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Bcl-x_L Deamidation Is a Critical Switch in the Regulation of the Response to DNA Damage

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Summary

The therapeutic value of DNA-damaging antineoplastic agents is dependent upon their ability to induce tumor cell apoptosis while sparing most normal tissues. Here, we show that a component of the apoptotic response to these agents in several different types of tumor cells is the deamidation of two asparagines in the unstructured loop of Bcl-x_L, and we demonstrate that deamidation of these asparagines imports susceptibility to apoptosis by disrupting the ability of Bcl-x_L to block the proapoptotic activity of BH3 domain-only proteins. Conversely, Bcl-x_L deamidation is actively suppressed in fibroblasts, and suppression of deamidation is an essential component of their resistance to DNA damage-induced apoptosis. Our results suggest that the regulation of Bcl-x_L deamidation has a critical role in the tumor-specific activity of DNA-damaging antineoplastic agents.

Introduction

DNA-damaging antineoplastic agents are effective because they induce tumor cell apoptosis (Schmitt and Lowe, 1999). Most tumor cells lack wild-type p53 activity (Evan and Vousden, 2001), and therefore p53-independent signaling pathways must be of primary importance in tumor cell apoptosis. These pathways are presumably intact in the cells of normal tissue; however, apoptotic signaling is suppressed in many of these cells. For example, fibroblasts arrest without undergoing apoptosis in response to DNA damage (Di Leonardo et al., 1994), and high concentrations of cisplatin-DNA adducts accumulate in many tissues, not just in the malignant tissue, during cisplatin treatment (Poirier et al., 1987, 1992; Terheggen et al., 1987), yet cisplatin treatment does not cause apoptosis in most normal tissue. We sought to determine the mechanism by which DNA-damaging antineoplastic agents selectively induce p53 null tumor cell apoptosis.

Because they play a central role in the apoptotic process, we focused our study on Bcl-2 family members. In response to DNA damage, the activity of individual proapoptotic BH3 domain-only Bcl-2 family members is upregulated (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). The BH3 domain-only proteins then activate Bak and Bax (Cheng et al., 2001; Wei et al., 2000, 2001). Activated Bak and Bax facilitate mitochondrial cytochrome c release, which initiates a cascade of caspase activation that results in cell death (Gross et al., 1999).

It has recently been demonstrated that the antiapoptotic Bcl-2 proteins Bcl-2 and Bcl-x_L inhibit apoptosis by blocking the activity of BH3 domain-only proteins (Cheng et al., 2001). The precise function of the antiapoptotic Bcl-2 family members in the response to DNA damage has yet to be defined, however. Critical unresolved issues include the following. (1) Why are there proteins that block the activity of BH3 domain-only proteins once they are activated? (2) After DNA damage, do antiapoptotic Bcl-2 proteins function merely as immutable buffers of proapoptotic activity or do they actively regulate the apoptotic process? (3) Do the antiapoptotic Bcl-2 family members all share a redundant role in the response to DNA damage or do any of them have a unique function?

In addressing these issues, we found that treatment of several types of tumor cells with DNA-damaging agents induces deamidation of two asparagines in the unstructured loop of Bcl-x_L. We demonstrate that deamidation completely disrupts the antiapoptotic activity of Bcl-x_L, and that this occurs because it disrupts the ability of Bcl-x_L to block the proapoptotic activity of BH3 domain-only Bcl-2 proteins. Importantly, we show that Bcl-x_L deamidation is actively suppressed in growth-arrested fibroblasts, which is indeed critical for the suppression of cisplatin-induced apoptosis in these cells.

Results

Rb-Mediated Arrest of Tumor Cells Suppresses a DNA-Damaging Antineoplastic Agent-Induced Modification of Bcl-x_L

Some antineoplastic agents damage the DNA of normal as well as tumor cells, but selectively cause tumor cell apoptosis. Most tumors lack Rb activity; however, Rb is present in all normal tissue (Nevins, 2001), and it is a potent antiapoptotic protein (Haas-Kogan et al., 1995). Therefore, we considered the possibility that Rb has a role in the tumor cell selectivity of such antineoplastic agents.

To begin to determine the mechanism by which DNA damage selectively induces tumor cell death, we generated a p53 null, Rb null osteosarcoma cell line (SAOS-2) in which Rb expression is inducible. Whereas SAOS-2 cells were susceptible to cisplatin-induced apoptosis, cisplatin-induced apoptosis was blocked by Rb expression (Figure 1A).

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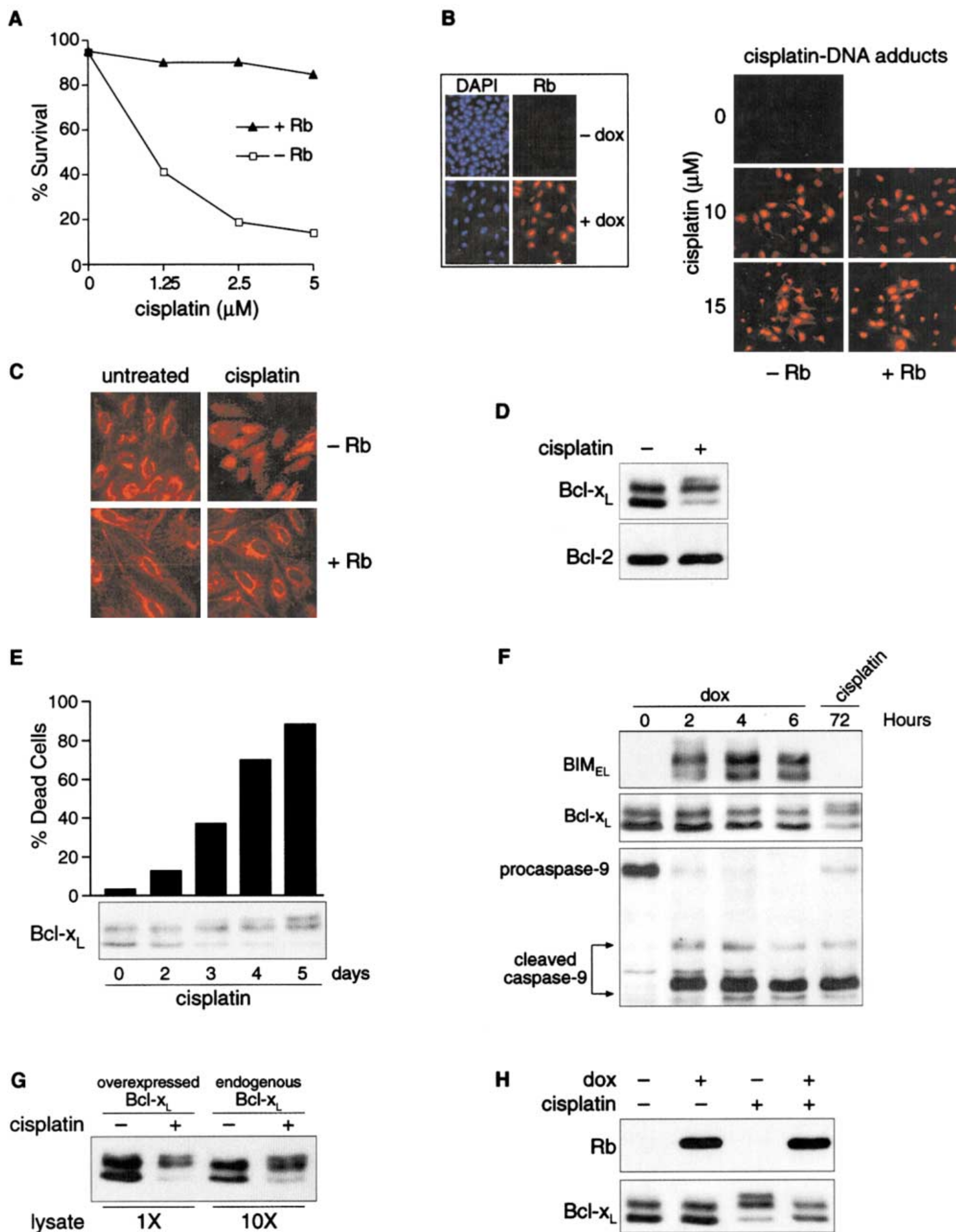

Figure 1. Cisplatin Induces an Rb-Suppressible Modification of Bcl-x_L

(A) Rb blocks cisplatin-induced death of SAOS-2 cells. Dose response of Rb-inducible SAOS-2 cells. Rb expression was induced by treatment with doxycycline (dox) for 36 hr prior to cisplatin treatment.

(B) Rb does not block cisplatin-DNA adduct formation in SAOS-2 cells. Left: immunostaining for Rb. Rb is expressed in almost all of the cells in the Rb-inducible SAOS-2 cell line treated with dox. Right: cisplatin-DNA adduct formation was detected using an antibody specific for cisplatin-DNA adducts (Tilby et al., 1991). Rb expression was induced as in (A).

(C) Rb blocks cisplatin-induced cytochrome c release. Rb expression was induced as in (A). Cells were then treated with 5 μM cisplatin for 96 hr and cytochrome c localization was assessed. DN-caspase-9 was stably expressed in these cells to facilitate visualization of cytochrome c release.

(D) Cisplatin treatment induces a modification of Bcl-x_L that is not detected in Bcl-2. Immunoblot of Bcl-x_L and Bcl-2 from untreated and cisplatin-treated (5 μM) SAOS-2 cells.

(E) The modification of Bcl-x_L correlates temporally with cisplatin-induced cell death. SAOS-2 cells were treated with 5 μM cisplatin.

(F) The cisplatin-induced modification of Bcl-x_L is not a consequence of apoptosis. BIM_{EL}-inducible SAOS-2 cells were induced with dox. Immunoblots of BIM, Bcl-x_L, and caspase-9 cleavage. An immunoblot of a lysate from cisplatin-treated cells is shown for comparison.

(G) Overexpressed Bcl-x_L undergoes the same modification as endogenous Bcl-x_L. Bcl-x_L was overexpressed in SAOS-2 cells, and then the cells were treated with cisplatin. Immunoblot of Bcl-x_L. 10-fold more lysate from the untransfected cells was used to facilitate the comparison with overexpressed Bcl-x_L.

(H) Rb blocks the cisplatin-induced modification of Bcl-x_L. Immunoblots of Rb and Bcl-x_L from Rb-inducible SAOS-2 cells treated with cisplatin (5 μM) as indicated. Rb expression was induced as in (A).

We next sought to identify cisplatin-induced proapoptotic signals that are suppressed by expression of Rb. Rb expression had no affect on cisplatin-DNA adduct formation (Figure 1B), and we have found that induction of Rb expression in SAOS-2 cells even as late as 12 hr after the cells were γ irradiated efficiently protected them from γ radiation-induced apoptosis (data not shown). We next found that Rb expression blocks cisplatin-induced mitochondrial cytochrome c release (Figure 1C). Based on these findings, we hypothesized that Rb suppresses a proapoptotic signal(s) that is either parallel to or downstream of DNA damage, but upstream of mitochondrial cytochrome c release during the cisplatin-induced apoptotic response in SAOS-2 cells.

Because mitochondrial cytochrome c release is regulated in large part by Bcl-2 proteins (Gross et al., 1999), we examined Bcl-2 family members during cisplatin-induced apoptosis. Cisplatin induced a modification of Bcl-x_L; in contrast, Bcl-2 appeared to be unaffected by cisplatin treatment (Figure 1D). The Bcl-x_L modification correlated temporally with death (Figure 1E). These findings suggested that Bcl-x_L has a role in the regulation of the SAOS-2 cell response to cisplatin.

We then sought to rule out the possibility that the modification of Bcl-x_L was simply an effect of apoptosis. We found that the Bcl-x_L modification that occurs during cisplatin-induced apoptosis does not occur during BIM-induced apoptosis (Figure 1F) or apoptosis induced by either E2F-1 overexpression or treatment with staurosporine (data not shown). Therefore, apoptosis per se does not cause the Bcl-x_L modification. More importantly, even though overexpression of Bcl-x_L blocks cisplatin-induced mitochondrial cytochrome c release and apoptosis (data not shown), we found that overexpressed Bcl-x_L is modified in cisplatin-treated cells in the same manner as the endogenous Bcl-x_L (Figure 1G), indicating that the modification occurs upstream of the mitochondria. These findings ruled out the possibility that the cisplatin-induced modification of Bcl-x_L is merely a result of mitochondrial dysfunction or apoptosis.

Because Rb blocks cisplatin-induced apoptosis in SAOS-2 cells, we examined the effect of Rb expression on the cisplatin-induced modification of Bcl-x_L. Expression of Rb suppressed the modification of Bcl-x_L (Figure 1H). Interestingly, neither p21^{CIP1} nor p27^{KIP1} had a similar effect (see Supplemental Data at http://www.cell.com/cgi/content/full/111/1/51/DC1). Therefore, we thought that suppression of the Bcl-x_L modification could be a component of the antiapoptotic activity of Rb. We address this possibility below.

We then assessed the effect of cisplatin treatment on endogenous Bcl-x_L in two additional p53 null, Rb null cell lines and in ovarian cancer cells that were freshly isolated from malignant ascites. Cisplatin induced the same modification of Bcl-x_L in these cells (Figure 2A). Additionally, Rb blocked cisplatin-induced apoptosis and the cisplatin-induced modification of Bcl-x_L in HTB-9 cells (data not shown), a Rb null, p53 null bladder cancer cell line. Finally, we found that the DNA-damaging agents etoposide and γ radiation induce the same modification of Bcl-x_L as cisplatin, and that Rb suppresses the effects of each (Figure 2B). Therefore, several DNA-damaging agents induce the same Rb-suppressible modification of Bcl-x_L in a range of tumor cells of disparate origin. This modification is not the result of mitochondrial dysfunction or apoptosis, and suppression of the Bcl-x_L modification by Rb correlates with the ability of Rb to inhibit apoptosis.

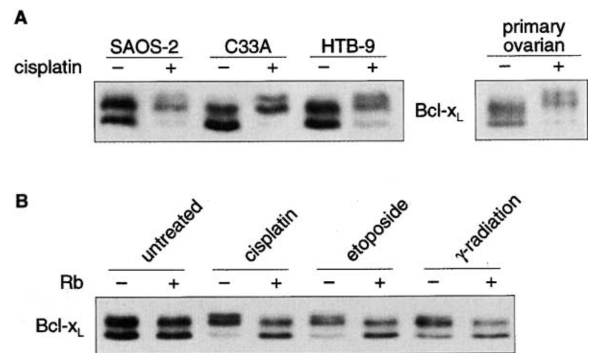

Figure 2. DNA-Damaging Agents Induce the Same Rb-Suppressible Modification of Bcl-x_L in p53 Null, Rb Null Tumor Cell Lines and Primary Ovarian Cancer Cells

(A) Cisplatin induces the same modification of Bcl-x_L in tumor cell lines and primary ovarian cancer cells. Immunoblot of the Bcl-x_L from an osteosarcoma cell line (SAOS-2), a cervical cancer cell line (C33A), bladder cancer cell line (HTB-9), and ovarian cancer cells that were treated with cisplatin (5 μM) as indicated.

(B) Different DNA-damaging agents induce the same Rb-suppressible modification of Bcl-x_L. Immunoblot of Bcl-x_L from Rb-inducible SAOS-2 cells treated with cisplatin (5 μM), etoposide (10 μM), or γ radiation (20 Gy). Rb expression was induced with dox for 36 hr prior to treatment with the DNA-damaging agents.

DNA-Damaging Agents Induce Deamidation of Bcl-x_L

Paclitaxel induces phosphorylation of Bcl-x_L and Bcl-2, and phosphorylation alters their migration during SDS-PAGE (Poruchynsky et al., 1998; Yamamoto et al., 1999). We compared the Bcl-x_L and Bcl-2 from SAOS-2 cells that were treated with DNA-damaging agents or paclitaxel. The migration of Bcl-x_L isolated from cells treated with DNA-damaging agents differed significantly from that of the Bcl-x_L isolated from paclitaxel-treated cells (Figure 3A). Furthermore, phosphatase treatment completely reverses the paclitaxel-induced modification of Bcl-x_L (Poruchynsky et al., 1998); however, phosphatase treatment had no effect on the DNA damage-induced modification of Bcl-x_L (data not shown). Finally, although the paclitaxel-induced modification of Bcl-2 was detectable, there was no detectable modification of the Bcl-2 in cells that had been treated with DNA-damaging agents (Figure 3A). These findings indicate that the modification of Bcl-x_L induced by DNA-damaging agents is different from that induced by paclitaxel.

Bacterially synthesized rat Bcl-x_L undergoes deamidation in vitro (Aritomi et al., 1997); however, the significance of Bcl-x_L deamidation has not been addressed. Deamidation is a posttranslational modification in which asparagines are converted to a mixture of aspartates and isoaspartates (Aswad et al., 2000). An asparagine is most susceptible to deamidation when it is followed by a glycine in an unstructured region of a protein (Robinson and Robinson, 2001). Two such sites are conserved in the nine upper vertebrate Bcl-x_L proteins listed

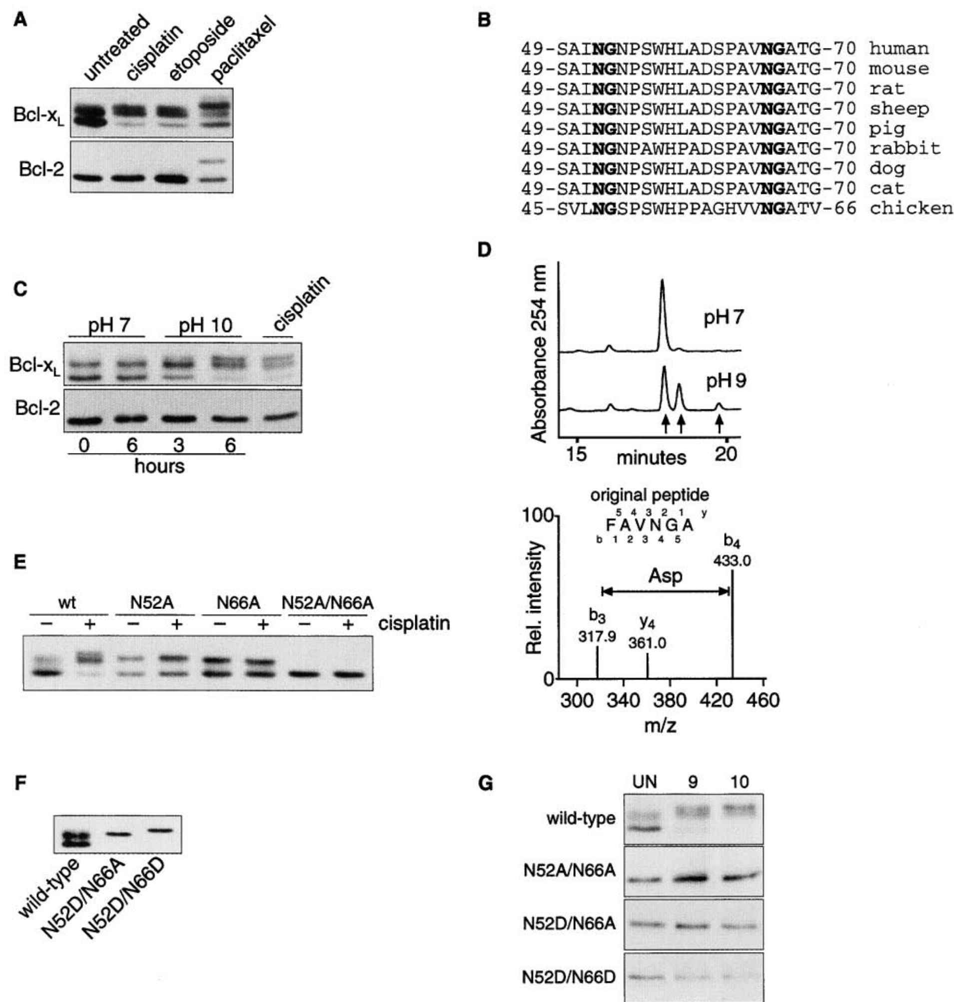

Figure 3. DNA Damage Induces Deamidation of Asparagines 52 and 66 of Bcl-x_L

(A) DNA-damaging agents and paclitaxel induce different modifications of Bcl-x_L. Immunoblots of Bcl-x_L and Bcl-2 from SAOS-2 cells treated with cisplatin (5 μM), etoposide (10 μM), or paclitaxel (100 nM).

(B) Potential deamidation sites are conserved in Bcl-x_L. Asparagine is most susceptible to deamidation when it is followed by glycine in an unstructured region of a protein. Two such sites are conserved in every upper vertebrate Bcl-x_L protein for which the sequence is available.

(C) Cisplatin treatment and incubation in an alkaline buffer seem to induce the same modification of Bcl-x_L. Immunoblot analysis of Bcl-x_L and Bcl-2 from SAOS-2 cell lysates that were incubated at pH 7 or pH 9 for the indicated time. An immunoblot of lysates from cisplatin-treated cells is shown for comparison.

(D) Incubation in an alkaline buffer causes deamidation of the asparagine in a peptide that corresponds to Bcl-x_L amino acids 64–68. Top: the peptide FAVNGA (phenylalanine was added to the N terminus of the Bcl-x_L sequence for quantitative analysis by absorption spectroscopy) was incubated for 24 hr at 37°C at the indicated pH and analyzed by reverse-phase HPLC. Bottom: electrospray ionization tandem mass spectrometric analysis of the new peaks of material observed in the alkaline reaction mixture demonstrated that Asn was converted to Asp or an isomer of Asp.

(E) Substitution of alanine for the asparagines at the potential deamidation sites blocks the modification of Bcl-x_L in both untreated and cisplatin-treated (5 μM) cells (see Results for details).

(F) Constitutively deamidated forms of Bcl-x_L migrate in the same position as the modified forms of Bcl-x_L when analyzed by SDS-PAGE (see Results for details).

(G) The alkalinization-induced modification of Bcl-x_L occurs at asparagines 52 and 66. Lysates made from SAOS-2 cells transfected with wild-type Bcl-x_L, Bcl-x_L (N52A/N66A), Bcl-x_L (N52D/N66A), or Bcl-x_L (N52D/N66D) were incubated at the indicated pH. Immunoblots of Bcl-x_L.

in GenBank (Figure 3B). Therefore, we considered the possibility that DNA-damaging agents induce the deamidation of Bcl-x_L.

Because alkaline conditions increase the rate of protein deamidation (Johnson et al., 1989; Robinson and Rudd, 1974), we first assessed the endogenous Bcl-x_L from a cell lysate incubated at an alkaline pH. Whereas Bcl-x_L was unaffected by incubation of the lysate in a buffer at neutral pH, we found that the Bcl-x_L from a lysate incubated in the same buffer at alkaline pH migrated in the same pattern as the Bcl-x_L from cisplatin-treated cells (Figure 3C). To explore the basis for the altered mobility, we incubated a peptide that corresponds to Bcl-x_L amino acids 64–68 in similar buffers. Tandem mass spectrophotometric analysis demonstrated that incubation of this peptide in alkaline buffer

Table 1. Predicted Deamidation Half-Lives of the Asparagines in Bcl-x_L

Asparagine	Predicted Deamidation Half-Life (Relative)
5	6,050
33	64
52	1
54	405
66	1
128	36,023
136	299
175	4,095
185	543

The relative predicted deamidation half-lives of the asparagines in Bcl-x_L were calculated using the formula developed by N.E. Robinson (Robinson, 2002). The calculations are based on the X-ray structure of Bcl-x_L (Muchmore et al., 1996) and were performed using the program at http://deamidation.entrewave.com.

caused deamidation of the asparagine that corresponds to asparagine 66 of Bcl-x_L (Figure 3D). These findings suggested that the cisplatin-induced modification of Bcl-x_L is deamidation.

We next generated constructs that express forms of human Bcl-x_L in which alanines are substituted for the two asparagines at the potential deamidation sites (residues 52 and 66) either individually, in constructs Bcl-x_L(N52A) and Bcl-x_L(N66A), or together, in construct Bcl-x_L(N52A/N66A). We found that replacement of either asparagine with alanine blocked the formation of the slowest migrating form of Bcl-x_L and replacement of both asparagines blocked the formation of the slowest and intermediate forms of Bcl-x_L (Figure 3E). Because these are the forms of Bcl-x_L that increase in cells that have been treated with DNA-damaging agents, these findings suggested that DNA damage induces deamidation of Bcl-x_L.

Aspartate is one of the major products of asparagine deamidation, and aspartate undergoes spontaneous conversion to the other major asparagine deamidation product, isoaspartate. However, aspartate and isoaspartate do not spontaneously convert back to asparagine (Aswad et al., 2000). Therefore, we were able to generate constructs that express constitutively deamidated forms of Bcl-x_L by substituting aspartates for the asparagines.

One construct expresses a protein in which an aspartate is substituted for asparagine 52 and alanine is substituted for asparagine 66, Bcl-x_L(N52D/N66A)—the equivalent of a Bcl-x_L protein that is deamidated at only one of the potential deamidation sites. The second construct expresses a form of Bcl-x_L in which aspartates replace both asparagine 52 and 66, Bcl-x_L(N52D/N66D)—the equivalent of a Bcl-x_L protein that is deamidated at both of the potential deamidation sites. When evaluated by SDS-PAGE, the singly deamidated form migrated with the intermediate Bcl-x_L band, and the Bcl-x_L protein that is deamidated at both sites migrated in the same position as the slowest migrating form of endogenous Bcl-x_L (Figure 3F). Because these are the forms of Bcl-x_L that increase in cells treated with DNA-damaging agents, these findings provided further evidence that DNA damage induces deamidation of Bcl-x_L.

We next assessed the effect of alkaline pH on wild-type Bcl-x_L, Bcl-x_L(N52A/N66A), Bcl-x_L(N52D/N66A), and Bcl-x_L(N52D/N66D). After incubation in the alkaline buffer, the migration of wild-type Bcl-x_L was altered as expected, but the migration of the mutant forms was unaffected (Figure 3G). Because replacement of Bcl-x_L asparagines 52 and 66 with alanines or aspartates blocks the alkaline-induced modification, these results provided further evidence that the cisplatin-induced modification of Bcl-x_L is deamidation of asparagines 52 and 66.

Finally, a method for predicting the deamidation rate of any asparagine in a protein for which the three-dimensional structure is known was recently published (Robinson, 2002). The predicted deamidation half-lives of Bcl-x_L asparagines 52 and 66 are extraordinarily short when compared with the other asparagines in Bcl-x_L (Table 1) and the asparagines in most other proteins (Robinson, 2002). Thus, the nature of the predicted deamidation half-lives of Bcl-x_L asparagines 52 and 66 serves as corroborative evidence that the cisplatin-induced modification of Bcl-x_L results from deamidation of Bcl-x_L asparagines 52 and 66.

When considered together, our results indicate that these mutant forms of Bcl-x_L are essentially the equivalent of the corresponding forms of Bcl-x_L found in untreated and cisplatin-treated tumor cells. Indeed, it is very likely that Bcl-x_L(N52D/N66D) is precisely the same as its corresponding endogenous form of Bcl-x_L.

Deamidation Disrupts Bcl-x_L Activity

We compared the antiapoptotic activity of the constitutively deamidated forms of Bcl-x_L with that of wild-type Bcl-x_L. Whereas wild-type Bcl-x_L efficiently blocked cisplatin-induced apoptosis in SAOS-2 cells, neither of the constitutively deamidated forms displayed significant antiapoptotic activity, even though they were expressed at levels exceeding that of the wild-type Bcl-x_L (Figure 4A). Therefore, deamidation of asparagines 52 and 66 disrupts the antiapoptotic activity of Bcl-x_L.

The loss of antiapoptotic activity upon deamidation is not simply due to the removal of the asparagines at positions 52 and 66 per se, as it has been demonstrated that deletion of large regions of Bcl-x_L that encompass these sites actually increases the antiapoptotic activity of Bcl-x_L (Chang et al., 1997), and we have found that replacement of asparagines 52 and 66 in Bcl-x_L with alanines to block deamidation [Bcl-x_L(N52A/N66A)] similarly increases the antiapoptotic activity of Bcl-x_L (data not shown). This indicates that the deamidation products at residues 52 and 66 actively disrupt Bcl-x_L activity. One possible explanation for this is that the introduction of negatively charged amino acids at these positions alters the tertiary structure of Bcl-x_L.

It was recently demonstrated that the antiapoptotic activity of Bcl-x_L is due to its ability to block the proapoptotic activity of BH3 domain-only proteins, and that Bcl-x_L must be able to bind the BH3 domain-only proteins to do so (Cheng et al., 2001). Therefore, we compared the ability of the nondeamidated and the constitutively deamidated forms of Bcl-x_L to bind to the BH3 domain-only protein BIM (O'Connor et al., 1998). We found that the nondeamidated form of Bcl-x_L binds over-

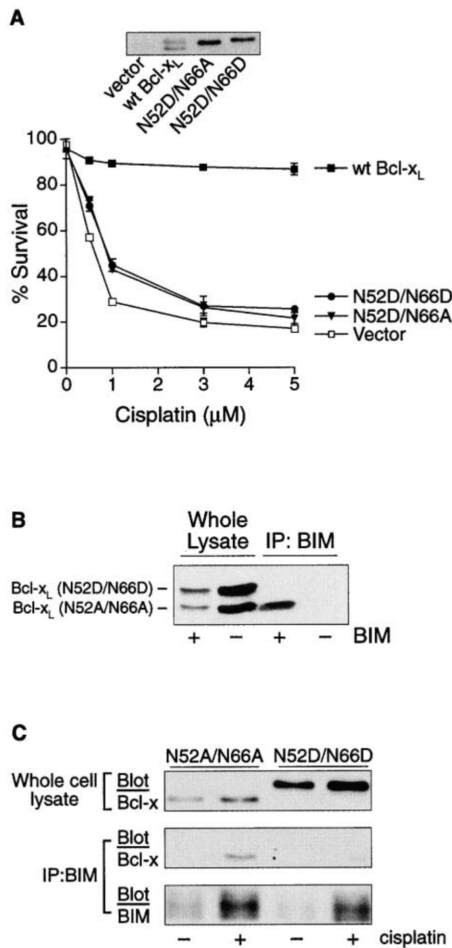

Figure 4. Deamidation Disrupts the Antiapoptotic Activity of Bcl-x_L

(A) Constitutively deamidated forms of Bcl-x_L fail to inhibit cisplatin-induced apoptosis. SAOS-2 cells were transfected with expression vectors for the wild-type or the constitutively deamidated forms of Bcl-x_L along with an expression vector for CD20 to identify transfected cells. Expression of the wild-type and the constitutively deamidated forms of Bcl-x_L was assessed by immunoblotting.

(B) Deamidation disrupts the interaction between Bcl-x_L and the BH3 domain-only protein BIM. Expression vectors for the form of Bcl-x_L that does not become deamidated, Bcl-x_L(N52A/N66A), and the form of Bcl-x_L that is constitutively deamidated, Bcl-x_L(N52D/N66D), were cotransfected in SAOS-2 cells. An expression vector for BIM (EE tagged) was transfected as indicated. Immunoprecipitation was performed with an EE tag antibody. Bcl-x_L forms that were coimmunoprecipitated with BIM were detected by immunoblotting. The protein levels are decreased in the far left lane because BIM induced apoptosis of a portion of the transfected cells.

(C) Deamidation disrupts the interaction between Bcl-x_L and endogenous BIM. $p53^{-/-}$, $bcl\text{-}x^{-/-}$ MEFs were transfected with expression vectors for either Bcl-x_L (N52A/N66A) or Bcl-x_L (N52D/N66D). The cells were then treated with 5 μM cisplatin for 24 hr to induce BIM expression. Immunoprecipitation was performed with an antibody for BIM. Total Bcl-x_L, coimmunoprecipitated Bcl-x_L, and BIM were detected by immunoblotting.

expressed BIM more readily than does the deamidated form (Figure 4B). We then took advantage of our finding that BIM is upregulated by cisplatin treatment of $p53^{-/-}$ mouse embryo fibroblasts (MEFs) (Figure 4C) to assess the ability of the nondeamidated and the constitutively deamidated forms of Bcl-x_L to bind to endogenous BIM. We found that whereas endogenous BIM binds to the nondeamidated form of Bcl-x_L, it fails to bind to Bcl-x_L once the Bcl-x_L is deamidated (Figure 4C). Thus, deamidation disrupts the antiapoptotic activity of Bcl-x_L at least in part by disrupting the ability of Bcl-x_L to block the proapoptotic activity of BH3 domain-only proteins.

The Antiapoptotic Activity of Rb Is Dependent upon Bcl-x_L in Tumor Cells and Fibroblasts

This work was initiated in an attempt to identify proapoptotic signals or events that are actively suppressed in the cells of normal tissue. Because Rb is absent or inactive in most tumors but present in all normal tissue (Nevins, 2001), we were intrigued by the finding that Rb expression in tumor cells suppresses the inactivating deamidation of Bcl-x_L (Figures 1H and 2B) and that the suppression of Bcl-x_L deamidation correlates with the cisplatin resistance afforded by Rb expression in both SAOS-2 (Figures 1A and 1H) and HTB-9 cells (data not shown). Therefore, we wanted to determine if suppression of Bcl-x_L deamidation is a component of the antiapoptotic activity of Rb.

Because deamidation completely inactivates the antiapoptotic function of Bcl-x_L, we first sought to determine the consequences of Bcl-x_L inactivation in Rb-arrested SAOS-2 cells. To do so, we transfected SAOS-2 cells with Bcl-x antisense (Figure 5A, left) and then arrested the cells by inducing Rb expression (Figure 5A, middle) before they were treated with cisplatin. We found that inhibition of Bcl-x_L expression rendered the growth-arrested cells sensitive to cisplatin (Figure 5A, right). Therefore, Rb-arrested SAOS-2 cells are dependent upon the presence of active Bcl-x_L to inhibit apoptosis.

The endogenous Rb in fibroblasts inhibits cisplatin-induced apoptosis. This is indicated by the finding that $rb^{-/-}$ MEFs are susceptible to cisplatin-induced apoptosis (Knudsen et al., 2000). Because we had found that the antiapoptotic effect of Rb is dependent upon the presence of active Bcl-x_L in SAOS-2 cells, we considered the possibility that the antiapoptotic effect of Rb in fibroblasts is also dependent upon the presence of active Bcl-x_L. Indeed, we found that inhibition of fibroblast Bcl-x_L expression rendered normal human diploid fibroblasts (NHDFs) susceptible to cisplatin-induced apoptosis (Figure 5B).

To confirm that the increased susceptibility of the fibroblasts was not an artifact of the antisense treatment, we assessed the cisplatin susceptibility of wild-type, $bcl\text{-}x^{-/-}$, and $bcl\text{-}x^{+/-}$ MEFs. We found that $bcl\text{-}x^{-/-}$ MEFs were significantly more susceptible to cisplatin treatment than wild-type MEFs (Figure 5C), indicating that the innate cisplatin resistance of wild-type fibroblasts is dependent upon the presence of active Bcl-x_L, just as it is dependent upon the presence of Rb. Furthermore, we found that the $bcl\text{-}x^{+/-}$ MEFs were of intermediate susceptibility (Figure 5C), which indicates that the level of active Bcl-x_L is a critical determinant of susceptibility to cisplatin. Because the endogenous Rb in fibroblasts inhibits apoptosis, these findings indicate that the effectiveness of Rb as an antiapoptotic protein is dependent upon the amount of active Bcl-x_L in fibroblasts.

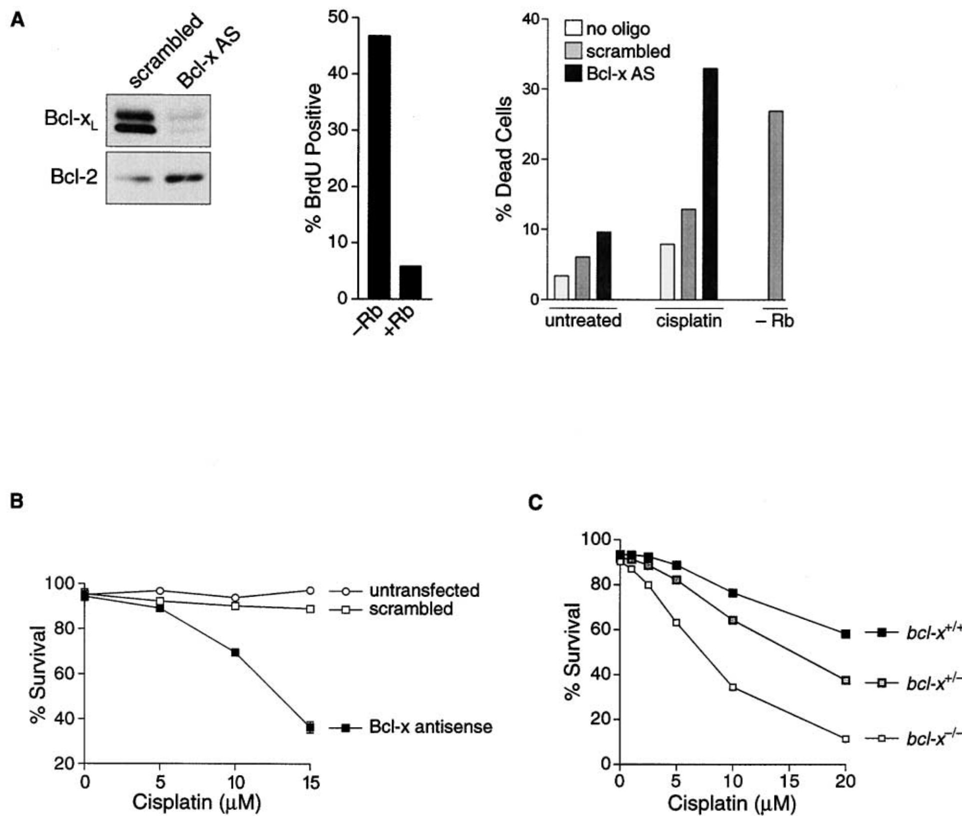

Figure 5. Bcl-x_L Is Necessary to Inhibit Cisplatin-Induced Apoptosis of Both Rb-Arrested Tumor Cells and Normal Fibroblasts

(A) The antiapoptotic activity of Rb in SAOS-2 cells is dependent upon the presence of Bcl-x_L. Left: Bcl-x antisense inhibits Bcl-x_L expression without affecting expression of Bcl-2. Rb-inducible SAOS-2 cells were treated with Bcl-x antisense, and then Rb expression was induced by dox for 36 hr. Immunoblot of Bcl-x_L and Bcl-2 from these cells. Middle: Rb expression arrests SAOS-2 cells. SAOS-2 cells in which Rb expression was induced as indicated. Cells were then labeled with BrdU for 8 hr. BrdU uptake was quantitated by flow cytometry. More than 90% of the cells that expressed Rb were in G_1 as assessed by propidium iodide staining and flow cytometry (data not shown). Right: Inhibition of Bcl-x_L expression renders Rb-arrested SAOS-2 cells susceptible to cisplatin. SAOS-2 cells were transfected as indicated and then Rb expression was induced as indicated for 36 hr prior to treatment with cisplatin (5 μM).

(B) NHDFs are dependent upon Bcl-x_L to maintain their innate resistance to cisplatin. Fibroblasts were transfected with Bcl-x antisense or control. Cells were treated with cisplatin for 24 hr.

(C) MEFs are dependent upon Bcl-x_L to maintain their innate resistance to cisplatin and the resistance correlates with *bcl-x* gene dosage. Dose response of *bcl-x*$^{+/+}$, *bcl-x*$^{+/-}$, and *bcl-x*$^{-/-}$ MEFs. Survival was assessed at 48 hr.

Suppression of Bcl-x_L Deamidation Is a Critical Component of the Innate Cisplatin Resistance of Fibroblasts

We had found that an Rb-mediated growth arrest suppresses Bcl-x_L deamidation in tumor cells (Figure 1H), so we considered the possibility that Rb might inhibit fibroblast apoptosis in part because it suppresses deamidation of Bcl-x_L. Therefore, we sought to determine the role of Rb in the regulation of deamidation of Bcl-x_L in fibroblasts.

Several DNA-damaging agents have been found to induce activation (dephosphorylation) of fibroblast Rb (Slebos et al., 1994). We wanted to confirm that Rb is active in cisplatin-treated MEFs. Rb rapidly became dephosphorylated upon cisplatin treatment (Figure 6A). Cyclin A expression is downregulated in response to DNA damage in an Rb-dependent manner (Knudsen et al., 1999). Cyclin A was downregulated by cisplatin treatment, confirming that Rb is active in cisplatin-treated MEFs (Figure 6A). Because DNA-damaging agents fail to activate Rb in cells lacking p53 (Slebos et al., 1994), we examined the Rb and cyclin A of cisplatin-treated *p53*$^{-/-}$ MEFs. Rb phosphorylation was unchanged and cyclin A expression was not suppressed by cisplatin treatment (Figure 6A).

Because p53 null MEFs fail to activate Rb in response to treatment with cisplatin (Figure 6A), we thought it notable that inactivation of p53 renders NHDFs and MEFs susceptible to DNA-damaging agents (Figure 5D; Fan et al., 1995; Hawkins et al., 1996) (we note that it has been reported that wild-type fibroblasts are more sensitive to DNA-damaging agents than *p53*$^{-/-}$ MEFs; however, the cells used in the study were transformed with the viral oncogene E1a [Lowe et al., 1993]). We hypothesized that *p53*$^{-/-}$ fibroblasts are susceptible to cisplatin-induced apoptosis, at least in part, because the lack of Rb activation in these cells would be permissive for Bcl-x_L deamidation in the same manner that the lack of Rb activation is permissive for expression of cyclin A in cisplatin-treated *p53*$^{-/-}$ MEFs.

Indeed, whereas cisplatin treatment had no effect on the Bcl-x_L in wild-type MEFs, the Bcl-x_L from cisplatin-

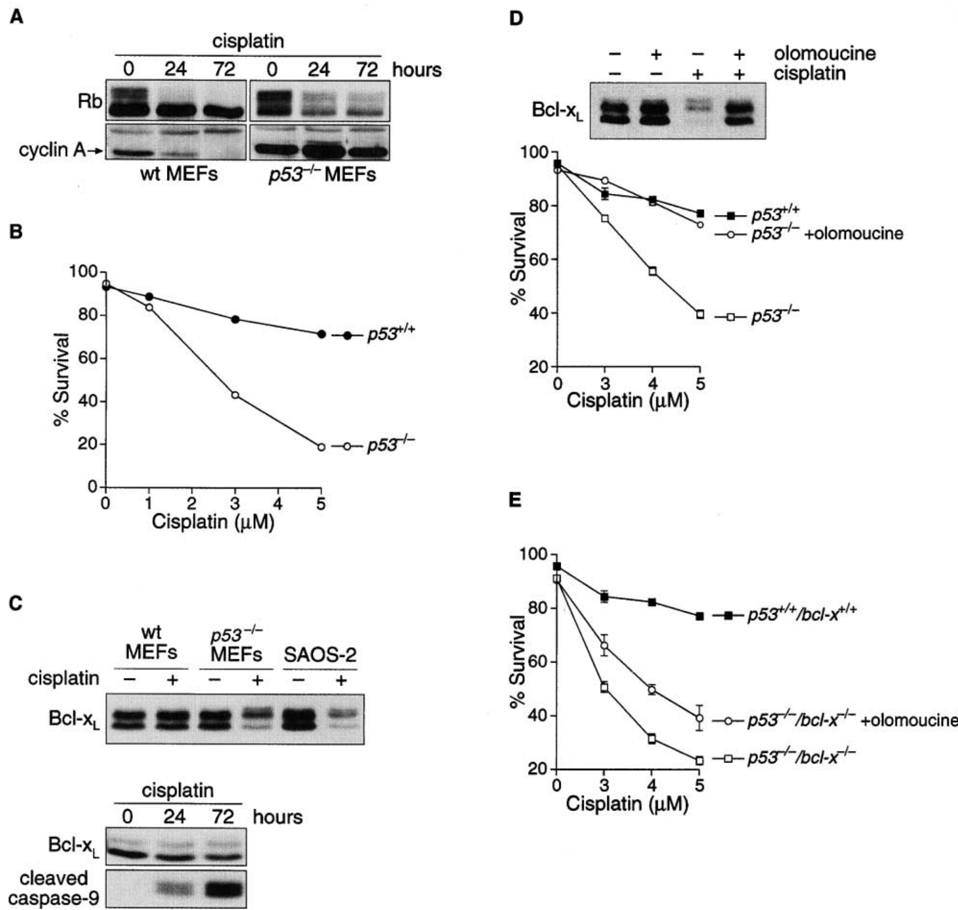

Figure 6. Suppression of Bcl-x_L Deamidation Is Necessary to Inhibit Cisplatin-Induced Apoptosis in Fibroblasts

(A) Rb is activated in a p53-dependent manner by cisplatin. MEFs were treated with cisplatin (10 μM) for the indicated time. The Rb in the MEFs was activated by cisplatin treatment, as indicated by the fact that it became dephosphorylated and the finding that expression of cyclin A was downregulated. These changes did not occur in *p53*$^{-/-}$ MEFs. The decreased levels of Rb in the cisplatin-treated *p53*$^{-/-}$ MEFs is likely to be due to caspase cleavage (Tan and Wang, 1998).

(B) Loss of p53 renders MEFs susceptible to cisplatin. Dose response of *p53*$^{-/-}$ and wild-type MEFs. Survival was assessed at 96 hr.

(C) Loss of p53 renders Bcl-x_L susceptible to cisplatin-induced deamidation. Top: immunoblot of Bcl-x_L from wild-type (wt) MEFs, *p53*$^{-/-}$ MEFs, and SAOS-2 cells treated with cisplatin (5 μM) as indicated. Bottom: Bcl-x_L deamidation is not a consequence of apoptosis in fibroblasts. Immunoblot of Bcl-x_L and cleaved caspase 9 from cisplatin-treated *bcl-x*$^{+/-}$ MEFs. Nearly all of the cells had died by 72 hr (data not shown).

(D) Olomoucine suppresses Bcl-x_L deamidation and renders *p53*$^{-/-}$ MEFs resistant to cisplatin. Top: immunoblot of Bcl-x_L from *p53*$^{-/-}$ MEFs treated with olomoucine and cisplatin as indicated. Bottom: dose response of *p53*$^{-/-}$ MEFs treated with cisplatin and olomoucine as indicated. Cells were maintained in olomoucine for 24 hr prior to and during cisplatin treatment.

(E) The olomoucine-induced cisplatin resistance of *p53*$^{-/-}$ MEFs is dependent upon active Bcl-x_L. Dose response of *p53*$^{-/-}$, *bcl-x*$^{-/-}$ MEFs treated with olomoucine and cisplatin as indicated. Cells were maintained in olomoucine for 24 hr prior to and during cisplatin treatment.

treated *p53*$^{-/-}$ MEFs migrated in precisely the same pattern as the Bcl-x_L from susceptible cisplatin-treated human tumor cells (Figure 6C). This was not an effect of apoptosis because the Bcl-x_L in *bcl-x*$^{+/-}$ MEFs remains unperturbed during cisplatin-induced apoptosis (Figure 6C). These results indicate that the Bcl-x_L in *p53*$^{-/-}$ MEFs is susceptible to inactivation by deamidation. In Rb-arrested SAOS-2 cells and fibroblasts, we found that active Bcl-x_L is a critical component of the resistance to DNA-damaging agents (Figures 5A–5C). We therefore sought to determine if the maintenance of Bcl-x_L activity by the suppression of deamidation is required for resistance to cisplatin-induced apoptosis in fibroblasts.

p53 causes Rb dephosphorylation by inducing expression of the cyclin-dependent kinase (cdk) inhibitor $p21^{CIP1}$; hence, in *p53*$^{-/-}$ MEFs, the failure to activate Rb in response to DNA damage is due to the lack of increased $p21^{CIP1}$ expression (Dulic et al., 1994; Harper et al., 1993, 1995). Therefore, we assessed the effect of cdk inhibition on the cisplatin susceptibility of *p53*$^{-/-}$ MEFs by treating them with the cdk inhibitor olomoucine. Strikingly, olomoucine rendered the *p53*$^{-/-}$ MEFs as resistant to cisplatin-induced apoptosis as wild-type MEFs (Figure 6D), and this correlated with the suppression of Bcl-x_L deamidation (Figure 6D). To determine if suppression of Bcl-x_L deamidation is necessary for the protective effect of the olomoucine on the *p53*$^{-/-}$ MEFs, we assessed the effect of olomoucine on the cisplatin sensitivity of *p53*$^{-/-}$ MEFs that lacked Bcl-x_L (*p53*$^{-/-}$, *bcl-x*$^{-/-}$ MEFs). Whereas olomoucine rendered *p53*$^{-/-}$

MEFs as resistant to cisplatin as wild-type MEFs, olomoucine failed to confer the same level of resistance to the *p53*$^{-/-}$, *bcl-x*$^{-/-}$ MEFs as it did to *p53*$^{-/-}$ MEFs (Figure 6E). Therefore, if Bcl-x_L is inactivated, olomoucine is ineffective in blocking cisplatin-induced apoptosis of MEFs that lack p53. Because deamidation of Bcl-x_L results in the inactivation of Bcl-x_L (Figure 4A), these results indicated that suppression of Bcl-x_L deamidation is critical for the suppression of cisplatin-induced apoptosis in fibroblasts.

Suppression of Bcl-x_L Deamidation Keeps BH3 Domain-Only Protein Activity in Check

It is notable that our experiments using Bcl-x antisense-treated and *bcl-x*$^{-/-}$ fibroblasts indicate that inactivation of Bcl-x_L only confers susceptibility to cisplatin-induced apoptosis—cisplatin must activate a second proapoptotic signal(s) before fibroblast apoptosis occurs. Therefore, it is notable that DNA damage in fibroblasts results in the upregulation of expression of the proapoptotic BH3 domain-only proteins NOXA and PUMA (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001), yet fibroblasts growth arrest without undergoing apoptosis in response to DNA damage (Di Leonardo et al., 1994). We hypothesized that the upregulation of BH3 domain-only protein expression is the second proapoptotic signal, and that functional Bcl-x_L is required to suppress their proapoptotic activity for fibroblasts to maintain resistance to apoptosis. Therefore, we examined Bcl-x_L regulation of NOXA activity during the fibroblast response to cisplatin.

We first established that NOXA is upregulated by a concentration of cisplatin that does not normally induce apoptosis of NHDFs (Figure 7A). We then examined the effects of cisplatin on NHDFs that were transfected with Bcl-x antisense alone or together with NOXA antisense. As we had found previously, inhibition of Bcl-x_L expression rendered the cells susceptible to cisplatin-induced apoptosis (Figure 7B). Because we had found that NOXA is upregulated upon treatment with cisplatin, this suggested that functional Bcl-x_L is required for the suppression of proapoptotic BH3 domain-only protein activity. Indeed, inactivation of NOXA markedly decreased the cisplatin susceptibility of the Bcl-x antisense-treated cells (Figure 7B), thereby confirming that Bcl-x_L suppresses the proapoptotic activity of NOXA. We note that NOXA antisense would not be expected to completely block the apoptotic response because at least one other BH3 domain-only protein, PUMA, is upregulated by DNA-damaging agents in fibroblasts (Nakano and Vousden, 2001; Yu et al., 2001). These results indicate that in fibroblasts, Bcl-x_L deamidation must be suppressed to maintain resistance to apoptosis because Bcl-x_L activity is necessary to suppress the proapoptotic activity of BH3 domain-only proteins once their expression is upregulated by DNA damage.

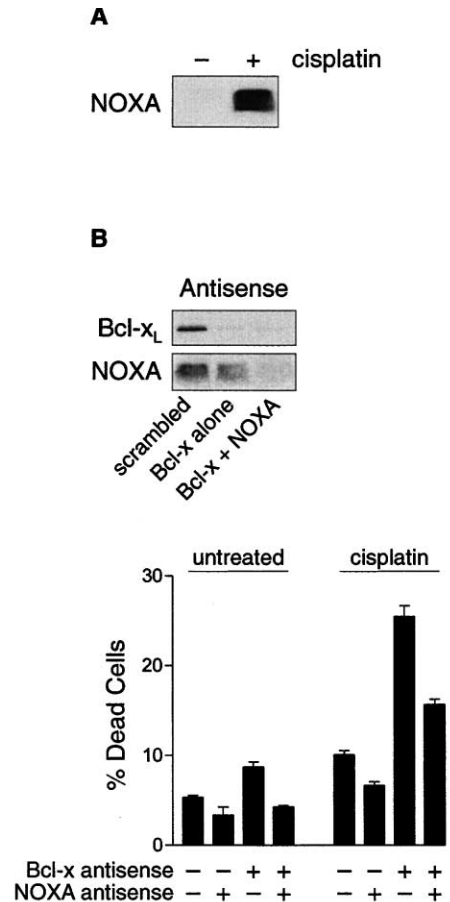

Figure 7. Bcl-x_L Keeps BH3 Domain-Only Protein Activity in Check

(A) NOXA is upregulated in NHDFs by a concentration of cisplatin (10 μM) that does not induce apoptosis. Immunoblot of NOXA from NHDFs treated as indicated.

(B) Bcl-x_L suppresses the proapoptotic activity of NOXA in NHDFs. Top: immunoblots of Bcl-x_L and NOXA from NHDFs treated with antisense as indicated. Immunoblot of Bcl-x_L is from untreated cells; immunoblot of NOXA is from cisplatin-treated (10 μM) cells. Bottom: NHDFs were treated with antisense and cisplatin (10 μM) as indicated. Plus sign indicates cells treated with indicated antisense. Minus sign indicates cells treated with indicated scrambled control.

Discussion

We show here that deamidation plays a critical role in a signal transduction pathway. Although it has been demonstrated that deamidation increases protein turnover (Flatmark, 1966; Robinson and Rudd, 1974), the fact that deamidation is of significance to the cell has never been firmly established. Because it is a relatively slow process, it has been proposed that deamidation functions as a molecular timer of biological events (Robinson and Robinson, 2001). Hence, in respect to the regulation of DNA damage-induced apoptosis, Bcl-x_L deamidation may serve as a chronometric buffer, affording the cell time to reverse low-level genotoxic stress-induced events.

In addition to its tumor-suppressor activity, Rb is a potent antiapoptotic protein—loss of Rb in normal fibroblasts confers sensitivity to DNA-damaging agents (Almasan et al., 1995; Knudsen et al., 2000), and reintroduction of Rb into Rb null tumor confers resistance to these agents (Haas-Kogan et al., 1995). Hence, we reasoned that Rb must suppress proapoptotic signals. We found that Rb suppresses the inactivating deamidation of Bcl-x_L, and indeed, our findings indicate that the anti-

apoptotic activity of Rb is dependent upon the ability of Rb to suppress Bcl-x_L deamidation. Finally, our data suggest that the inactivation of Rb increases the susceptibility of tumor cells to DNA-damaging agents in part because inactivation of Rb is permissive for Bcl-x_L deamidation.

In response to DNA damage, BH3 domain-only proteins are upregulated in fibroblasts (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001); however, fibroblasts growth arrest without undergoing apoptosis in response to DNA damage (Di Leonardo et al., 1994). It has been unclear how the proapoptotic activity of the BH3 domain-only proteins is suppressed in fibroblasts. Our results suggest a mechanism in which the proapoptotic activity of BH3 domain-only proteins is upregulated by DNA damage in cells regardless of whether or not the cell is destined to undergo apoptosis. In fibroblasts, Bcl-x_L deamidation is suppressed, and it must be suppressed to prevent cisplatin-induced apoptosis. In these cells, the proapoptotic activity of the BH3 domain-only proteins is kept in check by Bcl-x_L and other antiapoptotic Bcl-2 family members. However, in cells such as the *p53*$^{-/-}$ MEFs and the tumor cells we studied, Bcl-x_L is inactivated by deamidation in response to DNA damage, and inactivation of Bcl-x_L is sufficient to allow BH3 domain-only proteins to initiate the final stages of apoptosis. We note that we have not excluded the possibility that other antiapoptotic Bcl-2 family members are also inactivated in response to DNA damage, but, as we have demonstrated, inactivation of Bcl-x_L is sufficient to allow the apoptotic process to go to completion.

That Bcl-x_L serves a unique role in the response to DNA-damaging agents is consistent with recent findings demonstrating a correlation between Bcl-x_L expression and resistance to antineoplastic treatment (Amundson et al., 2000). When the 60 cell lines of the National Cancer Institute's Anticancer Drug Screen (NCI-ADS) were assessed for a correlation between expression of a number of cancer-related genes and resistance to chemotherapy, the strongest correlation between gene expression and resistance to a panel of standard chemotherapy agents was for Bcl-x_L. The correlation was stronger than the positive correlation with cytotoxicity that the NCI-ADS had reported for p53. Indeed, the authors referred to the correlation as "striking." The importance of Bcl-x_L in resistance to chemotherapy was further underscored by the finding of the same study that Bcl-2 and Bax levels have no correlation whatsoever with the degree of cellular sensitivity to chemotherapeutic agents. Our finding that resistance correlates with *bcl-x* gene dosage in MEFs suggests that Bcl-x_L truly has a functional role in the resistance to antineoplastic therapy, i.e., it is not just a marker for another cellular characteristic that imparts resistance.

Experimental Procedures

Plasmids

pSGL5-HA-Rb (Sellers et al., 1998), pSFFV-Bcl-x_L (Yang et al., 1995), pEF-EE-Bim_{EL} (O'Connor et al., 1998), pCDNA3-Flag-DN-caspase-9 (Duan et al., 1996), and pCMV-CD20 (van den Heuvel and Harlow, 1993) were generous gifts from W. Kaelin, S. Korsmeyer, A. Strasser, V. Dixit, and S. van den Heuvel, respectively. Mutations were made in pSFFV-Bcl-x_L by converting the codon for asparagine (AAT) to either alanine (GCT) or aspartate (GAT) using QuikChange (Stratagene).

Cell Culture and Transfections

SAOS-2 (ATCC HTB-85), C33A (ATCC HTB-31), MRC-5 fibroblasts (ATCC CCC-171), and MEFs were maintained in DMEM with 10% FBS. HTB-9 (ATCC HTB-9) cells were maintained in RPMI-1640 with 10% FBS. *p53*$^{-/-}$ mice were a generous gift from L. Donehower. Rb-inducible and Bim_{EL}-inducible cells were generated using T-REx (Invitrogen). The Rb-inducible cells were stably transfected with pCDNA3-Flag-DN-caspase-9 for the experiment depicted in Figure 1C. SAOS-2 cells and p53$^{-/-}$, *bcl-x*$^{-/-}$ MEFs were transfected with calcium phosphate. For survival assays, cells were cotransfected with the indicated Bcl-x_L plasmid and a CD20 expression vector.

Cells were treated with cisplatin for 96 hr (SAOS-2 cells) unless otherwise indicated, 48 hr (ovarian cancer cells and MEFs), or 24 hr (antisense experiments); 10 μM etoposide for 96 hr; 20 Gy γ radiation and harvested after 96 hr; or 100 nM paclitaxel for 36 hr unless otherwise indicated. 50 μM olomoucine was used when indicated. Survival was quantified by flow cytometry using the Live/Dead kit (Molecular Probes). Cells were labeled with an anti-CD20 antibody (Pharmingen) as indicated. For alkalinization experiments, cells were lysed in buffers containing 20 mM sodium phosphate and 0.2% Triton X-100 at the indicated pH and incubated at 37°C.

Antisense Treatment

Bcl-x antisense 5′-CTACGCTTTCCACGCACAGT-3′ and control 5′-CTCCGATGTCCCCTCAAAGT-3′ (Taylor et al., 1999) were used at 100 nM in SAOS-2 cells and 200 nM in fibroblasts. NOXA antisense and control (Calbiochem) were used at 50 nM. Oligonucleotides were transfected with Cytofectin GSV (Glen Research).

Immunoblotting

The following antibodies were used: anti-Bcl-x (B22630) and anti-Bcl-2 (B46620) from Transduction Laboratories, anti-Bim (AAP-330) and anti-mouse caspase-9 (AAM-139E) from Stressgen, anti-NOXA (IMG-349) from Imgenex, anti-human caspase-9 (9502) from NEB, anti-cyclin A (H-432) from Santa Cruz, and anti-pRb (14001A) from PharMingen.

Immunoprecipitation

SAOS-2 cells were transfected with pSFFV-Bcl-x_L(N52A/N66A) and pSFFV-Bcl-x_L(N52D/N66D) with pEF-EE-Bim_{EL}. Cells were lysed in 50 mM HEPES (pH 7.0), 250 mM NaCl, 1 mM EDTA, 0.2% NP-40, and Complete Protease Inhibitor (Roche). Lysates were incubated with 1 μg anti-EE antibody (MMS-115P) from Covance Research Products prebound to 20 μl Protein G Dynabeads (Dynal).

For immunoprecipitation of endogenous BIM, *p53*$^{-/-}$, *bcl-x*$^{-/-}$ MEFs were transfected with pCDNA3-Bcl-x_L(N52A/N66A) or pCDNA3-Bcl-x_L(N52D/N66D) and treated with 5 μM cisplatin 48 hr after transfection. Immunoprecipitation was performed with anti-BIM antibody (Ab-1) Oncogene.

Immunofluorescence Staining

DNA adducts were detected using the ICR4 antibody (a gift from Michael Tilby) and the TSA system (PerkinElmer Life Sciences). Cells were fixed in 4% paraformaldehyde, permeabilized in PBS with 0.2% Triton X-100, blocked in TNB blocking buffer, and treated with DNaseI in TNB buffer with 20 mM $MgCl_2$ for 1 hr. The cells were then incubated with ICR4 in TNB buffer, and then an immunofluorescence signal was generated. Anti-cytochrome c (556432 PharMingen) and anti-HA (3F10 Roche) were used to detect cytochrome c and induced Rb, respectively.

Peptide Analysis

The peptide FAVNGA (100 μM) was incubated for 24 hr at 37°C at the indicated pH in 20 mM sodium phosphate. HPLC (Fu et al., 2002) and mass spectrometric analysis (Fu et al., 2001) were performed as previously described.

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