The MUC1-C Oncoprotein Binds to the BH3 Domain of the Pro-apoptotic BAX Protein and Blocks BAX Function*

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Background: The MUC1-C oncprotein attenuates activation of the intrinsic apoptotic pathway by mechanisms that are not understood.

Results: MUC1-C binds directly to the pro-apoptotic BAX protein at the critical BH3 domain.

Conclusion: MUC1-C blocks BAX dimerization and BAX-mediated release of mitochondrial cytochrome c.

Significance: MUC1-C is a novel inhibitor of BAX function.

The pro-apoptotic BAX protein contains a BH3 domain that is necessary for its dimerization and for activation of the intrinsic apoptotic pathway. The MUC1 (mucin 1) heterodimeric protein is overexpressed in diverse human carcinomas and blocks apoptosis in the response to stress. In this study, we demonstrate that the oncogenic MUC1-C subunit associates with BAX in human cancer cells. MUC1-C-BAX complexes are detectable in the cytoplasm and mitochondria and are induced by genotoxic and oxidative stress. The association between MUC1-C and BAX is supported by the demonstration that the MUC1-C-cytoplasmic domain is sufficient for the interaction with BAX. The results further show that the MUC1-C cytoplasmic domain CQC motif binds directly to the BAX BH3 domain at Cys-62. Consistent with binding to the BAX BH3 domain, MUC1-C blocked BAX dimerization in response to (i) truncated BID in vitro and (ii) treatment of cancer cells with DNA-damaging agents. In concert with these results, MUC1-C attenuated localization of BAX to mitochondria and the release of cytochrome c. These findings indicate that the MUC1-C oncprotein binds directly to the BAX BH3 domain and thereby blocks BAX function in activating the mitochondrial death pathway.

BAX is a pro-apoptotic member of the BCL-2 family of proteins that is critical for activation of the intrinsic mitochondrial death pathway. BAX resides predominantly in the cytoplasm and, in response to pro-apoptotic stress signals, localizes to the mitochondrial outer membrane (MOM)². In the MOM, BAX forms a putative homo-oligomeric pore that induces cytochrome c release from the mitochondrial intermembrane space and damage to the mitochondrial network. BAX has nine α-helices; helix α2 includes the BH3 domain, which is critical for BAX function. In a structural model of the inactive BAX monomer, the BH3 domain is buried in the hydrophobic protein core. Activation of BAX is induced by engagement with a triggering BH3 helix and thereby exposure of its BH3 domain. The BH3 domain is critical for the formation of BAX homodimers and for killing. In this regard, dimerization of BAX results in its translocation to the mitochondrial membrane and the induction of apoptotic cell death. BAX has two cysteine residues, one of which resides at position 62 in the BH3 domain. The other cysteine residue is located at position 126 in the pore-forming region. These exposed cysteines are involved in the generation of disulfide bonds and, in turn, the formation of homodimers that promote translocation to mitochondria. Other work has shown that activation of BAX and its mitochondrial translocation are dependent on Cys-62 in the response to oxidative stress. These findings have supported a potential role for BAX as a sensor of redox stress. However, there are no reports that BAX Cys-62 is involved in protein interactions other than the formation of homodimers.

MUC1 (mucin 1) is overexpressed by diverse human carcinomas and confers a survival response to stress. Of importance to understanding its function in transformation, MUC1 consists of two subunits that are derived from autocleavage of a common protein precursor and that form, in turn, a stable heterodimeric complex at the cell surface. The extracellular MUC1 N-terminal subunit (MUC1-N) contains glycosylated tandem repeats that are a structural characteristic found in other mucin family members. The MUC1 C-terminal subunit (MUC1-C) is embedded in the apical membrane of normal secretory epithelial cells; however, with transformation, loss of polarity, and overexpression, MUC1-C accumulates in the cytoplasm and is targeted to the nucleus. MUC1-C consists of a 58-amino acid (aa) extracellular domain and a 72-aa cytoplasmic domain. The MUC1-C cytoplasmic domain (MUC1-CD) contains a CQC motif that contributes to the formation of dimers, interactions with other proteins, and localization to the nucleus. MUC1-CD also functions as a substrate for Src, glycogen synthase kinase 3β, protein kinase C (C2), and Abl. In addition, MUC1-CD interacts with certain effectors (such as p53, NF-κB, and STAT3) that have been linked to oncogenesis.
induced by genotoxic agents, reactive oxygen species, and hypoxia (14, 15, 27, 28).

In this study, we demonstrate that MUC1-CD interacts directly with BAX. We show that the MUC1-C cysteine residues in the CQC motif bind to BAX at Cys-62 in the BH3 domain. The functional significance of the MUC1-C-BAX interaction is supported by the demonstration that MUC1-C blocks BAX dimerization and function.

MATERIALS AND METHODS

Cell Culture—Human MCF-7 breast cancer cells (26) and HCT116 colon cancer cells (15, 18) were cultured in DMEM with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Human ZR-75-1 breast cancer cells were grown in RPMI 1640 medium with antibiotics and l-glutamine. HCT116 cells were transfected to stably express an empty pIREs-puro2 vector or one expressing MUC1 (15). MCF-7 and ZR-75-1 cells were infected with a retroviral vector expressing MUC1 siRNA or, as a control, with the empty vector as described (15). Cells were treated with doxorubicin (DOX; Sigma), hydrogen peroxide (H2O2; Sigma), the cell-penetrating GO-203 (R9CQCRRKN) and CP-2 (R9AQARRKN) peptides (AnaSpec), and vinblastine (VBL; Sigma).

Immunoprecipitation and Immunoblotting—Total cell and cytoplasmic lysates were prepared in Nonidet P-40 as described (26). In certain experiments, lysates were prepared in the presence of CHAPS buffer (10 mM HEPES, 150 mM NaCl, 1% CHAPS, and 5 mM PMSF). Soluble proteins were subjected to immunoprecipitation with anti-BAX (N-20) or anti-BAX (6A7) antibody (Santa Cruz Biotechnology). The precipitates and cell lysates were analyzed by immunoblotting with anti-MUC1-C (Lab Vision Corp.), anti-BAX, anti-cytochrome c (Abcam), and anti-β-actin (Sigma) antibodies. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

In Vitro Binding Assays—GST, GST-MUC1-CD, GST-MUC1-CD(1–45), GST-MUC1-CD(46–72), and GST-MUC1-CD(AQA) were purified as described (18, 25). GST-BAX (amino acids 1–192) and deletion mutants were generated by PCR amplification of GFP-BAX (plasmid 19741 (human BAX C3-EGFP), Addgene) and subcloning into pGEX-5X-1 (GE Healthcare). GST-BAX(1–192) and GST-BAX(1–90) were mutated at Cys-62 to alanine (C62A) by site-directed mutagenesis (Stratagene). GST and GST fusion proteins bound to glutathione beads were incubated with cell lysates or purified proteins, and the adsorbates were analyzed by immunoblotting. The GST fusion proteins were cleaved with thrombin or factor Xa to remove the GST moiety. In certain experiments, the binding assays were performed in the presence of 100 μM N-acetyl-cysteine (NAC; Calbiochem) or 100 μM DTT (Invitrogen). Binding assays were also performed in the presence of wild-type BAX BH3, BAX BH3(L36E) (AnaSpec), or BAX BH3(C62A) (Dana-Farber Cancer Institute Molecular Biology Core) peptides.

Isolation of Mitochondria—Mitochondria were isolated as described (29). Briefly, cells were homogenized in buffer A (10 mM HEPES (pH 7.4), 250 mM mannitol, 10 mM KCl, 5 mM MgCl2, 1 mM EGTA, 1 mM PMSF, and protease inhibitors) by 30 strokes of a Dounce homogenizer. The homogenate was centrifuged at 600 × g for 5 min, and the resulting supernatant was centrifuged at 10,000 × g for 10 min. The mitochondrial pellet was washed with buffer A. For cytochrome c release assays, 50 μg of mitochondria in 50 μl of buffer A was incubated with BAX and His-MUC1-CD for 1 h at 30 °C. The mitochondria were pelleted by centrifugation and resuspended in buffer A at a volume equal to that of the collected supernatant.

Detection of BAX Dimerization—Isolated mitochondria (50 μg) were suspended in 50 μl of buffer containing 10 mM HEPES (pH 7.5), 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 1 mM EGTA, and protease inhibitors and incubated with 10 nm truncated BID (tBID; Alexis Biochemicals) for 15 min at 30 °C as described (30). Freshly prepared disuccinimidyl suberate (Thermo Scientific) dissolved in Me2SO was added at a final concentration of 2 mM and incubated with rotation for 30 min at room temperature. Mitochondria were pelleted by centrifugation and dissolved in radioimmune precipitation assay buffer. Lysates were immunoprecipitated with anti-BAX monoclonal antibody (BD Biosciences), and the precipitates were immunoblotted with anti-BAX polyclonal antibody (Santa Cruz Biotechnology). For analysis of BAX dimerization in cells, washed cell pellets were resuspended in PBS with freshly prepared 1,6-bismaleimidohexane (Thermo Scientific) at a final concentration of 5 mM and incubated with rotation for 30 min at room temperature as described (31). The cells were pelleted, dissolved in radioimmune precipitation assay buffer, incubated on ice for 5 min, and centrifuged at 14,000 rpm for 10 min. Supernatants were analyzed by immunoblotting with anti-BAX antibody.

Confocal Microscopy—Cells were cultured on glass coverslips. For staining mitochondria, the cells were incubated in serum-free growth medium containing 100 nM MitoTracker Red CMXRos (Molecular Probes) for 30 min at 37 °C. Cells were then washed with complete growth medium, fixed with 4% paraformaldehyde for 30 min, and permeabilized in 1% BSA-supplemented PBS containing 0.5% Triton X-100 for 20 min at 25 °C. The fixed cells were incubated overnight with anti-BAX antibody at 4 °C and then with Alexa Fluor 647-conjugated anti-rabbit IgG secondary antibody (Invitrogen). Nuclei were stained with 1 mg/ml DAPI (Invitrogen). After mounting the coverslips, images were captured with a Yokogawa spinning disk confocal microscope.

Analysis of Apoptosis—Cells were incubated with propidium iodide and Alexa Fluor 488-conjugated annexin V (Invitrogen) and analyzed by flow cytometry.

RESULTS

MUC1-C Associates with BAX—The MUC1-C subunit blocks the apoptotic response to diverse forms of stress (15, 27). To determine whether MUC1-C interacts with the pro-apoptotic BAX protein, lysates from MCF-7 breast cancer cells were immunoprecipitated with anti-BAX antibody. The results from immunoblot analysis of the precipitates with an antibody against MUC1-C supported the association of these proteins (Fig. 1A). Similar results were obtained when analyzing lysates from ZR-75-1 breast cancer cells (Fig. 1B). These co-immuno-
precipitation experiments were performed with lysates prepared in the presence of the non-ionic Nonidet P-40 detergent. However, previous studies indicated that BCL-2 family members can engage in spurious interactions in non-ionic detergents (32). Accordingly, the association between MUC1-C and BAX was confirmed using lysates prepared in the zwitterionic detergent CHAPS (Fig. 1C). BAX is maintained in the cytosol (33). Analysis of the cytosolic fraction from MCF-7 cells further demonstrated the association between MUC1-C and BAX (Fig. 1D). Moreover, in pulldown experiments, incubation of the cytoplasmic fraction with MUC1-CD fused to GST demonstrated binding with BAX (Fig. 1E).

**MUC1-C Forms Complexes with Activated BAX**—To determine whether MUC1-C interacts with activated BAX in the response to stress, we treated ZR-75-1 cells with the genotoxic agent DOX. DOX treatment was associated with a modest increase in MUC1-C:BAX complexes (Fig. 2A). Similar results were obtained in the response to H$_2$O$_2$ (Fig. 2A). In mitochondrially mediated apoptosis, BAX undergoes a conformational change that can be detected with anti-BAX antibody 6A7 (34). Lysates from control and DOX-treated ZR-75-1 cells were thus immunoprecipitated with anti-BAX antibody 6A7. Analysis of the precipitates demonstrated that MUC1-C associated with activated BAX and that this interaction was induced by DOX exposure (Fig. 2B). Treatment of ZR-75-1 cells with hydrogen peroxide was also associated with increases in complexes of MUC1-C and activated BAX (Fig. 2C). The cytosolic form of BAX is targeted to mitochondria in the response to stress (33). In this context, co-immunoprecipitation analysis of purified mitochondria from DOX-treated cells further supported an interaction between MUC1-C and activated BAX (Fig. 2D). These findings indicate that MUC1-C associates with BAX in both the cytosol and mitochondria.

**FIGURE 1.** MUC1-C associates with BAX in cells. A and B, lysates from MCF-7 (A) and ZR-75-1 (B) breast cancer cells prepared in the presence of Nonidet P-40 were immunoprecipitated (IP) with anti-BAX antibody or control IgG. The precipitates and whole cell lysate (WCL) were immunoblotted (IB) with anti-MUC1-C antibody and, as a control, anti-BAX antibody. C, lysates prepared from MCF-7 cells in the presence of CHAPS were immunoprecipitated with anti-BAX antibody or control IgG. The precipitates were immunoblotted with anti-MUC1-C and anti-BAX antibodies. D, cytosolic lysate from MCF-7 cells was precipitated with anti-BAX antibody and control IgG. The precipitates were immunoblotted with the indicated antibodies. E, cytosolic lysate from MCF-7 cells was incubated with GST or GST-MUC1-CD bound to glutathione beads. The adsorbates were immunoblotted with anti-BAX antibody. Input of the GST proteins was analyzed by Coomassie Blue staining.

**FIGURE 2.** MUC1-C forms complex with activated BAX. A, ZR-75-1 cells were left untreated (Control) or were treated with 1 μM DOX for 24 h or with 0.3 mM H$_2$O$_2$ for 24 h. Anti-BAX precipitates were immunoblotted (IB) with the indicated antibodies. IP, immunoprecipitate. B and C, ZR-75-1 cells were left untreated or were treated with DOX (B) or H$_2$O$_2$ (C). Anti-BAX (6A7) precipitates were immunoblotted with the indicated antibodies. D, mitochondria were purified from ZR-75-1 cells left untreated or treated with DOX. Anti-BAX (6A7) precipitates were immunoblotted with the indicated antibodies.
MUC1-CD CQC Motif Binds Directly to BAX—To determine whether MUC1-CD binds directly to BAX, we incubated GST-MUC1-CD with recombinant purified BAX. The finding that GST-MUC1-CD, but not GST, bound to BAX indicated that the association was direct (Fig. 3A, left). These results were confirmed by demonstrating that GST-BAX bound directly to purified MUC1-CD (Fig. 3A, right). MUC1-CD contains a CQC motif (aa 1–3) that was considered as a potential site for binding to BAX. Indeed, mutation of the MUC1-CD CQC motif to AQA completely abrogated the BAX interaction (Fig. 3D). In addition, mutation of the individual cysteine residues to alanine (AQC and CQA) indicated that both cysteines contribute to the formation of MUC1-CD/BAX complexes (Fig. 3D). These results support a model in which the interaction between MUC1-CD and BAX is mediated by disulfide bonds. To lend further support to such a
MUC1-C Blocks BAX BH3 Domain

model, the binding studies were performed in the presence of the reducing agent NAC to inhibit oxidative disulfide bond formation. The results demonstrated that NAC blocked binding of GST-MUC1-CD and BAX (Fig. 3E, left). Moreover, NAC inhibited the interaction between GST-BAX and MUC1-CD (Fig. 3E, right).

MUC1-CD Binds to BAX Cys-62—BAX contains BH1–BH3 functional domains and a transmembrane domain (Fig. 4A). Deletion fragments encompassing BH3 (aa 1–90), BH1 (aa 91–130), and BH2 (aa 131–192) were purified to define the region responsible for the interaction with MUC1-CD (Fig. 4A). Binding studies demonstrated that, as found for full-length BAX(1–192), MUC1-CD formed complexes with BAX(1–90), but not BAX(91–130) or BAX(131–192) (Fig. 4B). Of note, preparations of both GST-BAX(1–192) and GST-BAX(1–90) contained degradation products as detected by Coomassie Blue staining (Fig. 4B, lower), which would be expected to decrease reactivity with MUC1-CD. BAX(1–90) includes the BH3 domain; consequently, to determine whether the BH3 domain contributes to the interaction, we incubated full-length BAX(1–192) and MUC1-CD in the absence and presence of a BH3 peptide (aa 55–74, STKKLECLKRGDELDSNM). The interaction between BAX(1–90) and GST-BAX(1–90/C62A) was incubated with MUC1-CD. The adsorbates were immunoblotted with anti-MUC1-C antibody. Input of the GST proteins was assayed by Coomassie Blue staining (Fig. 4C).

FIGURE 4. MUC1-CD binds to BAX at Cys-62 in BH3 domain. A, schema of the BAX protein. Highlighted are the BH1–BH3 and transmembrane (TM) domains and the BAX fragments used for GST fusion proteins. B, GST, GST-BAX(1–192) (full-length), and the indicated GST-BAX deletion mutants were incubated with MUC1-CD. Adsorbates were immunoblotted (IB) with anti-MUC1-C antibody. Input of the GST proteins was analyzed by Coomassie Blue staining. C, GST-BAX (left) and GST-BAX(1–90) (right) were incubated with MUC1-CD in the absence and presence of a BH3 peptide (aa 55–74, STKKLECLKRGDELDSNM). The adsorbates were immunoblotted with anti-MUC1-C antibody. D, positioning of BAX Cys-62 within the BH3 domain (aa 59–73). GST, GST-BAX(1–90), and GST-BAX(1–90/C62A) were incubated with MUC1-CD. The adsorbates were immunoblotted with anti-MUC1-C antibody. Input of the GST proteins was assayed by Coomassie Blue staining. E, GST-BAX(1–90) was incubated with MUC1-CD in the presence of the wild-type BH3, BH3(C62A), or BH3(L63E) peptide. The adsorbates were immunoblotted with anti-MUC1-C antibody.

MUC1-CD, the BH3(C62A) peptide had no apparent effect on these complexes (Fig. 4E). These results do not exclude the possibility that the C62A mutation causes a structural change in the BH3 domain that alters reactivity with MUC1-CD. Therefore, as a control, we tested a BH3 peptide in which the adjacent Leu-73 was altered to Glu (L63E). As found with the wild-type BH3 peptide and in contrast to BH3(C62A), the BH3(L63E) peptide blocked the interaction between BAX(1–90) and MUC1-CD (Fig. 4E). These findings support a model in which the MUC1-CD cysteine residues bind directly to Cys-62 in the BH3 domain.

MUC1-C Inhibitor GO-203 Blocks Binding to BAX in Vitro and in Cells—To confirm that both of the MUC1-CD cysteine residues are involved in binding to the BAX BH3 domain, we first showed that the MUC1-CD(AQA) mutant failed to bind to BAX(1–90) (Fig. 5A). MUC1-CD(AQC) and MUC1-CD(CQA) were also found to interact with BAX(1–90), indicating that either cysteine is capable of complexing with BAX Cys-62 (Fig. 5A). To further support the contention that the interaction between BAX and MUC1-CD is conferred by disulfide bond formation, we performed the binding studies in the presence of the reducing agent DTT. The results demonstrated that DTT blocked the interaction between GST-BAX or GST-BAX(1–90) and MUC1-C (Fig. 5B). Accordingly, we synthesized a peptide (designated GO-203) derived from MUC1-CD that contains the CQC motif (CQCRRKN) (Fig. 5C). As a control, a similar peptide (CP-2) was synthesized in which the CQC motif was altered to AQA (Fig. 5C). GO-203, but not CP-2, binds to MUC1-CD at the CQC motif and blocks that site (36). In this context, GO-203, but not the control peptide, blocked the interaction between MUC1-CD and full-length BAX(1–192)}
Similar results were obtained when MUC1-CD was incubated with BAX(1–90) in the presence of these peptides (Fig. 5D, left). To assess the effects on endogenous MUC1-C:BAX complexes, we treated MCF-7 cells with the GO-203 or CP-2 peptide. Of note, GO-203 and CP-2 contain a poly-Arg sequence to facilitate entry of these peptides into cells (37). The results showed that GO-203, but not CP-2, blocked the association of MUC1-C and BAX (Fig. 5E). These findings indicate that the MUC1-C CQC motif binds to the BAX BH3 domain in vitro and in cells.

**MUC1-C Blocks BAX Dimerization**—The BAX BH3 domain confers BAX dimerization (4). To assess the effects of MUC1-C on BAX dimerization, we performed experiments with HCT116 cells that were stably transfected with an empty pIRES-puro2 vector (HCT116/vector) or one expressing MUC1 (Fig. 6A). Studies were also performed with MCF-7 cells infected with a retroviral vector expressing MUC1 siRNA (MCF-7/MUC1 siRNA) or, as a control, an empty vector (MCF-7/vector) (15). Mitochondria were purified from the HCT116 and MCF-7 cells and treated with tBID to directly activate BAX. Analysis of BAX monomers and dimers was performed in the absence (Fig. 6B, left) and presence (Fig. 6B, right) of disuccinimidyl suberate, a cell-permeable homobifunctional protein cross-linker that has been used to detect BAX dimerization in mitochondria (30). The tBID-induced BAX dimers were detectable in the presence, but not in the absence, of disuccinimidyl suberate. Moreover, MUC1-C expression in the HCT116/MUC1 cells was associated with attenuation of tBID-induced BAX dimerization compared with that in the HCT116/vector cells (Fig. 6B, right). In MCF-7 cells, silencing MUC1-C resulted in an increase in tBID-induced BAX dimers (Fig. 6B, right), indicating that MUC1-C blocks BAX dimerization. To determine whether MUC1-C affects BAX dimerization in the response to cellular stress, we treated MCF-7/vector and MCF-7/MUC1 siRNA cells with the genotoxic agent VBL. The cells were also treated with 1,6-bismaleimidohexane, a homobifunctional cross-linking reagent that reacts with sulfhydryl groups and that has been used to detect BAX dimerization in cells (31). BAX dimers were detectable in VBL-treated, but not untreated, MCF-7/MUC1 siRNA cells (Fig. 6C). By contrast, BAX dimers were not detectable in VBL-treated MCF-7/vector cells (Fig. 6C), indicating that MUC1-C blocks BAX dimerization in the response to stress. To further assess the effects of MUC1-C on BAX function, we incubated BAX with purified mitochondria from MCF-7 cells. BAX treatment was associated with release of cytochrome c from the mitochondrial pellet to the supernatant (Fig. 6D). By contrast, incubation of mitochondria with both BAX and MUC1-CD demonstrated that MUC1-CD blocked BAX-mediated cytochrome c release (Fig. 6D). As found with wild-type BAX, incubation of purified mitochondria with BAX(C62A) was associated with release of cytochrome c (Fig. 6E). However, in contrast to wild-type BAX, MUC1-CD had little if any effect on BAX(C62A)-induced cytochrome c release (Fig. 6E). These findings indicate that binding of MUC1-C to BAX blocks its dimerization and function in inducing the release of mitochondrial cytochrome c.

**MUC1-C Blocks BAX Activation in Response to Stress**—To further assess the effects of MUC1-C on BAX activation, we...
first treated HCT116/vector and HCT116/MUC1 cells with DOX. BAX dimers were detectable in DOX-treated, but not untreated, HCT116/vector cells (Fig. 7A). Moreover, BAX dimers were not detectable in DOX-treated HCT116/MUC1 cells (Fig. 7A), confirming that MUC1-C blocks BAX dimerization. To determine whether binding of MUC1-C to BAX affects localization of BAX to mitochondria, studies were performed using HCT116 cells expressing MUC1 or the MUC1(AQA) mutant (Fig. 7B) (18). Co-immunoprecipitation analysis of lysates from HCT116/MUC1 and HCT116/MUC1(AQA) cells demonstrated that MUC1, and not MUC1(AQA), associated with BAX (Fig. 7C), also confirming that the MUC1-C CQC motif is required for binding to BAX in cells. As detected by confocal microscopy, DOX treatment of HCT116/vector cells was also associated with localization of BAX to mitochondria (Fig. 7D). Notably, this response was attenuated in HCT116/MUC1 cells, but not in HCT116/MUC1(AQA) cells (Fig. 7D), indicating that binding of MUC1-C to BAX blocks localization of activated BAX to mitochondria.

DOX-induced apoptosis of HCT116 cells was also abrogated by MUC1 (Fig. 8A). By contrast, MUC1(AQA) had little effect on the apoptotic response (Fig. 8A). These results were confirmed with the analysis of repetitive experiments (Fig. 8B). In addition, MUC1, but not MUC1(AQA), blocked the apoptotic response to hydrogen peroxide and VBL (Fig. 8, A and B). To extend these studies, we performed similar experiments with the MCF-7/vector and MCF-7/MUC1 siRNA cells. Here, silencing MUC1 was associated with a significant increase in the induction of apoptosis in response to treatment with DOX, hydrogen peroxide, and VBL (Fig. 8C). For confirmation of these results, we studied ZR-75-1 cells infected with the retroviral vector expressing MUC1 siRNA (ZR-75-1/MUC1 siRNA) or the empty vector (ZR-75-1/vector) (Fig. 8E). As found with MCF-7 cells, silencing MUC1 in the ZR-75-1 cells resulted in an increased apoptotic response to DOX, hydrogen peroxide, and VBL (Fig. 8E). These findings indicate that the inhibitory effects of MUC1-C on BAX activation are associated with MUC1-C-mediated attenuation of stress-induced apoptosis.

**DISCUSSION**

MUC1-C Oncoprotein Binds Directly to BAX BH3 Domain—
The BAX BH3 domain is necessary for BAX homodimerization and heterodimerization with BCL-2 (4). However, little is known about interactions of the BAX BH3 domain with proteins other than BCL-2 family members (38). Our results dem-
onstrate that the MUC1-C oncoprotein associates with BAX in cells. Our in vitro studies further demonstrate that MUC1-C binds directly to BAX. MUC1-CD includes two cysteines in a CQC motif that functions in the formation of MUC1-C homodimers and heterodimeric complexes with other proteins containing reactive cysteine residues (18). Our results further show that both of the cysteines in the MUC1-C CQC motif are functional in forming a complex with BAX and that this interaction is disrupted by reducing agents. These findings support a model in which binding of MUC1-C and BAX is conferred by disulfide bonds. BAX contains cysteine residues at positions 62 and 126. There is no evidence for binding of MUC1-CD to BAX Cys-126. However, the results Clearly demonstrate that the MUC1-C CQC motif cysteines bind directly to BAX at Cys-62 in the BH3 domain. Previous work has demonstrated that BAX Cys-62 is oxidized by reactive oxygen species and is potentially reactive to form disulfide bridges (9, 10). In this context, an in silico model has proposed that BAX forms homodimers as a result of disulfide bonds between Cys-62 and Cys-126 (9). To our knowledge, there are no other reports of proteins that interact with BAX at Cys-62. Accordingly, to confirm that MUC1-CD confers the association with BAX in cells, we treated cells with a cell-penetrating peptide that binds to the CQC motif and thereby blocks its reactivity. These findings could be explained, at least in part, by two potential mechanisms. In one model, binding of MUC1-C to BAX Cys-62 sterically blocks the availability of BH3 domains to form BAX homodimers. Alternatively, if BAX forms homodimers through interactions between Cys-62 and Cys-126 (9), then binding of MUC1-CD to BAX Cys-62 would block the availability of Cys-62 to form disulfide bonds with Cys-126. In both models, redox imbalance and oxidative stress, as has been commonly found in transformed cells (39), could promote the interaction between MUC1-CD and BAX Cys-62 and block BAX dimerization.

FIGURE 7. MUC1-C attenuates BAX localization to mitochondria. A, the indicated HCT116 cells were treated with 1 μM DOX for 24 h. Cells were left untreated (left) or were cross-linked with 1,6-bismaleimidohexane (BMH; right). Cell lysates were immunoblotted (IB) with the indicated antibodies. B, lysates from HCT116/vector, HCT116/MUC1, and HCT116/MUC1(AQA) cells were immunoblotted with the indicated antibodies. C, lysates from HCT116/MUC1 and HCT116/ MUC1(AQA) cells were subjected to immunoprecipitation (IP) with IgG or anti-BAX antibody. The precipitates were immunoblotted with the indicated antibodies. D, HCT116/vector, HCT116/MUC1, and HCT116/MUC1(AQA) cells were left untreated or were treated with 1 μM DOX for 24 h. The cells were stained with MitoTracker Red, fixed, incubated with anti-BAX antibody, and analyzed by confocal microscopy.

MUC1-C Blocks BAX BH3 Domain

The demonstration that the BAX BH3 domain is necessary for forming BAX homodimers (4) and our finding that MUC1-C binds to the BAX BH3 domain invoked the possibility that MUC1-C might block BAX dimerization. MUC1-C is expressed at the apical membrane of normal secretory epithelial cells (11). However, in carcinoma cells with loss of polarity and MUC1 overexpression, the MUC1-C subunit accumulates in the cytoplasm and localizes to the MOM (11). BAX also resides in the cytoplasm and mitochondria (33). In this respect, the interaction between MUC1-C and BAX (i) was detected in the cytoplasmic and mitochondrial fractions of breast cancer cells and (ii) was induced by genotoxic stress. In addition, MUC1-C blocked BAX dimerization in the response to tBID stimulation in vitro and in cells treated with genotoxic agents. These findings could be explained, at least in part, by two potential mechanisms. In one model, binding of MUC1-C to BAX Cys-62 sterically blocks the availability of BH3 domains to form BAX homodimers. Alternatively, if BAX forms homodimers through interactions between Cys-62 and Cys-126 (9), then binding of MUC1-CD to BAX Cys-62 would block the availability of Cys-62 to form disulfide bonds with Cys-126. In both models, redox imbalance and oxidative stress, as has been commonly found in transformed cells (39), could promote the interaction between MUC1-CD and BAX Cys-62 and block BAX dimerization. Previous work demonstrated that BAX dimerization results in translocation to mitochondria and induction of apoptosis (3). In our work, in addition to blocking BAX dimerization, MUC1-C attenuated localization of BAX to mitochondria in the response to stress. Thus, our results indicate that MUC1-C binds to the BAX BH3 domain and blocks, in turn, BAX dimerization and localization to the MOM.
MUC1-C Blocks BAX BH3 Domain

FIGURE 8. MUC1-C attenuates stress-induced apoptosis. A and B, HCT116/vector, HCT116/MUC1, and HCT116/MUC1(AQA) cells were left untreated or were treated with 1 μM DOX for 12 h, with 0.3 μM hydrogen peroxide for 12 h, or with 30 nm VBL for 24 h. The cells were incubated with propidium iodide (PI) and annexin V and analyzed by flow cytometry. The percentage of apoptotic cells is expressed as the mean ± S.D. of three determinations. C, MCF-7/vector and MCF-7/MUC1 siRNA cells were treated with 1 μM DOX for 12 h, with 0.3 μM hydrogen peroxide for 12 h, or with 30 nm VBL for 24 h. The percentage of apoptotic cells is expressed as the mean ± S.D. of three determinations. D, ZR-75-1/vector and ZR-75-1/MUC1 siRNA cells were treated with 1 μM DOX for 12 h, with 0.3 μM hydrogen peroxide for 12 h, or with 30 nm VBL for 24 h. The percentage of apoptotic cells is expressed as the mean ± S.D. of three determinations.

MUC1-C Functions as Anti-apoptotic Protein—Previous work using carcinoma cells has shown that MUC1 expression is associated with a block in the apoptotic response to genotoxic and oxidative stress (15, 27, 40). Other studies have shown that MUC1 also attenuates apoptosis in the response to hypoxia and glucose deprivation (28, 41), indicating that MUC1-C has a fundamental effect on the intrinsic apoptotic pathway. These findings have been attributed, at least in part, to localization of MUC1-C in the MOM and thereby a block in the release of mitochondrial apoptotic factors, such as cytochrome c (15). Our results provide further clarity regarding the effects of MUC1-C on stress-induced apoptosis. In an in vitro model of adding BAX to purified mitochondria, MUC1-CD blocked BAX-induced release of cytochrome c, indicating that the interaction between MUC1-C and BAX suppresses activation of the intrinsic apoptotic pathway. In concert with this model, MUC1-C attenuated stress-induced localization of BAX to mitochondria. By contrast, MUC1-C with the CQC-to-AQA mutation was ineffective in binding to BAX and blocking BAX localization to mitochondria. Moreover, MUC1-C, and not MUC1-C with the AQA mutation, was effective in blocking stress-induced apoptosis. BAX-mediated apoptosis is abrogated by interactions between the BAX BH3 domain and the anti-apoptotic BCL-2, BCL-xL, and MCL-1 proteins (4). Our results suggest that binding of MUC1-C to the BAX BH3 domain in the cytoplasm and/or in the MOM may have a similar effect. Recent studies have shown that BCL-xL retrotranslocates BAX from the mitochondria such that BAX is maintained in the cytoplasm (33). As noted above, little is known about the function of MUC1-C in the MOM and whether, like BCL-xL, it shuttles between the MOM and cytoplasm. By extension, further studies will be needed to define the role of mitochondrial MUC1-C in the regulation of apoptosis.

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