MUC1 oncoprotein blocks nuclear targeting of c-Abl in the apoptotic response to DNA damage

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The nonreceptor c-Abl tyrosine kinase binds to cytosolic 14-3-3 proteins and is targeted to the nucleus in the apoptotic response to DNA damage. The MUC1 oncoprotein is overexpressed by most human carcinomas and blocks the induction of apoptosis by genotoxic agents. Using human carcinoma cells with gain and loss of MUC1 function, we show that nuclear targeting of c-Abl by DNA damage is abrogated by a MUC1-dependent mechanism. The results demonstrate that c-Abl phosphorylates MUC1 on Tyr-60 and forms a complex with MUC1 by binding of the c-Abl SH2 domain to the pTyr-60 site. Binding of MUC1 to c-Abl attenuates phosphorylation of c-Abl on Thr-735 and the interaction between c-Abl and cytosolic 14-3-3. We also show that expression of MUC1 with a mutation at Tyr-60 (i) disrupts the interaction between MUC1 and c-Abl, (ii) relieves the MUC1-induced block of c-Abl phosphorylation on Thr-735 and binding to 14-3-3, and (iii) attenuates the MUC1 antiapoptotic function. These findings indicate that MUC1 sequesters c-Abl in the cytoplasm and thereby inhibits apoptosis in the response to genotoxic anticancer agents.

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Introduction

DNA damage activates a nuclear complex that consists in part of the nonreceptor c-Abl tyrosine kinase (Kharbanda *et al*, 1995b; Yuan *et al*, 1997). c-Abl binds to the p53 tumor suppressor in the response to genotoxic stress (Yuan *et al*, 1996a,b) and regulates p53 by preventing its nuclear export (Sionov *et al*, 2001). Other studies have demonstrated that c-Abl interacts with the p73 homolog of p53 in the apoptotic response of cells to DNA damage (Agami *et al*, 1999; Gong *et al*, 1999; Yuan *et al*, 1999). Nuclear c-Abl also activates MEK kinase-1 and thereby the proapoptotic c-Jun N-terminal

kinase (JNK) pathway (Kharbanda *et al*, 1995a,b, 2000). c-Abl contains three nuclear localization signals and one nuclear export signal in the carboxy-terminal region that are responsible for shuttling of c-Abl between the cytoplasm and nucleus (Wen *et al*, 1996; Taagepera *et al*, 1998). Recent work has shown that c-Abl is sequestered in the cytoplasm through binding to 14-3-3 proteins (Yoshida *et al*, 2005). Phosphorylation of c-Abl on Thr-735 by an as yet unidentified kinase serves as a site for direct binding to 14-3-3. In the response to DNA damage, activation of JNK induces phosphorylation of 14-3-3 and the release of c-Abl for targeting to the nucleus (Yoshida *et al*, 2005). In this regard, expression of a 14-3-3 mutant at the JNK phosphorylation site attenuates DNA damage-induced nuclear import of c-Abl and apoptosis. These findings have provided support for a model in which 14-3-3 is an important regulator of intracellular c-Abl localization and the apoptotic response to genotoxic stress.

The MUC1 transmembrane glycoprotein is expressed as a stable heterodimer following synthesis as a single polypeptide and cleavage into two subunits in the endoplasmic reticulum (Ligtenberg *et al*, 1992). MUC1 is normally localized to the apical borders of secretory epithelial cells (Kufe *et al*, 1984). With transformation and loss of polarity, the MUC1 heterodimer is aberrantly overexpressed on the entire cell membrane (Kufe *et al*, 1984). The MUC1 N-terminal subunit (MUC1-N) contains variable numbers of 20 amino-acid tandem repeats that are modified by O-linked glycans (Gendler *et al*, 1988; Siddiqui *et al*, 1988). MUC1-N is tethered to the cell membrane by the MUC1 C-terminal subunit (MUC1-C), which includes a transmembrane region and a 72 amino-acid cytoplasmic domain (MUC1-CD) (Merlo *et al*, 1989). MUC1-CD associates with β -catenin (Yamamoto *et al*, 1997; Li *et al*, 1998; Huang *et al*, 2005) and the p53 tumor suppressor (Wei *et al*, 2005). In addition, MUC1-CD is phosphorylated by the epidermal growth factor receptor (Li *et al*, 2001b), c-Src (Li *et al*, 2001a) and glycogen synthase kinase 3 β (Li *et al*, 1998). Other studies have shown that overexpression of MUC1 confers transformation (Li *et al*, 2003; Huang *et al*, 2005) and blocks the apoptotic response to genotoxic stress (Raina *et al*, 2004; Ren *et al*, 2004). In this regard and in addition to expression at the cell membrane, MUC1-C localizes to the mitochondrial outer membrane and inhibits DNA damage-induced release of apoptogenic factors (Ren *et al*, 2004, 2006). Mitochondrial targeting of MUC1-C is attenuated by mutation of the cytoplasmic domain at Tyr-46 (Ren *et al*, 2004, 2006). Moreover, expression of MUC1 with a Y46F mutation attenuates the antiapoptotic function of MUC1 in the response to DNA damage (Ren *et al*, 2004). These findings indicate that MUC1 blocks in part the apoptotic response to genotoxic anticancer agents by disrupting the intrinsic mitochondrial pathway.

The overexpression of MUC1 in most human carcinomas, the antiapoptotic function of MUC1 in the response of cancer cells to genotoxic anticancer agents and the role of c-Abl in inducing apoptosis in the DNA damage response prompted us

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to investigate whether MUC1 interacts with c-Abl. Our results demonstrate that MUC1 binds directly to c-Abl and sequesters c-Abl in the cytoplasm. We also show that MUC1 blocks nuclear targeting of c-Abl and thereby the apoptotic response to genotoxic anticancer agents.

Results

MUC1 attenuates DNA damage-induced targeting of c-Abl to the nucleus

To investigate whether MUC1 regulates nuclear targeting of c-Abl, HeLa cells were transfected to stably express an empty vector or MUC1. The MUC1 N-terminal (MUC1-N) and C-terminal (MUC1-C) subunits were detectable in HeLa/MUC1, but not HeLa/vector, cells (Figure 1A). Moreover, c-Abl levels were similar in the absence and presence of MUC1 (Figure 1A). Consistent with previous results (Yoshida *et al*, 2005), treatment of HeLa/vector cells with the DNA damaging agent adriamycin (ADR) was associated with targeting of c-Abl to the nucleus (Figure 1B). By contrast, there was little if any nuclear targeting of c-Abl in the ADR-treated HeLa/MUC1 cells (Figure 1B). As controls, the nuclear fractions were immunoblotted with antibodies against the nuclear lamin B and cytoplasmic I κ B α proteins (Figure 1B). Treatment of HeLa/vector and HeLa/MUC1 cells with cispla-

tin (CDDP) confirmed that MUC1 attenuates nuclear targeting of c-Abl by genotoxic anticancer agents (Figure 1C). In concert with these results, CDDP treatment was associated with a decrease in cytosolic c-Abl in HeLa/vector, but not HeLa/MUC1, cells (Figure 1D).

As reported previously, endogenous MUC1 expression was knocked-down with a MUC1siRNA in human ZR-75-1 breast cancer cells (Ren *et al*, 2004). Silencing MUC1 in the ZR-75-1 cells had no effect on c-Abl levels (Figure 2A). When ZR-75-1/vector cells were treated with CDDP, there was no detectable targeting of c-Abl to the nucleus (Figure 2B). However, in ZR-75-1/MUC1siRNA cells silenced for MUC1, nuclear targeting of c-Abl was increased about six-fold in response to CDDP treatment (Figure 2B). Cytosolic c-Abl was also decreased by CDDP treatment of ZR-75-1/MUC1siRNA, but not ZR-75-1/vector, cells (Figure 2C). To further analyze the effects of MUC1 on c-Abl localization, transient overexpression of GFP-tagged c-Abl in 293 cells resulted in targeting of c-Abl to the nucleus that was detectable constitutively and increased by ADR treatment (Supplementary Figure S1A). By contrast, constitutive and ADR-induced nuclear targeting of c-Abl was attenuated by coexpression of MUC1 (Supplementary Figure S1A). Similar results were obtained when the transfected cells were treated with CDDP (data not shown). The c-Abl mutant with deletion of the nuclear export signal (c-AblNES) localizes exclusively in the nucleus (Yoshida *et al*, 2005). Importantly, MUC1 expression had little effect on nuclear localization of GFP-c-Abl Δ NES (Supplementary Figure S1B). These findings indicate that MUC1 blocks targeting of cytosolic c-Abl to the nucleus in the response to genotoxic anticancer agents.

Figure 1 Exogenous MUC1 attenuates nuclear targeting of c-Abl in HeLa cells. (A) Lysates from HeLa cells were subjected to immunoblotting with anti-MUC1-N, anti-MUC1-C, anti-c-Abl or anti- β -actin. (B, C) HeLa/vector and HeLa/MUC1 cells were treated either with adriamycin (ADR) (B) or cisplatin (CDDP) (C) for 2 h. Nuclear fractions were subjected to immunoblot analysis with anti-c-Abl, anti-lamin B or anti-I κ B α . (D) HeLa/vector and HeLa/MUC1 cells were treated with CDDP for 2 h. Cytosolic extracts were subjected to immunoblot analysis with anti-c-Abl or anti- β -actin.

Figure 2 c-Abl is targeted to the nucleus by silencing MUC1 in ZR-75-1 cells. (A) Lysates from ZR-75-1/vector and ZR-75-1/MUC1siRNA cells were subjected to immunoblotting with anti-MUC1-N, anti-MUC1-C, anti-c-Abl or anti- β -actin. (B and C) ZR-75-1/vector and ZR-75-1/MUC1siRNA cells were treated with CDDP for 2 and 4 h. Nuclear (B) and cytosolic (C) fractions were subjected to immunoblot analysis with the indicated antibodies.

MUC1 associates with c-Abl at the cell membrane

To determine if MUC1 associates with c-Abl, lysates from HeLa/MUC1 cells were precipitated with anti-MUC1-N or, as a control, IgG. Immunoblot analysis of the precipitates with anti-c-Abl demonstrated that MUC1 associates with c-Abl (Figure 3A). Coimmunoprecipitation studies performed on ZR-75-1 cells confirmed that MUC1 forms a complex with c-Abl (Figure 3B). MUC1 is expressed at the cell membrane as a heterodimer of MUC1-N and MUC1-C. Consequently, we asked if c-Abl localizes to the cell membrane as a function of MUC1 expression. Immunoblot analysis showed a substantially higher level of c-Abl in HeLa/MUC1 cell membranes as compared to those purified from HeLa/vector cells (Figure 3C). Equal loading and purity of the cell membrane fraction was confirmed by immunoblotting with antibodies against the cell membrane-associated platelet-derived growth factor receptor, endoplasmic reticulum-associated calnexin and cytosolic I κ B α proteins (Figure 3C). Consistent with the results in HeLa cells, localization of c-Abl to the cell membrane was significantly decreased, but not completely abrogated, by silencing MUC1 in ZR-75-1 cells (Figure 3D).

Consistent with these results, c-Abl is known to bind to other cell membrane-associated proteins, such as scramblase 1 and caveolin 1 (Sun *et al*, 2001; Sanguinetti and Mastick, 2003). Confocal microscopy further showed colocalization of MUC1 and c-Abl at the cell membrane (Figure 3E). These findings indicate that c-Abl associates with MUC1 at the cell membrane.

c-Abl phosphorylates the MUC1 cytoplasmic domain

To identify the region of MUC1 that associates with c-Abl, studies were performed on HCT116 colon cancer cells. As found in other cell types (Kharbanda *et al*, 1995b; Yuan *et al*, 1997), treatment of the HCT116 cells with CDDP was associated with induction of c-Abl activity (Supplementary Figure S2). Consequently, we studied HCT116 cells that stably express the empty vector or a Flag-tagged MUC1 cytoplasmic domain (MUC1-CD) (Huang *et al*, 2003) (Figure 4A). Expression of Flag-MUC1-CD had no detectable effect on c-Abl levels (Figure 4A). Immunoblot analysis of anti-Flag precipitates with anti-c-Abl further showed that MUC1-CD is sufficient for the association with c-Abl (Figure 4B). To

Figure 3 MUC1 forms complexes with c-Abl at the cell membrane. (A, B). Lysates from HeLa/MUC1 (A) and ZR-75-1/vector (B) cells were subjected to immunoprecipitation (IP) with mouse IgG or anti-MUC1-N. The precipitates were immunoblotted with anti-c-Abl or anti-MUC1-N. (C) Cell membrane fractions from HeLa/vector and HeLa/MUC1 cells were subjected to immunoblot analysis with the indicated antibodies. (D) Cell membrane fractions from ZR-75-1/vector or ZR-75-1/MUC1siRNA cells were subjected to immunoblot analysis with the indicated antibodies. Immunoblots from three separate experiments were subjected to densitometric scanning to determine the effects of MUC1 silencing on localization of c-Abl to the cell membrane. The results are expressed as the percentage (mean $\pm$ s.d.) of c-Abl localization for ZR-75-1/vector cells (assigned a value of 100%) and ZR-75-1/MUC1siRNA cells. (E) Confocal microscopy of ZR-75-1/vector cells stained with anti-MUC1-C or anti-c-Abl. Nuclei were stained with TO-PRO-3.

Figure 4 c-Abl phosphorylates the MUC1 cytoplasmic domain. (A) Lysates from HCT116/vector and HCT116/Flag-MUC1-CD cells were immunoblotted with anti-MUC1-C, anti-c-Abl or anti-β-actin. (B) Lysates from HCT116/Flag-MUC1-CD cells were immunoprecipitated with mouse IgG or anti-Flag. The precipitates were immunoblotted with anti-c-Abl or anti-Flag. (C) Purified GST-MUC1-CD was cleaved with thrombin to remove the GST tag and then incubated with GST-c-Abl or GST-c-Abl(K-R) and [γ - 32 P]ATP for 15 min at 30°C. The reaction products were analyzed by SDS-PAGE and autoradiography (top panel) or by immunoblotting with anti-MUC1-C (lower panel). (D) Amino acid sequence of MUC1-CD (upper panel). Purified MUC1-CD or MUC1-CD(Y60F) was incubated with GST-c-Abl or GST-c-Abl(K-R) and [γ - 32 P]ATP for 15 min at 30°C. Reaction products were analyzed by SDS-PAGE and autoradiography or by immunoblotting with anti-MUC1-C (lower panels).

determine if MUC1-CD is a substrate for c-Abl, we incubated purified kinase-active c-Abl and MUC1-CD in the presence of [γ - 32 P]ATP. Analysis of the reaction products by SDS-PAGE and autoradiography showed phosphorylation of MUC1-CD (Figure 4C). As a control, there was no detectable phosphorylation when MUC1-CD was incubated with a purified kinase-inactive c-Abl(K-R) (Kharbanda *et al*, 1995b) (Figure 4C). MUC1-CD contains a consensus c-Abl phosphorylation site (YXXP) at Y⁶⁰TNP (Figure 4D). Consequently, we asked if c-Abl phosphorylation of MUC1-CD is decreased by a Y60>F mutation. Compared to MUC1-CD, c-Abl phosphorylation was substantially decreased with the MUC1-CD(Y60F) mutant (Figure 4D), indicating that c-Abl phosphorylates MUC1, at least in large part, on Tyr-60 in the cytoplasmic domain.

c-Abl SH2 domain binds to the MUC1 pY⁶⁰TNP site

MUC1 with the Y60F mutation was stably expressed in HCT116 cells. Immunoblot analysis of two separately isolated clones showed somewhat higher levels of MUC1(Y60F) as compared to the wild-type protein in stably transfected HCT116/MUC1 cells (Figure 5A). As found for MUC1, expression of the MUC1(Y60F) mutant had no effect on c-Abl levels (Figure 5A). Notably, c-Abl was detectable in anti-MUC1-N immunoprecipitates from HCT116/MUC1, but not HCT116/MUC1(Y60F), cells (Figure 5B). As a control, the Y60F mutation had no effect on the association between MUC1-N and MUC1-C (Figure 5B). Similar results were obtained with the two HCT116/MUC1(Y60F) clones (data not shown). Localization of c-Abl to the cell membrane was also decreased in HCT116/MUC1(Y60F), as compared to that in HCT116/MUC1, cells (Figure 5C). In 293 cells, binding of MUC1 to c-Abl was also attenuated by the Y60F mutation

(Figure 5D). To determine if the pY⁶⁰TNP motif functions as a binding site for the c-Abl SH2 domain, we incubated MUC1-CD or MUC1-CD(Y60F) with kinase-active truncated Abl (no SH2 domain) and ATP. Incubation of the reaction products with GST-c-Abl SH2 demonstrated that binding of the c-Abl SH2 domain to phosphorylated MUC1-CD is abrogated by the Y60F mutation (Figure 5E). To extend these results, we generated a c-Abl SH2 point mutant in which Arg-152 in the conserved FLVRES sequence was modified to Leu. *In vitro* studies demonstrated that the R152L mutation abrogates binding of GST-c-Abl SH2 to c-Abl phosphorylated MUC1-CD (Supplementary Figure S3A). We also found that MUC1 is effective in blocking nuclear targeting of GFP-c-Abl, but not GFP-c-Abl(R152L), in the response to DNA damage (Supplementary Figure 3B-D). These findings indicate that MUC1 and c-Abl interact directly through binding of the c-Abl SH2 domain to the MUC1 pYTNP motif.

MUC1 blocks binding of c-Abl to 14-3-3 proteins

c-Abl contains a RSVpT⁷³⁵LP sequence that confers binding to 14-3-3 proteins as a mechanism for regulating nuclear targeting of c-Abl in the response to DNA damage (Yoshida *et al*, 2005). To determine if MUC1 affects phosphorylation of Thr-735, anti-c-Abl precipitates from HeLa/vector and HeLa/MUC1 cells were immunoblotted with an anti-phospho-c-Abl-Thr-735 antibody. The results show that MUC1 blocks c-Abl Thr-735 phosphorylation (Figure 6A). In concert with these results, c-Abl Thr-735 phosphorylation was increased in ZR-75-1 cells silenced for MUC1 (Figure 6B). In addition, c-Abl Thr-735 phosphorylation was attenuated by expression of MUC1, but not MUC1(Y60F), in HCT116 cells, indicating that the interaction between MUC1 and c-Abl blocks phosphor-

Figure 5 c-Abl-SH2 domain binds to the MUC1 pY⁶⁰-TNP site. (A) Lysates from HCT116/MUC1 (clones A and B) and HCT116/MUC1(Y60F) (clones A and B) were subjected to immunoblotting with anti-MUC1-N, anti-MUC1-C, anti-c-Abl or anti-β-actin. (B) Lysates from HCT116/MUC1 and HCT116/MUC1(Y60F) cells were immunoprecipitated with mouse IgG and anti-MUC1-N. The precipitates were immunoblotted with anti-c-Abl, anti-MUC1-N and anti-MUC1-C. (C) Cell membrane fractions from HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) cells were immunoblotted with the indicated antibodies. (D) 293T cells were transfected with GFP and MUC1, GFP-c-Abl and MUC1, and GFP-c-Abl and MUC1(Y60F). Lysates were subjected to immunoprecipitation with anti-MUC1-N or control IgG and immunoblotting of the precipitates with anti-GFP and anti-MUC1-N. (E) Purified MUC1-CD and MUC1-CD(Y60F) were incubated in the presence or absence of recombinant truncated Abl (no SH2 domain) and 200 μM ATP for 30 min at 30°C. GST or GST-c-Abl-SH2 bound to GST-beads was then added and incubated for 1 h at 4°C. Adsorbed (upper panel) and input (lower panel) proteins were subjected to immunoblot analysis with anti-MUC1-C.

ylation of the Thr-735 site (Figure 6C). Consistent with a block in Thr-735 phosphorylation, we found that MUC1 inhibits the association between c-Abl and 14-3-3 in HeLa cells (Figure 6D) and ZR-75-1 cells (Figure 6E). MUC1 also blocked binding of c-Abl to 14-3-3 in HCT116 cells

(Figure 6F). By contrast, MUC1(Y60F) had little effect on binding of c-Abl to 14-3-3 (Figure 6F). These results indicate that binding of MUC1 and c-Abl blocks phosphorylation of c-Abl on Thr-735 and thereby the association between c-Abl and 14-3-3.

Binding of MUC1 and c-Abl attenuates DNA damage-induced apoptosis

HCT116/vector cells express a low level of nuclear c-Abl and, in the response to CDDP treatment, c-Abl was targeted to the nucleus (Figure 7A). As found in the other cell types, expression of MUC1 in HCT116 cells blocked nuclear targeting of c-Abl (Figure 7A). Moreover, there was less than a two-fold increase in nuclear MUC1-C levels in response to CDDP, consistent with the effects on c-Abl (Supplementary Figure S4). Importantly, however, MUC1(Y60F) had little if any effect on CDDP-induced targeting of c-Abl to the nucleus (Figure 7A). In concert with the block in nuclear targeting of c-Abl by wild-type MUC1, CDDP treatment increased binding of MUC1, but not MUC1(Y60F), with c-Abl (Figure 7B and data not shown). DNA damage activates the intrinsic apoptotic pathway with caspase-3-mediated cleavage of protein kinase Cδ (Emoto *et al*, 1995; Ghayur *et al*, 1996). Cleavage of PKCδ was attenuated by MUC1, but not the MUC1(Y60F) mutant (Figure 7C). Analysis of the cells for sub-G1 DNA further demonstrated that MUC1 blocks DNA damage-induced apoptosis and that this inhibition is relieved by the Y60F mutation (Supplementary Figure S5). Similar results were obtained in repetitive experiments (Figure 7D) and when the HCT116 cells were treated with ADR (data not shown). To assess the role of c-Abl in the induction of apoptosis, we treated the cells with CDDP in the absence and presence of STI571, an inhibitor of the c-Abl kinase function (Druker *et al*, 1996). STI571 significantly attenuated CDDP-induced apoptosis in HCT116/vector and HCT116/MUC1(Y60F) cells, but had no significant effect on HCT116/MUC1 cells (Figure 7E). To substantiate these results, c-Abl expression was downregulated in HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) cells by transient transfection with a pool of c-Abl siRNAs (Supplementary Figure S6A). Consistent with the effects of STI571, c-Abl downregulation was associated with attenuation of CDDP-induced apoptosis in HCT116/vector and HCT116/MUC1(Y60F) cells (Figure 7F, Supplementary Figure S6B and S6D). By contrast, silencing c-Abl in HCT116/MUC1 cells had no significant effect on the induction of apoptosis (Figure 7F, Supplementary Figure S6C). These findings demonstrate that binding of MUC1 to c-Abl and sequestration of c-Abl in the cytosol blocks the proapoptotic function of c-Abl in the response to DNA damage.

Selectivity of the MUC1 Tyr-60 site for the regulation of c-Abl

Previous work showed that the antiapoptotic effects of MUC1 are attenuated by mutation of Tyr-46 (Ren *et al*, 2004). To determine whether the loss of MUC1 function by the MUC1(Y60F) and MUC1(Y46F) mutants is due to similar or distinct mechanisms, we compared the effects of stably expressing these proteins in HCT116 cells (Supplementary Figure S7A). As found for MUC1(Y60F), the MUC1(Y46F) mutant had no apparent effect on c-Abl levels (Supplementary Figure S7A). Coimmunoprecipitation studies

Figure 6 MUC1 blocks c-Abl Thr-735 phosphorylation and binding of c-Abl to 14-3-3. (A–C) Lysates from HeLa/vector, HeLa/MUC1 (A), ZR-75-1/vector, ZR-75-1/MUC1siRNA (B), HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) (C) cells were immunoprecipitated with anti-c-Abl. The precipitates were immunoblotted with anti-phospho-c-Abl-Thr-735 and anti-c-Abl. (D–F) Cytoplasmic fractions of HeLa/vector, HeLa/MUC1 (D), ZR-75-1/vector, ZR-75-1/MUC1siRNA (E), HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) (F) cells were immunoprecipitated with anti-14-3-3. The precipitates were immunoblotted with anti-c-Abl and anti-14-3-3.

further demonstrated that, like MUC1, the MUC1(Y46F) mutant associates with c-Abl (Supplementary Figure S7B). Moreover, in contrast to MUC1(Y60F), MUC1(Y46F) blocked CDDP-induced targeting of cytosolic c-Abl to the nucleus (Supplementary Figures S7C and S7D). MUC1 localizes to the outer mitochondrial membrane and blocks the release of apoptogenic factors (Ren *et al*, 2004, 2006). In addition, mitochondrial localization of MUC1 is substantially attenuated by mutation of Tyr-46 (Ren *et al*, 2004, 2006), but not the Tyr-60 site (Supplementary Figure S7E). These findings indicate that MUC1 Tyr-60, and not Tyr-46, regulates c-Abl function. Conversely, MUC1 Tyr-46, and not Tyr-60, regulates mitochondrial localization.

Discussion

MUC1 binds directly to c-Abl

Nuclear c-Abl is activated by diverse genotoxic agents and induces apoptosis (Kharbanda *et al*, 1995b; Yuan *et al*, 1997). Conversely, overexpression of MUC1, as found in most human carcinomas, blocks the induction of apoptosis by genotoxic anticancer agents (Raina *et al*, 2004; Ren *et al*, 2004). The present studies demonstrate that MUC1 associates with c-Abl. MUC1 is expressed at the cell membrane as a heterodimer of the MUC1-N and MUC1-C subunits. MUC1-N extends beyond the cell glycocalyx and is tethered to the cell membrane by the MUC1-C transmembrane subunit. The observation that c-Abl is detectable in anti-MUC1-N precipitates suggested that c-Abl interacts with the cell membrane-associated MUC1 heterodimer. Colocalization of MUC1 and c-Abl at the cell membrane was confirmed by confocal microscopy. Moreover, cell membrane-associated c-Abl was increased by an MUC1-dependent mechanism. The results

further demonstrate that c-Abl interacts directly with the MUC1 cytoplasmic domain (MUC1-CD). The results show that c-Abl phosphorylates MUC1-CD on Tyr-60. In turn the pY⁶⁰TNP motif functions as a binding site for the c-Abl SH2 domain. Consistent with this mechanism of interaction, mutation of Tyr-60 abrogated binding of c-Abl to MUC1 *in vitro*. Moreover, stable expression of MUC1 with a Y60F mutation in cells abrogated the formation of MUC1–c-Abl complexes. These findings provide the first evidence for an interaction between the antiapoptotic MUC1 and proapoptotic c-Abl proteins.

MUC1 blocks binding of c-Abl to 14-3-3 proteins

Cytosolic 14-3-3 proteins bind to the consensus RSXpS/TXP sequence and disrupt nuclear import of binding proteins by blocking their nuclear localization signals (Muslin and Xing, 2000; Tzivion and Avruch, 2002). The c-Abl RSVT⁷³⁵LP motif conforms to the consensus 14-3-3 binding site and is phosphorylated by a presently unidentified kinase (Yoshida *et al*, 2005). In this context, c-Abl binds to 14-3-3 and mutation of Thr-735 abrogates this interaction (Yoshida *et al*, 2005). Using cells with gain and loss of MUC1 function, the present results demonstrate that MUC1 blocks phosphorylation of c-Abl on Thr-735. Importantly, phosphorylation of the c-Abl Thr-735 site was not blocked by MUC1 with the Y60F mutation, indicating that direct binding of MUC1 and c-Abl is responsible for maintaining Thr-735 in the unphosphorylated state. Consistent with these results, MUC1 blocked binding of c-Abl to 14-3-3 and the c-Abl-14-3-3 interaction was unaffected by MUC1(Y60F). These results indicate that MUC1 blocks phosphorylation of c-Abl on Thr-735 by direct binding to c-Abl. However, whereas the c-Abl Thr-735 kinase is not known, our results do not exclude the possibility that MUC1 may

Figure 7 Binding of MUC1 and c-Abl attenuates DNA damage-induced apoptosis. (A) HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) cells were treated with CDDP for 2 and 4 h. Nuclear lysates were immunoblotted with anti-c-Abl, anti-lamin B and anti-IκB α . Intensity of the signals was determined by densitometric scanning and compared to that for untreated cells. (B) Anti-MUC1-N precipitates from HCT116/MUC1 cells treated with CDDP for the indicated times were immunoblotted with anti-c-Abl and anti-MUC1-N. (C) HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) cells were treated with CDDP for indicated times. Lysates were immunoblotted with anti-PKC δ and anti- β -actin. FL: full-length. CF: cleaved fragments. (D) The results from sub-G1 DNA analysis of HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) cells treated with CDDP for 0 h (open bars), 12 h (solid bars) or 24 h (hatched bars) are expressed as the percentage apoptosis (mean $\pm$ s.d. of three separate experiments). (E) The results from sub-G1 DNA analysis of HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) cells left untreated (open bars) or treated with CDDP alone for 12 h (solid bars), STI571 alone for 12 h (shaded bars) or both CDDP and STI571 for 12 h (hatched bars) are expressed as the percentage apoptosis (mean $\pm$ s.d. of three separate experiments). (F) The results from sub-G1 DNA analysis of HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) cells transfected with the control CsiRNA or c-AblsiRNA and left untreated (open bars) or treated with CDDP for 12 h (solid bars) are expressed as the percentage of apoptosis (mean $\pm$ s.d. of three separate experiments). NS, not significant.

inhibit that kinase. JNK is activated in the response to DNA damage (Kharbanda *et al*, 1995b) and phosphorylates 14-3-3 as a mechanism for the release of Bax to mitochondria (Tsuruta *et al*, 2004). JNK phosphorylation of 14-3-3 similarly induces the release of c-Abl from 14-3-3 for targeting of c-Abl to the nucleus (Yoshida *et al*, 2005). However, our results support a model in which MUC1 subverts this mechanism for DNA damage-induced shuttling of c-Abl between the cytoplasm and nucleus by blocking binding of c-Abl to 14-3-3.

MUC1 blocks DNA damage-induced targeting of c-Abl to the nucleus and thereby the apoptotic response

Nuclear c-Abl functions in activating pro-apoptotic signals in the DNA damage response (Kharbanda *et al*, 1995b; Yuan *et al*, 1997). The Bcr-Abl fusion protein, which also contains the three NLSs, localizes to the cytoplasm (Wetzler *et al*, 1993) and confers survival (Raitano *et al*, 1997). Conversely, forced entrapment of Bcr-Abl in the nucleus induces apoptosis (Vigneri and Wang, 2001). The present studies demonstrate that MUC1 sequesters c-Abl, at least in large part, at the

cell membrane. Moreover and importantly, MUC1 blocks DNA damage-induced targeting of c-Abl to the nucleus. The results indicate that the direct binding between MUC1 and c-Abl is responsible for the block in localization of c-Abl to the nucleus. In this regard, expression of MUC1 with the Y60F mutation was ineffective in preventing DNA damage-induced nuclear targeting of c-Abl. Our results also provide support for a model in which binding of MUC1 to c-Abl blocks the apoptotic response to DNA damage. c-Abl-deficient cells are resistant to DNA damage-induced apoptosis (Yuan *et al*, 1997) and activation of nuclear c-Abl is essential for its proapoptotic function (Agami *et al*, 1999; Gong *et al*, 1999; Yuan *et al*, 1999; Yoshida *et al*, 2002). Thus, we found that the antiapoptotic effects of MUC1 are inhibited by blocking the c-Abl interaction with MUC1(Y60F). Moreover, DNA damage-induced apoptosis in the presence of MUC1(Y60F) was attenuated by inhibiting c-Abl activation with STI571 or by downregulating c-Abl expression. These findings indicate that the antiapoptotic effects of MUC1 are in large part dependent on sequestering c-Abl in the cytoplasm.

Why would MUC1 inhibit c-Abl-induced apoptosis?

MUC1 contributes to a physical barrier that protects epithelia from damage induced by diverse forms of stress that occurs at the interface with the external environment. In the epithelial stress response, damage induces the disruption of tight junctions, loss of polarity and the activation of a repair and survival program (Vermeer *et al*, 2003). The available evidence indicates that MUC1 contributes to this survival program through targeting of the MUC1-C subunit to the outer mitochondrial membrane where it blocks release of apoptogenic factors (Ren *et al*, 2004, 2006). In this context, targeting of MUC1 to mitochondria is attenuated by mutation of the Tyr-46 site (Ren *et al*, 2004, 2006). Moreover, the antiapoptotic function of MUC1 is attenuated by expression of MUC1(Y46F) (Ren *et al*, 2004, 2006). The present results indicate that MUC1 also contributes to the epithelial survival program by blocking nuclear targeting of c-Abl. Notably, MUC1(Y46F) and MUC1(Y60F) appear to inhibit the antiapoptotic function of MUC1 by different mechanisms. In contrast to MUC1(Y60F), the MUC1(Y46F) mutant binds to c-Abl and attenuates DNA damage-induced targeting of c-Abl to the nucleus. Conversely, unlike MUC1(Y46F), we found that MUC1(Y60F) localizes to mitochondria. These findings suggest that MUC1 blocks DNA damage-induced apoptosis by two mechanisms involving (i) localization to the mitochondrial outer membrane through phosphorylation of the Tyr-46 site and (ii) sequestration of c-Abl at the cell membrane through phosphorylation of the Tyr-60 site. The results suggest that both pathways in which MUC1 is phosphorylated on Tyr-46 by c-Src and on Tyr-60 by c-Abl are integral in conferring the MUC1 antiapoptotic function. Notably, c-Abl functions as a substrate and effector of c-Src in the response to growth factor stimulation (Plattner *et al*, 1999; Furstoss *et al*, 2002). As such, activation of the c-Src → c-Abl pathway may be integrated with MUC1 as a mechanism for attenuating signals that induce an apoptotic response. Thus, overexpression of MUC1 as found in diverse human tumors may confer resistance to genotoxic anticancer agents by exploiting physiologic mechanisms that evolved to protect normal epithelia from stress-induced death.

Materials and methods

Cell culture

Human HeLa cervical cancer, HCT116/vector, HCT116/MUC1, HCT116/MUC1(Y46F) colon cancer (Ren *et al*, 2002) and 293 cells were cultured in Dulbecco's modified medium with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Human ZR-75-1/vector and ZR-75-1/MUC1siRNA breast cancer cells (Ren *et al*, 2004) were grown in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum and antibiotics. Cells were treated with 1 µM adriamycin (ADR; Calbiochem), 100 µM cisplatin (CDDP; Sigma) or 10 µM STI571 (Gleevec; Novartis, Basel, Switzerland).

Cell transfections

HeLa cells were stably transfected with pCMV or pCMV-MUC1 using LipofectAMINE and selected in the presence of G418. The pIRESpuro2-MUC1(Y60F) and GFP-c-Abl(R152L) vectors were constructed by site-directed mutagenesis as described (Li *et al*, 2001a). HCT116 cells were transfected with pIRESpuro2-MUC1(Y60F) using LipofectAMINE and selected in the presence of puromycin (Calbiochem-Novabiochem Co.). 293 cells were transiently transfected with pIRESpuro2, pIRESpuro2-MUC1, pIRESpuro2-MUC1(Y60F), GFP, GFP-c-Abl, GFP-c-AblΔNES and GFP-c-Abl(R152L) using LipofectAMINE. For downregulation of c-Abl,

HCT116/vector, HCT116/MUC1 and HCT116/MUC1(Y60F) cells were seeded (5×10^5 /well) on six-well plates. After 24 h, the cells were transfected with 1 nM SMART pool c-Abl or nonspecific control pool siRNAs (Upstate Biotechnology Inc.) using LipofectAMINE. The cells were incubated for an additional 72 h and then harvested for analysis.

Subcellular fractionation

Nuclear fractions were prepared as described (Kharbanda *et al*, 1996). Cytosolic fractions were prepared as described (Hill *et al*, 2004). Cell membrane fractions were prepared with the Plasma Membrane Extraction Kit (BioVision Research Products). Mitochondria were purified as described (Ren *et al*, 2004, 2006).

Immunoprecipitations and immunoblot analysis

Cell lysates were prepared as described (Kharbanda *et al*, 1995b; Yoshida *et al*, 2005). Soluble proteins were incubated with anti-MUC1-N (Kufe *et al*, 1984), anti-MUC1-C (Ab5; Neomarker), anti-Flag (Sigma), anti-c-Abl (Ab3; Oncogene) or anti-14-3-3 (Abcam Inc.) for 2 h at 4°C, followed by precipitation with protein G-Sepharose beads for 1 h. Immune complexes and cell lysates (50 µg) were subjected to immunoblot analysis with anti-MUC1-N, anti-MUC1-C, anti-c-Abl (Calbiochem), anti-lamin B (Oncogene), anti-IκBα (Santa Cruz Biotechnology), anti-GFP (BD Biosciences), anti-Flag (Sigma), anti-β-actin (Sigma), anti-phospho-c-Abl-Thr-735 (Cell Signaling Technology), anti-14-3-3 (Santa Cruz Biotechnology), anti-PKCδ (Santa Cruz Biotechnology), anti-HSP60 (BD Biosciences), anti-PCNA (Oncogene) and anti-calnexin (Stressgen). The antigen-antibody complexes were visualized by chemiluminescence (NEN Life Science Products). Intensity of the signals was determined by densitometric scanning. Statistical significance was determined by the Student's *t*-test.

In vitro kinase assays

GST-c-Abl or GST-c-Abl(K-R) purified from baculovirus infected Sf9 cells (Yuan *et al*, 1999) were incubated in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 2 mM dithiothreitol, 0.1 mM sodium vanadate) with thrombin-cleaved and purified MUC1-CD or MUC1-CD(Y60F) and [γ -³²P]ATP (NEN Life Science Products) for 15 min at 30°C. Reaction products were analyzed by SDS-PAGE and autoradiography.

Immunofluorescence microscopy

Cells cultured on coverslips were fixed in 3.7% formaldehyde, washed with PBS, permeabilized in PBS containing 0.2% Triton X-100 and then postfixed in 3.7% formaldehyde. The cells were blocked with 10% goat serum and stained with anti-MUC1-C, followed by FITC-conjugated secondary antibody. The cells were then incubated with anti-c-Abl (24-11; Santa Cruz Biotechnology), followed by Texas Red-goat anti-mouse Ig conjugate (Jackson ImmunoResearch Laboratory). Nuclei were stained with 2 µM TO-PRO3 (Molecular Probes). Images were captured with a Zeiss LSM510 confocal microscope at 1024 × 1024 resolution.

Analysis of c-Abl kinase activity

Nuclear lysates were immunoprecipitated with anti-c-Abl (K-12) as described (Kharbanda *et al*, 1997). The precipitates were resuspended in kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂) containing [γ -³²P]ATP, GST-Crk(120-225) for 20 min at 30°C. The reaction products were analyzed by SDS-PAGE and autoradiography.

Binding studies

Purified MUC1-CD and MUC1-CD(Y60F) were incubated in the absence and presence of recombinant truncated Abl (kinase domain; New England Biolabs) and 200 µM ATP for 30 min at 30°C. GST-c-Abl SH2 (Yoshida *et al*, 2002) or GST-c-Abl SH2(R152L) bound to glutathione beads was then added for 1 h at 4°C. After washing, the precipitated proteins were subjected to immunoblot analysis.

Assessment of apoptosis

DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (Becton Dickinson). The percentage of cells with sub-G1 DNA was determined by the

MODFIT LT Program (Verity Software). Statistical, significance was determined using the unpaired Student's *t*-test and GraftPad software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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