phosphorylated upon cisplatin treatment (Fig. 5*A* and *B*). Consistent with our previous results, and despite inducing higher levels of nuclear RelA, neither UV-C, daunorubicin, nor etoposide treatment induced Thr^{505} phosphorylation (Fig. 5*B*; data not shown). Furthermore, the UCN-01–related Chk1 inhibitor Gö6976 (17, 27–29) inhibited both basal and cisplatin-induced RelA Thr^{505} phosphorylation (Fig. 5*C*). This latter result confirmed that in U-2 OS cells, cisplatin regulates NF-κB function in a manner strikingly similar to that seen with the ARF tumor suppressor.

Discussion

By a number of criteria, regulation of RelA transactivation by ARF and cisplatin are similar, if not identical. Both induce Thr^{505} phosphorylation, both require the ATR/Chk1 pathway, neither induce NF-κB DNA binding but rather modulate its transactivation, and both show similar target gene specificity,

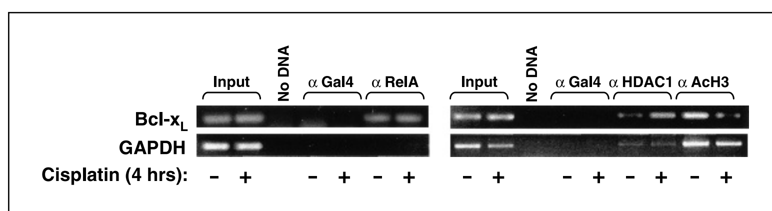

Figure 3. Cisplatin does not affect RelA binding to the *Bcl-x$_L$* promoter but does induce HDAC1 recruitment. Control or cisplatin-treated U-2 OS cells were harvested, and chromatin immunoprecipitation analysis was done with the indicated RelA, HDAC1, acetylated H3 (Lys^9 and Lys^{14}) or Gal4 control antibodies using primers to the *Bcl-x$_L$* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoters.

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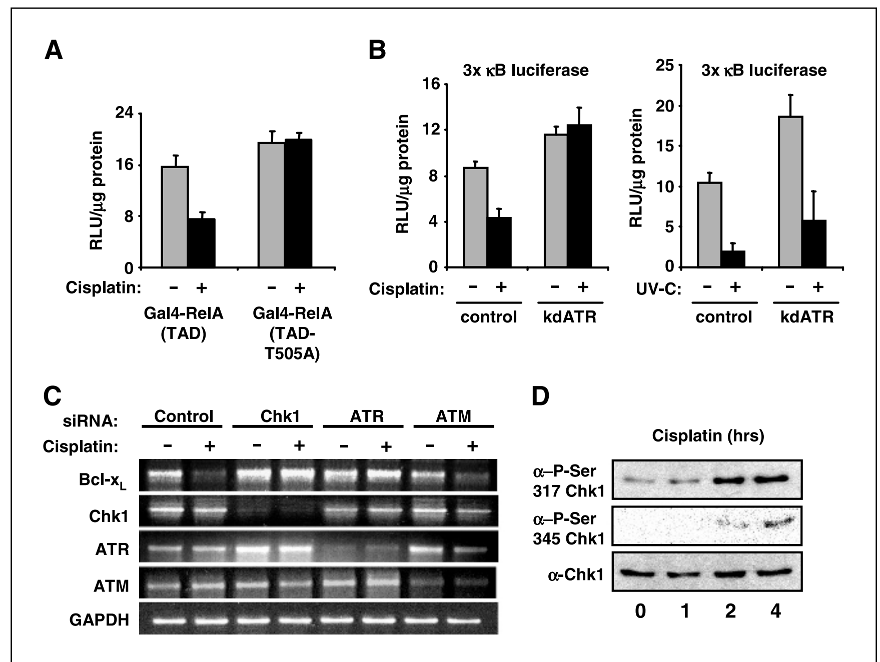

Figure 4. Cisplatin repression of NF-κB requires the ATR/Chk1 pathway. *A,* cisplatin represses the RelA transactivation domain in a Thr[505]-dependent manner. U-2 OS cells were transfected with 2 μg of Gal4 luciferase reporter plasmid together with expression plasmids encoding either the Gal4 RelA (TAD) or Gal4 RelA (TAD T505A) expression plasmids as indicated. Thirty-two hours after transfection, cells were either left untreated or stimulated for (16 hours) with 4 μg/mL cisplatin. Relative luciferase activity per microgram of protein. The RelA transactivation domain (*TAD*) used in this study encodes amino acids 428 to 551 of human RelA. *B,* cisplatin-mediated repression of NF-κB activity requires ATR. U-2 OS cells were transfected as in Fig. 1*A* and treated with either 4 μg/mL cisplatin or 40 J/m^2 UV-C for 0 or 8 hours. Cells also included 1 μg of kinase-dead ATR expression plasmid or a control plasmid as indicated. *C,* siRNAs targeting ATR and Chk1 abolish cisplatin-induced repression of *Bcl-x$_L$*. PCR analysis of *Bcl-x$_L$* expression was done following treatment of U-2 OS cells with the indicated siRNAs. Analysis of ATR, Chk1, and ATM expression, as well as the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control, is also included. *D,* cisplatin induces phosphorylation of Chk1. Whole-cell lysates were prepared from U-2 OS cells treated with 4 μg/mL cisplatin for the indicated times. Western blot analysis was done using the indicated Chk1 or phosphospecific Chk1 antibodies.

repressing *Bcl-x$_L$* but not *XIAP* expression. In contrast, UV-C and daunorubicin, both DNA-damaging agents, also induce Chk1 activity in U-2 OS cells (data not shown) but do not require ATR/Chk1 to repress NF-κB transactivation (15, 17). Moreover, another DNA-damaging agent, etoposide, is a potent inducer of NF-κB DNA-binding activity, which stimulates NF-κB transactivation and results in induction of *Bcl-x$_L$* and *XIAP*. There are a number of important conclusions from these observations. The first of these is that within a single cell line, the response of NF-κB to different chemotherapeutic drugs and DNA-damaging agents shows great diversity. If this rule is also applicable to other tumor types, which we consider highly probable, there are important implications for the role NF-κB plays in response to cancer chemotherapy. That is, it might not always reduce the efficacy of these treatments; therefore, routinely targeting it for inhibition might not always be the best clinical practice. Instead, some diagnostic indication of NF-κB status might be required. This could involve analysis of NF-κB subunit phosphorylation using phosphospecific antibodies (18, 30). The complexity of the NF-κB response to genotoxic damage is likely to be very diverse, varying not only between treatments but also between cell types and tumor stages. For example, although we do not observe cisplatin-induced NF-κB DNA binding in U-2 OS cells, an effect also observed previously in T-leukemia cells (31), it will induce NF-κB DNA binding in smooth muscle cells (32). Furthermore, cisplatin-induced down-regulation of NF-κB reporter plasmid activity was also observed in human hepatoma and ovarian cancer cells, although a mechanism for this effect was not described (33, 34). Interestingly, underlining the importance of cell type–specific effects, in both of these studies, cisplatin was shown to down-regulate *XIAP* expression, whereas *Bcl-x$_L$* was not investigated (33, 34). What determines these differences is unclear. In general, it could be significant that U-2 OS cells are a relatively differentiated osteosarcoma cell line and therefore can be expected to have retained many pathways lost in other more malignant cell types. Therefore, treatments targeting NF-κB in cancer, if not used as stand alone therapies, might be more effective if appropriately matched both with the class of therapy and the tumor type and stage. It is interesting to note that the combination of cisplatin and doxorubicin, which in our hands regulates RelA identically to its closely related congener daunorubicin, are commonly used therapies for osteosarcoma (21). Their effectiveness might partly result from their ability, at least in some tumor cells, to regulate RelA through distinct pathways, which result in similar functional effects (i.e., the repression of antiapoptotic genes expression). It will be interesting to investigate whether differences in patient responses to this treatment correlate to any extent with any differences in the effects of these drugs on NF-κB.

Another implication of our studies concerns the ATR/Chk1 checkpoint kinase pathway. That this pathway seems able to modulate RelA function only in response to certain inducers implies that scaffold or adaptor proteins must exist to target Chk1 to RelA only under certain circumstances. Exactly why this occurs with ARF and cisplatin but not UV-C, daunorubicin/doxorubicin, and etoposide is unclear, but similar adaptor proteins, such as BRCA1 tumor suppressor, are known to target ATR to a subset of its substrates (35). The identity of such Chk1 adaptor proteins will be of great interest. It could be expected that this protein or proteins might also target Chk1 to substrates other than RelA. Moreover, if a consequence of the ARF/cisplatin induced Chk1 adaptor is to neutralize the oncogenic and antiapoptotic functions of RelA, it could well be a tumor suppressor in its own right. Furthermore, it is possible that its presence or deletion in a tumor might act as a diagnostic indicator for NF-κB function, as described above.

Although we have focused on Thr[505] phosphorylation of RelA because this is a modification with proven functional effects (16, 17), it is probable that this is not the only post-translational effect induced by cisplatin treatment. For example, a recent report implied that dephosphorylation of RelA at Thr[435] by protein phosphatase 4 in response to cisplatin increases RelA transcriptional activity (36). Thr[435] is also located in the RelA

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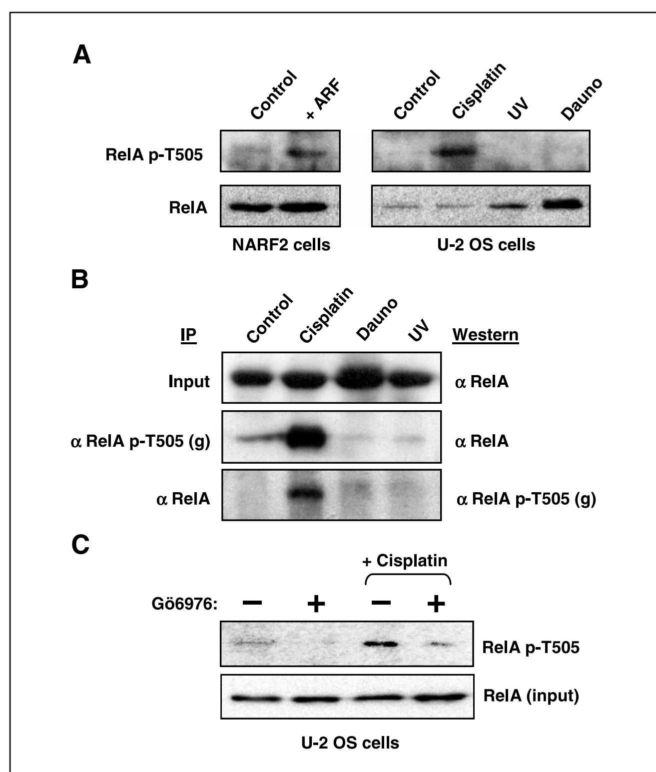

Figure 5. Cisplatin induces Thr^{505} phosphorylation of RelA. *A,* cisplatin induces Thr^{505} phosphorylation. Whole-cell lysates were prepared from NARF2 cells either treated with IPTG to induce ARF for 24 hours or nuclear protein extracts were prepared from U-2 OS cells stimulated with 4 μg/mL cisplatin (4 hours), 1 μmol/L daunorubicin (4 hours), or 40 J/m^2 UV-C (4 hours) or left untreated. Phosphorylated RelA was then immunopreciptated by incubating 100 μg of lysate with purified rabbit phospho-RelA T505A antibody. The immunoprecipitate was resolved by SDS-PAGE before Western blotting with an anti-RelA antibody (Santa Cruz Biotechnology). *B,* experiment was done as in (*A*), except that whole-cell lysates from U-2 OS cells were used in combination with the goat phospho-RelA T505A antibody and anti-RelA antibody (Santa Cruz Biotechnology). Both antibodies were used to either immunoprecipitate or Western blot RelA as indicated. *C,* cisplatin induced Thr^{505} phosphorylation is prevented by the Chk1 inhibitor Gö6976. Experiment was done as in (*A*), except that indicated cells were incubated with 1 μmol/L Gö6976 for 30 minutes before the addition of cisplatin and the preparation of whole-cell lysates. The same result is also seen with the goat phospho-RelA T505 antibody (data not shown).

transactivation domain and shows some evolutionary conservation. The relative contribution of phosphorylations at 435 and 505 to RelA activity is currently under investigation, but the absence of a Thr^{435} phosphospecific antibody prevented analysis in this study.

This report adds cisplatin to a group of cancer drugs that have diverse effects on NF-κB function. We propose that proapoptotic and antiapoptotic signals converge on RelA in response to a variety of stimuli, and that particular combinations of signals trigger distinct gene expression profiles (18, 30). This complexity of the NF-κB response poses challenges to future therapy based on its activity. Nonetheless, it remains highly likely that compounds targeting the NF-κB pathway, such as inhibitors of IκB kinase, will prove clinically useful in cancer treatment. For example, this will almost certainly be the case where tumors have an inflammatory origin or where the expression of tumor suppressors, such as ARF, has been lost. However, an increased understanding of the mechanisms regulating diverse NF-κB function in response to different treatments might help therapies be targeted more effectively. Furthermore, these studies could suggest alternative drug development pathways. Our demonstration that cisplatin essentially mimics the ability of ARF to regulate RelA activity suggests that it should be possible to develop other compounds targeting the same pathway but possibly with increased specificity and reduced toxicity. That a well-known and widely used chemotherapeutic drug seems to have the same mechanism of action on RelA as an important tumor suppressor, underlines the likely clinical importance of this pathway. It will be of interest therefore to determine how widely it becomes activated in response to other therapies and in other biological contexts.

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