Membrane Mucin Muc4 Induces Density-dependent Changes in ERK Activation in Mammary Epithelial and Tumor Cells

ROLE IN REVERSAL OF CONTACT INHIBITION*

Received for publication, May 19, 2006, and in revised form, July 26, 2006. Published, JBC Papers in Press, August 4, 2006, DOI 10.1074/jbc.M604858200

Vanessa Pino, Victoria P. Ramsauer, Pedro Salas, Coralie A. Carothers Carraway, and Kermit L. Carraway

From the Departments of Cell Biology and Anatomy and Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101

The membrane mucin Muc4 has been shown to alter cellular behavior through both anti-adhesive effects on cell-cell and cell-matrix interactions and its ability to act as an intramembrane ligand for the receptor tyrosine kinase ErbB2. The ERK pathway is regulated by both cell-matrix and cell-cell adhesion. An analysis of the effects of Muc4 expression on ERK phosphorylation in mammary tumor and epithelial cells, which exhibit both adhesion-dependent growth and contact inhibition of growth, showed that the effects are density dependent, with opposing effects on proliferating cells and contact-inhibited cells. In these cells, cell-matrix interactions through integrins are required for activation of the ERK mitogenesis pathway. However, cell-cell interactions via cadherins inhibit the ERK pathway. Expression of Muc4 reverses both of these effects. In contact-inhibited cells, Muc4 appears to activate the ERK pathway at the level of Raf-1; this activation does not depend on Ras activation. The increase in ERK activity correlates with an increase in cyclin D1 expression in these cells. This abrogation of contact inhibition is dependent on the number of mucin repeats in the mucin subunit of Muc4, indicative of an anti-adhesive effect. The mechanism by which Muc4 disrupts contact inhibition involves a Muc4-induced relocalization of E-cadherin from adherens junctions at the lateral membrane of the cells to the apical membrane. Muc4-induced abrogation of contact inhibition may be an important mechanism by which tumors progress from an early, more benign state to invasiveness.

Contact inhibition is the process by which epithelial cell proliferation is arrested at high cell densities (1). Contact inhibition of cell division occurs in culture when non-transformed cells, unlike cancerous cells, stop dividing after contacting neighboring cells on all sides (2). In adult tissues, contact inhibition is typically thought to be continuously active, and loss of contact inhibition is usually associated with abnormal growth states such as neoplastic transformation (3). Establishment of cell-cell contacts correlates with contact inhibition of mitosis and inhibition of responsiveness to stimuli by a mechanism that inhibits ERK1/2 phosphorylation and cyclin D1 accumulation (1) and that increases the levels of the cyclin-dependent kinase inhibitor p27 (4). Intercellular adhesion molecules such as E-cadherin are involved in the process of contact inhibition in epithelial cells and fibroblasts. Cell-cell contact mediated by E-cadherin homophilic interactions and associated catenins provides a possible mechanism by which cell growth is regulated by cell-cell contact (1, 5–7). Cell adhesion molecules mediate intercellular and cell-matrix interactions and modulate signal transduction. In many cells, E-cadherin acts as an adhesion-activated cell signaling receptor. Conversely, signaling pathways can directly affect the function of adhesion molecules, leading to changes in cell-cell and cell-matrix interactions (8).

Contact inhibition can be blocked by disruption of cell-cell interactions (1, 4). Many of the characterized disruptors of cell-cell interactions in the contact-inhibited state are receptor tyrosine kinases activated by growth and mobility factors. A well documented disruptor of the contact-inhibited state is the hepatocyte growth factor (HGF)/scatter factor. HGF activation of the c-Met receptor in contact-inhibited Madin-Darby canine kidney cells results in loss of contact inhibition characterized by an increase in phosphorylated ERK1/2, cyclin D1, proliferation, and scattering of these cells (1, 2, 9). This loss of contact inhibition correlates with an increase in apically accessible E-cadherin by a mechanism that involves relocalization of E-cadherin to the membrane domain in contact with the apical compartment, loss of tight junction integrity, and a decrease in polarization of previously well polarized Madin-Darby canine kidney cells. HGF abrogation of contact inhibition also correlates with modulation of the association of E-cadherin with β-catenin and modulation of E-cadherin and β-catenin tyrosine phosphorylation (10–12). HGF is a potent inducer of the epidermal-mesenchymal transition (EMT) in many epithelial systems, including mammary epithelium. In normal mammary epithelial cells, HGF expression is low; however, overexpression of HGF and its receptor c-Met occurs in many types of invasive breast carcino-

---

1 This work was supported in part by Grants CA 74072 and CA52498 from the National Institutes of Health and by the Sylvester Comprehensive Cancer Center of the University of Miami. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 The abbreviations used are: ERK, extracellular signal-regulated kinase; HGF, hepatocyte growth factor; EMT, epithelial-mesenchymal transition; ASGP, asialoglycoprotein; RIPA, radioimmuno precipitation assay; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase extracellular signal-regulated kinase kinase; JNK, c-Jun N-terminal kinase; siRNA, small interfering RNA.
Muc4 Reversal of Contact Inhibition

A high level of HGF expression and sustained activation of Met in breast carcinoma can promote increased EMT, invasion, and metastasis (13).

Because disruption of adherens junctions between cells has been shown to be responsible for loss of contact inhibition and because membrane mucins can disrupt cell-cell interactions, the question is raised whether the anti-adhesive properties of the membrane mucin Muc4 can disrupt contact inhibition by altering cell-cell interactions. In normal epithelial tissues, membrane mucins are expressed only on the apical surface of the cells. Rat Muc4 has been found to be expressed apically in the most accessible and vulnerable epithelia, where it has a protective function (14); however, in tumor cells, mucins are often overexpressed and localized over the whole cell surface, altering the adhesion properties of these cells and facilitating tumor progression and metastasis. Muc4 overexpression has been observed in many carcinomas of the breast, lung, pancreas, and colon (15, 16). Rat Muc4 is a heterodimeric glycoprotein composed of a mucin subunit (asialoglycoprotein (ASGP)-1) attached to the membrane via a transmembrane subunit (ASGP2). The complex is synthesized as a 300-kDa polypeptide precursor that is cleaved into the two subunits early in its transit to the cell surface (17, 18). ASGP2 consists of an 80-kDa polypeptide with ~17 N-linked oligosaccharides and two epidermal growth factor-like domains, both of which have the conserved amino acid residues of active growth factors in the epidermal growth factor family (19). Muc4 is a novel intramembrane, intracrine modulator of ErbB2 signaling. Its transmembrane subunit (ASGP2) interacts as an intramembrane ligand with the receptor tyrosine kinase ErbB2 to modulate its localization, phosphorylation, and signaling (20–23). Muc4 is proposed to influence cell differentiation and apoptosis via its effects on the ErbB2 receptor. Upon complex formation, specific phosphorylation sites are activated in ErbB2, and phosphorylation of the ErbB2-ErbB3 complex is potentiated when the cells are stimulated with the ErbB3 ligand neuregulin (21). The mucin subunit of Muc4 (ASGP1) consists of a highly O-glycosylated 220-kDa polypeptide of 12 imperfect tandem repeats of 117–124 amino acids each. The length of this subunit has been estimated to be ~500 nm (24). When expressed at the cell surface, ASGP1 provides an anti-recognition and anti-adhesive mechanism that prevents cell recognition by immune system components and that disrupts cell-cell and cell-extracellular matrix interactions (25–28). The anti-adhesive effect is dependent on the number of mucin repeats in the ASGP1 subunit and on the level of Muc4 expression. Muc4 blocks cell-cell adhesion and integrin-dependent cell-extracellular matrix interaction by a nonspecific steric effect (25). Thus, Muc4 has two potential mechanisms by which it might block contact inhibition, an anti-adhesive effect and activation of the receptor tyrosine kinase ErbB2.

In this study, we show that Muc4 can activate phosphorylation of ERK in cells that are contact-inhibited. No change in ErbB2 phosphorylation was observed upon Muc4 expression. Instead, the increased ERK phosphorylation correlates with the size of the mucin subunit of Muc4, indicating that the increase in signaling through the mitogenic pathway is a result of the anti-adhesive activity of Muc4. This study provides the first evidence that membrane mucin anti-adhesive effects alter cell signaling in ways that might influence tumor progression.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions**—The establishment of the MCF7 breast carcinoma cell line transfected with recombinant cDNAs for Muc4 analogs (MCF7 mh1) has been described previously (25). Briefly, MCF7 cells were stably transfected with a Muc4 construct containing five mucin repeats in the ASGP1 subunit and the complete ASGP2 subunit. Muc4 expression in this construct is under the control of a tetracycline-regulated promoter (Tet-Off system). ~30% of the cell population expresses Muc4 when induced. These cells were cultured in minimal essential medium containing 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1.5 g/liter sodium bicarbonate (American Type Culture Collection, Manassas, VA) supplemented with 10% fetal bovine serum, 0.01 mg/ml insulin, 100 units/ml penicillin/streptomycin, 150 μg/ml G418, and 40 μg/ml hygromycin. Muc4 expression was repressed by culturing in medium containing 2 μg/ml tetracycline. Muc4 expression was induced in these cells by removing tetracycline for 48 h. The non-tumorigenic MCF10 mammary epithelial cell line was purchased from American Type Culture Collection and cultured in serum-free mammary epithelial growth medium (Cambrex Corp., North Brunswick, NJ) supplemented with 100 ng/ml cholera toxin (Calbiochem). The metastatic SK-BR-3 mammary epithelial cell line was purchased from American Type Culture Collection and cultured in McCoy’s 5A medium with 1.5 mM L-glutamine and 10% fetal bovine serum. Cells were used at low passage numbers. MCF10A and SK-BR-3 cells were used between passages 2 and 10, and MCF7 cells between passages 9 and 15. For low density conditions, cells had reached 10% confluence at the time Muc4 was expressed. For contact-inhibited conditions, MCF7 and MCF10A cells had been cultured for 48 h after reaching confluence at the time Muc4 was expressed. Cells expressing Muc4 and control cells were starved in 0.1% fetal bovine serum for 24 h before being collected. Experiments done without starving the cells yielded similar results.

**Transient Transfections**—Recombinant cDNAs for Muc4 analogs containing the complete ASGP2 subunit but varying in the number of mucin repeats in the ASGP1 subunit (containing one, three, or five mucin repeats) have been described previously (25). MCF10A and SK-BR-3 cells were transiently transfected with these cDNAs using ExGen 500 (Fermentas Inc., Hanover, MD).

**Cell Lysate Preparation and Immunoblotting**—Cells were rinsed once with phosphate-buffered saline and lysed in the plate with RIPA (radioimmune precipitation assay) buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 1% SDS, and 1% Nonidet P-40) containing protease and phosphatase inhibitors (Sigma) for 20 min on ice. Cells were then scraped from the culture dishes to generate total cell lysates, which were then sedimented at 4 °C in a microcentrifuge at 14,000 rpm for 10 min. The protein concentration of each cell lysate was determined using the Advanced protein assay reagent (Cytoskeleton, Inc., Denver, CO). 2× Laemmli buffer containing β-mercaptoethanol (Bio-Rad) was added,
and the samples were boiled for 5 min. Equal amounts of proteins in cell lysates were loaded and separated by 8 or 10% SDS-PAGE at 120 V for 1 h. Proteins were transferred to a nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI) for 1 h at 100 V or overnight at 15 V. Immunoblot procedures were done according to the protocol for each antibody. Membranes were probed with primary antibodies against phospho-Thr\(^{202}/\)Tyr\(^{204}\) p44/p42 MAPK, p44/p42 MAPK, phospho-Ser\(^{217}/\)Ser\(^{221}\) MEK1/2, MEK1/2, phospho-Thr\(^{180}/\)Tyr\(^{182}\) p38, p38, phospho-Thr\(^{183}/\)Tyr\(^{185}\) JNK1/2, and JNK1/2 (Cell Signaling Technology, Inc., Beverly, MA); cyclin D1, p27, c-Raf, β-catenin, and E-cadherin (BD Biosciences); β-actin (Sigma); phospho-Tyr\(^{1248}\) ErbB2 (Upstate, Lake Placid, NY); phospho-Tyr\(^{1139}\) ErbB2 (BIOSOURCE, Camarillo, CA); human c-ErbB2 (DakoCytomation, Carpinteria, CA); and PY99 (Santa Cruz Biotechnology, Inc.). The secondary antibodies used were affinity-purified goat anti-mouse (Pierce) and anti-rabbit (Promega Biotechnology, Inc.). The secondary antibodies used were affinity-purified goat anti-mouse (Pierce) and anti-rabbit (Promega Biotechnology, Inc.).

Membranes were developed using the SuperSignal West Pico chemiluminescence substrate (Pierce) and exposed on x-ray film. The intensity of the bands was quantified by digitizing the image (Scion Image, Scion Corp.) from x-ray film.

**c-Raf Small Interfering RNA (siRNA) Treatments**—MCF7 cells were transfected with 100 nM c-Raf or control glyceraldehyde-3-phosphate dehydrogenase siRNA using DharmaFECT 1 siRNA transfection reagent (Dharmacon, Inc., Lafayette, CO) 48 h before Muc4 expression was induced. Where indicated, Muc4 expression was induced for 48 h by removal of tetracycline.

**Immunoprecipitations**—Contact-inhibited MCF7 cells induced to express Muc4 for 48 h or not induced to express Muc4 were lysed with RIPA buffer. Immunoprecipitation was done using the Catch and Release v2.0 immunoprecipitation kit (Upstate) according to the manufacturer’s protocol with antibody against E-cadherin or β-catenin. Immunoblotting with antibody against phosphorylated tyrosine (PY99) allowed us to determine the amount of phosphorylated β-catenin and E-cadherin. Membranes were stripped and reblotted with antibody against E-cadherin or β-catenin to determine the total amount immunoprecipitated and used for normalization.

**p21\(^{ras}\) Activation Assay**—This assay was performed following the manufacturer’s protocol (Upstate). The method is based on the ability of the Raf-1 Ras-binding domain to act as a specific trap to selectively precipitate p21\(^{ras}\) in its active GTP-bound state.

**Immunofluorescence and Confocal Microscopy**—MCF7 cells were grown to confluence on 6-mm Transwell clear filters (Corning Costar Corp., Cambridge, MA). Contact-inhibited MCF7 cells were processed for immunofluorescence 48 h after induction of Muc4 expression. Cells were washed three times with 1× cold phosphate-buffered saline with Ca\(^{2+}\) and Mg\(^{2+}\) and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were then rinsed and permeabilized with 0.2% Triton X-100 for 5 min, followed by quenching with 50 mM NH\(_4\)Cl. The cells were incubated with primary antibody in 1% bovine serum albumin at room temperature for 1 h. Anti-phospho-Thr\(^{202}/\)Tyr\(^{204}\) p44/p42, anti-Muc4 polyclonal (rat Muc4 C-peptide), mouse anti-Muc4 monoclonal (4F12), and anti-E-cadherin antibodies were used as primary antibodies for immunofluorescence. Hoechst stain was used to visualize the cell nuclei. Cells were then rinsed and incubated with the secondary antibody for 1 h in the dark. Alexa Fluor® 488- and Texas Red®-conjugated secondary antibodies (Molecular Probes, Eugene, OR) and fluorescein isothiocyanate- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as specified by the manufacturers. Cells were then mounted in 10% polyvinyl alcohol, 30% glycerol, 1% n-propyl gallate, and SlowFade\(^{TM}\) (Molecular Probes). The preparations were first observed under a Leica DM RB microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Leica Orthomat E microphotography system using a ×63 (1.4 numerical aperture) infinity-corrected objective. Laser confocal microscopy was performed with an LSM 510 microscope (Carl Zeiss Microlmaging GmbH) in the Imaging Core of the Diabetes Research Institute, University of Miami School of Medicine, equipped with two laser sources and an option of up to three channels. Cell monolayers were analyzed using a ×63 oil immersion objective. The images were collected using the LSM 510 software. The micrographs were selected to show cells expressing Muc4 among a population of cells that do not express Muc4 to highlight the differences among these cells.

**Cell Surface-selective Biotinylation**—MCF7 cells were grown to confluence on Transwell clear filters. Contact-inhibited MCF7 control cells or cells expressing Muc4 were biotinylated on the apical or basolateral surface 48 h after induction of Muc4 expression. Proteins on the apical surface of the cell were exposed to the biotinylation reagent for 15 min, whereas proteins on the basolateral surface were exposed to the reagent for 45 min. The biotinylation procedure, isolation of labeled proteins, and protein elution were carried out using the Pinpoint cell surface isolation kit (Pierce) according to the manufacturer’s protocol. Samples were analyzed by immunoblotting with antibody recognizing E-cadherin.

**RESULTS**

**Effect of Muc4 Overexpression on ERK Phosphorylation in MCF7 Cells Grown at Different Densities**—The effect of Muc4 on signaling through the ERK pathway was examined in MCF7 mh1 cells, which have been stably transfected with Muc4 under the control of a tetracycline-regulated promoter. Despite being carcinoma cells, MCF7 cells are characterized by a high degree of polarity, which is a stable differentiated feature of these cells (29). When plated at low densities, MCF7 mh1 cells proliferate until they reach confluence and then exhibit contact inhibition of proliferation. Immunoblot analysis indicated that, in low density proliferating MCF7 mh1 cells, the level of active ERK1/2 (p44/p42 phosphorylated at Tyr\(^{202}\) and Thr\(^{204}\)) was high, whereas in contact-inhibited MCF7 mh1 cells, this level was very low, which correlates with the low proliferative activity of these cells (Fig. 1, A and B). The total level of ERK1/2 was similar in low density proliferating cells and contact-inhibited cells. By removing tetracycline, Muc4 was expressed for 48 h in MCF7 mh1 cells plated at different densities. Muc4 overexpression in contact-inhibited cells led to a large induction of phosphorylation of ERK1/2 at Tyr\(^{202}\) and Thr\(^{204}\), whereas the total
levels of ERK1/2 remained unchanged (Fig. 1, A and B). In contrast, Muc4 overexpression in low density proliferating cells led to significant inhibition of phosphorylation of ERK1/2, whereas the total levels of ERK1/2 remained unchanged (Fig. 1, A and B). In proliferating cells at 50% confluency, overexpression of Muc4 did not have a pronounced effect on ERK1/2 phosphorylation or total ERK levels (Fig. 1, A and B).

To confirm that Muc4 overexpression in contact-inhibited MCF7 cells leads to an increase in ERK1/2 phosphorylation, we further analyzed low density proliferating and contact-inhibited MCF7 mh1 cells by immunofluorescence and confocal microscopy. Fig. 1C (first and second panels) shows that proliferating cells exhibited high levels of active ERK, whereas contact-inhibited cells showed no active ERK. This contact inhibition of ERK1/2 activation was abrogated only in cells overexpressing Muc4 as shown by co-localization of Muc4 and phospho-ERK (Fig. 1C, third panel). Fig. 1C (fourth panel) shows that active ERK in cells overexpressing Muc4 partially localized to the nucleus.

Effect of Muc4 Expression on p38 and JNK MAPK Phosphorylation in Contact-inhibited MCF7 Cells—

The effect of Muc4 expression on the activation of the other two MAPKs, p38 and JNK, was also determined. Changes in MCF7 cell density did not have a dramatic effect on the activation of p38 and JNK MAPKs, as they did on the activation of ERK-MAPK (Fig. 2), suggesting that p38 and JNK MAPKs do not have a role in contact inhibition of proliferation in these cells. Immunoblot analysis indicated that expression of Muc4 in contact-inhibited MCF7 cells had no effect on the phosphorylated or total levels of these kinases. Densitometry analysis of the immunoblots confirmed these results (data not shown).

Effect of Muc4 Expression on ERK Phosphorylation in MCF10A and SK-BR-3 Cells—To ensure that the Muc4-induced activation of the ERK-MAPK pathway in contact-inhibited MCF7 cells is a result of disrupting the contact-inhibited state, the effect of Muc4 overexpression on ERK activation was determined in two other mammary epithelial cell lines, MCF10A and SK-BR-3. MCF10A cells are non-transformed, well polarized mammary epithelial cells that display contact inhibition of proliferation at high culture densities. SK-BR-3 cells are malignant and have lost their ability to implement cell-cell contact-dependent inhibition of cell growth and proliferation and therefore form multilayer cultures at high culture densities. MCF10A and SK-
BR-3 cells were transiently transfected with an empty plasmid or with a recombinant cDNA for a Muc4 analog containing five mucin repeats in the ASGP1 subunit. As shown in Fig. 3 (A and B), contact-inhibited MCF10A cells had no active ERK, and Muc4 expression in these cells significantly increased ERK phosphorylation. In high density SK-BR-3 cells, ERK was significantly overphosphorylated in the absence of Muc4. Muc4 expression was unable to further increase ERK activation in high density SK-BR-3 cells, even though the levels of Muc4 expression in these cells were higher than those in contact-inhibited MCF10A cells. Thus, Muc4 is able to override the effect of contact inhibition on ERK phosphorylation, but has no effect on cells that are not contact-inhibited.

Muc4-induced Increase in ERK1/2 Phosphorylation in Contact-inhibited MCF7 Cells Correlates with Increase in Cyclin D1

Levels with No Change in the Cyclin-dependent Kinase Inhibitor p27—Contact-inhibited cells arrest at the G1/G0 phase of the cell cycle; therefore, we analyzed two important regulators of the G0/G1-S cell cycle transition, cyclin D1, and the cyclin-dependent kinase inhibitor p27. The levels of these regulators have been shown to be affected by contact inhibition (1, 4). Cyclin D1 levels are regulated by changes in E-cadherin-dependent cell-cell interactions in Madin-Darby canine kidney cells (1) and EMT/6 cells (4). Changes in p27 levels correlating with changes in E-cadherin-mediated cell-cell interactions have been documented in E-cadherin-expressing EMT/6 cells grown in three-dimensional cultures and in colon, lung, and breast cell lines grown in two- and three-dimensional cultures (4). Contact-inhibited MCF7 cells showed low proliferative activity and low levels of cyclin D1, a target of the ERK-MAPK pathway. Immunoblot analysis indicated that Muc4 overexpression in these cells resulted in an increase in the total levels of cyclin D1 (Fig. 4, A and B). In contrast, the total levels of the cyclin-dependent kinase inhibitor p27 were not affected by Muc4 expression in contact-inhibited MCF7 cells (Fig. 4, C and D). The failure to detect a change in the total levels of p27 in this study may be due to the fact that our MCF7 cultures were two-dimensional monolayer cultures as opposed to three-dimensional cultures.

Muc4 Induction in Contact-inhibited MCF7 Cells Also Activates MEK1/2—The dual function kinase MEK is upstream of ERK in the Ras-ERK pathway. Immunoblot analysis indicated that the Muc4-induced increase in ERK1/2 phosphorylation in contact-inhibited MCF7 cells correlated with an induction of activation of the ERK kinases MEK1 and MEK2, as reflected by an increase in MEK1/2 phosphorylation at Ser217 and Ser221 without any changes in the total levels of these kinases (Fig. 5, A and B). The failure to detect a change in the total levels of p27 in this study may be due to the fact that our MCF7 cultures were two-dimensional monolayer cultures as opposed to three-dimensional cultures.

Muc4 overexpression activates ERK in contact-inhibited MCF10A cells but does not affect ERK activation in high density SK-BR-3 cells. A, contact-inhibited MCF10A cells and high density SK-BR-3 cells were transiently transfected either with an empty vector (−, mock transfection) or with a recombinant cDNA for a Muc4 analog containing the complete ASGP2 subunit and five mucin repeats in the ASGP1 subunit (Rep5). 48 h after transfection, cells were lysed in RIPA buffer and analyzed by immunoblotting with antibodies recognizing phospho-ERK1/2, ERK1/2, and Muc4 (4F12). B, the levels of phosphorylated ERK1/2 were normalized to the total levels of ERK1/2. The results shown are representative of three independent experiments.
dehydrogenase siRNA for 48 h before inducing Muc4 expression. Fig. 6 (B and C) shows that the Muc4-induced increase in ERK1/2 phosphorylation was abrogated in cells treated with c-Raf siRNA but not in cells treated with control siRNA or transfection reagent only. The results suggest that the protein kinase Raf-1 is the first enzyme in the Raf-MEK-ERK-MAPK cascade targeted by Muc4.

Muc4-induced Abrogation of Contact Inhibition Is Not Mediated by a Change in the Phosphorylation Levels of ErbB2—The reversal of contact inhibition induced by Muc4 may result from the interaction of its ASGP2 subunit with ErbB2 and the subsequent change in the phosphorylation patterns of this receptor tyrosine kinase. In A375 and CaCo2 cells, ErbB2 phosphorylation at Tyr1248 and Tyr1139 is induced as a result of the ErbB2 interaction with Muc4 (21, 22, 23). To determine whether the interaction of Muc4 with ErbB2 and the resulting specific ErbB2 phosphorylation at these residues correlate with abrogation of contact inhibition in MCF7 cells, the levels of ErbB2 and phospho-ErbB2 were determined by immunoblotting of contact-inhibited MCF7 cells overexpressing Muc4 and control cells. The results in Fig. 7 (A and B) show that Muc4-induced abrogation of contact inhibition did not correlate with changes in the levels of phosphorylation at these residues or with changes in the total levels of ErbB2 in these cells. Densitometry analysis of the immunoblots confirmed these results.

Muc4-induced Abrogation of Contact Inhibition of ERK Phosphorylation Is Dependent on the Size of the ASGP1 Subunit of Muc4—To test the hypothesis that the anti-adhesive effect of Muc4 caused by the sterical hindrance of its mucin subunit (ASGP1) may result in disruption of cell-cell interactions and abrogation of contact inhibition, we transiently transfected contact-inhibited MCF10A non-tumorigenic mammary epithelial cells with recombinant cDNAs for Muc4 analogs containing the complete ASGP2 subunit but varying numbers of mucin repeats in the ASGP1 subunit (containing one, three, or five mucin repeats) and determined the effect of the expression of each one of these constructs on the activity of ERK1/2 in these cells. Comparable levels of Muc4 were expressed by the cells transfected with the different constructs. Immunoblot analysis indicated that the increase in ERK1/2 activity caused by Muc4 expression in contact-inhibited MCF10A cells was proportional to the number of mucin repeats in the ASGP1 subunit of Muc4 (Fig. 8, A and B). The construct containing five mucin repeats in the ASGP1 subunit caused the largest induction of ERK activation, whereas the construct containing one mucin repeat in the ASGP1 subunit had no effect on ERK activation.
This result directly parallels our previous observations on the repression of adhesion by these Muc4 analogs (25). Moreover, these data show that Muc4 anti-adhesive effects alter cell signaling and that the effect on signaling is dependent on the number of mucin repeats in the ASGP1 subunit of Muc4.

**Muc4 Overexpression Does Not Affect the Total Cellular or Tyrosine Phosphorylation Levels of E-cadherin and β-Catenin in Contact-inhibited MCF7 Cells**—Engagement of E-cadherin and its associated catenins in cell-cell adherens junctions has been shown to regulate contact inhibition in many cell lines, and Muc4 is known to disrupt cell-cell interactions. Reduction in E-cadherin levels and changes in the tyrosine phosphorylation levels of E-cadherin and β-catenin are known to affect cell-cell adhesion (12); therefore, we analyzed the effect of Muc4 expression on the total cellular and tyrosine phosphorylation levels of E-cadherin and β-catenin. Immunoblot analysis indicated that Muc4 expression did not affect the total levels of E-cadherin or β-catenin in contact-inhibited cells (data not shown). Immunoprecipitation of E-cadherin or β-catenin followed by immunoblot analysis with antibody against phospho-

**FIGURE 6.** A. Muc4-induced activation of ERK1/2 is independent of p21ras activation. Equal amounts of total protein extract from contact-inhibited MCF7 cells overexpressing Muc4 and from control cells were used to perform the p21ras activity assay. Active Ras (Ras-GTP) was pulled down using 10 µg of the Ras assay reagent (glutathione S-transferase fusion protein corresponding to the human Ras-binding domain of Ras-1 bound to glutathione-agarose beads) for 45 min at 4°C. The beads were pelleted, washed, resuspended in 40 µl of Laemmli buffer, and boiled for 5 min. The supernatant was used as a control. The supernatant and extract from beads were loaded onto a gel, and SDS-PAGE was performed. Membranes were probed with antibody recognizing p21ras. The results shown are representative of three independent experiments. B. Muc4-induced activation of ERK1/2 is dependent on Raf-1 activation. Contact-inhibited MCF7 cells were transfected with 100 nM c-Raf or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA 48 h before Muc4 expression was induced. Where indicated, Muc4 expression was induced for 48 h by removal of tetracycline. Cells were lysed in RIPA buffer and analyzed by immunoblotting with antibodies recognizing phospho-ERK1/2, ERK1/2, and c-Raf. C, the levels of the phosphorylated protein were normalized to the total levels of the protein. The results shown are representative of three independent experiments.

**FIGURE 7.** Muc4 overexpression in MCF7 cells does not increase ErbB2 phosphorylation at Tyr1248 or Tyr1139. Muc4 expression was induced for 48 h in contact-inhibited MCF7 cells where indicated. Cells were lysed in RIPA buffer and analyzed by immunoblotting with antibodies recognizing ErbB2 phosphorylated at Tyr1248 (PY1248 ErbB2; A) and Tyr1139 (PY1139 ErbB2; B) and ErbB2. The results shown are representative of three independent experiments.

**FIGURE 8.** Correlation between the number of mucin repeats in the ASGP1 subunit of Muc4 and ERK activation in contact-inhibited MCF10A cells expressing Muc4. A, contact-inhibited MCF10A cells were transiently transfected either with an empty vector (mock transfection) or with recombinant cDNAs for Muc4 analogs containing the complete ASGP2 subunit but varying in the number of mucin repeats in the ASGP1 subunit (one (Rep1), three (Rep3), or five (Rep5) repeats). 48 h after transfection, cells were lysed in RIPA buffer and analyzed by immunoblotting with antibodies recognizing phospho-ERK1/2, ERK1/2, and Muc4 (4F12). B, the levels of phosphorylated ERK1/2 were normalized to the total levels of ERK1/2. The results shown are representative of three independent experiments.

Muc4 Overexpression Disrupts Cell-Cell Adhesion and Alters Localization of E-cadherin from the Lateral to the Apical Sur-
Muc4 Reversal of Contact Inhibition

FIGURE 9. A, Muc4 overexpression in contact-inhibited MCF10A cells disrupts cell-cell interactions. Contact-inhibited MCF10A cells were transiently transfected either with an empty vector (mock transfection) or with a recombinant cDNA for a Muc4 analog containing the complete ASGP2 subunit and five mucin repeats in the ASGP1 subunit (Rep5). 48 h after transfection, the morphology of the cells was analyzed using a motorized upright Leica DM RB epifluorescence/transmitted light microscope controlled by SlideBook software. Image acquisition was performed with a Hamamatsu ORCA high resolution CCD camera. White areas correspond to detached groups of cells. B, MCF7 cells display apical-basolateral polarity as shown by the localization of the basolateral marker Na⁺K⁺-ATPase. Immunofluorescence analysis was performed using mouse monoclonal antibody recognizing Na⁺K⁺-ATPase. Cy3-conjugated goat anti-mouse and Alexa Fluor 577-conjugated goat anti-rabbit secondary antibodies were used. Images were analyzed by laser confocal microscopy. Confocal sections are shown: x-y, x-z, and y-z planes. x-z sections are shown with the apical side up. y-z sections are shown with the apical side to the right. C and D, Muc4 overexpression in contact-inhibited MCF7 cells disrupts cell-cell interactions and induces a partial relocalization of E-cadherin from the lateral adherens junctions to the apical surface of these cells. Muc4 expression was induced for 48 h in contact-inhibited MCF7 cells. Immunofluorescence analysis was performed using mouse monoclonal antibody recognizing E-cadherin and rabbit polyclonal antibody recognizing Muc4 (C-peptide). Cy3-conjugated goat anti-mouse and Alexa Fluor 577-conjugated goat anti-rabbit secondary antibodies were used. Images were analyzed by laser confocal microscopy. x-z confocal sections are shown with the apical side up. y-z confocal sections are shown with the apical side to the right. Arrows point to E-cadherin relocalized to the apical surface of the cells. The micrograph in C was selected to show a single cell in the population expressing Muc4. Note that the Muc4-expressing cell rounded up and exhibited a partial relocalization of E-cadherin from the lateral adherens junctions to the apical surface and disruption of these junctions. The micrograph in D was selected to show a cluster of cells within the population expressing Muc4 and to highlight disruption of cell-cell interactions. Note that Muc4 expression caused dissociation of the cell pair from the remainder of the layer, although they were still associated. These cells exhibited a partial relocalization of E-cadherin from the lateral surface at the adherens junctions to the apical surface and disruption of interactions of these cells with the rest of the cells in the monolayer because of a disturbance in the integrity of the adherens junctions. E, cell surface-selective biotinylation indicates that Muc4 overexpression increases the amount of apically accessible E-cadherin in MCF7 cells. Muc4-expressing MCF7 cells and control cells were biotinylated on the apical (A) or basolateral (BL) surface. Biotinylated proteins were isolated using immobilized NeutrAvidin, eluted with sample buffer containing 50 mM dithiothreitol, and analyzed by SDS-PAGE, followed by immunoblotting with antibody recognizing E-cadherin.

face of Contact-inhibited Cells—Microscopy analysis of contact-inhibited MCF10A cells showed that Muc4 expression in these cells disrupted cell-cell interactions and caused groups of cells to detach from the monolayer, round up, and eventually lose contact with the substrate (Fig. 9A). After observing the dramatic effect of Muc4 expression on MCF10A cell-cell adhesion, we analyzed the consequences of Muc4 expression on MCF7 cell-cell adhesion in a contact-inhibited monolayer and the effect of Muc4 on E-cadherin localization in these cells. MCF7 cells displayed a well-defined apical-basolateral polarity as shown by the localization of the basolateral marker Na⁺K⁺-ATPase in a monolayer of these cells (Fig. 9B). To determine whether Muc4 expression alters E-cadherin localization, we analyzed the effect of Muc4 expression on the localization of E-cadherin by immunofluorescence and confocal microscopy of MCF7 cells, focusing on regions of the cell layers with limited Muc4 expression, in which the effects of Muc4 could be visualized more easily. Fig. 9C shows a single cell overexpressing Muc4 among a population of cells not expressing Muc4. This cell exhibited a more rounded morphology compared with cells not expressing Muc4, which exhibited a more cobblestone shape (x-y plane). Fig. 9D shows a cluster of cells expressing Muc4 among a group of cells not expressing Muc4. The Muc4-expressing cells rounded up and lost contact with other cells in

---

VOLUME 281 • NUMBER 39 • SEPTEMBER 29, 2006
29418 JOURNAL OF BIOLOGICAL CHEMISTRY
Muc4 Reversal of Contact Inhibition

FIGURE 10. Model for Muc4-induced abrogation of contact inhibition in mammary epithelial cells. In normal contact-inhibited mammary epithelial cells, Muc4 expression is limited to the apical surface; adherens and tight junctions are intact; and the levels of active ERK and cyclin D1 are minimal. When Muc4 is overexpressed in these cells, its location is not limited to the apical surface; it is also found in the lateral surface of the cells, where it causes disruption of the adherens junctions and partial relocalization of E-cadherin to the apical surface of the cells. Contact inhibition is then abrogated in these cells, and the ERK-MAPK pathway is activated at the level of Raf-1.

The Muc4-induced increase in apically accessible E-cadherin in cells that overexpress Muc4 suggest that tight junctions are also disrupted in these cells. Because E-cadherin is an essential component of adherens junctions and because its engagement has been shown to control contact inhibition, we propose that Muc4-induced disruption of E-cadherin localization leads to disruption of the contact-inhibited state, as reflected by an increase in ERK1/2 activity and cyclin D1 levels. The Muc4-induced increase in ERK1/2 activity in previously contact-inhibited Muc4-expressing cells correlates with an increase in the activity of the ERK kinases MEK1 and MEK2 and is dependent on Raf-1 activity but is independent of Ras activity. Therefore, Muc4 appears to activate the ERK-MAPK pathway at the level of Raf-1. This is the first reported case in which an anti-adhesive effect of a membrane mucin is implicated in a cell signaling mechanism that can promote tumor growth and progression.

The Muc4-induced increase in apically accessible E-cadherin and modulation of adherens and tight junction integrity in normal mammary epithelial cells result in a loss of functional and structural cell polarity, which leads to increased ERK-MAPK activation and cyclin D1 levels, a phenotype seen in carcinomas. Muc4-induced abrogation of contact inhibition and induction of epithelial de-differentiation may be an important step in normal epithelial cell transformation to carcinomas. In tumor mammary epithelial cells, Muc4-induced disruption of cadherin-based cell-cell adhesions may not only contribute to further de-differentiation but may also allow tumor cells to escape the confines of the main tumor mass and invade into their local environment, therefore contributing to metastasis. Loss of intracellular adhesion has been widely reported to lead to the gain of a motile and invasive phenotype. Loosening of intercellular junctions between cells could facilitate separation, spreading, and migration of epithelial cells during processes such as wound resealing and organ development and regeneration. Muc4-induced abrogation of contact inhibition may contribute to these physiological processes.
The effect of Muc4 on the ERK-MAPK pathway of low density proliferating MCF7 mammary epithelial cells is less directly relevant to tumor progression but may provide additional insights into Muc4 membrane mucin functions and will be the focus of future studies. Muc4 overexpression in these cells leads to a significant inhibition of ERK1/2 phosphorylation, whereas the total levels of ERK1/2 remain unchanged. The working hypothesis is that the Muc4 effect on adhesion-dependent signaling is due to the disruption of integrin-mediated cell-extracellular matrix interaction caused by Muc4. These interactions are required for activation of MAPK pathways, in particular the ERK-MAPK pathway.

Many normal and tumor mammary epithelial cells exhibit both adhesion-dependent signaling and contact inhibition, and Muc4 is able to abrogate both of these effects. Muc4 relieves contact inhibition by blocking cell-cell interactions and blocks the cell-extracellular matrix adhesion necessary for activation of the mitogenic pathways. This is the first reported case in which an anti-adhesive effect of a membrane mucin is implicated in a cell signaling mechanism that can promote tumor growth and progression.

Acknowledgment—We thank Brigitte Shaw (Imaging Core of the Diabetes Research Institute, University of Miami School of Medicine) for assistance.

REFERENCES
1. Li, S., Gerrard, E., and Balkovetz, D. (2004) Am. J. Physiol. 287, C432–C439
2. Balkovetz, D. (1999) Life Sci. 64, 1393–1401
3. Fagotto, F., and Gumbiner, B. M. (1996) Dev. Biol. 180, 445–454
4. St. Croix, B., Sheehan, C., Rak, J. W., Florenes, V. A., Slingerland, J. M., and Kerbel, R. S. (1998) J. Cell Biol. 142, 557–571
5. Aoki, J., Umeda, M., Takio, K., Taitani, K., Utsumi, H., Sasaki, M., and Inoue, K. (1991) J. Cell Biol. 115, 1751–1761
6. Takahashi, K., and Suzuki, K. (1996) Exp. Cell Res. 226, 214–222
7. Kandikonda, S., Oda, D., Niederman, R., and Sorkin, B. C. (1996) Cell Adhes. Commun. 4, 13–24
8. Cavallaro, U., and Christofori, G. (2004) Nat. Rev. Cancer 4, 18–32
9. Ishibashi, K., Sasaki, S., Sakamoto, H., Nakamura, Y., Hata, T., Nakamura, T., and Marumo, F. (1992) Biochem. Biophys. Res. Commun. 191, 960–965
10. Balkovetz, D., Pollack, A., and Mostov, K. (1997) J. Biol. Chem. 272, 3471–3477
11. Nusrat, A., Parkos, C., Bacarra, A., Godowski, P., and Madara, J. (1994) J. Clin. Investig. 93, 2056–2065
12. Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M., and Ito, F. (1994) Cell Adhes. Commun. 1, 295–305
13. Elliott, B. E., Hung, W. L., Boag, A. H., and Tuck, A. B. (2002) Can. J. Physiol. Pharmacol. 80, 91–102
14. Carraway, K. L., Perez, A., Idris, N., Jepson, S., Arango, M., Komatsu, M., Haq, B., Price-Schiavi, S. A., Zhang, J., and Carothers Carraway, C. A. (2002) Prog. Nucleic Acid Res. Mol. Biol. 71, 149–185
15. Nagy, P., Friedlander, E., Tanner, M., Kapanen, A. L., Carraway, K. L., Isola, J., and Jovin, T. M. (2005) Cancer Res. 65, 473–482
16. Moniaux, N., Andrianifahanana, M., Brand, R. E., and Batra, S. K. (2004) Br. J. Cancer 91, 1633–1638
17. Sheng, Z. Q., Hull, S. R., and Carraway, K. L. (1990) J. Biol. Chem. 265, 8505–8510
18. Hull, S. R., Sheng, Z., Vanderpuye, O., David, C., and Carraway, K. L. (1990) Biochem. J. 265, 121–129
19. Sheng, Z., Wu, K., Carraway, K. L., and Fregien, N. (1992) J. Biol. Chem. 267, 16341–16346
20. Ramsauer, V. P., Carothers Carraway, C. A., Salas, P. J., and Carraway, K. L. (2003) J. Biol. Chem. 278, 30142–30147
21. Carraway, K. L., III, Rossi, E. A., Komatsu, M., Price-Schiavi, S. A., Huang, D., Guy, P. M., Carvajal, M. E., Fregien, N., Carothers Carraway, C. A., and Carraway, K. L. (1999) J. Biol. Chem. 274, 5263–5266
22. Jepson, S., Komatsu, M., Haq, B., Arango, M. E., Huang, D., Carothers Carraway, C. A., and Carraway, K. L. (2002) Oncogene 21, 7524–7532
23. Ramsauer, V. P., Pino, V., Carothers Carraway, C. A., Salas, P., and Carraway, K. L. (2006) Mol. Biol. Cell 7, 2931–2941
24. Wu, K., Fregien, N., and Carraway, K. L. (1994) J. Biol. Chem. 269, 11950–11955
25. Komatsu, M., Carraway, C. A., Fregien, N. L., and Carraway, K. L. (1997) J. Biol. Chem. 272, 33245–33254
26. Komatsu, M., Tatsumi, T., Altman, N. H., Carothers Carraway, C. A., and Carraway, K. L. (2000) Int. J. Cancer 87, 480–486
27. Komatsu, M., Jepson, S., Arango, M. E., Carothers Carraway, C. A., and Carraway, K. L. (2001) Oncogene 20, 461–470
28. Komatsu, M., Yee, L., and Carraway, K. L. (1999) Cancer Res. 59, 2229–2236
29. Van Deurs, B., Zou, Z. Z., Briand, P., Balslev, Y., and Petersen, O. W. (1987) J. Histochem. Cytochem. 5, 461–469