The MUC1 Oncoprotein Activates the Anti-apoptotic Phosphoinositide 3-Kinase/Akt and Bcl-xL Pathways in Rat 3Y1 Fibroblasts*

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The MUC1 transmembrane glycoprotein is overexpressed by most human carcinomas. Overexpression of MUC1 confers transformation; however, the signaling pathways activated by this oncoprotein are largely unknown. The present studies demonstrated that MUC1-induced transformation of 3Y1 fibroblasts is associated with increased levels of phospho-Akt and phospho-Bad. The finding that LY294002 blocks MUC1-mediated increases in phospho-Akt and phospho-Bad supports the involvement of phosphoinositide 3-kinase (PI3K) as an upstream effector of this response. We also show that MUC1 increases the expression of the anti-apoptotic Bcl-x_L protein (but not Bcl-2) by a PI3K-independent mechanism. In concert with these results, MUC1 attenuated (i) the loss of mitochondrial transmembrane potential, (ii) mitochondrial cytochrome c release, (iii) activation of caspase-9, and (iv) induction of apoptosis by the antimetabolite, 1-β-D-arabinofuranosylcytosine. Similar results were obtained with the anti-cancer agent, gemcitabine. These findings indicate that expression of MUC1 in 3Y1 cells activates the anti-apoptotic PI3K/Akt and Bcl-x_L pathways.

The phosphoinositide 3-kinase (PI3K)³/Akt signaling pathway is of importance in promoting cell survival. PI3K, which consists of a p85 regulatory subunit and a p110 catalytic subunit, phosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to PI(3,4,5)P3 through its pleckstrin-homology domain (2, 3). Phosphorylation of Akt by PDK1 localizes at cell membrane sites by direct binding to PI(3,4,5)P3 through their pleckstrin-homology domains (2, 3). Phosphorylation of Akt by PDK1 stimulates Akt activity (4). In turn, Akt phosphorylates and inactivates the Forkhead-related transcription factor 1 by retaining it in the cytosol in a complex with 14-3-3 proteins. Similarly, Akt phosphorylates Bad, induces binding of Bad to 14-3-3 proteins, and prevents Bad from interacting with the anti-apoptotic Bcl-2 and Bcl-x_L proteins (5). The activity of the pro-death caspase-9 protease is inhibited by Akt-mediated phosphorylation (6). Akt can also confer cell survival by indirect regulation of NF-κB through 1kB kinase (7, 8) and of p53 through Mdm2 (9, 10). These findings, and the demonstration that PI3K/Akt signaling is dysregulated in human malignancies, have provided support for the significance of this pathway in conferring tumor cell survival (11).

The human D3/MUC1 transmembrane glycoprotein is expressed on the apical borders of normal secretory epithelial cells (12). The MUC1 protein is synthesized as a single polypeptide that is cleaved in the endoplasmic reticulum into N-terminal and C-terminal subunits, which then reside as heterodimers at the apical cell surface (13, 14). The large (>250 kDa) N-terminal ectodomain (N-ter) contains variable numbers of 20-amino acid tandem repeats that are heavily glycosylated (15, 16). The smaller (<25 kDa) C-terminal subunit (C-ter) consists of an extracellular domain of 58 amino acids, a 28-amino acid transmembrane domain, and a 72-amino acid cytoplasmic tail (17). The MUC1 C-ter forms a complex with β-catenin that localizes to the nucleus (18–22). Glycogen synthase kinase 3β (GSK3β), a substrate of Akt (23), phosphorylates the MUC1 C-ter on serine in a SPY site adjacent to that for β-catenin binding and inhibits the formation of MUC1-β-catenin complexes (24). Other studies have shown that phosphorylation of MUC1 by the epidermal growth factor receptor (25), c-Src (26), and protein kinase Cδ (PKCδ) (27) induces binding of MUC1 and β-catenin. These findings indicate that MUC1 functions by integrating signals from growth factor receptors, which also activate PI3K/Akt (1) and the Wnt pathway.

Overexpression of MUC1 is sufficient to induce the transformation of rat 3Y1 fibroblasts (20). The present studies demonstrated that expression of MUC1 in 3Y1 cells is associated with the activation of the PI3K/Akt pathway. We also showed that MUC1 up-regulates Bcl-x_L expression and attenuates the apoptotic response of 3Y1 cells to the anti-cancer agents, 1-β-D-arabinofuranosylcytosine (araC) and gemcitabine.

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† The abbreviations used are: PI3K, phosphoinositide 3-kinase; PI, phosphatidylinositol; PDK1, phosphoinositide-dependent kinase 1; GSK3β, glycogen synthase kinase 3β; PKCδ, protein kinase Cδ; TUNEL, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling; araC, 1-β-D-arabinofuranosylcytosine; PI(4,5)P2, phosphatidylinositol 4,5-diphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatidylinositol 3,4,5-triphosphate 3-phosphatase; MUC1 N-ter, MUC1 N-terminal subunit; MUC1 C-ter, MUC1 C-terminal subunit.

MATERIALS AND METHODS

Cell Culture—Rat 3Y1 fibroblasts stably expressing the empty vector or full-length MUC1 (20) were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Two independently selected clones (A and B) were used for both 3Y1/vector and 3Y1/MUC1 cells (20). Cells were treated with 25 μM LY294002 (Sigma), 10 μM araC (Sigma), or 50 μM gemcitabine ( Eli Lilly).

Immunoblot Analysis—Whole cell lysates and cytoplasmic fractions were prepared from subconfluent cells as described in Refs. 24 and 28. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-phospho-Akt (Cell Signaling,
Beverly, MA), anti-Akt (Cell Signaling), anti-phospho-Bad (Cell Signaling), anti-Bad (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PI3K p85 (Upstate Biotechnology, Lake Placid, NY), anti-PTEN (29), anti-Bcl-xL (Santa Cruz Biotechnology), anti-Bcl-2 (BD Biosciences), anti-MUC1 N-ter (DF3) (12), anti-MUC1 C-ter (Neomarkers, Fremont, CA), anti-cytochrome c (30), anti-caspase-9 (H-83; Santa Cruz Biotechnology), and anti-PKC (C-20; Santa Cruz Biotechnology).

Assessment of Mitochondrial Transmembrane Potential—Cells were incubated with 50 ng/ml Rhodamine 123 (Molecular Probes, Eugene, OR) for 15 min at 37 °C. After washing with phosphate-buffered saline, the samples were analyzed by flow cytometry using 488 nm excitation and measurement of emission through a 575/26 (ethidium) band-pass filter.

Isolation of Mitochondria—Mitochondria were purified as described in Refs. 31 and 32.

Apoptosis Assays—For TUNEL staining, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, 0.1% sodium citrate for 2 min, and processed according to the manufacturer’s instructions (In Situ Cell Death detection kit, fluorescein; Roche Applied Science). For analysis of sub-G1 DNA, the cells were washed with phosphate-buffered saline, fixed in 70% ethanol, incubated with 2.5 μg/ml propidium iodide and 50 μg/ml RNase, and then monitored by FACSscan using a Modfit program.

RESULTS

MUC1 Activates the PI3K/Akt Signaling Pathway—To determine whether MUC1 affects the PI3K/Akt pathway, lysates from 3Y1 cells stably expressing the empty vector or MUC1 were subjected to immunoblotting with anti-phospho-Akt. The levels of phospho-Akt were substantially increased in 3Y1/MUC1 cells compared with that in 3Y1/vector cells (Fig. 1A). Similar results were obtained in separately isolated 3Y1/vector and 3Y1/MUC1 clones (Fig. 1A). The demonstration that MUC1 has little if any effect on Akt protein levels indicated that Akt is activated in the 3Y1/MUC1 cells (Fig. 1A). Akt phosphorylates and inactivates the pro-apoptotic function of Bad (5). Immunoblot analysis of the lysates with an anti-phospho-Bad antibody demonstrated that MUC1 markedly increases levels of phosphorylated Bad (Fig. 1B). As a control, MUC1 had little effect on Bad protein levels (Fig. 1B). Akt is regulated in part by the PI3K-mediated formation of PI(3,4,5)P3 and degradation of this PI by the PTEN phosphatase (33). To determine whether MUC1 affects the expression of PI3K, the lysates were probed with an anti-PI3K p85 antibody. MUC1 had no detectable effect on PI3K p85 expression (Fig. 1C). Moreover, PTEN levels were similar in the 3Y1/vector and 3Y1/MUC1 cells (Fig. 1C). Significantly, however, treatment of the 3Y1/MUC1 cells with the PI3K inhibitor LY294002 was associated with down-regulation of both phospho-Akt and phospho-Bad levels (Fig. 1D). These findings indicate that MUC1 activates the PI3K/Akt pathway.

MUC1 Up-regulates Bcl-xL Expression—Bad promotes apoptosis by forming a heterodimer with Bcl-xL (5). To determine whether MUC1 also affects Bcl-xL expression, the lysates were analyzed by immunoblotting with anti-Bcl-xL. The levels of Bcl-xL were increased in 3Y1/MUC1 cells as contrasted to 3Y1/vector cells (Fig. 2A). The demonstration that MUC1 has little
lysate (WCL) blotting with anti-Bcl-xL, anti-MUC1 N-ter, and anti-HSP60. Whole cell anti-MUC1 N-ter. Cell lysates were analyzed by immunoblotting with anti-Bcl-xL, anti-Bcl-2, and anti-Bad (37, 38). In this context, Bcl-xL levels were increased in the mitochondria of 3Y1/MUC1 as contrasted to 3Y1/vector cells (Fig. 2C). As a control, there was no detectable MUC1 N-ter in the 3Y1 mitochondrial lysates (Fig. 2C). Immunoblot analysis of the mitochondrial HSP60 protein was also included as a control for equal loading of the lanes (Fig. 2C). These results indicate that MUC1 increases mitochondrial Bcl-xL levels by a PI3K-independent mechanism.

MUC1 Activates Akt and Bcl-xL Signaling—Increases in phospho-Bad and Bcl-xL protect against apoptosis induced by activation of the intrinsic mitochondrial pathway (38, 39). To assess the effects of MUC1 on chemosensitivity, we treated 3Y1 cells with araC, an antimetabolite that induces the release of mitochondrial cytochrome c and thereby apoptosis (40, 41). Exposure of 3Y1/vector-A and -B cells to araC was associated with a decrease in mitochondrial transmembrane potential (Δψm) (Fig. 3A). By contrast, there was no decrease in Δψm when 3Y1/MUC1 cells were exposed to araC (Fig. 3A). Indeed, as shown previously (38), overexpression of Bcl-xL confers an increase in Δψm in response to araC-induced genotoxic stress (Fig. 3A). araC treatment is also associated with the release of cytochrome c into the cytosol of 3Y1/vector (and not 3Y1/MUC1) cells (Fig. 3B). In concert with these results, activation of caspase-9 was attenuated in 3Y1/MUC1 (as contrasted to 3Y1/vector) cells (Fig. 3B). Moreover, caspase-3-mediated cleavage of PKCδ (42) was attenuated in the MUC1 expressing cells (Fig. 3C). Similar results were obtained in the separately isolated 3Y1/vector-B and 3Y1/MUC1-B cells (data not shown). These findings indicate that MUC1 attenuates araC-induced activation of the intrinsic apoptotic pathway.

MUC1 Activates the PI3K/Akt Pathway—To determine whether MUC1 affects induction of apoptosis by araC, the 3Y1/vector and 3Y1/MUC1 cells were analyzed for TUNEL staining and sub-G1 DNA. Treatment with araC was associated with a substantial induction of TUNEL staining in 3Y1/vector (but not 3Y1/MUC1) cells (Fig. 4A). Moreover, araC treatment of 3Y1/vector cells was associated with over 40% apoptosis as determined by sub-G1 DNA analysis (Fig. 4B). By contrast, araC-induced apoptosis was significantly attenuated in the 3Y1/MUC1 cells (Fig. 4B). Similar results were obtained in multiple experiments with the 3Y1/MUC1-A cells (Fig. 4C) and in the other separately isolated 3Y1/MUC1-B clone (Fig. 4D).

To extend these observations to other anti-cancer agents, we treated the 3Y1/vector and 3Y1/MUC1 cells with gemcitabine. As determined by the analysis of sub-G1 DNA and as found with araC, gemcitabine-induced apoptosis was attenuated in 3Y1/MUC1 (as contrasted to 3Y1/vector) cells (data not shown). These findings demonstrated that MUC1 attenuates the induction of 3Y1 cells apoptosis by araC and gemcitabine.

**DISCUSSION**

*MUC1 Activates the PI3K/Akt Pathway*—PI3K is aberrantly activated in diverse human carcinomas (1, 11, 36). Interaction of the PI3K p85 subunit with phosphoryrosine residues of activated growth factor receptors or adaptor proteins results in stimulation of the PI3K p110 catalytic subunit. Growth factor-
induced binding of Ras to p110 also stimulates PI3K activity and the formation of PI(3,4,5)P3. Direct binding of PI3K p110 to mutant forms of activated Ras can thus contribute to dysregulation of PI3K activity. Loss of the PTEN phosphatase, which dephosphorylates PI(3,4,5)P3, represents another mechanism for aberrant activation of PI3K and, similar to mutant Ras, has been widely found in human cancers (1, 11). Akt and other pleckstrin homology domain-containing proteins accumulate at sites of PI3K activation by binding to PI(3,4,5)P3. Importantly, Akt-mediated phosphorylation and inactivation of a number of downstream effectors, such as Bad, can contribute to the malignant phenotype (1, 11).

The present results demonstrated that overexpression of the MUC1 oncoprotein in 3Y1 fibroblasts is associated with activation of the PI3K/Akt pathway. MUC1 is overexpressed by most human carcinomas of the breast and other epithelia (12). Importantly, overexpression of MUC1 confers anchorage-independent growth and tumorigenicity (19, 43). MUC1 functions as a substrate for c-Src, Lyn, and PKCα/h254 (19, 26, 27) but has not been shown previously to be responsible for the activation of specific kinases. Indeed, although the precise mechanisms responsible for the transforming effects of MUC1 remain unknown, the demonstration that MUC1 activates PI3K/Akt signaling provides new insights into how MUC1 may contribute to the malignant phenotype. The available evidence indicates that MUC1 functions as a coreceptor for the ErbB family of growth factor receptors (21, 25, 44). Overexpression of MUC1 could therefore aberrantly stimulate ErbB receptor-mediated activation of PI3K and Akt. Moreover, the MUC1 cytoplasmic tail contains a YHPM motif, which after tyrosine phosphorylation

![Figure 3](http://www.jbc.org/)

**Fig. 3.** MUC1 blocks activation of the intrinsic apoptotic pathway. A, the indicated cells were left untreated (Control) or treated with araC for 24 h, stained with Rhodamine 123, and analyzed by flow cytometry. B, 3Y1/vector and 3Y1/MUC1 cells were treated with araC for the indicated times. Cytosolic lysates were analyzed by immunoblotting (IB) with anti-cytochrome c, anti-caspase-9, and anti-β-actin. C, lysates from 3Y1/vector and 3Y1/MUC1 cells treated with araC for the indicated times were analyzed by immunoblotting with anti-PKCα and anti-β-actin. FL, full-length; CF, catalytic fragment. The asterisk denotes nonspecific bands.
represents a potential binding site for the Src homology 2 domain of the PI3K p85 subunit. As such, MUC1 might also function as a cell membrane docking site for PI3K activation.

**MUC1 Activates the Anti-apoptotic Bcl-xL Pathway**—The present work also demonstrated that MUC1 activates the Bcl-xL pathway in 3Y1 cells. Bcl-xL was identified as a Bcl-2-related protein that functions as a regulator of cell death (37). Like Bcl-2, Bcl-xL prevents the release of cytochrome c from mitochondria by maintaining the integrity of the outer membrane (38, 45–47). In the setting of growth factor withdrawal and decreases in metabolic rate, Bcl-xL also blocks activation of the intrinsic apoptotic pathway in the response of cells to genotoxic stress (39, 47, 49). Importantly, tumor cells exhibiting a multidrug-resistant phenotype overexpress Bcl-xL and not Bcl-2 (39, 49). These findings have suggested that Bcl-xL is selectively up-regulated to protect cells against genotoxic and other forms of stress. In this context, overexpression of Bcl-xL overrides Bad-mediated inactivation of Bcl-xL and thereby the induction of apoptosis (5).

In certain settings, up-regulation of Bcl-xL occurs in response to activation of the PI3K/Akt pathway (34, 35). The finding, however, that exposure of the 3Y1/MUC1 cells to LY294002 has little if any effect on Bcl-xL expression indicates that MUC1 up-regulates Bcl-xL by a PI3K-independent mechanism. The demonstration that MUC1 N-ter is not detectable in mitochon-

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**Fig. 4.** MUC1 attenuates araC-induced apoptosis. A and B, 3Y1/vector-A and 3Y1/MUC1-A cells were treated with araC for 24 h and analyzed for TUNEL staining (A, left panels) by bright field (A, right panels), and for sub-G1 DNA (B). C, the results from sub-G1 DNA analysis of 3Y1/vector-A (solid bars) and 3Y1/MUC1-A (open bars) cells treated with araC for the indicated times are expressed as the percentage apoptosis (mean ± S.D. of three separate experiments). D, 3Y1/vector-B (solid bars) and 3Y1/MUC1-B (open bars) cells were treated with araC for the indicated times and analyzed for sub-G1 DNA. The results are presented as the percentage apoptosis (mean ± S.D. of three separate experiments).
Bcl-xL pathways. Activation of these anti-apoptotic signals may cell context. These findings further indicate that overexpression of MUC1 C-ter to mitochondrial Bcl-xL is not mediated by a direct interaction with full-length MUC1. In concert with MUC1-mediated activation of both PI3K/Akt and Bcl-xL pathways and provide, at least in part, a mechanism for an anti-apoptotic phenotype of MUC1-expressing cells. The present results did not exclude the possibility that MUC1 attenuates apoptosis by other mechanisms. In this regard, MUC1 signaling is activated by cytokines in myeloma cells (19) and by ErbB receptors in carcinoma cells (21, 25). In HCT116 carcinoma cells, enforced expression of MUC1 has no detectable effect on the PI3K/Akt or Bcl-xL pathways (50). Moreover, in carcinoma cells, localization of MUC1 C-ter to mitochondria is associated with attenuation of the apoptotic response to diverse types of stress (50, 51). Thus, MUC1 can block apoptosis by different mechanisms that are dependent on cell context. These findings further indicate that overexpression of MUC1 in certain human tumors could confer resistance to anti-cancer agents by the activation of the PI3K/Akt and Bcl-xL pathways. Activation of these anti-apoptotic signals may also contribute to MUC1-induced transformation (20). However, the present data did not exclude the possibility that MUC1 activates other events that are responsible for induction of a malignant phenotype.

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