Human MUC1 oncoprotein regulates p53-responsive gene transcription in the genotoxic stress response

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Summary

The MUC1 oncoprotein is aberrantly overexpressed by most human carcinomas. The present work demonstrates that MUC1 associates with the p53 tumor suppressor, and that this interaction is increased by genotoxic stress. The MUC1 cytoplasmic domain binds directly to p53 regulatory domain. Chromatin immunoprecipitation assays demonstrate that MUC1 coprecipitates with p53 on the p53-responsive elements of the *p21* gene promoter and coactivates *p21* gene transcription. Conversely, MUC1 attenuates activation of *Bax* transcription. In concert with these results, MUC1 promotes selection of the p53-dependent growth arrest response and suppresses the p53-dependent apoptotic response to DNA damage. These findings indicate that MUC1 regulates p53-responsive genes and thereby cell fate in the genotoxic stress response.

Introduction

The human DF3/MUC1 integral membrane glycoprotein is expressed on the apical borders of normal secretory epithelial cells (Kufe et al., 1984). With transformation and loss of polarity, MUC1 is found at high levels in the cytosol and over the entire surface of carcinoma cells (Kufe et al., 1984; Perey et al., 1992). MUC1 is expressed as a heterodimer following synthesis as a single polypeptide and cleavage in the endoplasmic reticulum (Ligtenberg et al., 1992). The MUC1 N-terminal subunit (N-ter) consists of variable numbers of 20 amino acid tandem repeats that are modified by O-glycans (Gendler et al., 1988; Siddiqui et al., 1988). MUC1 N-ter is tethered to the cell membrane through dimerization with the C-terminal subunit (C-ter), which consists of a 58 amino acid extracellular domain, a 28 amino acid transmembrane domain, and a 72 amino acid cytoplasmic tail (Merlo et al., 1989). MUC1 interacts with members of the ErbB family (Li et al., 2001b, 2003c; Schroeder et al., 2001) and is targeted to the nucleus and mitochondria (Li et al., 2003c; Ren et al., 2004). MUC1 also associates with β-catenin (Yamamoto et al., 1997), and this interaction is regulated by GSK3β-, Src-, and PKCδ-mediated phosphorylation of the MUC1 cytoplasmic domain (MUC1-CD) (Li et al., 1998, 2001a, 2003a, 2001b; Ren et al., 2002b). Overexpression of MUC1 is sufficient to confer transformation (Huang et al., 2003; Li et al., 2003b; Schroeder et al., 2004) and to attenuate oxidative and genotoxic stress-induced apoptosis (Ren et al., 2004; Yin et al., 2004; Yin and Kufe, 2003).

The p53 tumor suppressor functions in the cellular response to stress by inducing growth arrest, DNA repair, senescence, differentiation, or apoptosis (Levine, 1997). Genotoxic stress, oxidative damage, hypoxia, nucleotide depletion, heat shock, and oncogene expression are associated with stabilization of p53 and induction of p53-mediated transcription. Selective transactivation of p53 target genes dictates the induction of apoptosis or a growth arrest and repair response (Chao et al., 2000; Jimenez et al., 2000). Genes encoding death receptors Fas/CD95 and DR5 (Muller et al., 1998; Wu et al., 1997) or proapoptotic effectors such as Bax, Noxa, and Puma (Miyashita and Reed, 1995; Oda et al., 2000a; Yu et al., 2001) contribute to the induction of a p53-dependent apoptotic response. Alternatively, the growth arrest response to p53 activation is mediated in large part by induction of the *p21* gene (El-Deiry et al., 1993). p21 plays a role in promoting cell cycle progression and preventing apoptosis (Asada et al., 1999; Dong et al., 2004; Dupont et al., 2003; Weiss, 2003; Zhang et al., 2003). The choice of cell fate is influenced by growth factor stimulation, proliferation status, and extent of damage (Vousden and Lu, 2002; Wahl and Carr, 2001). Promoter selectivity for p53-induced apoptosis is also influenced by phosphoryla-

SIGNIFICANCE

The MUC1 oncoprotein is aberrantly overexpressed in about 800,000 of the 1.3 million tumors diagnosed annually in the United States. MUC1 localizes to mitochondria and blocks activation of the intrinsic apoptotic pathway. The present studies demonstrate that MUC1 binds directly to the p53 tumor suppressor and coactivates DNA damage-induced transcription of the *p21* gene. By contrast, MUC1 attenuates activation of the proapoptotic *Bax* gene. The demonstration that MUC1 both promotes the p53-dependent growth arrest response and suppresses the p53-dependent apoptotic response to DNA damage further indicates that overexpression of MUC1 by human tumors could be of importance to cell fate selection in the activation of p53 by genotoxic anticancer agents.

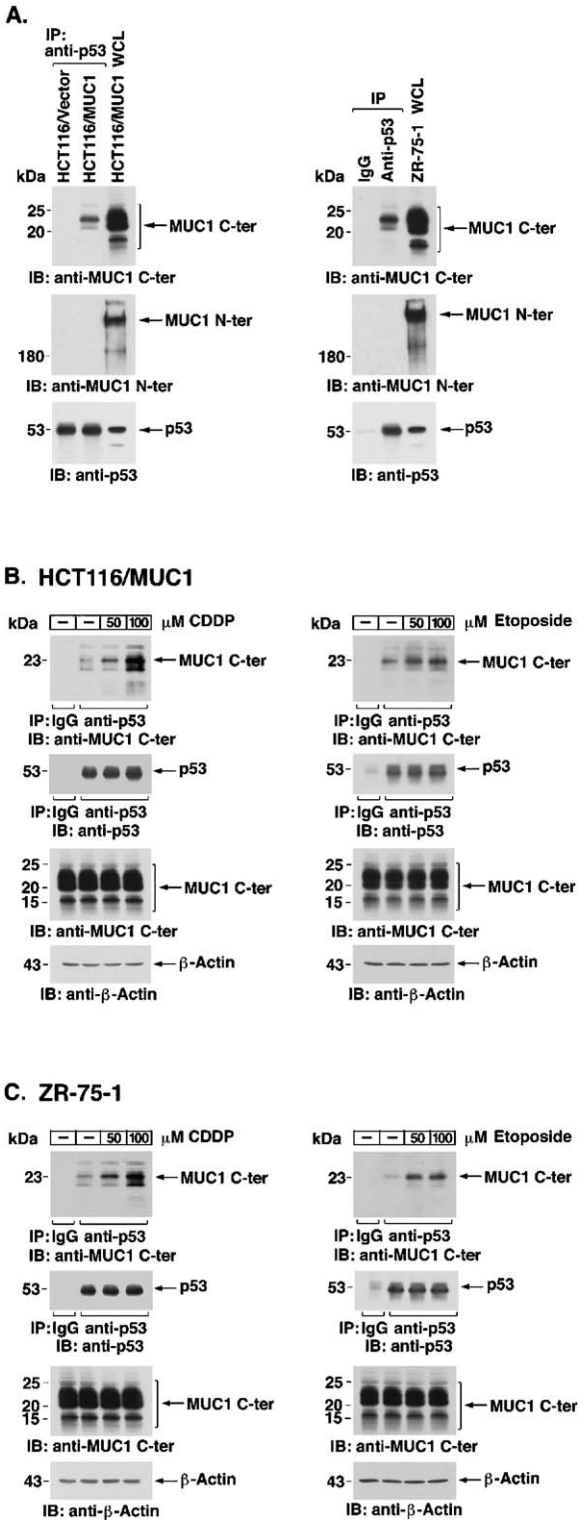

Figure 1. MUC1 associates with p53

A: Lysates from HCT116/vector and HCT116/MUC1 cells were immunoprecipitated with anti-p53. The precipitates were analyzed by immunoblotting with anti-MUC1 C-ter, anti-MUC1 N-ter, and anti-p53 (left). Anti-p53 and control IgG immunoprecipitates from ZR-75-1 cells were immunoblotted with the indicated antibodies (right). WCL, whole cell lysate.

B and C: HCT116/MUC1 (**B**) and ZR-75-1 (**C**) cells were treated with 50 or 100 μM cisplatin (CDDP; left) or etoposide (right) for 24 hr. Lysates were immunoprecipitated with IgG or anti-p53 and the precipitates were analyzed by immunoblotting with anti-MUC1 C-ter and anti-p53 (upper 2 panels). Lysates were directly immunoblotted with anti-MUC1 C-ter and anti-β-actin (lower 2 panels).

tion of p53 on serines 20 and 46 (Jack et al., 2002; Oda et al., 2000b) and by interactions between p53 and members of the ASPP family (Samuels-Lev et al., 2001), the JMY p300 binding protein (Shikama et al., 1999), the E2F transcription factor (Hsieh et al., 2002), and the p53 family members p73/p63 (Flores et al., 2002). Although certain insights are thus available regarding the signals that activate p53-mediated apoptosis, less is known about the mechanisms responsible for selection of the p53- and p21-dependent growth arrest response.

The present results demonstrate that MUC1 binds directly to p53 and promotes p53-dependent cell fate selection of the growth arrest and survival responses to genotoxic anticancer agents.

Results

MUC1 associates with p53

To investigate whether MUC1 associates with p53, lysates from HCT116/vector and HCT116/MUC1 cells were immunoprecipitated with anti-p53. Immunoblot analysis of the precipitates with anti-MUC1 C-ter (reacts with the C-terminal 17 amino acids of the MUC1 cytoplasmic domain [MUC1-CD]) showed that the ~23 kDa MUC1 C-ter coprecipitates with p53 (Figure 1A, left). In contrast, there was no detectable MUC1 N-ter in the anti-p53 immunoprecipitates (Figure 1A, left). Similar studies performed on human ZR-75-1 breast cancer cells confirmed coimmunoprecipitation of p53 and MUC1 (Figure 1A, right). Densitometric scanning of the MUC1 signals obtained from the whole cell lysates as compared to that after immunoprecipitation of the lysates with anti-p53 indicate that 4% and 5% of total MUC1 C-ter associates with p53 in HCT116/MUC1 and ZR-75-1 cells, respectively. Treatment of HCT116/MUC1 cells with cisplatin or etoposide resulted in an increase in the association of p53 and MUC1 (Figure 1B). Increased binding of endogenous MUC1 and p53 was also found in ZR-75-1 cells treated with cisplatin (Figure 1C, left) or etoposide (Figure 1C, right). Similar results were obtained with human MCF-7 breast and LNCaP prostate cancer cells (Supplemental Figures S1A and S1B). These findings demonstrate that MUC1 associates with p53 constitutively and that this interaction is increased in the response to DNA damage.

MUC1-CD binds directly to p53

To define the sequences of MUC1-CD (amino acids 1–72) and p53 involved in the association, we prepared vectors expressing wild-type and deletion mutants of both proteins (Figure 2A, schema). Incubation of purified ^{35}S-labeled p53 with purified GST or GST-MUC1-CD demonstrated binding to MUC1-CD (Figure 2A). Deletion of MUC1-CD from amino acids 9 to 46 abrogated the interaction, while MUC1-CD (1–51) was sufficient to form a complex with p53 in vitro (Figure 2A). To confirm these findings, we expressed p53 and MUC1-CD deletion mutants in cells. As found in vitro, p53 formed complexes with MUC1-CD (1–72), but not with MUC1-CD (△9–46) (Figure 2B).

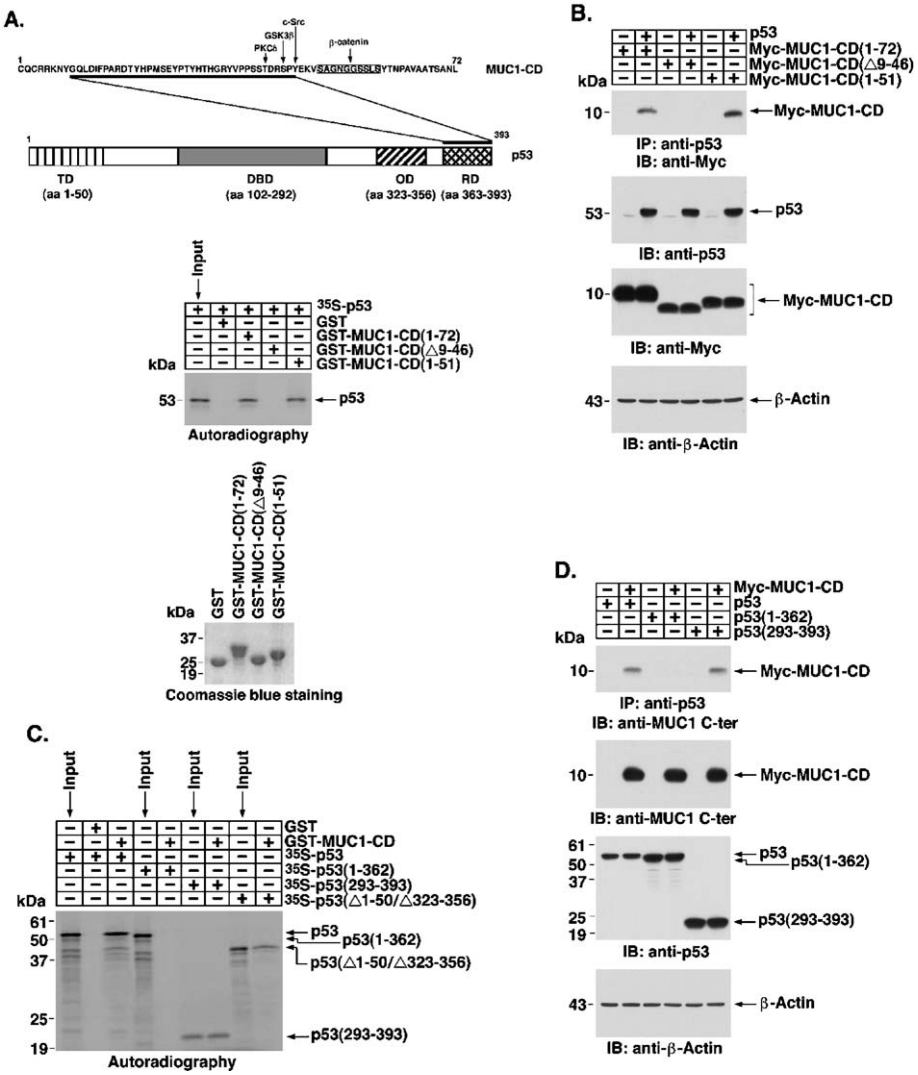

Figure 2. MUC1-CD binds directly to the p53 regulatory domain

A: Schema depicting the structures of MUC1-CD and p53. Highlighted on MUC1-CD are phosphorylation sites and the β-catenin binding site (upper panel). GST, GST-MUC1-CD (1–72), or the indicated GST-MUC1-CD deletion mutants were bound to glutathione agarose and incubated with ^{35}S-labeled p53. The adsorbates were analyzed by SDS-PAGE and autoradiography (lower panel). Input of the GST and GST-MUC1-CD proteins was assessed by Coomassie blue staining.

B: Anti-p53 immunoprecipitates from U2OS cells transfected with 2 μg p53 and 1 μg Myc-MUC1-CD or the indicated deletion mutants were immunoblotted with anti-Myc (upper panel). Lysates were directly immunoblotted with anti-p53, anti-Myc, and anti-β-actin (lower 3 panels).

C: GST or GST-MUC1-CD were bound to glutathione agarose and incubated with ^{35}S-labeled p53 or the indicated p53 deletion mutants. The adsorbates were analyzed by SDS-PAGE and autoradiography.

D: U2OS cells were transfected with 1 μg Myc-MUC1-CD and 2 μg p53 or the indicated deletion mutants. Anti-p53 (Ab-2/Ab-6) immunoprecipitates were analyzed by immunoblotting with anti-MUC1 C-ter (upper panel). Lysates were directly immunoblotted with anti-MUC1 C-ter, anti-p53, and anti-β-actin (lower 3 panels).

In addition, we found in vivo binding of p53 to MUC1-CD (1–51) (Figure 2B). To define the region within p53, GST-MUC1-CD was incubated with full-length p53 and certain deletion mutants. Deletion of the p53 C terminus (amino acids 363–393) abrogated binding to MUC1-CD (Figure 2C). In concert with these results, MUC1-CD formed complexes with p53 (293–393) and p53 (△1–50/△323–356) in vitro (Figure 2C). Expression of Myc-MUC1-CD and the p53 deletion mutants in cells demonstrated that, as found in vitro, the interaction with MUC1-CD is abrogated by expression of p53 (1–362) (Figure 2D). The results also show that MUC1-CD associates with p53 (293–393) in cells (Figure 2D). These findings indicate that MUC1-CD (amino acids 9–46) binds directly to the p53 regulatory domain (amino acids 363–393).

MUC1 occupies the p53-responsive *p21* gene promoter

To determine if MUC1 is present in the p53 transcription complex, we performed chromatin immunoprecipitation (ChIP) assays on the *p21* promoter with anti-MUC1 C-ter or a control IgG. Immunoprecipitation of the two p53-responsive elements (p53REs) in the *p21* promoter was analyzed by semiquantitative PCR. Using HCT116/MUC1 and ZR-75-1 cells, occupancy of both *p21* promoter p53REs by MUC1 was clearly detectable in anti-MUC1, and not the control IgG, precipitates (Figure 3A, left). By contrast, there was no detectable MUC1 associated with a control region (CR) of the *p21* promoter downstream to the p53REs (Figure 3A, left). There was also no detectable MUC1 associated with the *p21* proximal promoter (PP) (Figure 3A, right). To determine if MUC1 occupies the *p21* promoter with p53, the anti-MUC1 complexes were released, reimmunoprecipitated with anti-p53, and then analyzed by PCR (Re-ChIP). The results show that anti-p53 precipitates the *p21* p53REs after their release from anti-MUC1, indicating that MUC1 occupies these elements with p53 (Figure 3B). A kinetic analysis after treatment of HCT116/MUC1 and ZR-75-1 cells with cisplatin or etoposide demonstrated that MUC1 occupancy of *p21* p53RE2 increases in response to DNA damage and reaches maximal levels at 8–12 hr of drug exposure (Figure 3C). Moreover, as expected from other work (Espinosa et al., 2003), treatment of HCT116/vector cells with cisplatin or eto-

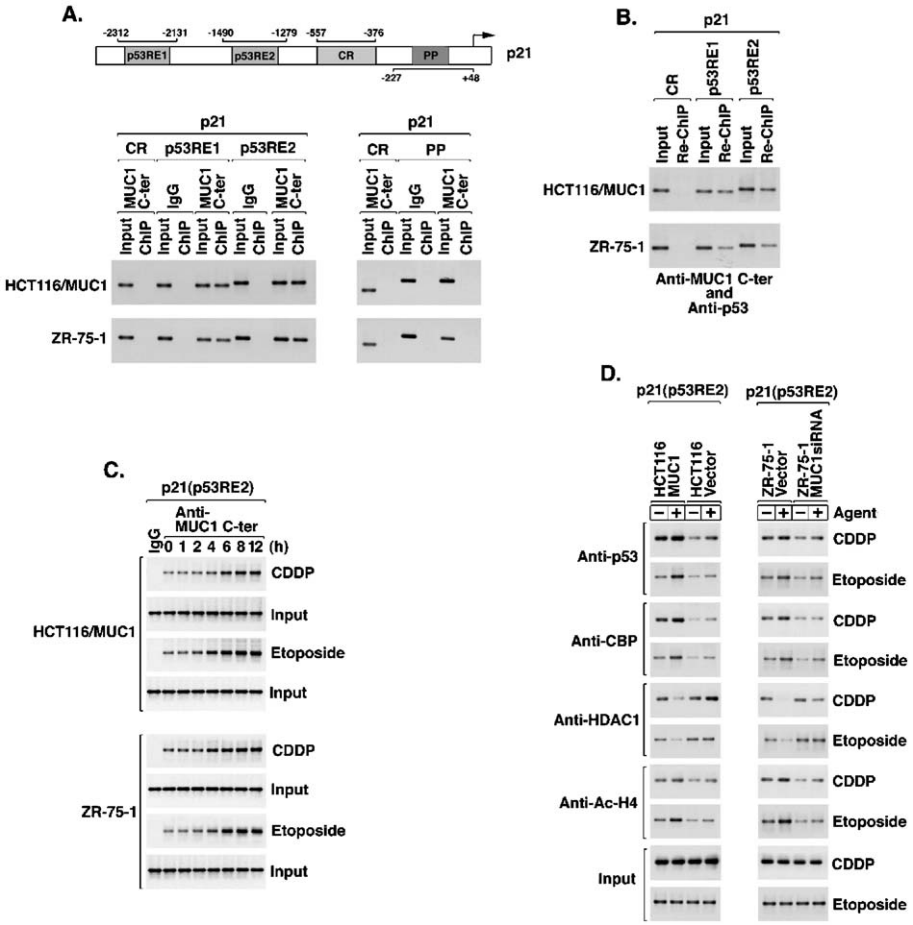

Figure 3. MUC1 C-ter occupies p53-responsive elements in the *p21* promoter

A: Soluble chromatin from HCT116/MUC1 and ZR-75-1 cells was immunoprecipitated with anti-MUC1 C-ter or a control IgG. The final DNA extractions were amplified by PCR using primers that cover the indicated p53-response elements (p53REs; −2281 to −2262 and −1395 to −1376), a control region (CR), or the proximal promoter (PP; TATA box located at −46 to −43) region of the *p21* gene.

B: In Re-ChIP experiments, soluble chromatin from HCT116/MUC1 or ZR-75-1 cells was immunoprecipitated with anti-MUC1 C-ter, eluted with DTT, diluted with Re-ChIP buffer, reimmunoprecipitated with anti-p53, and analyzed for the indicated *p21* promoter sequences.

C: HCT116/MUC1 and ZR-75-1 cells were treated with 50 μM cisplatin or etoposide for the indicated times. Soluble chromatin was immunoprecipitated with anti-MUC1 C-ter and analyzed for *p21* p53RE2 sequences.

D: HCT116/MUC1 and HCT116/vector cells (left) or ZR-75-1/vector and ZR-75-1/MUC1siRNA (right) were left untreated or treated with 50 μM cisplatin or etoposide for 8 hr. Soluble chromatin was immunoprecipitated with the indicated antibodies. The precipitates were analyzed for *p21* p53RE2 promoter sequences.

poside increased p53 occupancy of the *p21* p53RE2 (Figure 3D, left). However, occupancy of the *p21* p53RE2 by p53 was higher constitutively and after DNA damage in HCT116/MUC1 cells (Figure 3D, left). The CREB binding protein (CBP) functions as a histone acetyltransferase and coactivator of transcription. MUC1 expression was associated with an increase in CBP occupancy of the *p21* p53RE2, indicating that MUC1 is associated with recruitment of CBP to the p53 transcription complex (Figure 3D, left). In addition, occupancy of the *p21* promoter by the histone deacetylase HDAC1 was decreased in the response of HCT116/MUC1 cells to cisplatin or etoposide as compared to that found in HCT116/vector cells (Figure 3D, left). Acetylation of histone H4 was also more pronounced on the *p21* promoter in MUC1-positive, as compared to MUC1-negative, HCT116 cells (Figure 3D, left). ChIP studies of the *p21* promoter in ZR-75-1/vector cells, which express endogenous MUC1, and MUC1-negative ZR-75-1/MUC1siRNA cells further demonstrated that MUC1 increases p53 and CBP occupancy, decreases HDAC1 occupancy, and increases histone H4 acetylation (Figure 3D, right). These findings indicate that endogenous, as well as exogenous, MUC1 is detectable on the p53REs in the *p21* promoter, and that MUC1 occupancy is associated with increased acetylation of histone H4.

MUC1 occupies the *Bax* proximal promoter

To assess binding of MUC1 to other p53-responsive genes, ChIP analyses were performed on the p53RE in the *Bax* promoter (Figure 4A). The results show that p53, and not MUC1, occupancy of the *Bax* p53RE is detectable in HCT116/MUC1 and ZR-75-1 cells (Figure 4A, left). Similar results were obtained after treatment of these cells with cisplatin or etoposide (data not shown). In contrast, p53 and MUC1 occupancy of the *Bax* proximal promoter (PP) was detectable in both HCT116/MUC1 and ZR-75-1 cells (Figure 4A, right). The results also show that anti-p53 precipitates the *Bax* proximal promoter after release from anti-MUC1 (Figure 4B, left). An antibody against the TATA binding protein (TBP) also precipitated the Bax proximal promoter after release from anti-MUC1 (Figure 4B, right), indicating that MUC1 occupies this region with the basal transcription apparatus. MUC1 occupancy of the *Bax* proximal promoter was increased through 4–6 hr and maximal at 6–12 hr after treatment with cisplatin or etoposide (Figure 4C). Analysis of the *Bax* proximal promoter following treatment with cisplatin or etoposide also demonstrated that MUC1 expression is associated with (1) increases in p53 binding and (2) decreases in occupancy of TFIIB and TAFII250, but not TBP (Figure 4D). These findings indicate that MUC1 occupies the *Bax* proximal promoter with p53 and interferes with assembly of the basal transcription apparatus in the DNA damage response.

MUC1-CD regulates transactivation of the *p21* and *Bax* promoters

To determine if MUC1 affects activation of the *p21* promoter, HCT116/vector and HCT116/MUC1 cells were transfected with

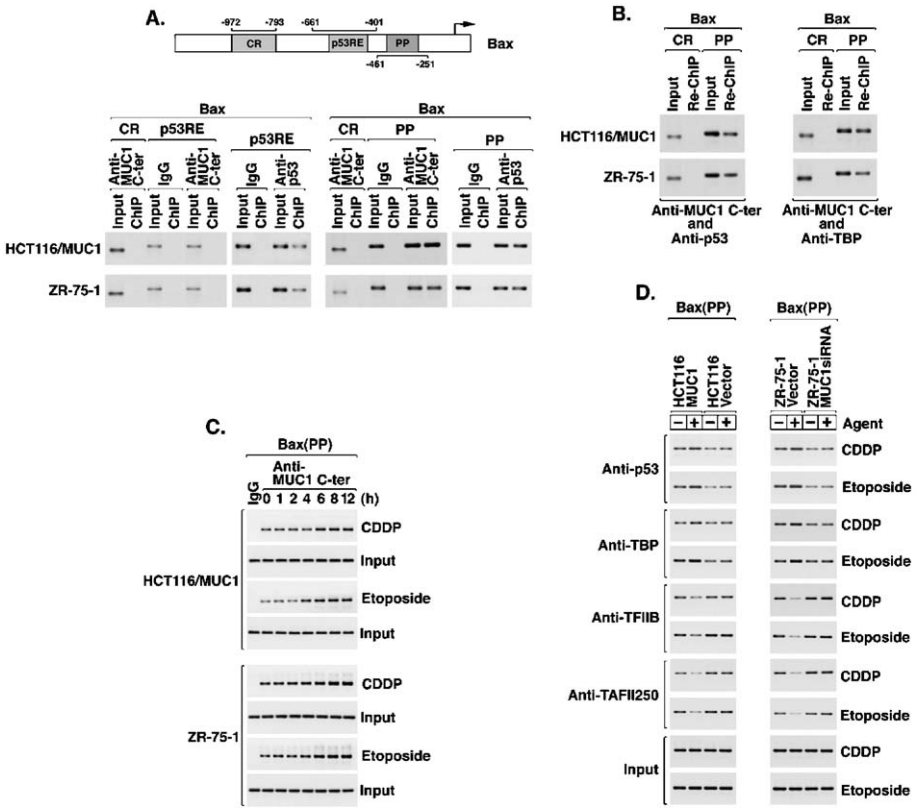

Figure 4. MUC1 C-ter occupies the proximal promoter in the *Bax* gene

A: Soluble chromatin from HCT116/MUC1 and ZR-75-1 cells was immunoprecipitated with anti-MUC1 C-ter or a control IgG. The final DNA extractions were amplified by PCR using primers that cover a control region (CR), the p53-response element (p53RE; −474 to −465), or the proximal promoter (PP; TATA box located at −398 to −395) region of the *Bax* gene.

B: In Re-ChIP experiments, soluble chromatin from HCT116/MUC1 or ZR-75-1 cells was immunoprecipitated with anti-MUC1 C-ter, eluted with DTT, diluted with Re-ChIP buffer, reimmunoprecipitated with anti-p53 or anti-TBP, and analyzed for *Bax* CR and PP sequences.

C: HCT116/MUC1 and ZR-75-1 cells were treated with 50 μM cisplatin or etoposide for the indicated times. Soluble chromatin was immunoprecipitated with anti-MUC1 C-ter and analyzed for *Bax* PP sequences.

D: HCT116/MUC1 and HCT116/vector cells (left) or ZR-75-1/vector and ZR-75-1/MUC1siRNA (right) were left untreated or treated with 50 μM cisplatin or etoposide for 8 hr. Soluble chromatin was immunoprecipitated with the indicated antibodies. The precipitates were analyzed for *Bax* PP sequences.

a *p21* promoter-Luc reporter vector (p21-Luc). Basal levels of *p21* promoter activity were increased in HCT116/MUC1 as compared to HCT116/vector cells (Figure 5A, left). In addition, cisplatin- or etoposide-induced activation of p21-Luc was enhanced as a result of MUC1 expression (Figure 5A, left). For comparison, similar studies were performed with the *Bax* promoter-Luc reporter (Bax-Luc). In contrast to p21-Luc, basal and DNA damage-induced levels of Bax-Luc activity were decreased by MUC1 expression (Figure 5A, right). Similar results were obtained with the ZR-75-1/vector and ZR-75-1/MUC1 siRNA cells (Figure 5B). Immunoblot analysis of lysates from control and cisplatin- or etoposide-treated HCT116 cells demonstrated that constitutive and drug-induced expression of p21 is increased in HCT116/MUC1, as compared to HCT116/vector, cells (Figure 5C). By contrast, levels of Bax were attenuated in control and cisplatin- or etoposide-treated HCT116/MUC1 cells (Figure 5C). MUC1 expression was also associated with selective increases in p21 protein in ZR-75-1 cells (Figure 5D). These findings indicate that MUC1 selectively coactivates *p21* gene transcription in the response to DNA damage.

MUC1 activates the growth arrest and survival response to DNA damage

Consistent with the increases in p21 expression, treatment of both HCT116/vector and HCT116/MUC1 cells with cisplatin or etoposide resulted in G1 arrest of cell cycle progression (Supplemental Figure S2A). However, the HCT116/MUC1 cells exhibited greater accumulation in G1 phase than that found with HCT116/vector cells (Supplemental Figure S2A). Additional experiments performed at 12 to 48 hr of drug exposure confirmed that MUC1 increases the percentage of cells that exhibit G1 phase arrest (Figure 6A). Treatment of ZR-75-1/vector and ZR-75-1/MUC1siRNA cells with cisplatin or etoposide provided further evidence for involvement of MUC1 in conferring an increase in the arrest of cells at G1 phase (Supplemental Figure S2B and Figure 6B). Activation of the p53-induced growth arrest response can be irreversible or associated with repair and survival (Oren, 2003; Weiss, 2003). To distinguish between these potential outcomes, we treated the HCT116 cells with cisplatin or etoposide and monitored survival by colony formation. Clonogenic survival was higher for cisplatin- or etoposide-treated HCT116/MUC1 as compared to HCT116/vector cells (Supplemental Figure S2C and Figure 6C). Clonogenic survival of ZR-75-1 cells following cisplatin or etoposide treatment (Supplemental Figure S2D and Figure 6D) was also increased by MUC1 expression, indicating that this response is not cell-type dependent. These findings indicate that MUC1 promotes growth arrest and survival in the response to DNA damage.

MUC1 regulates the p53-dependent growth arrest and apoptotic responses to DNA damage in HCT116 cells

To determine if the effects of MUC1 on growth arrest and survival are dependent on p53, HCT116/p53$^{-/-}$ cells (Bunz et al., 1998) were transfected to express an empty vector or MUC1. Immunoblot analysis of two separately isolated HCT116/p53$^{-/-}$/MUC1 clones confirmed expression of MUC1 N-ter and C-ter at levels similar to that in HCT116/MUC1 cells (Figure 7A). As controls, immunoblotting was also performed on ly-

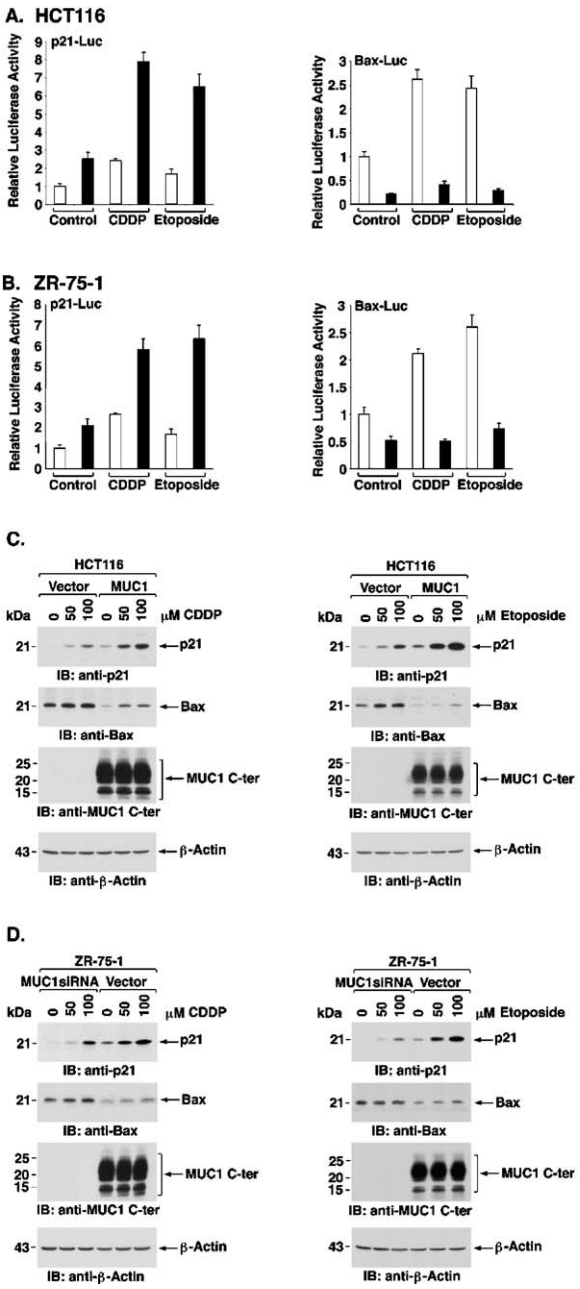

Figure 5. MUC1 regulates activation of the p21-Luc and Bax-Luc reporters

A: HCT116/vector (open bars) and HCT116/MUC1 cells (solid bars) were transfected with the p21-Luc reporter (left) or the Bax promoter-Luc reporter (right). At 24 hr after transfection, the cells were left untreated or treated with 10 μM CDDP or etoposide for 24 hr and then assayed for luciferase activity. The results are expressed as the fold activation (mean ± SD of 3 separate experiments) compared to that obtained with untreated HCT116/vector cells (assigned a value of 1). Similar results were obtained with the separately isolated HCT116/vector-B and HCT116/MUC1-B cells (data not shown).

B: ZR-75-1/MUC1siRNA (open bars) and ZR-75-1/vector (solid bars) cells were transfected with p21-Luc (left) or Bax-Luc (right), incubated for 24 hr, and then treated with 10 μM CDDP or etoposide for 24 hr. Luciferase activity is presented as the fold activation (mean ± SD of 3 separate experiments) compared to that obtained with untreated ZR-75-1/MUC1siRNA cells (assigned a value of 1).

C and D: The indicated cells were left untreated or treated with 50 or 100 μM cisplatin (left) or etoposide (right) for 24 hr. Lysates were analyzed by immunoblotting with the indicated antibodies.

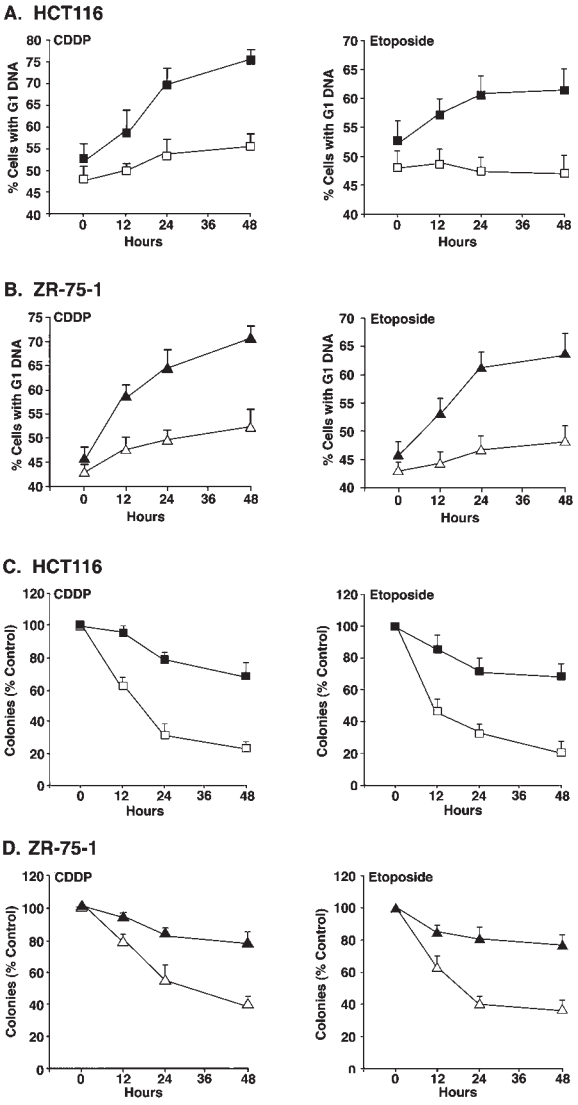

Figure 6. MUC1 activates the G1 growth arrest response to DNA damage

A: HCT116/vector (□) and HCT116/MUC1 (■) cells treated with 25 μM cisplatin (left) or 12.5 μM etoposide (right) for the indicated times were analyzed by flow cytometry. The results are presented as the percentage (mean ± SD of three separate experiments) of cells in G1 phase.

B: ZR-75-1/vector (▲) and ZR-75-1/MUC1siRNA (△) cells treated with 25 μM cisplatin (left) or 12.5 μM etoposide (right) for the indicated times were analyzed by flow cytometry. The results are presented as the percentage (mean ± SD of three separate experiments) of cells in G1 phase.

C: HCT116/vector (□) and HCT116/MUC1 (■) cells were treated with 50 μM cisplatin or etoposide for the indicated times. The cells were then incubated in the absence of drug for 8 days and stained with crystal violet. Results are expressed as the number (mean ± SD of three separate experiments) of colonies.

D: ZR-75-1/vector (▲) and ZR-75-1/MUC1siRNA (△) cells were treated with 50 μM cisplatin or etoposide for the indicated times. The cells were then incubated in the absence of drug for 8 days and stained with crystal violet. Results are expressed as the number (mean ± SD of three separate experiments) of colonies.

sates from HCT116 and HCT116/p53$^{-/-}$ cells stably transfected with the empty vector (Figure 7A). Importantly, and in contrast to HCT116/MUC1 cells, MUC1 occupancy of the p53REs in the *p21* promoter was undetectable in HCT116/

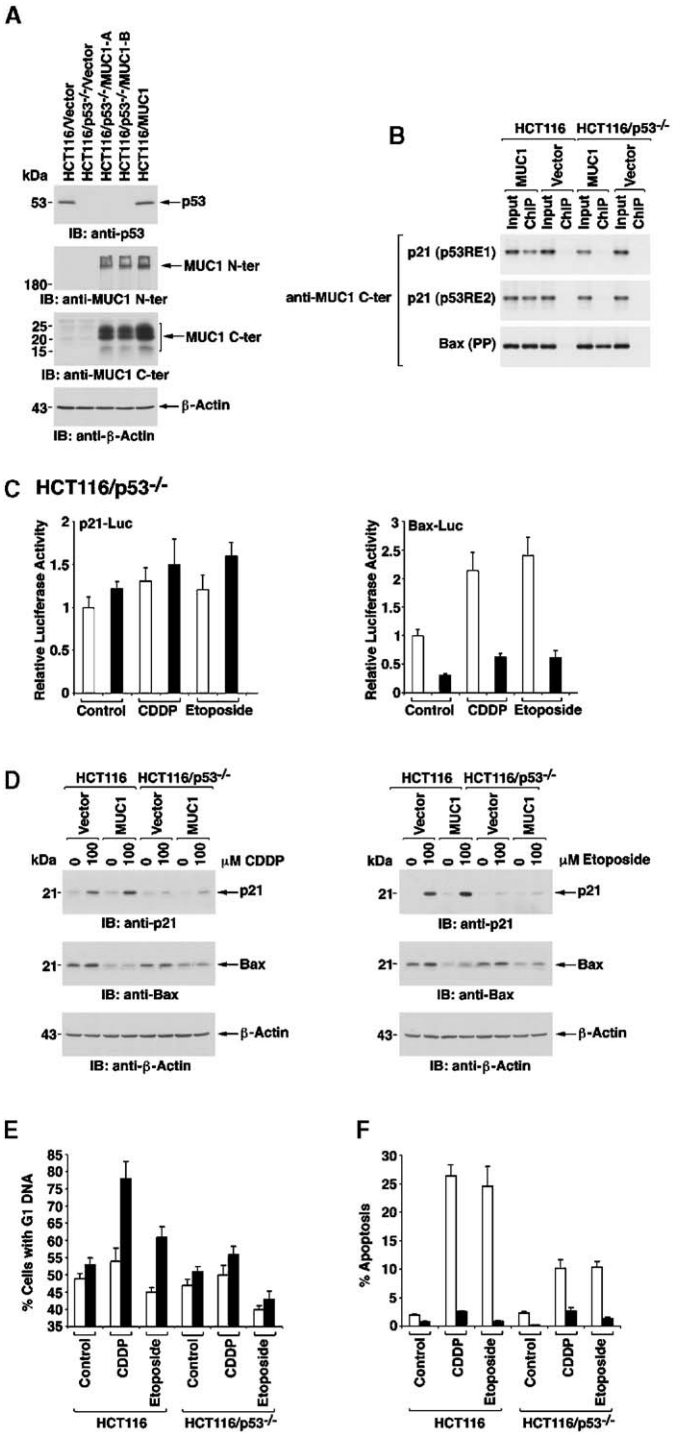

Figure 7. MUC1 regulates the p53-dependent, DNA damage-induced growth arrest and apoptotic responses in HCT116 cells

A: HCT116/p53$^{-/-}$ cells were transfected to stably express MUC1. Lysates from two independently selected HCT116/p53$^{-/-}$/MUC1 clones were subjected to immunoblotting with the indicated antibodies. Lysates from HCT116/vector, HCT116/p53$^{-/-}$/vector, and HCT116/MUC1 cells were included as controls.

B: Soluble chromatin from the indicated cells was immunoprecipitated with anti-MUC1 C-ter and analyzed for p21 p53RE and Bax PP sequences.

C: HCT116/p53$^{-/-}$/vector (open bars) and HCT116/p53$^{-/-}$/MUC1 cells (solid bars) were transfected with the p21-Luc (left) or Bax-Luc (right) reporter. At 24 hr after transfection, the cells were left untreated or treated with 10 μM cisplatin or etoposide for 24 hr and then assayed for luciferase activity. The results are expressed as the fold activation (mean ± SD of 3 separate experiments) compared to that obtained with untreated HCT116/p53$^{-/-}$/vector cells (assigned a value of 1). Similar results were obtained with the separately isolated HCT116/p53$^{-/-}$/MUC1 clones (data not shown).

D: The indicated cells were left untreated or treated with 100 μM cisplatin (left) or etoposide (right) for 24 hr. Lysates were analyzed by immunoblotting with the indicated antibodies.

E: MUC1-negative HCT116/vector and HCT116/p53$^{-/-}$/vector cells (open bars) or MUC1-positive HCT116/MUC1 and HCT116/ p53$^{-/-}$/MUC1 (solid bars) cells were treated with 25 μM cisplatin or 12.5 μM etoposide for 48 hr and analyzed by flow cytometry (Supplemental Figure S3A). The results are presented as the percentage (mean ± SD of three separate experiments) of cells in G1 phase.

F: MUC1-negative HCT116/vector and HCT116/p53$^{-/-}$/vector cells (open bars) or MUC1-positive HCT116/MUC1 and HCT116/ p53$^{-/-}$/MUC1 (solid bars) cells were treated with 50 μM cisplatin or 25 μM etoposide for 72 hr and analyzed by flow cytometry (Supplemental Figure S3B and S3C). The results are presented as the percentage (mean ± SD of three separate experiments) of cells with sub-G1 DNA.

p53$^{-/-}$/MUC1 cells (Figure 7B), indicating that association of MUC1 with these p53REs is dependent on p53. In contrast, the absence of p53 in HCT116/p53$^{-/-}$/MUC1 cells had little effect on MUC1 C-ter occupancy of the *Bax* proximal promoter (Figure 7B). Similar results were obtained with the two independently isolated HCT116/p53$^{-/-}$/MUC1 cell clones and in the response to DNA damage (data not shown). In addition, compared to HCT116/MUC1 cells (Figure 5A, left), the effects of MUC1 on DNA damage-induced activation of p21-Luc were substantially decreased in HCT116/p53$^{-/-}$/MUC1 cells (Figure 7C, left). Conversely, MUC1 was as effective in suppressing Bax-Luc expression in both HCT116/MUC1 (Figure 5A, right) and HCT116/p53$^{-/-}$/MUC1 cells (Figure 7C, right). Consistent with these results, the effects of MUC1 on DNA damage-induced expression of p21 were p53-dependent, while suppression of Bax by MUC1 was independent of p53 (Figure 7D). The effects of MUC1 on DNA damage-induced growth arrest were also dependent on p53 (Supplemental Figure S3A and Figure 7E). The results further demonstrate that MUC1 attenuates p53-dependent, as well as p53-independent, apoptosis in response to DNA damage (Supplemental Figures S3B and S3C and Figure 7F).

MUC1 silencing attenuates the p53-dependent growth arrest response and increases the p53-dependent apoptotic response to DNA damage in ZR-75-1 cells

p53 was knocked down in the ZR-75-1/vector and ZR-75-1/MUC1siRNA cells by infection with an adenovirus expressing a p53siRNA (Ad.p53siRNA) (Figure 8A). Knocking down p53 reduced MUC1 occupancy of the p53REs in the *p21* promoter (Figure 8B), consistent with a p53-dependent mechanism. However, knocking down p53 had little effect on MUC1 occupancy of the *Bax* proximal promoter (Figure 8B). In addition, MUC1 increased DNA damage-induced p21-Luc activation in ZR-75-1 cells by a p53-dependent mechanism (Figure 8C, left, compared to Figure 5B, left). Moreover, MUC1-mediated suppression of Bax-Luc was similar in ZR-75-1 cells with basal (Figure 5B, right) or knocked down (Figure 8C, right) levels of p53 expression. The effects of MUC1 on DNA damage-induced expression of p21 were attenuated in the absence of p53 (Figure 8D), while MUC1 suppressed Bax expression inde-

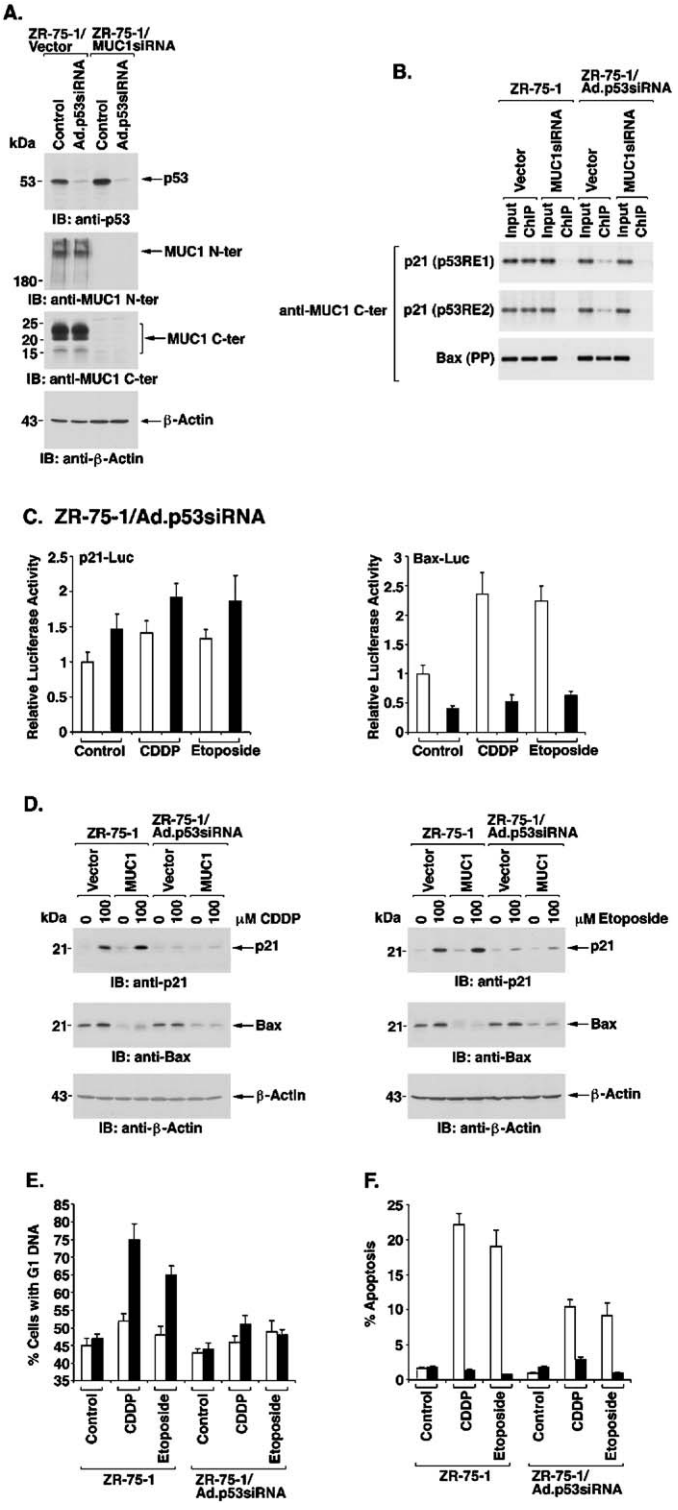

Figure 8. Silencing MUC1 in ZR-75-1 cells attenuates the p53-dependent growth arrest response and potentiates the p53-dependent apoptotic response to DNA damage

A: ZR-75-1/vector and ZR-75-1/MUC1siRNA cells were infected with a control empty adenovirus or one expressing a p53siRNA (Ad.p53siRNA) for 48 hr. Lysates were analyzed by immunoblotting with the indicated antibodies.

B: Soluble chromatin from the indicated cells was immunoprecipitated with anti-MUC1 C-ter and analyzed for p21 p53RE and Bax PP sequences.

C: ZR-75-1/MUC1siRNA (open bars) and ZR-75-1/vector cells (solid bars) were infected with Ad.p53siRNA for 24 hr and then transfected with the p21-Luc (left) or Bax-Luc (right) reporter. At 24 hr after transfection, the cells were left untreated or treated with 10 μM cisplatin or etoposide for 24 hr and then assayed for luciferase activity. The results are expressed as the fold activation (mean ± SD of 3 separate experiments) compared to that obtained with untreated ZR-75-1/MUC1siRNA/Ad.p53siRNA cells (assigned a value of 1). Similar results were obtained with the separately isolated ZR-75-1/vector and ZR-75-1/MUC1siRNA clones infected with Ad.p53siRNA (data not shown).

D: ZR-75-1/MUC1siRNA and ZR-75-1/vector cells infected with Ad.p53siRNA for 48 hr were left untreated or treated with 100 μM cisplatin (left) or etoposide (right) for 24 hr. Lysates were analyzed by immunoblotting with the indicated antibodies.

E: ZR-75-1/MUC1siRNA and ZR-75-1/MUC1siRNA/Ad.p53siRNA cells (open bars) or ZR-75-1/vector and ZR-75-1/vector/Ad.p53siRNA (solid bars) were treated with 25 μM cisplatin or 12.5 μM etoposide for 48 hr and analyzed by flow cytometry (Supplemental Figure S4A). The results are presented as the percentage (mean ± SD of three separate experiments) of cells in G1 phase.

F: ZR-75-1/MUC1siRNA and ZR-75-1/MUC1siRNA/Ad.p53siRNA cells (open bars) or ZR-75-1/vector and ZR-75-1/vector/Ad.p53siRNA (solid bars) were treated with 50 μM cisplatin or 25 μM etoposide for 72 hr and analyzed by flow cytometry (Supplemental Figures S4B and S4C). The results are presented as the percentage (mean ± SD of three separate experiments) of cells with sub-G1 DNA.

pendently of changes in p53 levels (Figure 8D). In concert with these results, MUC1 potentiated DNA damage-induced growth arrest in large part by a p53-dependent mechanism (Supplemental Figure S4A and Figure 8E). MUC1 also blocked p53-dependent and p53-independent apoptosis in the response of ZR-75-1 cells to DNA damage (Supplemental Figures S4B and S4C and Figure 8F).

Discussion

MUC1 interacts directly with p53

The MUC1 heterodimer is aberrantly overexpressed in the cytosol and on the entire cell membrane of diverse human carcinomas (Kufe et al., 1984; Perey et al., 1992). The MUC1 C-terminal subunit also localizes to the nucleus and mitochondria (Li et al., 2003a, 2003b, 2003c; Ren et al., 2004; Wen et al., 2003). However, the mechanisms responsible for disassociation of the MUC1 subunits, and whether this occurs in the ER, cytosol, and cell membrane, are presently not known. The present studies demonstrate that MUC1 C-ter, and not N-ter, interacts with p53. Binding of MUC1 and p53 was detectable constitutively and increased in the response to DNA damage. The results also demonstrate that MUC1 is detectable with p53 on promoters of the p53-responsive *p21* and *Bax* genes. MUC1 does not contain a prototypical nuclear localization signal (NLS) and, as such, does not bind to importin α (unpublished data). However, like other proteins without a NLS that enter the nucleus (Kau et al., 2004), MUC1 does bind to importin β (unpublished data). Our results indicate that a region of the MUC1 cytoplasmic tail (amino acids 9–46) functions as a site for direct binding to the p53 regulatory domain. Ref-1 binds to the p53 regulatory domain and enhances p53 DNA binding activity (Jayaraman et al., 1997). Activation of c-Abl by genotoxic agents is associated with binding of c-Abl to the p53 regulatory domain and, depending on cell context, contributes to induction of an apoptotic or growth arrest response (Khar-

banda et al., 1995; Nie et al., 2000; Yuan et al., 1996, 1997). Binding of the p53 regulatory domain to the Werner syndrome protein also activates p53-mediated apoptosis (Blander et al., 1999; Spillare et al., 1999). Conversely, binding of the BRCA1 protein to the p53 regulatory domain induces cell cycle arrest (MacLachlan et al., 2002). These findings indicated that binding of MUC1 to the p53 regulatory domain might also contribute to cell fate selection in the p53 response to stress.

MUC1 coactivates *p21* gene transcription

Chromatin-bound p53 is present at similar extents on genes that control apoptosis or growth arrest during induction of both responses (Kaeser and Iggo, 2002; Szak et al., 2001), indicating that selective binding of p53 to a promoter is not per se a determinant for choice of cell fate. Consistent with these findings, recruitment of the transcriptional coactivators p300/CBP and hADA3 has been shown to dictate selective activation of p53 target genes, including *p21* (Dumaz and Meek, 1999; Espinosa and Emerson, 2001; Lambert et al., 1998; Wang et al., 2001). The present results demonstrate that MUC1 is detectable with p53 on the p53REs in the *p21* promoter and that MUC1 occupancy of these elements is increased by DNA damage and dependent on p53 (Supplemental Figure S5). In addition, the finding that MUC1 coactivates *p21* transcription can be explained, at least in part, by MUC1-induced recruitment of CBP and not HDAC1 to the *p21* promoter (Supplemental Figure S5). Binding of CBP to p53 is necessary for histone acetylation (Barlev et al., 2001) and p53-mediated activation of *p21* transcription (Liu et al., 2003; Mujtaba et al., 2004). In this regard, MUC1-induced recruitment of CBP to the *p21* promoter was associated with increased acetylation of histone H4. Moreover, MUC1 expression was associated with activation of both the *p21* promoter-Luc reporter and the endogenous *p21* gene in the response to genotoxic stress. These findings indicate that the interaction between MUC1 and p53 contributes to the recruitment of CBP and thereby activation of *p21* transcription.

MUC1 attenuates activation of the *Bax* gene in response to DNA damage

Binding of p53 to the *Bax* promoter in cells is lower compared with the *p21* promoter (Kaeser and Iggo, 2002). The present studies demonstrate that, in contrast to the *p21* promoter, there is no detectable MUC1 occupancy of the p53-responsive element in the *Bax* promoter (Supplemental Figure S5). There was also no detectable MUC1 binding to the *Bax* p53RE after DNA damage. Surprisingly, however, we found that MUC1 precipitates with the *Bax* proximal promoter, and that this association is increased by DNA damage. Coprecipitation of MUC1 with TBP further indicated that MUC1 occupies the region of the *Bax* proximal promoter that includes the basal transcription complex (Supplemental Figure S5). Notably, p53 interacts with TBP on certain genes (Chang et al., 1995; Farmer et al., 1996; Horikoshi et al., 1995; Liu et al., 1993; Seto et al., 1992) and was detectable with MUC1 on the *Bax* proximal promoter, suggesting that MUC1 may be targeted to this region by a p53-dependent mechanism. However, decreases in p53 levels had little effect on MUC1 occupancy of the *Bax* proximal promoter. Thus, like p53, MUC1 may also associate with basal transcription factors. In this context, MUC1 occupancy of the *Bax* proximal promoter had no apparent effect on TBP, but decreased occupancy by TFIIB and TAFII250 (Supplemental Figure S5). Moreover, MUC1 expression was associated with attenuation of *Bax* gene activation by a p53-independent mechanism, and derepression of the *Bax* promoter in the absence of MUC1 was observed in both p53-positive and p53-negative cells. The findings with *p21* and *Bax* thus indicate that MUC1 selectively regulates transcription of p53-responsive genes by DNA damage-induced binding of MUC1 in a promoter-specific manner.

MUC1 is of functional importance in regulation of the p53-dependent growth arrest and apoptotic responses

Our results demonstrate that MUC1 promotes the growth arrest response to DNA damage by a mechanism dependent on p53. MUC1 also suppressed p53-dependent and p53-independent apoptotic responses to DNA damage. MUC1 attenuates DNA damage-induced activation of the intrinsic apoptotic pathway by a mechanism that is at least in part due to its mitochondrial localization (Ren et al., 2004). Thus, MUC1 may block apoptosis that is p53-dependent or p53-independent by suppressing both *Bax* activation and the release of apoptogenic factors from mitochondria. MUC1 also interacts with β-catenin and GSK3β, effectors of the antiapoptotic canonical Wnt signaling pathway (Li et al., 1998; Yamamoto et al., 1997). Crosstalk exists between p53 and β-catenin signaling (Damalas et al., 1999, 2001; Sadot et al., 2001), and MUC1 may function in integrating these pathways. In this regard, DNA damage decreases the interaction between MUC1 and β-catenin (unpublished data) and, as shown in the present work, increases binding of MUC1 and p53. Coimmunoprecipitation studies indicate that MUC1 forms independent complexes with β-catenin and with p53 (unpublished data). However, it is not presently known if the same pool of MUC1 interacts with these proteins. Further studies will be needed to determine if MUC1 attenuates stress-induced apoptosis by participating in both p53- and β-catenin-dependent pathways.

Does the MUC1-p53 interaction contribute to a physiologic defense mechanism exploited by human tumors?

MUC1 functions in providing a protective barrier against damage to the apical borders of epithelial cells. The MUC1 cytoplasmic tail also signals environmental stress at the apical membrane to the interior of the cell. The epithelial stress response is associated with loss of polarity and activation of a heregulin-induced repair program (Vermeer et al., 2003). Heregulin also targets MUC1 to the nucleus (Li et al., 2003c) and mitochondria (Ren et al., 2004). Thus, under physiologic conditions, stress-induced nuclear and mitochondrial targeting of MUC1 could contribute to a transient p53-mediated growth arrest response, suppression of *Bax* activation, and attenuation of the intrinsic apoptotic pathway. With transformation and loss of polarity, MUC1 is constitutively expressed in the nucleus and mitochondria (Li et al., 2003b, 2003c; Ren et al., 2004). Thus, human tumors that overexpress MUC1 may have a survival advantage to genotoxic and potentially other forms of stress by exploiting physiologic mechanisms that evolved for the repair of damaged epithelia.

Experimental procedures

Cell culture

Human HCT116/vector, HCT116/MUC1 (Ren et al., 2002b), HCT116/p53$^{-/-}$ (Bunz et al., 1998), and human MCF-7 breast cancer cells were grown in

Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 μg/ml streptomycin, 100 units/ml penicillin, and 2 mM L-glutamine. Human ZR-75-1 breast and human LNCaP prostate carcinoma cells were cultured in RPMI 1640 medium supplemented with 10% HI-FBS, antibiotics, and L-glutamine. Human U2OS osteosarcoma cells were grown in minimal essential medium with 10% HI-FBS and antibiotics. Cells were treated with cisplatin (CDDP; Sigma) or etoposide (Sigma).

Immunoblotting and immunoprecipitation

Lysates were prepared from subconfluent cells as described (Wei et al., 2003). Immunoblot analysis was performed with anti-p53 (Ab-2, Ab-6; Oncogene Research Products), anti-MUC1 N-ter (DF3) (Kufe et al., 1984), anti-MUC1 C-ter (Ab-5; Neomarkers, Freemont, CA), anti-β-actin (Sigma), anti-Myc (Ab-1; Oncogene Research Products), anti-Bax (Santa Cruz Biotechnology), and anti-p21 (Santa Cruz Biotechnology). Lysates were also subjected to immunoprecipitation with anti-p53 or anti-Myc, and the immune complexes were analyzed by immunoblotting.

Plasmid construction and transfection

MUC1-CD (1–72) and deletions thereof were generated by PCR using pIRESpuro2-MUC1 as a template (Li et al., 2001a). To generate vectors expressing GST-MUC1-CD, the PCR products were digested with BamHI/NotI and cloned into corresponding sites of pGEX-4T-3. Transfections were performed in 60 mm dishes using Fugene-6 (Roche Applied Science) or, for the luciferase assays, in 24-well plates using the calcium phosphate method (Invitrogen). Cells were transfected with the *p21* (2.4 kbp HindII fragment)-Luc reporter (Ren et al., 2002a) or the *Bax* promoter (370 bp SmaI/SacI fragment)-luciferase (Luc) reporter (Miyashita and Reed, 1995) and an internal control LacZ expression plasmid (pCMV-LacZ) (Wei et al., 2001). Luciferase assays were performed with the Luciferase Assay System (Promega Corp.) at 40 hr after transfection. Luciferase activity was normalized to that obtained for LacZ and presented as relative luciferase activity. HCT116/p53$^{-/-}$ cells were stably transfected with pIRES-puro-2 or pIRES-puro-2-MUC1 as described (Ren et al., 2004).

GST pulldown assays

GST and GST fusion proteins were purified by binding to glutathione-agarose beads (Sigma). ^{35}S-labeled p53 prepared in TNT reactions (Promega Corp.) was incubated with GST or the GST fusion proteins for 90 min at 4°C. After washing, the adsorbed proteins were resolved by SDS-PAGE and analyzed by autoradiography or Coomassie blue staining.

Chromatin immunoprecipitation (ChIP) and Re-ChIP assays

ChIP assays were performed as described (Shang et al., 2000) using anti-MUC1 C-ter, anti-p53 (Ab-6; Oncogene Research Products), anti-CBP (C-1; Santa Cruz Biotechnology), anti-HDAC1 (Upstate Biotechnology Inc.), anti-Ac-H4 (Upstate Biotechnology Inc.), anti-TBP (58C9; Santa Cruz Biotechnology), anti-TFIIB (IIB8; Santa Cruz Biotechnology), or anti-TAFII250 (6B3; Santa Cruz Biotechnology). For Re-ChIP assays, complexes from the primary ChIP were eluted with 10 mM DTT for 30 min at 37°C and diluted 20 times with Re-ChIP buffer (20 mM Tris-HCl [pH 8.1], 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), followed by reimmunoprecipitation with the indicated second antibodies, and subjected again to the ChIP procedure. For PCR, 2 μl from a 50 μl DNA extraction were used with 30–38 cycles of amplification. The primers for the *p21* promoter p53-responsive elements (p53RE1 and p53RE2) have been described (Liu et al., 2003). The primers for the *p21* control region (CR) were (5′-GGTGCTTCTGGGAGAGGTGAC; 3′-TGACCCACTCTGGCAGGCAAG) and for the *p21* proximal promoter (PP) were (5′-GGAAGTGCCCTCCTGCAGCAC; 3′-CGGCGAATCCGCGCCCAGCTC). Primers used for the *Bax* promoter p53RE were (5′-GATTGGGCCAC TGCACTCCAG; 3′-TGACTAAAAACTGAGTGG), for the *Bax* control region (CR) were (5′-CCTGCTGATCTATCAGCACAG; 3′-GCTGGTCTCTGAACT CCCAGA), and for the *Bax* proximal promoter (PP) were (5′-CGTGGGCTA TATTGCTAGATC; 3′-GTCCAATCGCAGCTCTAATGC).

Flow cytometry

Cells were fixed with 80% ice-cold ethanol, incubated in PBS containing 30 μg/ml RNase (Roche Applied Science) for 60 min at 37°C, and then stained with propidium iodide (Sigma) for 30 min at room temperature in the dark. DNA content was analyzed by flow cytometry (Coulter EPICS XL-MCL).

Colony formation assays

Aliquots containing 500 cells/well were plated onto 6-well plates containing 2 ml/well complete medium and incubated for 18–24 hr at 37°C. The cells were treated with CDDP or etoposide for 12–48 hr, washed, and incubated for 8 days. Resulting colonies were stained with crystal violet and counted manually.

Apoptosis assays

Apoptotic cells were quantitated by analysis of sub-G1 DNA and propidium iodide staining as described (Ren et al., 2004).

Downregulation of p53

ZR-75-1/vector and ZR-75-1/MUC1siRNA cells were seeded (5 × 10^5/well) on 6-well plates. After 24 hr, the cells were infected with an empty adenovirus or one expressing human p53 siRNA (2 × 10^9 Ad.p53siRNA particles/well; IMGENEX, San Diego, CA). The cells were incubated for 48–72 hr and then harvested for analysis.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/2/167/DC1/.

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