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MUC1-C Oncoprotein Functions as a Direct Activator of the Nuclear Factor-κB p65 Transcription Factor

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Abstract

Nuclear factor-κB (NF-κB) is constitutively activated in diverse human malignancies. The mucin 1 (MUC1) oncoprotein is overexpressed in human carcinomas and, like NF-κB, blocks cell death and induces transformation. The present studies show that MUC1 constitutively associates with NF-κB p65 in carcinoma cells. The MUC1 COOH-terminal subunit (MUC1-C) cytoplasmic domain binds directly to NF-κB p65 and, importantly, blocks the interaction between NF-κB p65 and its inhibitor IκBα. We show that NF-κB p65 and MUC1-C constitutively occupy the promoter of the *Bcl-xL* gene in carcinoma cells and that MUC1-C contributes to NF-κB–mediated transcriptional activation. Studies in nonmalignant epithelial cells show that MUC1-C interacts with NF-κB in the response to tumor necrosis factor-α stimulation. Moreover, tumor necrosis factor-α induces the recruitment of NF-κB p65-MUC1-C complexes to NF-κB target genes, including the promoter of the *MUC1* gene itself. We also show that an inhibitor of MUC1-C oligomerization blocks the interaction with NF-κB p65 *in vitro* and in cells. The MUC1-C inhibitor decreases MUC1-C and NF-κB p65 promoter occupancy and expression of NF-κB target genes. These findings indicate that MUC1-C is a direct activator of NF-κB p65 and that an inhibitor of MUC1 function is effective in blocking activation of the NF-κB pathway. [Cancer Res 2009;69(17):7013–21]

Introduction

The nuclear factor-κB (NF-κB) proteins (RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52) are ubiquitously expressed transcription factors. In the absence of stimulation, NF-κB proteins localize to the cytoplasm in complexes with IκBα and other members of the IκB family of inhibitor proteins (1). Phosphorylation of IκBα by the high molecular weight IκB kinase (IKKα, IKKβ, and IKKγ) complex induces ubiquitination and degradation of IκBα and thereby release of NF-κB for nuclear translocation. In turn, activation of NF-κB target genes contributes to tumor development through regulation of inflammatory responses, cellular proliferation, and survival (2). NF-κB p65, like other members of the family, contains a NH_2-terminal Rel homology domain (RHD) that is responsible for dimerization and DNA binding. The RHD also functions as a binding site for ankyrin repeats in the IκBα protein, which blocks the NF-κB p65 nuclear localization signal. The NF-κB-IκBα complexes shuttle between the nucleus and the cytoplasm (1). Activation of the canonical NF-κB pathway, for example, in the cellular response to tumor necrosis factor-α (TNF-α), induces IKKβ-mediated phosphorylation of IκBα and its degradation, with a shift in the balance of NF-κB p65 to the nucleus. The nuclear NF-κB dimers engage κB consensus sequences, as well as degenerate variants, in promoter and enhancer regions (3, 4). Activation of NF-κB target genes is then further regulated by posttranslational modification of NF-κB p65 and its interaction with transcriptional coactivators (1). One of the many NF-κB target genes is *IκBα*, the activation of which results in *de novo* synthesis of IκBα and termination of the NF-κB transcriptional response.

Human mucin 1 (MUC1) is a heterodimeric glycoprotein that is aberrantly overexpressed by diverse carcinomas and certain hematologic malignancies. Overexpression of MUC1 confers anchorage-independent growth and tumorigenicity by mechanisms involving, at least in part, constitutive activation of the Wnt/β-catenin and NF-κB pathways (5–7). The MUC1 NH_2-terminal subunit (MUC1-N), which contains variable numbers of extensively glycosylated tandem repeats, is dispensable for inducing a malignant phenotype (6). In this regard, the transmembrane MUC1 COOH-terminal subunit (MUC1-C) functions as a receptor (8) and contains a 72–amino acid cytoplasmic domain (MUC1-CD) that is sufficient for inducing transformation (6). The MUC1-C subunit is also targeted to the nucleus by a process dependent on its oligomerization (9). MUC1-CD functions as a substrate for phosphorylation by the epidermal growth factor receptor (10), c-Src (11), glycogen synthase kinase-3β (12), and c-Abl (13). MUC1-CD also stabilizes the Wnt effector, β-catenin, through a direct interaction and thereby contributes to transformation (6). Other studies have shown that MUC1-CD interacts directly with IKKβ and IKKγ and contributes to activation of the IKK complex (7). Significantly, constitutive activation of NF-κB p65 in human carcinoma cells is down-regulated by silencing MUC1, indicating that MUC1-CD has a functional role in regulation of the NF-κB p65 pathway (7). These findings have also suggested that MUC1-CD function could be targeted with small molecules to disrupt NF-κB signaling in carcinoma cells.

The present studies show that MUC1-CD binds directly to NF-κB p65 and blocks the interaction between NF-κB p65 and IκBα. We show that the MUC1-C subunit associates with NF-κB p65 on the promoters of NF-κB target genes and promotes NF-κB–mediated transcription. The results also show that an inhibitor of MUC1-C oligomerization blocks the MUC1 interaction with NF-κB p65 and constitutive activation of the NF-κB pathway.

Materials and Methods

Cell culture. Human ZR-75-1 breast cancer and U-937 leukemia cells were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine

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serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Human HeLa cervical and MCF-7 breast carcinoma cells were grown in DMEM with 10% fetal bovine serum, antibiotics, and L-glutamine. Human MCF-10A breast epithelial cells were grown in mammary epithelial cell growth medium (Lonza) and treated with 20 ng/mL TNF-α (BD Biosciences). Transfection of the MCF-10A cells with small interfering RNA (siRNA) pools (Dharmacon) was done in the presence of Lipofectamine 2000 (Invitrogen). Cells were treated with 5 μmol/L GO-201 and CP-1 synthesized by the MIT Biopolymer Laboratory (14).

Immunoprecipitation and immunoblotting. Lysates from subconfluent cells were prepared as described (15). Soluble proteins were precipitated with anti–NF-κB p65 (Santa Cruz Biotechnology). The immunoprecipitates and cell lysates were subjected to immunoblotting with anti-p65 (Santa Cruz Biotechnology), anti-p65(NLS) (clone 12H11; Millipore) anti–MUC1-C (Ab5; Lab Vision), anti-IκBα (Santa Cruz Biotechnology), anti–Bcl-xL (Santa Cruz Biotechnology), and anti–β-actin (Sigma). Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare Biosciences) and enhanced chemiluminescence (GE Healthcare).

***In vitro* binding assays.** GST, GST-MUC1-CD, GST-MUC1-CD(1-45), GST-MUC1-CD(46-72), and GST-MUC1-CD(mSRM) were prepared as described (6, 7) and incubated with p65 and certain p65 deletion mutants. Purified GST-MUC1-CD was cleaved with thrombin to remove the GST moiety. GST-IκBα (Millipore) was incubated with p65(186-306) for 2 h at 25°C in the absence and presence of purified MUC1-CD. Adsorbates to glutathione-conjugated beads were analyzed by immunoblotting.

Immunofluorescence confocal microscopy. Cells were fixed and permeabilized as described (13). Incubation with anti–MUC1-C and anti–NF-κB p65 in blocking buffer was done overnight at 4°C. The cells were blocked with 10% goat serum and stained with anti–MUC1-C followed by FITC-conjugated secondary anti-hamster antibody. The cells were then incubated with anti–NF-κB p65 followed by Texas red–conjugated

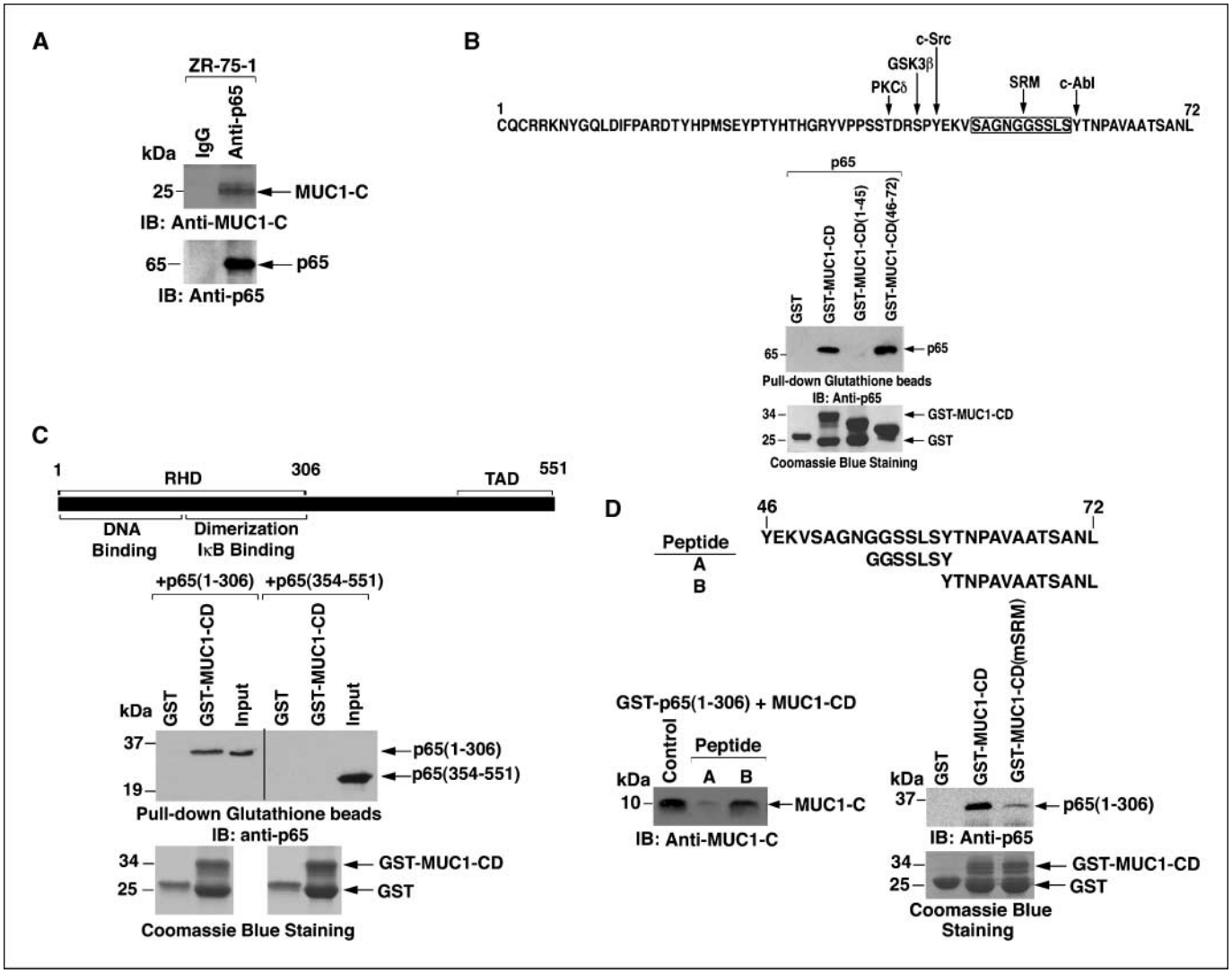

Figure 1. MUC1-C associates with NF-κB p65. *A,* lysates from ZR-75-1 cells were immunoprecipitated with anti-p65 or a control IgG. The precipitates were immunoblotted with anti–MUC1-C and anti-p65. *B,* amino acid sequence of the MUC1 cytoplasmic domain is shown with the protein kinase Cδ (*PKCδ*), glycogen synthase kinase-3β (*GSK3β*), c-Src, and c-Abl phosphorylation sites and the serine-rich motif (*SRM*). GST and the indicated MUC1-CD fusion proteins bound to glutathione beads were incubated with purified p65. The adsorbates were immunoblotted with anti-p65. Input of the GST and GST-MUC1-CD fusion proteins was assessed by Coomassie blue staining. *C,* schematic representation of the NF-κB p65 protein. *TAD,* transactivation domain. GST and GST-MUC1-CD bound to glutathione beads were incubated with purified p65(1-306) or p65(354-551). The adsorbates and inputs were immunoblotted with anti-p65. *D,* amino acid sequences of peptides derived from MUC1-CD(46-72). GST-p65(1-306) bound to glutathione beads was incubated with MUC1-CD in the absence (*Control*) and presence of the indicated A and B peptides at a 20-fold excess compared with MUC1-CD. The adsorbates were immunoblotted with anti–MUC1-C (*left*). GST, GST-MUC1-CD, and GST-MUC1-CD(mSRM) bound to glutathione beads were incubated with p65(1-306). The adsorbates were immunoblotted with anti-p65 (*right*).

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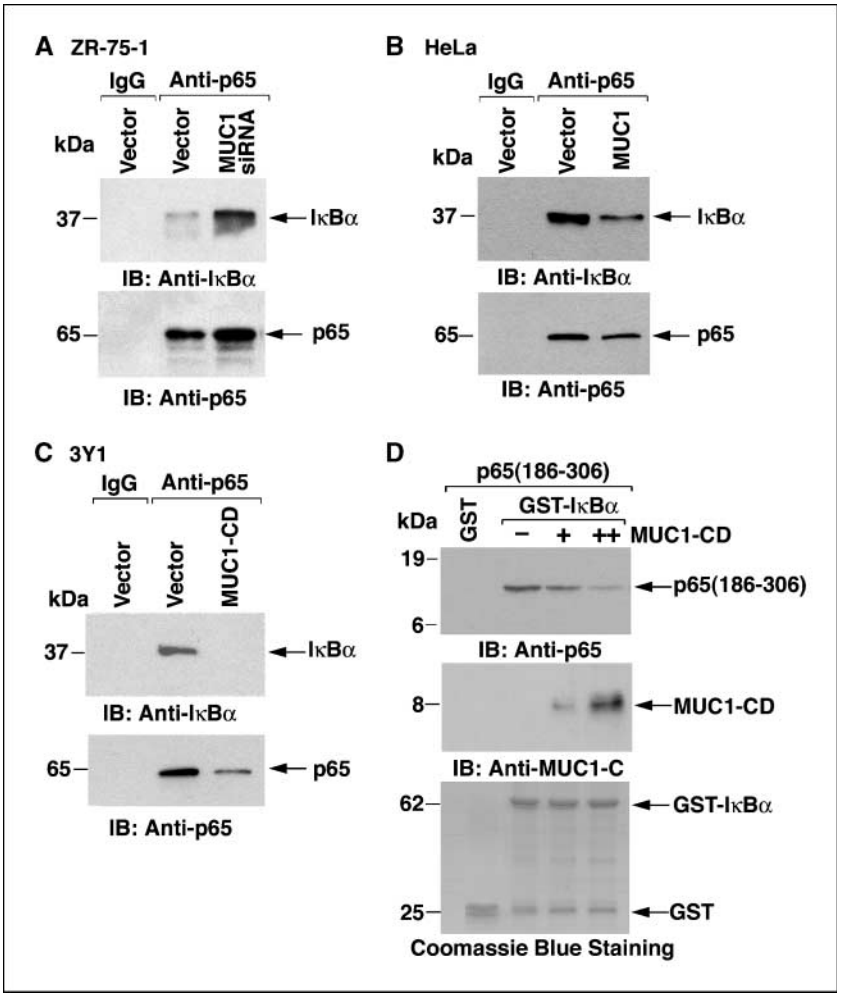

Figure 2. MUC1 attenuates binding of IκBα and NF-κB p65. Cytosolic lysates from the indicated ZR-75-1/vector and ZR-75-1/MUC1siRNA (*A*), HeLa/vector and HeLa/MUC1 (*B*), and 3Y1/vector and 3Y1/MUC1-CD (*C*) cells were immunoprecipitated with anti-p65 or a control IgG. The precipitates were immunoblotted with antibodies against IκBα and p65. *D,* GST and GST-IκBα bound to glutathione beads were incubated with p65(186-306) in the absence and presence of increasing amounts of MUC1-CD. The adsorbates were immunoblotted with anti-p65 (*top*). Input of the MUC1-CD was assessed by immunoblotting with anti–MUC1-C (*middle*). Input of the GST and GST-IκBα proteins was assessed by Coomassie blue staining (*bottom*).

anti-mouse immunoglobulin conjugate (Jackson ImmunoResearch Laboratories). Nuclei were stained with 2 μmol/L TO-PRO-3. Images were captured with a Zeiss LSM510 confocal microscope at 1,024 × 1,024 resolution.

Chromatin immunoprecipitation assays. Soluble chromatin was prepared as described (16) and precipitated with anti-p65, anti–MUC1-C, or a control nonimmune IgG. For re–chromatin immunoprecipitation assays, complexes from the primary chromatin immunoprecipitation were eluted with 10 mmol/L DTT, diluted in re–chromatin immunoprecipitation buffer, and reimmunoprecipitated with anti-p65. For PCR, 2 μL from a 50 μL DNA extraction were used with 25 to 35 cycles of amplification.

Luciferase assays. Cells were transfected with NF-κB-Luc (7) or pMUC1-Luc (17) and SV-40-*Renilla*-Luc (Promega) in the presence of Lipofectamine 2000. After 48 h, the cells were lysed in passive lysis buffer. Lysates were analyzed for firefly and *Renilla* luciferase activities using the dual luciferase assay kit (Promega).

Results

MUC1-C interacts directly with NF-κB p65. To determine whether MUC1 interacts with NF-κB, anti–NF-κB p65 precipitates from ZR-75-1 breast cancer cells were immunoblotted with an antibody against the MUC1-C subunit cytoplasmic domain. The results show that MUC1-C coprecipitates with NF-κB p65 (Fig. 1*A*). Similar findings were obtained with lysates from MCF-7 breast cancer cells, which also overexpress endogenous MUC1 (Supplementary Fig. S1*A*). To determine whether the MUC1-N subunit is necessary for the association, studies were done on U-937 cells that stably express exogenous MUC1-C and not MUC1-N (18). The coprecipitation of NF-κB p65 and MUC1-C in these cells showed that MUC1-N is dispensable for the interaction (Supplementary Fig. S1*B*). Incubation of ZR-75-1 cell lysates with GST or a GST fusion protein containing the 72–amino acid MUC1-CD further showed that MUC1-CD associates with NF-κB p65 (Supplementary Fig. S1*C*). To determine whether MUC1 binds directly to NF-κB, we incubated GST, GST-MUC1-CD, GST-MUC1-CD(1-45), or GST-MUC1-CD(46-72) with purified recombinant NF-κB p65 (Fig. 1*B*). Analysis of the adsorbates showed that GST-MUC1-CD, and not GST, binds to NF-κB p65 (Fig. 1*B*). Incubation of MUC1-CD deletion mutants further showed that this interaction is mediated by MUC1-CD(46-72) and not MUC1-CD(1-45) (Fig. 1*B*).

NF-κB p65 is a 551–amino acid protein that includes a NH_2-terminal RHD and a COOH-terminal transactivation domain (Fig. 1*C*). Incubation of GST-MUC1-CD with purified NF-κB deletion mutants showed binding to p65(1-306) and not p65(354-551) (Fig. 1*C*). To further define the MUC1-CD and NF-κB sequences responsible for the interaction, we incubated GST-p65(1-306) with MUC1-CD in the presence of peptides derived from MUC1-CD(46-72) (Fig. 1*D*). The results show that the GGSSLSY, and not the YNTPAVAATSANL, peptide blocks the interaction between MUC1-CD and p65(1-306) (Fig. 1*D, left*). Moreover, incubation of GST-MUC1-CD with mutation of the serine-rich

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motif (SAGNGGSSLS to AAGNGGAAAA) substantially decreased the interaction with p65(1-306) (Fig. 1*D, right*), further supporting dependence on the GGSSLSY sequence for the interaction.

The results further show that MUC1-CD binds to p65(1-180) (Supplementary Fig. S2*A*). As a control, there was no detectable interaction of GST-IκBα and p65(1-180) (Supplementary Fig. S2*A*). In that regard, IκBα binds to sequences just upstream to the nuclear localization signal at amino acids 301 to 304 (19, 20). Notably, however, both MUC1-CD and IκBα formed complexes with p65(186-306) (Supplementary Fig. S2*B*). These findings indicate that, like IκBα, MUC1-CD binds directly to the NF-κB p65 RHD.

MUC1-CD competes with IκBα for binding to NF-κB p65. The conserved RHD is responsible for DNA binding, dimerization, and association with the IκB-inhibitory proteins (21, 22). To determine whether binding of MUC1 to the RHD region affects the association with IκBα, we first studied ZR-75-1 cells that are stably silenced for MUC1 with a MUC1siRNA (Supplementary Fig. S3). Silencing of MUC1 was associated with increased binding of NF-κB p65 and IκBα (Fig. 2*A*). In addition, stable expression of exogenous MUC1 in HeLa cells (7) decreased the interaction between NF-κB p65 and IκBα (Fig. 2*B*). Stable expression of MUC1-CD in 3Y1 cells (6) was also sufficient to block binding of NF-κB p65 and IκBα (Fig. 2*C*), confirming that the MUC1-C cytoplasmic domain, and not other regions of this subunit, is responsible for the interaction. To determine whether MUC1 directly affects binding of NF-κB p65 and IκBα, we performed competition studies in which binding of IκBα to p65(186-306) was assessed in the presence of MUC1-CD. As expected, binding of IκBα to p65(186-306) was detectable in the absence of MUC1-CD (Fig. 2*D*). Significantly, however, the addition

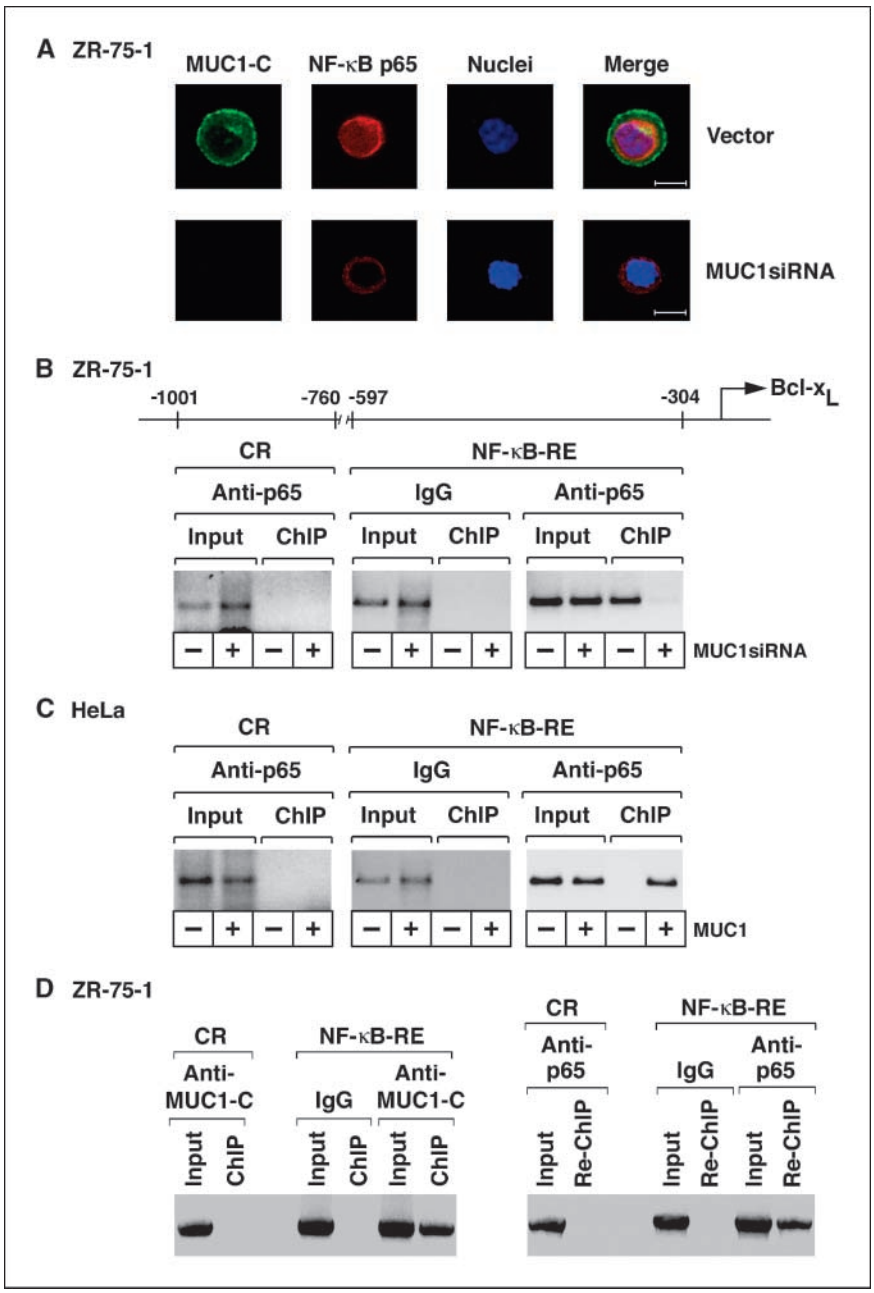

Figure 3. MUC1-C promotes occupancy of NF-κB p65 on the *Bcl-xL* gene promoter. *A,* ZR-75-1/vector and ZR-75-1/MUC1siRNA cells were fixed and double stained with anti–MUC1-C (*green*) and anti–NF-κB p65 (*red*). Nuclei were stained with TO-PRO-3. *B* and *C,* soluble chromatin from ZR-75-1/vector, ZR-75-1/MUC1siRNA (*B*), HeLa/vector, and HeLa/MUC1 (*C*) cells were immunoprecipitated with anti-p65 or a control IgG. The final DNA extractions were amplified by PCR with pairs of primers that cover the NF-κB-RE (-597 to -304) or control region (*CR*; -1,001 to -760) in the *Bcl-xL* promoter. *D,* soluble chromatin from ZR-75-1 cells was immunoprecipitated with anti–MUC1-C or a control IgG and analyzed for *Bcl-xL* NF-κB-RE or control region sequences (*left*). In re–chromatin immunoprecipitation (*ChIP*) experiments, the anti–MUC1-C precipitates were released, reimmunoprecipitated with anti-p65, and then analyzed for *Bcl-xL* promoter sequences (*right*).

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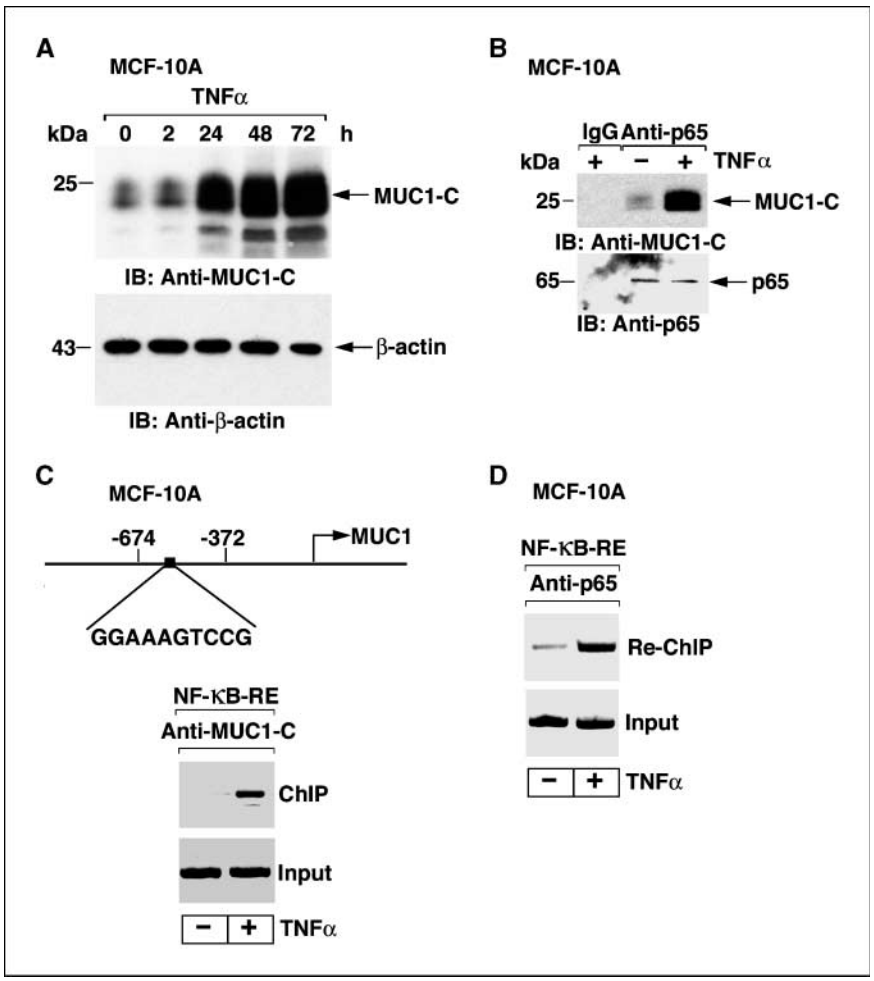

Figure 4. MUC1-C interacts with NF-κB p65 in the response of MCF-10A cells to TNF-α. *A,* MCF-10A cells were stimulated with 20 ng/mL TNF-α for the indicated times. Lysates were immunoblotted with anti–MUC1-C and anti–β-actin. *B,* lysates from MCF-10A cells left untreated or stimulated with 20 ng/mL TNF-α for 24 h were subjected to immunoprecipitation with anti-p65 or a control IgG. The precipitates were immunoblotted with the indicated antibodies. *C,* soluble chromatin from MCF-10A cells left untreated and stimulated with 20 ng/mL TNF-α for 24 h was immunoprecipitated with anti–MUC1-C and then analyzed for *MUC1* NF-κB binding motif promoter sequences. *D,* in re–chromatin immunoprecipitation experiments (*ChIP*), the anti–MUC1-C precipitates were released, reimmunoprecipitated with anti-p65, and then analyzed for *MUC1* NF-κB binding motif promoter sequences.

of increasing amounts of MUC1-CD was associated with a progressive decrease in the interaction of IκBα and p65(186-306) (Fig. 2*D*). These findings indicate that NF-κB p65 forms mutually exclusive complexes with IκBα and MUC1-CD.

MUC1-C associates with NF-κB p65 in the nucleus. Confocal analysis of ZR-75-1 cells showed nuclear colocalization of MUC1-C and NF-κB p65 (Fig. 3*A*). In addition and consistent with MUC1-CD competing for binding to NF-κB p65, silencing MUC1 in the ZR-75-1 cells was associated with localization of nuclear NF-κB p65 to the cytoplasm (Fig. 3*A*). Previous studies showed that MUC1 contributes to the up-regulation of Bcl-xL expression (7). To determine if MUC1-C affects the NF-κB p65 transcription complex, we performed chromatin immunoprecipitation assays with anti-p65. Immunoprecipitation of the NF-κB responsive element (RE) in the promoter of the *Bcl-xL* gene (GGGACTGCCC; -367 to -358; ref. 23) was analyzed by semiquantitative PCR. In ZR-75-1 cells, occupancy of the *Bcl-xL* promoter by NF-κB p65 was decreased by silencing MUC1 (Fig. 3*B*). As a control, there was no detectable signal in immunoprecipitates performed with nonimmune IgG (Fig. 3*B*). There was also no detectable NF-κB p65 occupancy of a control region (-1,001 to -760) of the *Bcl-xL* promoter upstream to the NF-κB-RE (Fig. 3*B*). Analysis of HeLa cells further showed that expression of exogenous MUC1 is associated with increased NF-κB p65 occupancy of the *Bcl-xL* promoter (Fig. 3*C*). To determine whether MUC1-C is present in the NF-κB transcription complex, chromatin immunoprecipitation assays were done with anti–MUC1-C. Using chromatin from ZR-75-1 cells, MUC1-C occupancy was detectable on the NF-κB-RE and not on the control region (Fig. 3*D, left*). In re–chromatin immunoprecipitation assays, the anti–MUC1-C complexes were released, reimmunoprecipitated with anti-p65, and then analyzed by PCR. Anti-p65 precipitated the NF-κB-RE region after release from anti–MUC1-C (Fig. 3*D, right*), indicating that MUC1-C is constitutively present in the *Bcl-xL* promoter region occupied by the NF-κB transcription complex.

Inducible interaction of NF-κB p65 and MUC1-C in MCF-10A breast epithelial cells. The nonmalignant MCF-10A breast epithelial cells (24, 25) express endogenous MUC1 but at levels lower than that found in breast carcinoma cells (7). We found, however, that stimulation of the MCF-10A cells with TNF-α is associated with a substantial up-regulation of MUC1 expression (Fig. 4*A*). In contrast to breast cancer cells, the MCF-10A cells exhibited little, if any, constitutive interaction between NF-κB p65 and MUC1-C (Fig. 4*B*). In turn, stimulation of the MCF-10A cells with TNF-α induced the interaction between NF-κB p65 and MUC1-C (Fig. 4*B*). NF-κB engages consensus and degenerate κB binding sequences (5′-GGGRNWYYCC-3′, where R is a purine, N is any base, W is an adenine or thymine, and Y is a pyrimidine). The *MUC1* promoter contains such a potential sequence for NF-κB binding (5′-GGAAAGTCCG-3′; -589 to -580; ref. 26; Fig. 4*C*). Chromatin immunoprecipitation analysis of TNF-α–stimulated,

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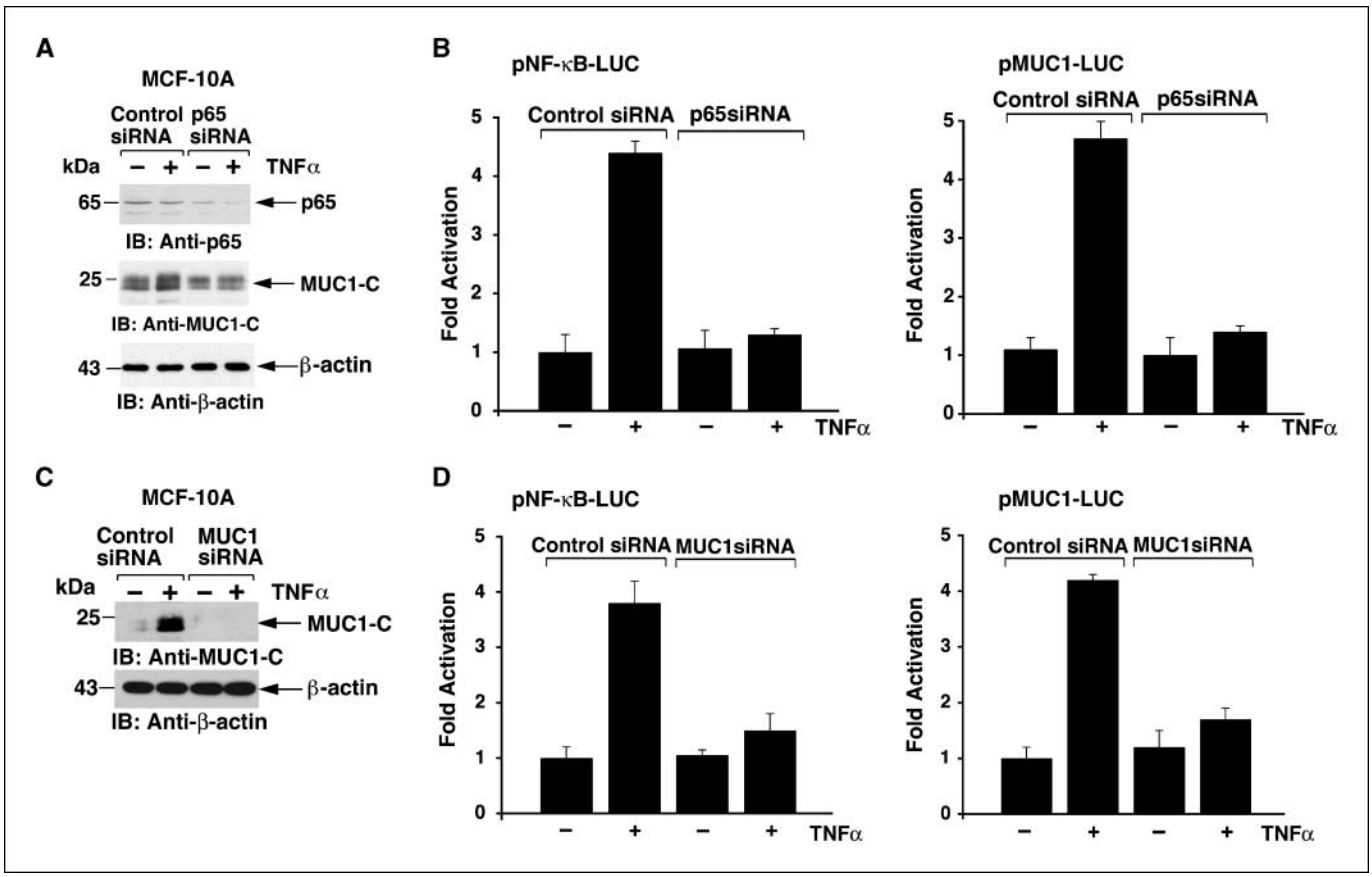

Figure 5. MUC1-C promotes NF-κB p65-mediated activation of the *MUC1* promoters. *A* and *B,* MCF-10A cells were transfected with control or p65siRNA pools for 72 h. The transfected cells were left untreated or stimulated with TNF-α for 24 h. Lysates were immunoblotted with the indicated antibodies (*A*). The cells were then transfected to express a NF-κB-Luc reporter or a MUC1 promoter-Luc reporter (pMUC1-Luc) and, as a control, the SV-40-*Renilla*-Luc plasmid (*B*). *C* and *D,* MCF-10A cells were transfected with control or MUC1siRNA pools for 72 h. The transfected cells were left untreated or stimulated with TNF-α for 24 h. Lysates were immunoblotted with the indicated antibodies (*C*). The cells were then transfected to express a NF-κB-Luc reporter or a MUC1 promoter-Luc reporter (pMUC1-Luc) and, as a control, the SV-40-*Renilla*-Luc plasmid (*D*). Luciferase activity was measured at 48 h after transfection. The results are expressed as the fold activation (mean ± SD from three separate experiments) compared with that obtained with cells transfected with the control siRNA and left untreated (assigned a value of 1).

but not unstimulated, MCF-10A cells showed MUC1-C occupancy of the *MUC1* promoter NF-κB binding motif (Fig. 4*C*). Re–chromatin immunoprecipitation analysis further showed that NF-κB p65 and MUC1-C occupy the same region of the *MUC1* promoter (Fig. 4D). These findings indicate that, in contrast to breast cancer cells, the interaction between NF-κB p65 and MUC1-C and their occupancy of the NF-κB binding motif in the *MUC1* promoter is inducible in MCF-10A cells.

Effects of MUC1 on NF-κB p65-mediated transcriptional activation. To determine whether MUC1 affects activation of NF-κB–mediated transcription, we silenced NF-κB p65 in control and TNF-α–stimulated MCF-10A cells (Fig. 5*A*). Silencing NF-κB p65 attenuated TNF-α–induced increases in MUC1-C expression (Fig. 5*A*), consistent with a potential role for NF-κB p65 in activating *MUC1* gene transcription. As expected, silencing NF-κB p65 attenuated TNF-α–induced activation of the NF-κB-Luc reporter (Fig. 5*B, left*). Significantly, TNF-α–induced activation of the MUC1 promoter-Luc (pMUC1-Luc) was also attenuated by silencing NF-κB p65 (Fig. 5*B, right*). To assess the effects of MUC1-C, we silenced MUC1 expression in the MCF-10A cells with a MUC1siRNA (Fig. 5*C*). Consistent with the effects of MUC1 on NF-κB p65 occupancy of the NF-κB-RE, silencing MUC1 attenuated TNF-α–induced activation of the NF-κB-Luc reporter (Fig. 5*D, left*). Moreover, silencing MUC1 attenuated activation of the pMUC1-Luc reporter (Fig. 5*D, right*). These findings indicate that MUC1 promotes NF-κB p65-mediated transcriptional activation of the *MUC1* promoter.

Targeting MUC1-CD blocks NF-κB p65 function. To further define the role of MUC1 in NF-κB p65 function, we synthesized a peptide (GO-201) corresponding to MUC1-CD(1-15), which blocks oligomerization and thereby function of the MUC1-C cytoplasmic domain (9, 14). In addition, a control peptide, designated CP-1, was synthesized in which the CQC motif was mutated to AQA (Fig. 6*A*). A poly-D-arginine transduction domain was included in the synthesis to facilitate entry of the peptides into cells (ref. 27; Fig. 6*A*). GO-201 blocked the interaction between MUC1-CD and NF-κB p65 *in vitro*, indicating that MUC1-CD oligomerization is necessary for forming complexes with p65 (Fig. 6*A, left*). By contrast, CP-1 had little, if any, effect on this interaction (Fig. 6*A, left*). Treatment of MCF-10A cells with GO-201, but not CP-1, also blocked the TNF-α–induced interaction between MUC1-C and NF-κB p65 (Fig. 6*A, right*). Chromatin immunoprecipitation analysis of the *MUC1* promoter further showed that treatment with GO-201 decreased TNF-α–induced MUC1-C and NF-κB p65 occupancy of the NF-κB binding motif (Fig. 6*B*). In concert with these results, treatment with GO-201 decreased TNF-α–induced MUC1

expression (Fig. 6*C*). GO-201 also attenuated TNF-α–induced Bcl-xL expression (Fig. 6*C*). These findings indicate that disruption of MUC1-C function with GO-201 attenuates (*a*) nuclear targeting of MUC1-C and (*b*) NF-κB p65-mediated activation of MUC1 and Bcl-xL expression.

Discussion

MUC1 binds to NF-κB p65 and blocks the IκBα interaction. The present work shows that the MUC1-C subunit associates with NF-κB p65 in cells and that the MUC1-C cytoplasmic domain binds directly to p65. More detailed binding studies showed that MUC1-CD(46-72) forms complexes with p65(1-306), but not p65(354-551), indicating that MUC1-CD interacts with the p65 RHD. This observation was confirmed with binding of MUC1-CD to p65(1-180) and p65(186-306). Studies using MUC1-CD(46-72)–derived peptides and a MUC1-CD(mSRM) mutant further identified the GGSSLSY sequence as responsible for conferring the interaction. Structural analysis of NF-κB and IκBα cocrystals has shown that IκBα ankyrin repeats interact with amino acid residues just preceding the nuclear localization signal that resides at the COOH terminus of the NF-κB p65 RHD (19, 20). Binding of IκBα to this region of the NF-κB p65 RHD sterically masks the nuclear localization signal (amino acids 287-300) and thereby targeting of NF-κB p65 to the nucleus. The finding that, like IκBα, MUC1-CD binds to p65(186-306) invoked the possibility that the MUC1-C subunit may interfere with the interaction between IκBα and NF-κB p65. Indeed, studies in cells with gain and loss of MUC1 expression indicated that MUC1 competes with IκBα for binding to NF-κB p65 and that MUC1-CD is sufficient for such competition. In concert with these results, silencing endogenous MUC1 in ZR-75-1 cells is associated with targeting of nuclear NF-κB p65 to the cytoplasm. Moreover, direct binding studies with purified proteins confirmed that MUC1-CD blocks the interaction between NF-κB p65 and IκBα. Whether MUC1-CD masks the nuclear localization signal is not known at this time, and like studies with IκBα (19, 20),

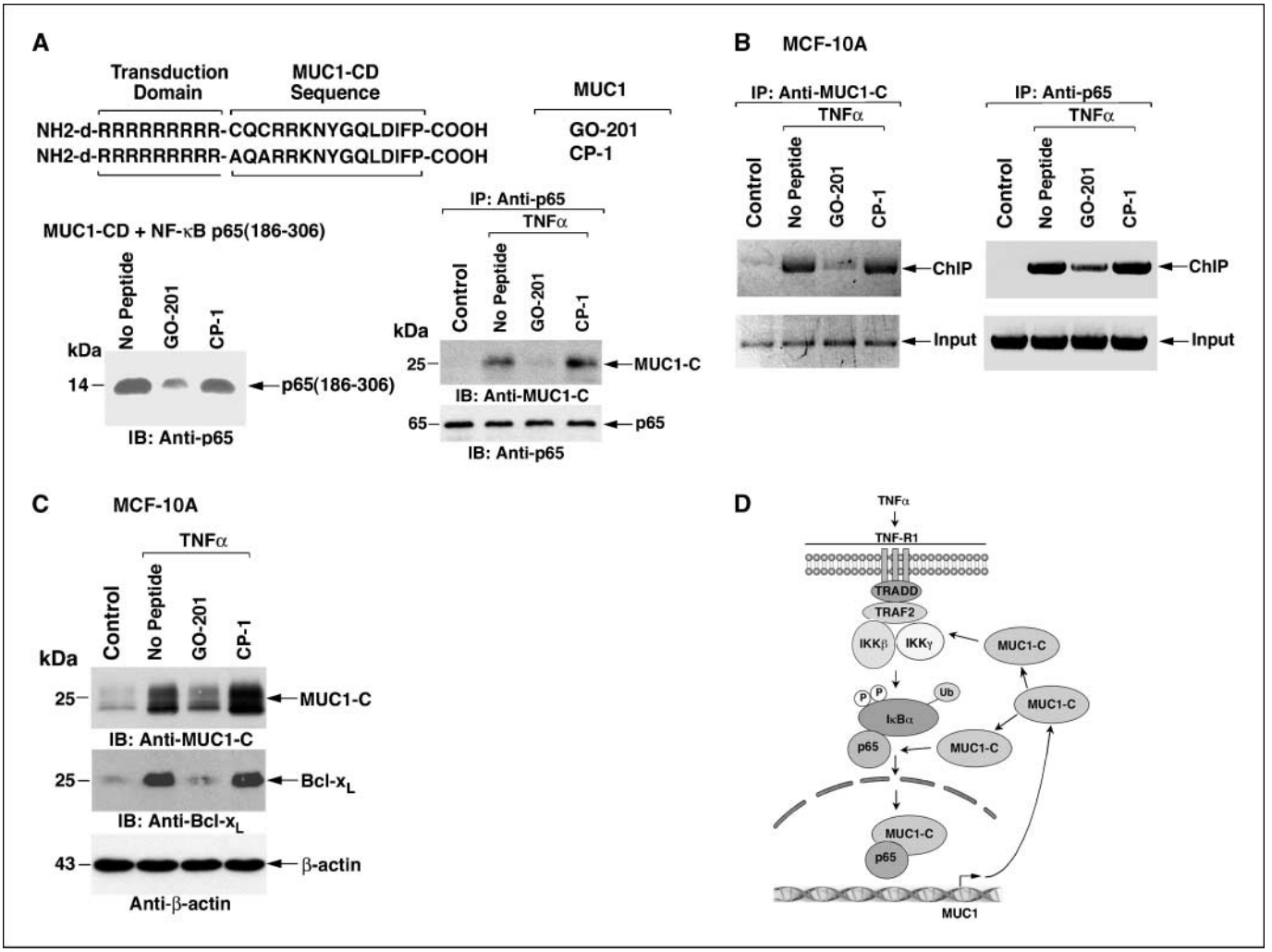

Figure 6. GO-201 blocks the interaction between MUC1 and NF-κB p65. *A,* sequence of GO-201 and CP-1 with the poly-D-arginine transduction domain. GST-MUC1-CD was incubated with purified NF-κB p65 (186-306) in the presence of GO-201 or CP-1 for 1 h at room temperature. Adsorbates to glutathione beads were immunoblotted with anti-p65 (*left*). MCF-10A cells were left untreated or stimulated with TNF-α in the presence of 5 μmol/L GO-201 or CP-1 added each 24 h for 72 h. Anti-p65 precipitates were immunoblotted with the indicated antibodies (*right*). *B* and *C,* MCF-10A cells were left untreated or stimulated with TNF-α in the presence of 5 μmol/L GO-201 or CP-1 added each 24 h for 72 h. Soluble chromatin was precipitated with anti–MUC1-C (*left*) or anti-p65 (*right*) and then analyzed for *MUC1* NF-κB binding motif promoter sequences (*B*). Lysates were immunoblotted with the indicated antibodies (*C*). *D,* model for the proposed effects of MUC1-C on activation of the NF-κB pathway through interactions with IKKs and p65 in an autoinductive regulatory loop. The available findings do not exclude the possibility that MUC1-C forms complexes that include both IKKβ and p65.

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cocrystals of MUC1-CD and the NF-κB RHD may be necessary to address this issue. NF-κB p65 interacts with multiple proteins that affect DNA binding and transcription (28). However, to our knowledge, there are no reports of proteins that interact with the NF-κB p65 RHD and interfere with binding of IκBα. Thus, based on these findings, the overexpression of MUC1-C in human malignancies could subvert the cytoplasmic retention of NF-κB p65 by competitively blocking the NF-κB p65-IκBα interaction.

MUC1 increases occupancy of NF-κB p65 on NF-κB target genes. Nuclear NF-κB activates IκBα expression in a negative feedback loop that promotes the formation of new NF-κB-IκBα complexes and shuttling of NF-κB back to the cytoplasm (1). In this context, the association of MUC1-C with NF-κB p65 could attenuate down-regulation of NF-κB signaling by blocking the interaction with IκBα. The present results provide support for a model in which binding of MUC1-C to NF-κB p65 results in targeting of NF-κB p65 to the promoters of NF-κB target genes (Fig. 6*D*). Stimulation of MCF-10A epithelial cells with TNF-α was associated with binding of MUC1-C to NF-κB p65 and occupancy of these complexes on the NF-κB-RE in the *Bcl-xL* gene promoter. In ZR-75-1 cells, NF-κB p65 occupancy of the *Bcl-xL* NF-κB-RE was detectable constitutively and decreased by silencing MUC1. In concert with the findings obtained for the *Bcl-xL* NF-κB-RE, occupancy of the *MUC1* NF-κB binding motif by NF-κB p65 and MUC1-C was constitutively detectable in ZR-75-1 breast cancer cells and inducible in MCF-10A epithelial cells. These findings and the demonstration that, like NF-κB p65, silencing of MUC1 attenuates activation of the NF-κB-Luc and pMUC1-Luc reporters indicate that MUC1-C is of importance to activation of the NF-κB p65 transcriptional function. Previous work has shown that down-regulation of NF-κB signaling is delayed in the absence of IκBα (29, 30); thus, overexpression of MUC1 in human tumors could confer similar effects by inhibiting the NF-κB p65-IκBα interaction. Further studies will be needed to determine whether the MUC1-C-p65 complexes that occupy gene promoters are formed in the nucleus or whether these complexes are transported from the cytoplasm and, if so, by what import mechanism.

Disruption of the NF-κB p65-MUC1-C interaction with the MUC1 inhibitor GO-201. Previous work first showed that the MUC1-CD contains a CQC motif that mediates the formation of cell surface heterodimeric complexes (31). The MUC1-C subunit also forms oligomers by a mechanism dependent on a CQC motif (9). MUC1-C oligomerization is necessary for its interaction with importin β and targeting to the nucleus (9). The GO-201 inhibitor, derived from the MUC1 cytoplasmic domain that includes the CQC motif, blocks oligomerization of MUC1-CD *in vitro* and of MUC1-C in cells (14). GO-201 also blocks nuclear localization of MUC1-C and induces death of human breast cancer cells (14). The present results show that GO-201 blocks the direct binding of MUC1-CD and NF-κB p65 *in vitro*, indicating that MUC1-CD oligomerization is, at least in part, necessary for the interaction. The TNF-α–induced association of NF-κB p65 and MUC1-C in MCF-10A cells was also blocked by treatment with GO-201. The specificity of GO-201 is further supported by the lack of an effect of the CP-1 control on the interaction between MUC1-CD and NF-κB p65 *in vitro* and in cells. These findings and those showing that the GGSSLSY motif mediates the interaction with p65 lend support to a potential model in which MUC1-CD oligomerization at the CQC sequence is associated with conformational changes in the cytoplasmic domain that are in turn necessary for direct binding of p65 at the downstream GGSSLSY region. Blocking the NF-κB p65-MUC1-C interaction with GO-201 was also associated with a decrease in occupancy of NF-κB p65 on the NF-κB binding motif in the *MUC1* promoter and a decrease in MUC1 expression. GO-201 also decreased Bcl-xL expression. These findings thus provide support for the potential importance of the NF-κB p65-MUC1-C interaction in targeting of NF-κB p65 to the promoters of NF-κB target genes.

Does the MUC1-C-NF-κB p65 interaction contribute to a physiologic defense mechanism exploited by human tumors? TNF-α stimulation of TNF receptor 1 induces the formation of cell membrane complexes that lead to the activation of (*a*) NF-κB and survival or (*b*) caspase-8 and apoptosis (32, 33). The overexpression of MUC1, as found in human breast carcinomas (34), blocks activation of caspase-8 and apoptosis in the response to TNF-α and other death receptor ligands (18). In MCF-10A cells, MUC1-C interacts with caspase-8 and FADD as an induced response to death receptor stimulation and blocks recruitment of caspase-8 to the death receptor complex (18). Other work has shown that MUC1-C associates with and activates the IKK complex (ref. 7; Fig. 6*D*). MUC1-CD(1-45) interacts directly with IKKβ (7) and the present work shows that the GGSSLSY region in MUC1-CD(46-72) binds to p65. Therefore, it is possible that MUC1-C may form complexes that include both IKKβ and p65. Previous work showed that enforced expression of MUC1 in COS cells is associated with increases in NF-κB activation (35). Moreover, mutation of the seven tyrosines in the MUC1 cytoplasmic domain attenuated NF-κB activity, indicating that MUC1-CD is responsible for this effect (35). As shown in the present work, TNF-α–induced up-regulation of MUC1-C expression in MCF-10A cells directly contributes to the activation of NF-κB p65. Thus, MUC1-C can activate the NF-κB pathway through direct interactions with both IKKs and p65 and thereby promote a survival response (Fig. 6*D*). In addition, the up-regulation of MUC1-C protects against the induction of apoptosis by blocking caspase-8 activation (18). The present findings also indicate that, through binding to NF-κB p65, MUC1-C can contribute to activation of the *MUC1* gene in an autoinductive loop and, as a result, prolong survival, albeit in a reversible manner. In this regard, MUC1 may play a physiologic role in transiently dictating cell fate in the inducible response to death receptor stimulation. Conversely, irreversible activation of MUC1 expression in carcinoma cells through a MUC1-C-NF-κB p65 regulatory loop could confer a phenotype that is stably resistant to cell death through persistent activation of NF-κB p65 and inhibition of caspase-8. Irreversible activation of a MUC1-C-NF-κB p65 loop and the up-regulation of prosurvival NF-κB target genes could also contribute to the MUC1-induced block in the apoptotic response of human carcinoma cells to genotoxic, oxidative, and hypoxic stress (15, 17, 36–38). Thus, a physiologic mechanism designed to protect epithelial cells during death receptor stimulation may have been exploited by human carcinomas for survival under adverse conditions.

Disclosure of Potential Conflicts of Interest

D. Raina and S. Kharbanda are employees and shareholders of Genus Oncology. D. Kufe is a founder of Genus Oncology and a consultant to the company. The other authors disclosed no potential conflicts of interest.

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References

1. Hayden MS, Ghosh S. Shared principles in NF-κB signaling. Cell 2008;132:344–62.
2. Karin M, Lin A. NF-κB at the crossroads of life and death. Nat Immunol 2002;3:221–7.
3. Hoffman A, Natoli G, Ghosh G. Transcriptional regulation via the NF-κB signaling module. Oncogene 2006;25:6706–16.
4. Chen FE, Ghosh G. Regulation of DNA binding by Rel/NF-κB transcription factors: structural views. Oncogene 1999;18:6845–52.
5. Li Y, Liu D, Chen D, Kharbanda S, Kufe D. Human DF3/MUC1 carcinoma-associated protein functions as an oncogene. Oncogene 2003;22:6107–10.
6. Huang L, Chen D, Liu D, Yin L, Kharbanda S, Kufe D. MUC1 oncoprotein blocks GSK3β-mediated phosphorylation and degradation of β-catenin. Cancer Res 2005;65:10413–22.
7. Ahmad R, Raina D, Trivedi V, et al. MUC1 oncoprotein activates the IκB kinase β complex and constitutive NF-κB signaling. Nat Cell Biol 2007;9:1419–27.
8. Ramasamy S, Duraisamy S, Barbashov S, Kawano T, Kharbanda S, Kufe D. The MUC1 and galectin-3 oncoproteins function in a microRNA-dependent regulatory loop. Mol Cell 2007;27:992–1004.
9. Leng Y, Cao C, Ren J, et al. Nuclear import of the MUC1-C oncoprotein is mediated by nucleoporin Nup62. J Biol Chem 2007;282:19321–30.
10. Li Y, Ren J, Yu W-H, et al. The EGF receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and β-catenin. J Biol Chem 2001;276:35239–42.
11. Li Y, Kuwahara H, Ren J, Wen G, Kufe D. The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3β and β-catenin. J Biol Chem 2001;276:6061–4.
12. Li Y, Bharti A, Chen D, Gong J, Kufe D. Interaction of glycogen synthase kinase 3β with the DF3/MUC1 carcinoma-associated antigen and β-catenin. Mol Cell Biol 1998;18:7216–24.
13. Raina D, Ahmad R, Kumar S, et al. MUC1 oncoprotein blocks nuclear targeting of c-Abl in the apoptotic response to DNA damage. EMBO J 2006;25:3774–83.
14. Raina D, Ahmad R, Joshi M, et al. Direct targeting of the MUC1 oncoprotein blocks survival and tumorigenicity of human breast carcinoma cells. Cancer Res 2009;69:5133–41.
15. Ren J, Agata N, Chen D, et al. Human MUC1 carcinoma-associated protein confers resistance to genotoxic anti-cancer agents. Cancer Cell 2004;5:163–75.
16. Wei X, Xu H, Kufe D. MUC1 oncoprotein stabilizes and activates estrogen receptor α. Mol Cell 2006;21:295–305.
17. Yin L, Kufe D. Human MUC1 carcinoma antigen regulates intracellular oxidant levels and the apoptotic response to oxidative stress. J Biol Chem 2003;278:35458–64.
18. Agata N, Kawano T, Ahmad R, Raina D, Kharbanda S, Kufe D. MUC1 oncoprotein blocks death receptor-mediated apoptosis by inhibiting recruitment of caspase-8. Cancer Res 2008;68:6136–44.
19. Jacobs M, Harrison SC. Structure of an IκBα/NF-κB complex. Cell 1998;95:749–58.
20. Huxford T, Huang D, Malek S, Ghosh G. The crystal structure of the IκBα/NF-κB complex reveals mechanisms of NF-κB inactivation. Cell 1998;95:759–70.
21. Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionary conserved mediators of immune responses. Annu Rev Cell Dev Biol 1998;16:225–60.
22. Chen LF, Greene WC. Shaping the nuclear action of NF-κB. Mol Cell Biol 2004;5:392–401.
23. Grillot D, Gonzalez-Garcia M, Ekhterae D, et al. Genomic organization, promoter region analysis, and chromosome localization of the mouse *bcl-x* gene. J Immunol 1997;158:4750–7.
24. Soule HD, Maloney TM, Wolman SR, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 1990;50:6075–86.
25. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol 2001;3:785–92.
26. Lagow EL, Carson DD. Synergistic stimulation of MUC1 expression in normal breast epithelia and breast cancer cells by interferon-γ and tumor necrosis factor-α. J Cell Biochem 2002;86:759–72.
27. Fischer PM. Cellular uptake mechanisms and potential therapeutic utility of peptidic cell delivery vectors: progress 2001-2006. Med Res Rev 2007;27:755–95.
28. Natoli G, Saccani S, Bososio D, Marazzi I. Interactions of NF-κB with chromatin: the art of being at the right place at the right time. Nat Immunol 2005;6:439–45.
29. Gerondakis S, Grumont R, Gugasyan R, et al. Unravelling the complexities of the NF-κB signalling pathway using mouse knockout and transgenic models. Oncogene 2006;25:6781–93.
30. Pasparakis M, Luedde T, Schmidt-Supprian M. Dissection of the NF-κB signalling cascade in trangenic and knockout mice. Cell Death Differ 2006;13:861–72.
31. Zrihan-Licht S, Baruch A, Elroy-Stein O, Keydar I, Wreschner DH. Tyrosine phosphorylation of the MUC1 breast cancer membrane proteins. Cytokine receptor-like molecules. FEBS Lett 1994;356:130–6.
32. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 2003;114:181–90.
33. Schneider-Brachert W, Tchikov V, Neumeyer J, et al. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity 2004;21:415–28.
34. Kufe D, Inghirami G, Abe M, Hayes D, Justi-Wheeler H, Schlom J. Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumors. Hybridoma 1984;3:223–32.
35. Thompson EJ, Shanmugam K, Hattrup CL, et al. Tyrosines in the MUC1 cytoplasmic tail modulate transcription via the extracellular signal-regulated kinase 1/2 and nuclear factor-κB pathways. Mol Cancer Res 2006;4:489–97.
36. Raina D, Kharbanda S, Kufe D. The MUC1 oncoprotein activates the anti-apoptotic PI3K/Akt and Bcl-xL pathways in rat 3Y1 fibroblasts. J Biol Chem 2004;279:20607–12.
37. Yin L, Huang L, Kufe D. MUC1 oncoprotein activates the FOXO3a transcription factor in a survival response to oxidative stress. J Biol Chem 2004;279:45721–7.
38. Yin L, Kharbanda S, Kufe D. Mucin 1 oncoprotein blocks hypoxia-inducible factor 1α activation in a survival response to hypoxia. J Biol Chem 2007;282:257–66.

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