Mucin 1 Oncoprotein Blocks Hypoxia-inducible Factor 1α Activation in a Survival Response to Hypoxia*

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Resistance of carcinoma cells to hypoxic stress is of importance to the growth of solid tumors. The mucin 1 (MUC1) oncoprotein is aberrantly overexpressed by most human carcinomas; however, there is no known relationship between MUC1 and the hypoxic stress response. The present work has demonstrated that MUC1 attenuates activation of hypoxia-inducible factor-1α (HIF-1α), a regulator of gene transcription in the response of cells to hypoxic stress. In cells with stable gain and loss of MUC1 function, we have shown that MUC1 up-regulates prolyl hydroxylase 3 (PHD3) expression and promotes HIF-1α degradation. PHD activity is attenuated by increases in reactive oxygen species (ROS) generated in the hypoxic stress response. Our results further demonstrate that MUC1 blocks hypoxia-induced increases in ROS and thereby potentiates PHD-mediated HIF-1α suppression. Importantly, MUC1 also blocks hypoxia-induced apoptosis and necrosis by suppressing accumulation of ROS. These findings indicate that MUC1 attenuates HIF-1α activation in a survival response to hypoxic stress.

Solid tumors outgrow their blood supply, resulting in regions with decreased oxygen tension (1, 2). Cancer cells, however, adapt to survive and proliferate in hypoxic environments (3). The hypoxia-inducible factor 1 (HIF-1)2 mediates adaptive changes in the response to hypoxia by regulating gene transcription. HIF-1 is a heterodimer of the tightly regulated HIF-1α subunit and the constitutively expressed HIF-1β subunit. The stability of HIF-1α is regulated through oxygen-dependent trans-4-hydroxylation of prolines by prolyl hydroxylase domain containing proteins PHD1, PHD2, and PHD3 (4–6). Hydroxylation of HIF-1α on Pro-402 and Pro-564 in the oxygen-dependent degradation domain promotes binding of the von Hippel-Lindau protein and the formation of an E3 ubiquitin ligase complex that targets HIF-1α for proteosomal degradation (7). Conversely, β-hydroxylation of Asn-803 attenuates the HIF-1α transactivation function by decreasing association with the CREB-binding protein/p300 coactivators (8). PHD activity is also dependent on ferrous iron (FeII) and is decreased by accumulation of ROS, which converts FeII to FeIII (9). The inhibition of PHD activity under hypoxic conditions or by hypoxia-induced ROS thus results in the stabilization of HIF-1α and activation of HIF-1 target genes. HIF-1 induces the expression of diverse genes that play adaptive roles to hypoxia by increasing angiogenesis, invasion, and resistance to apoptosis (7). Under more severe or prolonged hypoxic conditions, HIF-1 contributes to an apoptotic or necrotic response by stabilization of p53 (10) and induction of the pro-death Bcl-2 family members BNIP3 and NIX (11).

Mucin 1 (MUC1) is a heterodimeric mucin that is expressed on the apical borders of normal secretory epithelial cells (12). MUC1 is translated as a single polypeptide that undergoes autocleavage into two subunits (13–15). The >250-kDa MUC1 N-terminal ectodomain consists of variable numbers of heavily glycosylated tandem repeats that extend beyond the glycoplyxl (16, 17). The MUC1 N-terminal subunit is tethered at the cell membrane to the C-terminal subunit (MUC1-C) that consists of a 58-amino-acid extracellular domain, a 28-amino-acid transmembrane domain and a 72-amino-acid cytoplasmic tail (18). With transformation and loss of polarity, MUC1 is expressed at high levels over the entire carcinoma cell surface (12). MUC1 associates with members of the ErbB family of receptor tyrosine kinases (19–21) and integrates ErbB signaling with the Wnt pathway through direct interactions with β-catenin (22–25). Phosphorylation of the MUC1 cytoplasmic domain by glycogen synthase kinase 3β, c-Src, and protein kinase Cδ regulates binding of MUC1 and β-catenin (23, 24, 26). Other studies have demonstrated that MUC1-C accumulates in the cytosol of transformed cells and is targeted to the nucleus (21, 25, 27–30) and mitochondria (31, 32). Importantly, overexpression of MUC1 is sufficient to induce anchorage-independent growth and tumorigenicity (25, 27, 33, 34). Overexpression of MUC1 also suppresses H2O2-induced increases in ROS levels and confers resistance to the induction of apoptosis by oxidative stress (35, 36).

The findings that HIF-1α is stabilized by ROS (9, 37) and that MUC1 blocks accumulation of ROS (35, 36) prompted us to investigate whether MUC1 regulates the HIF-1α pathway. The results demonstrate that MUC1 attenuates hypoxia-induced activation of HIF-1α by up-regulating PHD3 and suppressing increases in ROS. The results also demonstrate that MUC1-dependent suppression of ROS blocks hypoxia-induced apoptosis and necrosis.

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The abbreviations used are: HIF-1α, hypoxia-inducible factor 1α; PHD, prolyl hydroxylase; MUC1-C, MUC1 C-terminal subunit; MUC1-CD, MUC1 cytoplasmic domain; siRNA, small interfering RNA; CsiRNA, control siRNA; MUC1siRNA, MUC1-specific siRNA; ROS, reactive oxygen species; E3, ubiquitin-protein isopeptide ligase; CREB, cAMP-response element-binding protein; RT, reverse transcription; DiOC6(3), 3,3′,3′,3′-dihexyloxacarbocyanine iodide; DCFH-DA, 5-(and -6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA).
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FIGURE 1. MUC1 attenuates HIF-1α activation in HCT116 cells. A–C, the indicated HCT116/vector, HCT116/MUC1, HCT116/MUC1-CD, and HCT116/MUC1(Y46F) cells were cultured under hypoxic (H) conditions (1% O2) for the indicated times or 24 h in C. Lysates were immunoblotted ( IB) with anti-HIF-1α, anti-MUC1-C, and anti-β-actin. D, the indicated HCT116 cells were exposed to hypoxia in the absence (−) and presence (+) of 25 μM MG132 for 8 h. Lysates were immunoblotted with anti-HIF-1α and anti-β-actin. N, normoxic.

MATERIALS AND METHODS

Cell Culture—Human HCT116/vector, HCT116/MUC1, HCT116/MUC1-CD, and HCT116/MUC1(Y46F) colon cancer cell clones (24, 31) were grown in Dulbecco’s modified Eagle’s medium (high glucose; Cellgro, Inc., Herndon, VA) supplemented 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 cell clones expressing an empty vector, a control siRNA, or a MUC1siRNA that targets the sequence 5'- AGTTCAGTGCCCAGCTCTAC-3' (31, 36) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum with antibiotics and L-glutamine. Cells were seeded, grown for 24 h, and then placed in a modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA) flushed with a gas mixture containing 1% O2, 5% CO2, and the balance N2. Cells were also treated with 25 μM MG132 (Calbiochem), 600 μM CoCl2 (Sigma-Aldrich), or 500 units/ml catalase (Sigma).

Immunoblot Analysis—Cells were lysed as described previously (35) and analyzed by immunoblotting with anti-HIF-1α (BD Biosciences), anti-PHD1, anti-PHD3 (Bethyl Laboratories, Montgomery, TX), anti-PHD2 (Novus Biologicals, Littleton, CO), anti-β-actin (Sigma), and anti-MUC1-C (Ab5; NeoMarkers Inc., Fremont, CA). Antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

Revertase Transcription (RT)-PCR—Total cellular RNA was extracted in TRizol dissolved in RNase-free water and incubated for 10 min at 55°C. HIF-1α (5'-CTCAGAAGTCGCCATCGGACAGCTCA-3' and 5'-CCCTGCAGT-AGGTCTGC-3'), and PHD3 (5'-TGACACATTTCTCCAGTGTT-C-3' and 5'-GCCCTGCAGT-AGGTCTGC-3')-specific primers were designed to amplify a 460-bp fragment. Primers for β-actin were used as a control (38). The RNA was reverse-transcribed and amplified using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen). Amplified fragments were analyzed by electrophoresis in 2% agarose gels.

Measurement of ROS Levels—Cells were incubated with 5 μM DCFH-DA (Molecular Probes) for 20 min at 37°C to assess H2O2-mediated oxidation to the fluorescent compound 2',7'-dichlorofluorescin. Fluorescence of oxidized 2',7'-dichlorofluorescin was measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm by flow cytometry (BD Biosciences).

Silencing of MUC1 and PHD3—Cells were seeded at 3 × 104 cells/60-mm well. After 24 h, the cells were transfected with control siRNA, MUC1siRNA, or PHD3siRNA pools (siGENOME SMART pool reagents; Dharmacon RNA Technologies) for 72 h.

Results

MUC1 Attenuates Activation of HIF-1α in the Response of HCT116 Cells to Hypoxia—HCT116 colon cancer cells are null for MUC1 expression (31). To determine whether MUC1 affects the response to hypoxia, we analyzed HIF-1α levels in HCT116 cells expressing an empty vector or exogenous MUC1. Exposure of HCT116/vector cells to hypoxic conditions was associated with increases in HIF-1α that were maximal at 6 h and remained elevated for 24 h (Fig. 1A). By contrast, hypoxia-induced increases in HIF-1α were attenuated in HCT116 cells expressing MUC1 (Fig. 1B).

Assays of Apoptosis and Necrosis—Sub-G1 DNA content was assessed by staining ethanol-fixed and citrate buffer-permeabilized cells with propidium iodide and monitoring by flow cytometry (BD Biosciences). Sub-G1 DNA content was assessed by staining ethanol-fixed and citrate buffer-permeabilized cells with propidium iodide and monitoring by flow cytometry (BD Biosciences). For assessment of necrosis, cells were incubated in 1 μg/ml propidium iodide/phosphate-buffered saline for 5 min at room temperature and then monitored by flow cytometry as described previously (36).

RESULTS

MUC1 Attenuates Activation of HIF-1α in the Response of HCT116 Cells to Hypoxia—HCT116 colon cancer cells are null for MUC1 expression (31). To determine whether MUC1 affects the response to hypoxia, we analyzed HIF-1α levels in HCT116 cells expressing an empty vector or exogenous MUC1. Exposure of HCT116/vector cells to hypoxic conditions was associated with increases in HIF-1α that were maximal at 6 h and remained elevated for 24 h (Fig. 1A). By contrast, hypoxia-induced increases in HIF-1α were attenuated in HCT116 cells expressing MUC1 (Fig. 1B).
that stably overexpress MUC1 (Fig. 1A). Similar results were obtained with separately isolated HCT116/vector and HCT116/MUC1 clones (supplemental Fig. S1A), indicating that clonal selection is not responsible for the attenuation of HIF-1α activation. To define the region of MUC1 that regulates HIF-1α levels, we performed studies on HCT116 cells stably overexpressing the MUC1 cytoplasmic domain (MUC1-CD) (33). Exposure of separately isolated HCT116/MUC1-CD clones to hypoxic conditions demonstrated that MUC1-CD is sufficient to attenuate HIF-1α activation (Fig. 1B and supplemental Fig. S1B). Members of the c-Src family of non-receptor tyrosine kinases are activated by ROS (39) and phosphorylate the MUC1 cytoplasmic domain on Tyr-46 (24, 28). In this regard, stable overexpression of MUC1 with a mutation at Tyr-46 (Y46F) in the cytoplasmic domain (31) reversed (although not completely) the suppressive effects of MUC1 on HIF-1α activation (Fig. 1C). HIF-1α is destabilized under normoxic conditions by O2-dependent proteosomal degradation (40–42). To define the level at which MUC1 regulates HIF-1α expression, RT-PCR was performed to assess HIF-1α gene transcription. MUC1 overexpression was associated with little effect on HIF-1α mRNA levels under normoxic and hypoxic conditions (supplemental Fig. S1C), indicating that MUC1 disrupts HIF-1α stabilization in the response to hypoxia. In concert with these results, attenuation of HIF-1α degradation with the proteosome inhibitor MG132 was associated with hypoxia-induced HIF-1α levels in HCT116/MUC1 and HCT116/MUC1-CD cells that were comparable with those in HCT116/vector cells (Fig. 1D). These findings indicate that MUC1 blocks hypoxia-induced stabilization of HIF-1α in HCT116 cells.

**Silencing Endogenous MUC1 Increases HIF-1α Activation in the Hypoxic Stress Response**—To assess the effects of silencing endogenous MUC1 on HIF-1α, we studied ZR-75-1 cells expressing an empty vector, a control siRNA (CsiRNA) or a MUC1siRNA. As shown previously (31), the MUC1siRNA stably down-regulates endogenous MUC1 expression (Fig. 2A). Silencing MUC1 in ZR-75-1 cells was associated with substantially higher HIF-1α levels in response to hypoxia (Fig. 2A). The effects of down-regulating MUC1 were similar in two separately isolated ZR-75-1/MUC1siRNA clones (Fig. 2B). RT-PCR demonstrated that silencing MUC1 is associated with increases in HIF-1α mRNA levels under normoxic and hypoxic conditions (Fig. 2C). MG132 treatment of hypoxic ZR-75-1/CsiRNA and ZR-75-1/vector cells that overexpress endogenous MUC1 was associated with an increase in HIF-1α levels (Fig. 2D). However, the effects of MG132 were substantially more pronounced in the ZR-75-1/MUC1siRNA cells (Fig. 2D). These findings indicate that MUC1 attenuates HIF-1α gene transcription and hypoxia-induced stabilization of HIF-1α in ZR-75-1 cells. Whereas MUC1 conferred destabilization of HIF-1α in both HCT116 and ZR-75-1 cells, our subsequent studies focused on signals responsible for HIF-1α degradation.

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**FIGURE 2. Silencing MUC1 in ZR-75-1 cells increases HIF-1α activation.** A, ZR-75-1/vector and ZR-75-1/MUC1siRNA cells were exposed to hypoxia for the indicated times. Lysates were immunoblotted with anti-HIF-1α, anti-MUC1-C, and anti-β-actin. B and C, the indicated ZR-75-1 cells were cultured under normoxic (N) or hypoxic (H) conditions for 24 h. Lysates were immunoblotted with the indicated antibodies (B). Total cellular RNA was amplified with HIF-1α- and β-actin-specific primers (C). The intensities of the signals were determined by densitometric scanning and are expressed as the relative signal intensity (RSI) compared with that obtained with normoxic ZR-75-1/CsiRNA cells. D, the indicated ZR-75-1 cells were exposed to hypoxia in the absence (−) and presence (+) of 25 μM MG132 for 8 h. Lysates were immunoblotted with anti-HIF-1α and anti-β-actin.
MUC1 Regulates PHD Expression in HCT116 Cells—The constitutive degradation of HIF-1α under normoxic conditions is dependent on the abundance of PHD1, PHD2, and PHD3 (5, 6, 43, 44). In this context, down-regulation of PHDs during hypoxia is important for stabilization of HIF-1α (5, 40, 41, 45). Immunoblot analysis of HCT116 cell lysates demonstrated that MUC1 overexpression has little if any effect on levels of the 43-kDa PHD1 protein (Fig. 3A). However, MUC1 overexpression was associated with down-regulation of the 46-kDa PHD2 (Fig. 3A). MUC1 overexpression was also associated with increases in levels of the 27-kDa PHD3 protein (Fig. 3A). In addition, hypoxia had little effect on the MUC1-induced increases in PHD3 (Fig. 3A and supplemental Fig. S2A). Similar results were obtained with the separately isolated HCT116/vector and HCT116/MUC1 cells (supplemental Fig. S2A). Increases in PHD3 levels were also found in HCT116 cells overexpressing MUC1-CD (Fig. 3B and supplemental Fig. S2B). By contrast, there was no apparent effect on PHD3 expression in the HCT116/MUC1(Y46F) cells (Fig. 3C). To determine whether MUC1 attenuates HIF-1α activation by up-regulating PHD3, we treated the HCT116 cells with CoCl₂, an inhibitor of PHD activity (46). The results show that CoCl₂-induced HIF-1α activation is similar in the absence and presence of MUC1 or MUC1-CD (Fig. 3D). To further assess whether PHD3 is involved in the regulation of HIF-1α in HCT116/MUC1 cells, we transiently down-regulated PHD3 expression with a PHD3 siRNA pool (Fig. 3E). Silencing PHD3 was associated with increases in HIF-1α (Fig. 3E). These findings indicate that MUC1 up-regulates PHD3 and thereby decreases HIF-1α in HCT116 cells.

Silencing MUC1 in ZR-75-1 Cells Decreases PHD3 Expression—Analysis of PHD levels in ZR-75-1 cells demonstrated that silencing of MUC1 has little effect on PHD1 or PHD2 levels (Fig. 4A). By contrast, silencing MUC1 was associated with a substantial down-regulation of PHD3 (Fig. 4A). A role for MUC1 in up-regulating PHD3 expression was confirmed in the ZR-75-1/CsiRNA cells and both ZR-75-1/MUC1siRNA clones (Fig. 4B). As a control, silencing MUC1 with a MUC1 siRNA pool was also associated with decreases in PHD3 and increases in HIF-1α levels (Fig. 4C), indicating that the results observed are not due to off-target effects of the MUC1siRNA. Analysis of PHD3 mRNA levels by RT-PCR further indicated that the down-regulation of PHD3 protein associated with silencing MUC1 is conferred by a post-transcriptional mechanism (supplemental Fig. S3). MUC1-mediated attenuation of HIF-1α activation was reversed in large part by CoCl₂ (Fig. 4D), consistent with regulation by a PHD-dependent mech-

![FIGURE 3. MUC1 up-regulates PHD3 expression in HCT116 cells. A and B, HCT116/vector, HCT116/MUC1, and HCT116/MUC1-CD cells were cultured under hypoxic conditions for the indicated times. Lysates were immunoblotted (IB) with antibodies against PHD1–3. NS, nonspecific band. C, the indicated HCT116 cells were cultured under normoxic (N) or hypoxic (H) conditions for 24 h. Lysates were immunoblotted with the indicated antibodies. D, the indicated HCT116 cells were incubated in the absence (−) and presence (+) of 600 μM CoCl₂ for 24 h. Lysates were immunoblotted with the indicated antibodies. E, HCT116/MUC1 cells were transfected with control CsiRNA or PHD3 siRNA pools. After 72 h, the cells were cultured under hypoxic conditions for 6 h. Lysates were immunoblotted with the indicated antibodies.](image-url)
anism. Moreover, silencing PHD3 was associated with increases in HIF-1α levels (Fig. 4E). These findings in ZR-75-1 cells indicate that MUC1 increases PHD3 levels and that this response contributes to the attenuation of hypoxia-induced HIF-1α activation.

**MUC1 Suppresses Hypoxia-induced Increases in ROS**—ROS stabilize HIF-1α by inhibiting PHD activity (9, 37). Because hypoxia increases ROS and MUC1 suppresses ROS (35, 36), we asked whether MUC1 affects ROS levels in the response to hypoxia. Hypoxia was associated with significant increases in ROS levels in HCT116/vector cells (Fig. 5A). By contrast, hypoxia-induced increases in ROS were suppressed in HCT116/MUC1 and HCT116/MUC1-CD cells (Fig. 5A and data not shown). Moreover, in concert with ROS-induced suppression of PHD activity, culture of HCT116/vector cells in the presence of the H2O2 scavenger catalase attenuated hypoxia-induced HIF-1α activation (Fig. 5B). In experiments with ZR-75-1/vector and ZR-75-1/CsiRNA cells that overexpress endogenous MUC1, hypoxia treatment was associated with increases in ROS (Fig. 5C). However, hypoxia-induced increases in ROS were substantially higher in ZR-75-1 cells with MUC1 silencing (Fig. 5C). In addition, catalase treatment of the ZR-75-1/MUC1siRNA cells attenuated activation of HIF-1α in the response to hypoxia (Fig. 5D). These findings indicate that MUC1 suppresses increases in ROS and HIF-1α activation in the response of cells to hypoxia.

**MUC1 Attenuates the Apoptotic Response of HCT116 Cells to Hypoxia**—Hypoxic stress is associated with loss of mitochondrial transmembrane potential (Δψm) and cell death. To determine whether MUC1 regulates the Δψm in response to hypoxia, HCT116/vector and HCT116/MUC1 cells were assayed for uptake of the Δψm-sensitive dye DiOC6(3). Staining of HCT116/vector cells with DiOC6(3) demonstrated two populations with low (28%) and high levels of fluorescence (Fig. 6A). Exposure of the HCT116/vector cells to hypoxia was associated with an increase in the low fluorescence population to 85% (Fig. 6A). By contrast, DiOC6(3) fluorescence was low in only 3–4%
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FIGURE 5. MUC1 attenuates hypoxia-induced increases in ROS and suppresses HIF-1α activation. A, the indicated HCT116 cells were cultured under normoxic (solid bars) or hypoxic (open bars) conditions for 48 h, incubated with DCFH-DA, and then analyzed by flow cytometry. The results (mean ± S.D. of three separate experiments) are expressed as ROS levels under hypoxic conditions relative to that under normoxic conditions (assigned a value of 1). B, the indicated HCT116 cells were cultured under hypoxic conditions for 6 h in the absence (−) and presence (+) of catalase. Lysates were immunoblotted with anti-HIF-1α and anti-β-actin. C, the indicated ZR-75-1 cells were cultured under normoxic (solid bars) and hypoxic (open bars) conditions for 72 h, incubated with DCFH-DA, and then analyzed by flow cytometry. The results (mean ± S.D. of three separate experiments) are expressed as ROS levels under hypoxic conditions relative to that under normoxic conditions (assigned a value of 1). D, the indicated ZR-75-1 cells were cultured under hypoxic conditions for 6 h in the absence (−) and presence (+) of catalase. Lysates were immunoblotted with anti-HIF-1α and anti-β-actin.

of HCT116/MUC1 cells under both normoxic and hypoxic conditions (Fig. 6A). Uptake of DiOOC6(3) was substantially lower in HCT116 cells expressing MUC1-CD under hypoxia as compared with that in HCT116/vector cells (Fig. 6A). In addition, expression of MUC1 with the Y46F mutation was associated with low fluorescence in 43 and 81% of cells under normoxic and hypoxic conditions, respectively (Fig. 6A). These results were confirmed in repetitive experiments and in the separately isolated cell clones (Fig. 6B). To determine whether MUC1 protects against hypoxia-induced apoptosis, the HCT116 cells were analyzed for sub-G1 DNA content. Under hypoxic conditions, 35% of HCT116/vector cells exhibited sub-G1 DNA (Fig. 6C). By contrast, the apoptotic response to hypoxia was substantially attenuated in the HCT116/MUC1 and HCT116/MUC1-CD cells (Fig. 6C). Notably, MUC1(Y46F) was ineffective in protecting against hypoxia-induced apoptosis (Fig. 6C). Similar results were obtained in repetitive experiments and in the separately isolated cell clones (Fig. 6D). The results also demonstrate that catalase suppresses hypoxia-induced apoptosis of the MUC1-null HCT116 cells (Fig. 6E). These findings indicate that MUC1 protects against loss of Δψm and induction of apoptosis in response to hypoxia and that these effects are mediated in part by suppressing hypoxia-induced disruption of redox balance.

MUC1 Silencing Increases Sensitivity to Hypoxia-induced Cell Death—To assess the effects of knocking down MUC1, ZR-75-1 cells were exposed to hypoxic conditions and monitored for uptake of DiOC6(3). Unlike HCT116 cells, there was little effect of hypoxia on Δψm in ZR-75-1 cells positive or negative for MUC1 expression (data not shown). However, previous work has shown that hypoxia-induced cell death of some transformed cells is blocked by buffer systems in the culture medium (47). In this regard, ZR-75-1/MUCsiRNA (but not ZR-75-1/CsiRNA or ZR-75-1/vector) cells grown in medium without bicarbonate responded to hypoxia with decreases in Δψm (Fig. 7A and B). There was no apparent increase in sub-G1 DNA in association with the loss of Δψm (data not shown), indicating that ZR-75-1 cells positive or negative for MUC1 are null for an apoptotic response to hypoxia. Necrosis is also associated with decreases in Δψm and is distinguished from apoptosis by an early loss of plasma membrane integrity (48, 49). To determine whether ZR-75-1 cells exhibit a necrotic response, uptake of propidium iodide was used to assess cell membrane integrity. Flow cytometric analysis of hypoxia-treated ZR-75-1/CsiRNA and ZR-75-1/vector cells demonstrated little if any necrotic response (Fig. 7C). By contrast, propidium iodide staining was substantially increased by hypoxic exposure of ZR-75-1/MUCsiRNA cells (Fig. 7C and D). The results also demonstrate that catalase suppresses hypoxia-induced necrosis of MUC1-silenced ZR-75-1 cells (Fig. 7E). These findings indicate that MUC1 protects ZR-75-1 cells from hypoxia-induced loss of Δψm and necrosis and that the necrotic response is mediated by disruption of redox balance.

DISCUSSION

MUC1 Attenuates HIF-1α Activation by Hypoxia—HIF-1α is specifically activated by hypoxia, a response that results in the dimerization of HIF-1α and HIF-1β to form the HIF-1 transcription factor. The broad array of genes activated by HIF-1 vary as a function of cell context and can dictate survival or death depending on the extent of hypoxia (7). Our results from HCT116 cells expressing exogenous MUC1 indicate that MUC1 attenuates the activation of HIF-1α by hypoxia. The demonstration that HIF-1α activation is also attenuated in HCT116/MUC1-CD cells indicates that the MUC1 cytoplasmic domain is sufficient for regulating this hypoxic stress response. Moreover, mutation of Tyr-46 → Phe in the MUC1 cytoplasmic domain partially blocked the negative regulatory effects of MUC1 on HIF-1α activation. The present results further demonstrate that silencing MUC1 in ZR-75-1 cells is asso-
MUC1 Regulates the PHD3 → HIF-1α Pathway—HIF-1α is subject to ubiquitination and proteasomal degradation under non-hypoxic conditions. O$_2$-dependent hydroxylation of HIF-1α by PHD1–3 facilitates binding of von Hippel-Lindau protein and formation of an E3 ligase complex with elongin B, elongin C, and Cullin 2 (50). Other studies have shown that the contribution of the PHDs is dependent in large part on the intracellular abundance of each enzyme (44). Our results demonstrate that MUC1 has little effect on PHD1 levels. MUC1 decreased PHD2 expression in HCT116 cells and had no effect on PHD2 levels in ZR-75-1 cells, indicating that this response is dependent on cell context. We also found that expression of MUC1 in both HCT116 and ZR-75-1 cells is associated with derepression of HIF-1α activation in the hypoxia response. The MUC1 cytoplasmic domain functions in the coactivation of β-catenin- and p53-mediated gene transcription and thereby the activation of growth and survival responses (25, 29, 33). MUC1-CD also activates pFOXO3a-mediated gene transcription in the survival response to oxidative stress (36). In HCT116 cells, MUC1 had little effect on HIF-1α mRNA levels and attenuated HIF-1α activation by increasing HIF-1α degradation. In ZR-75-1 cells, MUC1 expression was associated with both decreases in HIF-1α mRNA levels and increases in HIF-1α degradation. These findings indicate that MUC1 suppresses HIF-1α activation by multiple mechanisms that are dictated by cell context in the response to hypoxia.

MUC1 attenuates hypoxia-induced loss of mitochondrial transmembrane potential and apoptosis in HCT116 cells. A and B, the indicated HCT116 cells were cultured under normoxic and hypoxic conditions for 48 h, incubated with DiOC6(3), and then analyzed by flow cytometry (A). The results are expressed as the relative percentage of normoxia (solid bars)- or hypoxia (open bars)-exposed cells in M1 (mean ± S.D. for three separate experiments) compared with that obtained with HCT116/vector cells cultured under normoxic conditions (assigned a value of 1) (B). C and D, the indicated HCT116 cells were cultured under normoxic or hypoxic conditions for 48 h and then analyzed for sub-G1 DNA (C). The results are expressed as the percentage (mean ± S.D. for three separate experiments) of normoxia (solid bars)- and hypoxia (open bars)-exposed cells with sub-G1 DNA (D). E, the indicated HCT116 cells were cultured under normoxic conditions (solid bars) or hypoxic conditions in the absence (open bars) and presence (shaded bars) of catalase for 48 h and then analyzed for sub-G1 DNA. The results are expressed as the percentage (mean ± S.D. for three separate experiments) of cells with sub-G1 DNA.

FIGURE 6. MUC1 attenuates hypoxia-induced loss of mitochondrial transmembrane potential and apoptosis in HCT116 cells. A and B, the indicated HCT116 cells were cultured under normoxic and hypoxic conditions for 48 h, incubated with DiOC6(3), and then analyzed by flow cytometry (A). The results are expressed as the relative percentage of normoxia (solid bars)- or hypoxia (open bars)-exposed cells in M1 (mean ± S.D. for three separate experiments) compared with that obtained with HCT116/vector cells cultured under normoxic conditions (assigned a value of 1) (B). C and D, the indicated HCT116 cells were cultured under normoxic or hypoxic conditions for 48 h and then analyzed for sub-G1 DNA (C). The results are expressed as the percentage (mean ± S.D. for three separate experiments) of normoxia (solid bars)- and hypoxia (open bars)-exposed cells with sub-G1 DNA (D). E, the indicated HCT116 cells were cultured under normoxic conditions (solid bars) or hypoxic conditions in the absence (open bars) and presence (shaded bars) of catalase for 48 h and then analyzed for sub-G1 DNA. The results are expressed as the percentage (mean ± S.D. for three separate experiments) of cells with sub-G1 DNA.
increases in PHD3 levels. Notably, increased expression of PHD3 is sufficient to attenuate HIF-1α activation under hypoxic conditions (45). In this regard, transient silencing of PHD3 in HCT116/MUC1 and ZR-75-1/vector cells was associated with increases in HIF-1α, consistent with involvement of PHD3 in down-regulating HIF-1α. We also treated cells with CoCl2, an inhibitor of PHD activity, to confirm that MUC1 attenuates HIF-1α activation through the PHDs. Treatment of HCT116 cells with CoCl2 was associated with HIF-1α activation that was similar in the presence and absence of MUC1 expression. CoCl2 also derepressed the effects of MUC1 on HIF-1α activation in ZR-75-1 cells. These results and the effects of silencing PHD3 therefore indicate that MUC1 suppresses hypoxia-induced HIF-1α activation through a PHD3-dependent mechanism. The E3 ubiquitin ligases Siah1/2 regulate stability of the PHDs. Siah2-null cells exhibit increased PHD3 lev-
els and suppression of HIF-1α activation during hypoxia (45). Our results demonstrate that MUC1 up-regulates PHD3, at least in large part, by a post-transcriptional mechanism. Further studies will thus be needed to determine whether MUC1 contributes to the regulation of Siah1/2 function and thereby PHD3.

**MUC1 Suppresses Hypoxia-induced Increases in ROS**—The PHDs require O2, 2-oxoglutarate, and ferrous iron (FeII) for activity. HIF-1α is associated with increases in mitochondrial ROS generation (51). Accumulation of ROS increases the conversion of FeII to FeIII and thereby reduces activity of the PHDs (9). Our previous work showed that MUC1 suppresses H2O2-induced increases in ROS at least in part by up-regulation of catalase expression (35). We also found that MUC1-CD is sufficient to suppress the increases in ROS associated with H2O2 treatment (36). Moreover, these effects of MUC1 on redox balance are blocked by mutation of the MUC1 cytoplasmic domain at Tyr-46,3 indicating that phosphorylation of this site is essential for MUC1 function. In this context, MUC1 is phosphorylated on Tyr-46 by the c-Src tyrosine kinase (24), which is activated in the response of cells to oxidative stress (39). The present results demonstrate that MUC1 also suppresses hypoxia-induced accumulation of ROS. Increases in ROS reduce PHD activity and thereby promote HIF-1α activation (9). Thus, consistent with the suppression of ROS, MUC1 and MUC1-CD (but not MUC1(Y46F)) blocked hypoxia-induced HIF-1α activation.

Moreover, in support of a redox-dependent mechanism, treatment of the MUC1-null HCT116/vector cells with catalase to scavenge ROS attenuated hypoxia-induced activation of HIF-1α. Catalase also suppressed hypoxia-induced HIF-1α activation in ZR-75-1 cells silenced for MUC1. These findings indicate that MUC1 up-regulates PHD3 expression and suppresses ROS accumulation, both of which contribute to attenuation of HIF-1α activation in the response to hypoxia.

**MUC1 Protects Against Hypoxia-induced Cell Death**—Over-expression of MUC1 confers resistance to apoptosis in the cellular response to genotoxic and oxidative stress (29, 31, 32, 35, 36, 52). The present work demonstrates that MUC1 blocks hypoxia-induced apoptosis of HCT116 cells. Hypoxia-induced loss of ρψm (53) was also blocked by a MUC1-dependent mechanism. MUC1-C is targeted to mitochondria and integrates into the mitochondrial outer membrane (31, 32); however, it is not known whether mitochondrial MUC1-C contributes to regulation of the ρψm. MUC1-CD, which accumulates in the cytosol, may also interact with cytosolic effectors of pathways that regulate permeabilization of the mitochondrial outer membrane. The importance of the MUC1 cytoplasmic domain is further supported by the demonstration that the MUC1(Y46F) mutant has no effect on the PHD3->HIF-1α pathway and is ineffective in blocking hypoxia-induced apoptosis. Treatment of the MUC1-null HCT116 cells with catalase suppressed hypoxia-induced apoptosis, supporting the importance of ROS in mediating the death of these cells. Our results also demonstrate that MUC1 protects against hypoxia-induced necrosis of ZR-75-1 cells. Silencing of MUC1 in ZR-75-1 cells induced a necrotic response to hypoxia that was rescued by catalase treatment and enhanced buffering of the culture medium. Previous work had shown that, when uncoupled from acidosis, hypoxia is insufficient to decrease viability of certain tumor cells (47). Taken together with those observations, the present results indicate that MUC1 protects ZR-75-1 cells from hypoxia-induced increases in ROS and acidosis and thereby a necrotic response. Additional studies will thus be needed in this regard to determine whether MUC1 can protect cells against acidic conditions in the absence of hypoxia. Hypoxic regions with acidosis are commonly found in solid tumors and adaptation to hypoxic conditions is critical for tumor growth and survival (54, 55). Our findings that MUC1 protects against hypoxia-induced cell death may therefore be of importance to the survival of carcinoma cells that aberrantly overexpress MUC1 in primary and metastatic tumors.

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**REFERENCES**

1. Vaupel, P., Thews, O., Kelleher, D. K., and Hoeckel, M. (1998) *Adv. Exp. Med. Biol.* **454**, 591–602
2. Hockel, M., and Vaupel, P. (2001) *J. Natl. Cancer Inst.* **93**, 266–276
3. Wouters, B. G., and Brown, J. M. (1997) *Radiat. Res.* **147**, 541–550
4. Bruick, R. K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 9082–9087
5. Epstein, A. C., Gleadle, J. M., Neill, L. A., Hewitson, K. S., O’Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. I., and Ratcliffe, P. J. (2001) *Cell* **107**, 43–54
6. Yu, F., White, S. B., Zhao, Q., and Lee, F. S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 9630–9635
7. Semenza, G. L. (2003) *Nat. Rev. Cancer* **3**, 721–732
8. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. (2002) *Science* **295**, 858–861
9. Gerald, D., Berra, E., Frapart, Y. M., Chan, D. A., Giaccia, A. J., Mansuy, D., Pouyssegur, J., Yaniv, M., and Mechta-Grigoriou, F. (2004) *Cell* **118**, 781–794
10. An, W. G., Kanekal, M., Simon, M. C., Maltepe, E., Blagosklonny, M. V., and Neckers, L. M. (1998) *Nature* **392**, 405–408
11. Sowter, H. M., Ratcliffe, P. J., Watson, P., Greenberg, A. H., and Harris, A. L. (2001) *Cancer Res.* **61**, 6699–6703
12. Kufe, D., Inghirami, G., Abe, M., Hayes, D., Justi-Wheeler, H., and Schlom, J. (1984) *Hybridoma* **3**, 223–232
13. Ligtenberg, M. J., Kruijshaar, L., Buijs, F., van Meijer, M., Litvinov, S. V., and Hilkens, J. (1992) *J. Biol. Chem.* **267**, 6171–6177
14. Levitin, F., Stern, O., Weiss, M., Gil-Henn, C., Ziv, R., Prokocimer, Z., Smorodinsky, N. I., Rubinstein, D. B., and Wreschner, D. H. (2005) *J. Biol. Chem.* **280**, 33374–33386
15. Maaco, B., Johansson, D. G., Hansson, G. C., and Hard, T. (2006) *Nat. Struct. Mol. Biol.* **13**, 71–76
16. Siddiqui, I., Abe, M., Hayes, D., Shani, E., Yunis, E., and Kufe, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2320–2323
17. Gendler, S., Taylor-Papadimitriou, J., Duhui, T., Rothbard, J., and Burchell, J. A. (1988) *J. Biol. Chem.* **263**, 12820–12823
18. Merlo, G., Siddiqui, J., Cropp, C., Liscia, D. S., Lidereau, R., Callahan, R., and Kufe, D. (1989) *Cancer Res.* **49**, 6966–6971
19. Li, Y., Ren, J., Yu, W.-H., Li, G., Kuwahara, H., Yin, L., Carraway, K. L., and Kufe, D. (2001) *J. Biol. Chem.* **276**, 35239–35242
20. Schroeder, J., Thompson, M., Gardner, M., and Gendler, S. (2001) *J. Biol. Chem.* **276**, 13057–13064
21. Li, Y., Yu, W.-H., Ren, I., Huang, L., Kharbanda, S., Loda, M., and Kufe, D., unpublished data.
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(2003) Mol. Cancer Res. 1, 765–775
22. Yamamoto, M., Bharti, A., Li, Y., and Kufe, D. (1997) J. Biol. Chem. 272, 12492–12494
23. Li, Y., Bharti, A., Chen, D., Gong, J., and Kufe, D. (1998) Mol. Cell. Biol. 18, 7216–7224
24. Li, Y., Kuwahara, H., Ren, J., Wen, G., and Kufe, D. (2001) J. Biol. Chem. 276, 6061–6064
25. Huang, L., Chen, D., Liu, D., Yin, L., Kharbanda, S., and Kufe, D. (2005) Cancer Res. 65, 10413–10422
26. Ren, J., Li, Y., and Kufe, D. (2002) J. Biol. Chem. 277, 17616–17622
27. Li, Y., Kuwahara, H., Ren, J., Wen, G., and Kufe, D. (2001) J. Biol. Chem. 276, 6061–6064
28. Li, Y., Chen, W., Ren, J., Yu, W., Li, Q., Yoshida, K., and Kufe, D. (2003) Cancer Biol. Ther. 2, 187–193
29. Wei, X., Xu, H., and Kufe, D. (2005) Cancer Cell 7, 167–178
30. Wei, X., Xu, H., and Kufe, D. (2006) Mol. Cell 21, 295–305
31. Ren, J., Agata, N., Chen, D., Li, Y., Yu, W., Huang, L., Raina, D., Chen, W., Kharbanda, S., and Kufe, D. (2004) Cancer Cell 5, 163–175
32. Ren, J., Bharti, A., Raina, D., Chen, W., Ahmad, R., and Kufe, D. (2006) Oncogene 25, 20–31
33. Huang, L., Ren, J., Chen, D., Li, Y., Kharbanda, S., and Kufe, D. (2003) Cancer Biol. Ther. 2, 702–706
34. Schroeder, J. A., Masri, A. A., Adriance, M. C., Tessier, J. C., Kotlarczyk, K. L., Thompson, M. C., and Gendler, S. J. (2004) Oncogene 23, 5739–5747
35. Yin, L., and Kufe, D. (2003) J. Biol. Chem. 278, 35458–35464
36. Yin, L., Huang, L., and Kufe, D. (2004) J. Biol. Chem. 279, 45721–45727
37. Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11715–11720
38. Ben-Ezra, J., Johnson, D. A., Rossi, J., Cook, N., and Wu, A. (1991) J. Histochem. Cytochem 39, 351–354
39. Nakashima, I., Kato, M., Akhand, A. A., Suzuki, H., Takeda, K., Hossain, K., and Kawamoto, Y. (2002) Antioxid. Redox Signal. 4, 517–531
40. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001) Science 292, 464–468
41. Jakkkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Science 292, 468–472
42. Yu, W. H., Woessner, J. F., Jr., McNeish, J. D., and Stamenkovic, I. (2002) Genes Dev. 16, 307–323
43. Bruck, R. K., and McKnight, S. L. (2001) Science 294, 1337–1340
44. Appelhoff, R. J., Tian, Y. M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004) J. Biol. Chem. 279, 38458–38465
45. Nakayama, K., Frew, I. J., Hagensen, M., Skals, M., Habelhah, H., Bhoumik, A., Kadoya, T., Erdjument-Bromage, H., Tempst, P., Frappell, P. B., Bountell, D. D., and Ronai, Z. (2004) Cell 117, 941–952
46. Schofield, C. J., and Ratcliffe, P. J. (2004) Nat. Rev. Mol. Cell Biol. 5, 343–354
47. Schmaltz, C., Hardenbergh, P. H., Wells, A., and Fisher, D. E. (1998) Mol. Cell. Biol. 18, 2845–2854
48. Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., and Nicotera, P. (1997) J. Exp. Med. 185, 1481–1486
49. Nicotera, P., Leist, M., and Ferrando-May, E. (1998) Toxicol. Lett. 102–103, 139–142
50. Safran, M., and Kaelin, W. G., Jr. (2003) J. Clin. Investig. 111, 779–783
51. Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M., and Schumacker, P. T. (2000) J. Biol. Chem. 275, 25130–25138
52. Raina, D., Kharbanda, S., and Kufe, D. (2004) J. Biol. Chem. 279, 20607–20612
53. Steinbach, J. P., Klumpp, A., Wolburg, H., and Weller, M. (2004) Cancer Res. 64, 1575–1578
54. Helmlinger, G., Yuan, F., Dellian, M., and Jain, R. K. (1997) Nat. Med. 3, 177–182
55. Harris, A. (2002) Nat. Rev. Cancer 2, 38–47