The CCAAT/enhancer-binding protein β (C/EBPβ)4 is a leucine-zipper transcription factor that regulates growth and differentiation of hematopoietic and epithelial cells (1). Multiple isoforms of human C/EBPβ (p55, p45/42, and p20) are expressed as a result of alternative sites for translation initiation (2). The C/EBPβ p55 and p45/42 isoforms (LAP1 and LAP2) function as transcriptional activators, whereas the p20 isoform (LIP) acts as a repressor (3). C/EBPβ plays an important role in mammary gland development by regulating stem cell reprogramming activity and specifying luminal cell fate (4, 5). Dysregulation of C/EBPβ has also been associated with the development and metastatic progression of breast cancer (6–9). Inactive C/EBPβ is maintained in a closed conformational state that interferes with binding of the basic region to DNA (10, 11). In contrast, ERK-mediated phosphorylation of C/EBPβ on Thr-235 results in an open conformation with induction of its transactivation function (10–12). These findings have linked activation of C/EBPβ to the MEK → ERK signaling pathway.

The aldehyde dehydrogenase (ALDH) superfamily of enzymes plays an important role in cellular signaling and protection by catalyzing the oxidation of aldehydes (13). The ALDH1A1 isoform has been a particular focus of study as a marker for both normal and cancer stem cells (14). ALDH1A1 functions in part by oxidizing retinol to retinoic acid, a regulator of gene transcription and inducer of cellular differentiation (15). In this respect, high expression of ALDH1A1 in normal hematopoietic stem cells has been linked to retinoid metabolism and control of self-renewal capacity (16). ALDH1A1 has also been identified as a marker of normal and malignant mammary stem cells that regulates retinoid signaling and breast cancer stem cell differentiation (17, 18). Despite the potential role of ALDH1 in self-renewal and differentiation, little is known about the regulation of ALDH1A1 expression in cancer cells. Studies in human K562 erythroleukemia and Hep3B hepatoma cells demonstrated that the ALDH1A1 promoter contains a positive regulatory region (−91 to +53 bp to the transcription start site) with a CCAAT box as the major cis-acting element (19). Other work in mouse hepatoma cells showed that the retinoic acid receptor α (RARα) transactivates the ALDH1A1 promoter by binding to a RA response-like element (RARE) located at positions −91 to −75 bp (20). In addition, C/EBPβ has been shown to transactivate the ALDH1A1 promoter by interacting with the CCAAT box that resides at −75 to −71 bp adjacent to the RARE (20). These
findings and the demonstration that ERK activates C/EBPβ invoked the possibility that ERK→C/EBPβ signaling may contribute to induction of ALDH1A1 expression in cancer cells.

Mucin 1 (MUC1) is a transmembrane protein that is aberrantly overexpressed in most human breast cancers (21, 22). Studies of MUC1 function have been directed by the findings that MUC1 undergoes auto-cleavage into two subunits that, in turn, form a stable non-covalent complex at the cell membrane (22). The MUC1 N-terminal subunit (MUC1-N) contains glicosylated tandem repeats that are a physical characteristic of the mucin family members. MUC1-N is positioned extracellularly in a heterodimeric complex with the MUC1 C-terminal subunit (MUC1-C) that spans the cell membrane (21, 22). The MUC1-N/MUC1-C heterodimer resides at the apical membrane of non-transformed epithelial cells. However, with stress or transformation and thereby loss of polarity, the MUC1-N/MUC1-C complex is expressed over the entire cell membrane, where it interacts with receptor tyrosine kinases (RTKs), such as EGFR, HER2, and others (23–25). The weight of evidence indicates that MUC1 occurs as part of a complex with the RTK activation. In this regard, targeting MUC1-C down-regulates HER2 activation and its downstream signals in breast cancer cells (26). The MUC1-C cytoplasmic domain also contains a YHPrm site that, when phosphorylated on tyrosine, interacts with PI3K and contributes to activation of the AKT pathway (27, 28). In addition, the MUC1-C cytoplasmic domain contains a PYTPNp site that interacts with GRB2, linking MUC1-C to SOS and the RAS pathway (29). Moreover, the MUC1-C subunit localizes to the nucleus, where it interacts with transcription factors, such as NF-κB, and promotes expression of genes involved in growth and survival (30–32).

The present studies demonstrate that MUC1-C induces ERK signaling and thereby phosphorylation and activation of C/EBPβ. We also show that a complex of MUC1-C and C/EBPβ occupies the ALDH1A1 promoter and induces ALDH1A1 expression. These findings support a novel MUC1-C→ERK→C/EBPβ pathway that up-regulates ALDH activity in breast cancer cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human MDA-MB-468 and MCF-7 breast cancer cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM-l-glutamine. SKBR3 breast cancer cells were grown in McCoy’s 5A medium containing FBS, antibiotics, and 1-glutamine. MDA-MB-468 and SKBR3 cells were infected with a lentiviral vector expressing a MUC1 shRNA or, as a control, with a scrambled shRNA vector (CshRNA) as described (33). Cells were also infected with a lentiviral vector expressing C/EBPβ shRNA (Sigma). MCF-7 cells were stably transfected with a control pH-RCMV-GFP vector or one expressing MUC1-C. Cells were treated with the (i) MUC1-C inhibitor GO-203 (28, 34), (ii) MEK inhibitor U0126 (Sigma-Aldrich) (35) or (iii) NF-κB pathway inhibitor BAY11-7085 (Santa Cruz Biotechnology) (36).

**Immunoprecipitation and Immunoblotting**—Whole cell lysates were prepared in Nonidet P-40 lysis buffer as described (31). Immunoprecipitation of soluble proteins was performed with anti-C/EBPβ (Santa Cruz Biotechnology) or anti-MUC1-C (CT2; LabVision). Precipitates and cell lysates not subjected to precipitation were analyzed by immunoblotting with anti-MUC1-C (LabVision), anti-phospho-MEK, anti-MEK, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-C/EBPβ (Cell Signaling Technology), anti-C/EBPβ, anti-NF-κB p65 (Santa Cruz Biotechnology), and anti-β-actin (Sigma). Detection of immune complexes was achieved using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

**Quantitative RT-PCR**—For qRT-PCR analysis of C/EBPβ and ALDH1A1 mRNA levels, cDNA synthesis was performed with 1μg of total RNA using the Thermoscript RT-PCR system (Invitrogen). cDNA samples were amplified using the SYBR green qPCR assay kit (Applied Biosystems) and the ABI Prism 7000 Sequence Detector (Applied Biosystems). Primers used for qRT-PCR detection of C/EBPβ and ALDH1A1 are listed in *supplemental Table S1*. Statistical significance was determined by the Student’s t test.

**Immunofluorescence Microscopy**—Cells plated on cover slips were fixed in 4% paraformaldehyde/2% sucrose solution for 15 min and permeabilized in 0.5% Triton X-100/PBS for 5 min. The cells were then washed twice with PBS, incubated with anti-MUC1-C or anti-p-C/EBPβ (Abcam) antibodies diluted in 1% BSA/PBS for 30 min at 37°C, washed three times with PBS and incubated with appropriate conjugated secondary antibodies diluted in PBS for 25 min at 37°C. Following two washes with PBS, the coverslips were mounted on slides with Prolong Gold Antifade mounting reagent containing DAPI (Invitrogen) and stored in the dark at 4°C overnight to cure before visualization. The slides were visualized using a Leica SP5X: Laser Scanning Confocal microscope. Images were processed using Image software program (NIH).

**Direct Binding Assays**—GST-tagged human C/EBPβ was generated by subcloning C/EBPβ from pBabe-puro-LAP2 plasmid vector (Addgene) into the pGEX-5X-1 plasmid (GE Healthcare). GST-C/EBPβ(C35A) and GST-C/EBPβ(C184A) were generated from GST-C/EBPβ by site-directed mutagenesis (Stratagene). Purified MUC1-CD, MUC1-CD(1–45), MUC1-CD(46–72), and MUC1-CD(AQA) were prepared by expressing the appropriate GST-fusion protein and cleavage of the GST tag with thrombin as described (37). GST and GST fusion proteins bound to glutathione beads were incubated with purified proteins. The adsorbates were analyzed by immunoblotting with anti-C/EBPβ or anti-MUC1-C cytoplasmic domain antibodies CD1 (38) or CT2 (Ab5; LabVision).

**Promoter-Reporter Assays**—Cells were transfected with pGL3, pGL3-pALDH1A1-Luc or pGL3-pALDH1A1(CCAAT→GAGTC; mut)-Luc and, as an internal control, SV-40 Renilla-Luc (Promega) in the presence of Superfect (Qiagen). After 48 h, the cells were lysed in passive lysis buffer. The lysates were analyzed using the dual luciferase assay kit (Promega).

**Chromatin Immunoprecipitation (ChIP) Assays**—Soluble chromatin was prepared as described (32) and precipitated with anti-C/EBPβ or a control nonimmune IgG. For re-ChIP assays, C/EBPβ complexes from the primary ChIP were eluted and reimmunoprecipitated with anti-MUC1-C as described (39). The SYBR green qPCR assay kit was used for real time ChIP qPCR with the ABI Prism 7000 Sequence Detector (Applied Biosystems). The primers used for qPCR of the ALDH1A1 pro-
**MUC1-C Induces ALDH1A1 Expression**

**RESULTS**

### MUC1-C Induces ERK-mediated C/EBPβ Phosphorylation—C/EBPβ is an auto-repressed transcription factor that is activated by ERK-mediated phosphorylation on Thr-235 (10, 12, 41). MUC1-C has been linked to activation of the Ras→MEK→ERK pathway (29, 42, 43). To determine whether MUC1-C plays a role in the regulation of C/EBPβ, we stably silenced MUC1 in triple-negative MDA-MB-468 breast cancer cells (Fig. 1A). Down-regulation of MUC1-C was associated with a marked decrease in p-MEK and p-ERK levels (Fig. 1A). In concert with ERK-mediated phosphorylation of C/EBPβ on Thr-235, abundance of p-C/EBPβ (p45/42; LAP2) was also decreased by MUC1-C silencing (Fig. 1A). By contrast, there was no detectable phosphorylation of the C/EBPβ p20 isoform (LIP) in MDA-MB-468/GshRNA or MDA-MB-468/MUC1shRNA cells (data not shown). Treatment with the MUC1-C inhibitor, GO-203, was similarly associated with down-regulation of MEK and ERK phosphorylation (Fig. 1B). In addition, GO-203 treatment resulted in a 55 ± 2% decrease in p-C/EBPβ levels (Fig. 1B, legend). In HER2-overexpressing SKBR3 breast cancer cells, targeting MUC1-C with silencing or treatment with GO-203 is associated with down-regulation of HER2 activation (26). Silencing MUC1 in SKBR3 cells also resulted in decreased activation of MEK, ERK and C/EBPβ (Fig. 1C). In addition, treatment of SKBR3 cells with GO-203 was associated with suppression of p-MEK and p-ERK levels and a 54 ± 5% decrease in the abundance of p-C/EBPβ (Fig. 1D). As a control for involvement of ERK, treatment of the MDA-MB-468 and SKBR3 cells with the MEK inhibitor U0126 decreased both ERK and C/EBPβ phosphorylation (Fig. 1, E and F). Collectively, these findings demonstrate that MUC1-C promotes ERK-dependent phosphorylation of C/EBPβ on Thr-235.

### Overexpression of MUC1-C Induces C/EBPβ Expression and Activation—Studies were also performed on MCF-7 breast cancer cells that express MUC1-C and C/EBPβ at levels lower than those found in MDA-MB-468 and SKBR3 cells (Fig. 2A). Here, stable overexpression of MUC1-C in MCF-7 cells was associated with increases in C/EBPβ protein (Fig. 2B, left). qRT-PCR further demonstrated that MUC1-C increases C/EBPβ mRNA levels (Fig. 2B, right). Similar results have been obtained with a separate set of MCF-7 cells that were transduced with lentiviruses expressing an empty vector or MUC1-C, indicating that the increase in C/EBPβ expression is not due to clonal selection (data not shown). Previous studies have linked NF-κB to activation of the C/EBPβ gene (44). In addition, MUC1-C interacts with NF-κB p65 and promotes NF-κB-mediated gene transcription (30). To determine whether MUC1-C induces C/EBPβ expression by a NF-κB-dependent mechanism, we silenced NF-κB p65 in the MCF-7/MUC1-C cells (Fig. 2C). Silencing p65 was associated with down-regulation of C/EBPβ mRNA and protein (Fig. 2C, left and right). Treatment of MCF-7/MUC1-C cells with the NF-κB inhibitor BAY11-7085 also resulted in decreases in abundance of C/EBPβ protein (Fig. 2D, left). Diverse cell types respond to BAY11-7085 with increases in ERK phosphorylation (45). Similar results were obtained in the MCF-7/MUC1-C cells (Fig. 2D, right), indicating that MUC1-C induces C/EBPβ protein levels by an NF-κB-dependent, ERK-independent pathway. With regard to MUC1-C-induced activation of the MEK→ERK pathway, overexpression of MUC1-C was associated with increases in p-MEK and p-ERK levels (Fig. 2E, left). In addition, we found that the MUC1-C-induced C/EBPβ protein is phosphorylated on Thr-235 (Fig. 2E, left) and that treatment with the MEK inhibitor U0126 blocks this modification (Fig. 2E, right). These results demonstrate that overexpression of MUC1-C in MCF-7 cells induces both C/EBPβ abundance and ERK-mediated phosphorylation of C/EBPβ on Thr-235. Taken together with the finding that targeting MUC1-C in MDA-MB-468 and SKBR3 cells results in down-regulation of p-C/EBPβ, but not C/EBPβ protein, indicates that MUC1-C regulates C/EBPβ expression and activation by different mechanisms that are dependent on cell context.

### MUC1-C Forms a Complex with C/EBPβ—As noted above, MUC1-C binds directly to NF-κB p65 and promotes NF-κB-mediated gene transcription (30). Other work has shown that NF-κB associates with C/EBPβ and contributes to the activation of C/EBPβ target genes (46). To determine whether MUC1-C also associates with C/EBPβ, we first assayed for the presence of MUC1-C complexes that contain C/EBPβ. The results of coimmunoprecipitation studies demonstrate that C/EBPβ associates with MUC1-C in MDA-MB-468 cells (Fig. 3A, left). Moreover, analysis of anti-MUC1-C precipitates showed that MUC1-C associates with p-C/EBPβ (Fig. 3A, right). To determine whether MUC1-C preferentially binds phosphorylated C/EBPβ, we performed similar studies on MDA-MB-468 cells that had been treated with the MEK inhibitor U0126 to decrease C/EBPβ phosphorylation as shown in Fig. 1E. The results demonstrate that coimmunoprecipitation of MUC1-C and C/EBPβ is comparable in the absence and presence of U0126, indicating that ERK phosphorylation of
FIGURE 1. **Targeting MUC1-C with silencing or GO-203 treatment down-regulates ERK-mediated C/EBPβ phosphorylation.**

**A.** MDA-MB-468 cells were infected with lentiviruses to stably express a control scrambled CshRNA or a MUC1 shRNA. Lysates from MDA-MB-468/CshRNA and MDA-MB-468/MUC1shRNA cells were immunoblotted with the indicated antibodies. **B.** MDA-MB-468 cells were left untreated or treated with 5 μM GO-203 for 48 h. Lysates were immunoblotted with the indicated antibodies. Densitometric scanning of the p-C/EBPβ signals demonstrated that GO-203 treatment is associated with a 55 ± 2% decrease in p-C/EBPβ protein (mean ± S.D. of three determinations). **C.** SKBR3 cells were infected with lentiviruses to stably express a control scrambled CshRNA or a MUC1 shRNA. Lysates from SKBR3/CshRNA and SKBR3/MUC1shRNA cells were immunoblotted with the indicated antibodies. **D.** SKBR3 cells were left untreated or treated with 5 μM GO-203 for 48 h. Lysates were immunoblotted with the indicated antibodies. Densitometric scanning of the p-C/EBPβ signals demonstrated that GO-203 treatment is associated with a 54 ± 5% decrease in p-C/EBPβ protein (mean ± S.D. of three determinations). **E** and **F**, MDA-MB-468 (**E**) and SKBR3 (**F**) cells were treated with DMSO as a control or 20 μM U0126 for 48 h. Lysates were immunoblotted with the indicated antibodies.
C/EBPβ is not necessary for the association with MUC1-C (Fig. 3B). Confocal microscopy further demonstrated that MUC1-C colocalizes with p-C/EBPβ in the cytoplasm and nucleus of MDA-MB-468 cells (Fig. 3C). Similar findings were obtained with SKBR3 cells (Fig. 3, D, left and right, and E). In addition, the demonstration that MUC1-C associates with C/EBPβ (Fig. 3F, left) and p-C/EBPβ (Fig. 3F, right) in MCF-7/MUC1-C cells confirmed that MUC1-C and C/EBPβ form complexes in multiple cell types.

MUC1-C Cytoplasmic Domain Binds Directly to C/EBPβ—C/EBPβ (LAP2) is a 323-aa protein that contains proline-rich and bZIP domains (Fig. 4A). To determine whether the association is direct, we incubated GST or GST-C/EBPβ with purified MUC1-CD. Binding of GST-C/EBPβ, but not GST, to MUC1-CD supported a direct interaction (Fig. 4B). The MUC1-C cytoplasmic domain consists of 72 amino acids that is detectable with antibodies CD1 and CT2 directed against the N- and C-terminal regions, respectively (Fig. 4C). To further define the basis for the interaction, we detected binding of C/EBPβ to the MUC1-CD(1–45), but not the MUC1-CD(46–72), fragment (Fig. 4D, left and right). MUC1-CD(1–45) contains a CQC motif that confers homodimerization of the MUC1-C subunit and is the target of GO-203 (Fig. 4C) (28, 34, 37). Mutation of the CQC motif to AQA abrogated binding of MUC1-CD and C/EBPβ, indicating that the Cys residues are necessary for the interaction (Fig. 4E). Accordingly, we analyzed binding of MUC1-CD to C/EBPβ that was mutated at selected Cys sites (Fig. 4F). By contrast, the C/EBPβ C184A mutant had no effect
These findings demonstrate that the MUC1-CD CQC motif binds directly to C/EBPβ/H9252 at Cys-35.

MUC1-C Promotes C/EBPβ-mediated Activation of the ALDH1A1 Promoter—The ALDH1A1 gene promoter includes a CCAAT box at position -75 to -71 upstream to the transcription start site (19) (Fig. 5A). Given the above findings that MUC1-C activates C/EBPβ, we first asked if silencing MUC1 in MDA-MB-468 cells affects induction of an ALDH1A1 promoter-luciferase reporter (pALDH1A1-Luc) (Fig. 5A). As compared with MDA-MB-468/CshRNA cells, pALDH1A1-Luc activity was significantly decreased in the MDA-MB-468/MUC1shRNA cells (Fig. 5B, left). In concert with these results, silencing MUC1 in MDA-MB-468 cells was also associated with down-regulation of ALDH1A1 mRNA levels (Fig. 5B, right). Similar results were obtained in SKBR3/CshRNA and SKBR3/MUC1shRNA cells (supplemental Fig. S1A), indicating that MUC1-C contributes to activation of ALDH1A1 gene transcription. In MCF-7 cells, overexpression of MUC1-C was associated with activation of pALDH1A1-Luc (Fig. 5C, left) and a marked increase in ALDH1A1 mRNA levels (Fig. 5C, right). To extend this analysis, studies were performed with pALDH1A1-Luc in which the CCAAT box was mutated to GAGTC (pALDH1A1(mut)-Luc). Mutation of the CCAAT box was associated with a substantial decrease in reporter activity in both MDA-MB-468 (supplemental Fig. S2A) and MCF-7/MUC1-C (supplemental Fig. S2B) cells, confirming that the C/EBPβ binding site is of importance for MUC1-C-mediated transcriptional activation. Phosphorylation of C/EBPβ on Thr-235 is necessary for the C/EBPβ transcriptional function (10–12, 47). Indeed, treatment of MCF-7/MUC1 cells with U0126, which decreases p-C/EBPβ levels (Fig. 2E, right), resulted in down-regulation of ALDH1A1 expression (supplemental Fig. S3). The MCF-7/MUC1-C cells were also transiently silenced for C/EBPβ to confirm that MUC1-C induces ALDH1A1 expression by a C/EBPβ-dependent mechanism (Fig. 5D). Indeed, down-regulation of C/EBPβ levels was associated with decreases in (i) ALDH1A1 protein (Fig. 5D), (ii) pALDH1A1-Luc activity (Fig. 5E, left) and (iii) ALDH1A1 mRNA levels (Fig. 5E, right). These results...
collectively indicate that MUC1-C activates the ALDH1A1 gene by a C/EBPβ-mediated mechanism.

**MUC1-C Occupies the ALDH1A1 Promoter with C/EBPβ**—To further define the role of MUC1-C in the induction of ALDH1A1 expression, we performed chromatin immunoprecipitation (ChIP) studies of the ALDH1A1 promoter. Analysis of chromatin from MDA-MB-468/CshRNA and MDA-MB-468/MUC1shRNA cells demonstrated that C/EBPβ occupancy of the ALDH1A1 promoter is significantly decreased by silencing MUC1 (Fig. 6A). Re-ChIP studies further demonstrated that C/EBPβ occupies the ALDH1A1 promoter with MUC1-C (Fig. 6B). Similar results were obtained with SKBR3/CshRNA and SKBR3/MUC1shRNA cells (Fig. 6, C and D), indicating that MUC1-C associates with C/EBPβ on the ALDH1A1 promoter and increases C/EBPβ occupancy. With regard to MCF-7 cells, overexpression of MUC1-C increased C/EBPβ occupancy on the ALDH1A1 promoter (Fig. 6E). Moreover, re-ChIP studies demonstrated the presence of complexes containing C/EBPβ and MUC1-C (Fig. 6F). These findings and those above indicate that MUC1-C (i) associates with C/EBPβ on the ALDH1A1 promoter, (ii) increases C/EBPβ occupancy, and (iii) activates ALDH1A1 gene transcription.

**MUC1-C Up-regulates ALDH Activity**—The demonstration that MUC1-C activates the ALDH1A1 promoter and increases ALDH1A1 mRNA levels invoked the possibility that MUC1-C confers up-regulation of ALDH activity. In this respect, silencing MUC1 in MDA-MB-468 (Fig. 7A, left) and SKBR3 (Fig. 7A, right) cells was associated with a decrease in ALDH1A1 protein. Additionally, overexpression of MUC1-C in MCF-7 cells resulted in a marked induction of ALDH1A1 abundance (Fig. 7B). Assessment of ALDH activity in MDA-MB-468 cells by the aldefluor assay further demonstrated that silencing MUC1 decreases the ALDH-positive population from 86% to 16% (Fig. 7C, left). These results were confirmed by analyzing repetitive determinations (Fig. 7C, right). Comparable decreases in the ALDH-positive population were obtained as a consequence of silencing MUC1 in SKBR3 cells (supplemental Fig. S4, left and right). In concert with these effects of silencing MUC1, overexpression of MUC1-C in MCF-7 cells was associated with a substantial increase in the ALDH-positive population (Fig. 7D, left and right). These findings demonstrate that MUC1-C induces ALDH activity, which as noted above has been identified as a marker of normal and malignant mammary stem cells (17, 18).

To determine whether the MUC1-C→ALDH1A1 pathway is activated in breast cancer stem cell-like populations, MCF-7 cells were grown as first (M1) and second (M2) passage mammospheres. Analysis of cells from M2 mammospheres demonstrated a marked increase in MUC1-C expression as compared with that in MCF-7 cells grown as an adherent monolayer (Fig. 7E). In addition, up-regulation of MUC1-C in the M2 cells was associated with increases in p-C/EBPβ, C/EBPβ, and ALDH1A1 levels (Fig. 7E). Similar results were observed in cells from M1 mammo-
spheres (data not shown), indicating that the MUC1-C subunit interacts with GRB2 and SOS, and thereby promotes activation of RAS→MEK→ERK signaling (29, 42, 43). Accordingly, silencing of MUC1-C suppressed p-MEK and p-ERK levels, which in turn decreased C/EBPαThr-235 phosphorylation (Fig. 7F). In MCF-7 cells, overexpression of MUC1-C was associated with up-regulation of p-ERK levels, confirming the role of MUC1-C in activation of the ERK pathway (Fig. 7F). Under these circumstances, we also found that MUC1-C induces ERK-mediated phosphorylation of C/EBPβ on Thr-235. Based on these results, we conclude that MUC1-C induces ERK-mediated activation of C/EBPβ signaling.

The MUC1-C subunit localizes to the nucleus where it interacts with certain transcription factors, such as NF-κB, and promotes their transactivation functions (22, 30, 50). The results of

DISCUSSION

C/EBPβ is an essential regulator of epithelial cell growth and differentiation in the mammary gland (48, 49). In addition, mammary epithelial cells from C/EBPβ-null mice are defective in repopulating activity and commitment to the luminal cell lineage (5), supporting a role for C/EBPβ in regulation of the mammary stem cell. The present studies demonstrate that MUC1 contributes to the induction of C/EBPβ activity (Fig. 7F). The C/EBPβ p55 isoform (LAP1) is not expressed in breast cancer cell lines (6); thus, the present work focused on the interaction between MUC1 and the C/EBPβ p45/42 isoform (LAP2). In contrast to the transcriptional activator LAP2, the p20 isoform (LIP) functions as a repressor (3).
MUC1-C Induces ALDH1A1 Expression

FIGURE 6. MUC1-C associates with C/EBPβ on the ALDH1A1 promoter. A and B, soluble chromatin from MDA-MB-468/CshRNA and MDA-MB-468/MUC1shRNA cells was precipitated with anti-C/EBPβ and analyzed for ALDH1A1 promoter sequences (A). In the re-ChIP experiments, anti-C/EBPβ precipitates were released, reimmunoprecipitated with anti-MUC1-C, and then analyzed for ALDH1A1 promoter sequences (B). The final DNA samples for ChIP (A) and re-ChIP (B) were amplified by qPCR with pairs of primers for the C/EBPβ binding region (BR; −178 to −66) or a control region (CR; −4898 to −4815). The results (mean ± S.D. of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control. C and D, soluble chromatin from SKBR3/CshRNA and SKBR3/MUC1shRNA cells was precipitated with anti-C/EBPβ and analyzed for ALDH1A1 promoter BR or CR regions (C). In the re-ChIP experiments, anti-C/EBPβ precipitates were released, reimmunoprecipitated with anti-MUC1-C, and then analyzed for ALDH1A1 promoter BR or CR regions (C). In the re-ChIP experiments, anti-C/EBPβ precipitates were released, reimmunoprecipitated with anti-MUC1-C, and then analyzed for ALDH1A1 promoter BR or CR regions (F). The results (mean ± S.D. of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control.

Normal human mammary epithelial cells with stem cell properties have increased ALDH activity (17). Additionally, for breast cancers, high ALDH activity has been used to identify the cancer stem cells that is predictive of metastatic disease (5). The present studies lend support to the contention that MUC1-C also interacts with C/EBPβ. In this respect and as assessed by coimmunoprecipitation experiments, complexes of MUC1-C and C/EBPβ were detectable in breast cancer cells. Moreover, confocal microscopy demonstrated colocalization of MUC1-C and C/EBPβ in the cytoplasm and nucleus. As additional support for the interaction, studies with recombinant proteins demonstrated direct binding of the MUC1-C cytoplasmic domain to C/EBPβ. The finding that mutation of (i) MUC1-C Cys residues in the CQC motif or (ii) C/EBPβ Cys-35 abrogates the interaction between MUC1-C and C/EBPβ, further indicated that direct binding is mediated through the formation of disulfide bonds. C/EBPβ Cys-35 is located in the transactivation domain and is involved in intramolecular disulfide bonds that regulate C/EBPβ activity (51). In this way, it is conceivable that binding of MUC1-C could contribute to C/EBPβ activation by direct interaction with Cys-35; however, other studies will be needed to address such a potential mechanism. Among others, C/EBPβ drives the expression of genes, such as β-casein, that encode milk proteins (52). However, from the perspective of C/EBPβ involvement in mammary stem cells (5), the identification of a CCAAT box in the ALDH1A1 promoter prompted the present studies of C/EBPβ-mediated activation of the ALDH1A1 gene. Consistent with the interaction between MUC1-C and C/EBPβ, we found that MUC1-C associates with C/EBPβ on the ALDH1A1 promoter and increases C/EBPβ occupancy. MUC1-C also increased ALDH1A1 promoter activation and ALDH1A1 expression. Moreover, mutation of the CCAAT box in the ALDH1A1 promoter-reporter abrogated MUC1-C-induced activation, confirming the importance of this C/EBPβ binding site. Notably, the human ALDH1A3 gene promoter also contains a CCAAT box (−71 to −67 bp) that could be similarly activated by C/EBPβ (53). Indeed, like ALDH1A1, expression of ALDH1A3 is induced by a MUC1-C-dependent mechanism (data not shown). ALDH1A3 has been identified as a marker of breast cancer stem cells that is predictive of metastatic disease (54). Further studies will therefore be needed to determine whether MUC1-C-mediated ALDH1A3 expression is conferred by a MUC1-C→ERK→C/EBPβ pathway.
port the potential involvement of MUC1-C in the ALDH1+/H11001 phenotype of breast cancer stem cells. In other studies of breast cancer cell lines, MUC1 has been detected in "side populations" of cells that express the ABCG2 transporter, a marker of putative stem/progenitor cells (56, 57). In addition, recent work has demonstrated that MUC1-C induces characteristics, such as the epithelial-mesenchymal transition and mammosphere formation, which are associated with breast cancer stem cells (58).

In the present work, analysis of MCF-7 cells growing as M1 and M2 mammospheres demonstrated activation of MUC1-C/C/EBP/H9252 signaling, consistent with up-regulation of this pathway in a stem cell-like population that expands under anchorage-independent conditions. Analysis of human breast tumors of different subtypes and histologic stages has further shown that MUC1 is detectable in 10% of cells in all HER2/H11001 and luminal A tumors and in most basal-like tumors (59, 60). However, it is not known if the MUC1-positive cells in primary tumors also express ALDH1 or other markers that would signify stem cell populations. Moreover, it is conceivable that if breast cancer stem cells express MUC1, then certain subtypes of tumors, for example of the luminal lineage, may contain differentiated progenitors that are also MUC1-positive. Thus, additional studies are needed to determine whether MUC1-C and ALDH1A1 are coexpressed in stem cells from primary breast tumors.

Acknowledgment—We thank Dr. Mark Ewen for insightful comments about C/EBP structure-function relationships.

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