Transgenic MUC1 Interacts with Epidermal Growth Factor Receptor and Correlates with Mitogen-activated Protein Kinase Activation in the Mouse Mammary Gland*

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MUC1 is a large (>400 kDa), heavily glycosylated transmembrane protein that is aberrantly expressed on greater than 90% of human breast carcinomas and subsequent metastases. The precise function of MUC1 overexpression in tumorigenesis is unknown, although various domains of MUC1 have been implicated in cell adhesion, cell signaling, and immunoregulation. Stimulation of the MDA-MB-468 breast cancer line as well as mouse mammary glands with epidermal growth factor results in the co-immunoprecipitation of MUC1 with a tyrosine-phosphorylated protein of ~180 kDa. We have generated transgenic lines overexpressing full-length (MMF), cytoplasmic tail deleted (ΔCT), or tandem repeat deleted (ΔTR)-human MUC1 under the control of the mouse mammary tumor virus promoter to further examine the role of MUC1 in signaling and tumorigenesis. Immunoprecipitation experiments revealed that full-length transgenic MUC1 physically associates with all four erbB receptors, and co-localizes with erbB1 in the lactating gland. Furthermore, we detected a sharp increase in ERK1/2 activation in MUC1 transgenic mammary glands compared with Muc1 null and wild-type animals. These results point to a novel function of increased MUC1 expression, potentiation of erbB signaling through the activation of mitogenic MAP kinase pathways.

The transmembrane mucin MUC1 (DF3, CD227, episialin, PEM) is a heavily O-glycosylated protein expressed on most secretory epithelium, including the mammary gland as well as some hematopoetic cells. MUC1 is expressed abundantly in the lactating mammary gland in addition to being overexpressed in greater than 90% of human breast carcinomas and metastases (1). In the normal mammary gland, MUC1 is expressed mainly on the apical surface of glandular epithelium and is believed to play a role in anti-adhesion and immune protection (2-4). In breast cancer, MUC1 is overexpressed, underglycosylated, and apical localization is lost (2). Mice lacking Muc1 demonstrate no overt phenotypic developmental abnormalities in the mammary gland, but when crossed with the tumorigenic MMTV°-mTag transgenic line (5), mammary gland tumor growth was significantly slowed. Additionally, these Muc1-null/MMTV-mTag transgenics demonstrated a trend toward decreased metastasis, showing that the absence of Muc1 results in both reduced tumor growth and spread (6).

MUC1 is transcribed as a large precursor gene product, which, upon translation, is cleaved in the endoplasmic reticulum, yielding two separate proteins that form a heterodimeric complex, bound together by noncovalent interactions (7). The larger of the two components (the “mucin-like” subunit) contains most of the extracellular domain, including the signal sequence, tandem repeats, as well as some degenerate repeats. The tandem repeats consist of 30 to 90 repeat sequences of 20 amino acids, rich in serine and threonine residues. Approximately 50–90% of the mass of MUC1 is derived from O-glycosylation that occurs on these amino acids (8)). The second component of the heterodimer consists of an extracellular stem (where the two protein products are joined), the hydrophobic transmembrane domain, and a short, 72-amino acid cytoplasmic domain. The cytoplasmic domain contains potential docking sites for Src homology domain 2 containing proteins, as well as a variety of putative kinase recognition sites and is tyrosine-phosphorylated in vitro (9, 10). There are 7 tyrosine residues in the cytoplasmic tail, which are highly conserved across species (10).

MUC1 interacts with a number of proteins implicated in neoplasia through both its tandem repeat and cytoplasmic domains. The tandem repeat region can act as a ligand for intercellular adhesion molecule 1 on human umbilical vein endothelial cell monolayers, indicating a potential role in metastatic intravasation (11, 12). Additionally, MUC1 binds β-catenin and GSK3β, through motifs in the cytoplasmic tail similar to those found in the adenomatous polyposis coli protein, leading to a reduction in the binding of β-catenin to E-cadherin in ZR-75-1 breast carcinoma cells (13, 14). This could potentially subvert E-cadherin-mediated cell adhesion in epithelial cells, promoting cell migration (13). Additionally, studies in MCF-7 breast carcinoma cells demonstrated that upon phosphorylation, MUC1 can bind Grb2/SOS (15), signaling mediators of a number of receptor tyrosine kinases.

One family of transmembrane tyrosine kinases, erbB receptors (including erbB1 or epidermal growth factor receptor (EGFR), erbB2, erbB3, and erbB 4) are expressed dynamically during mammary gland development (16) and are commonly...
implicated in breast cancer initiation and progression in both humans and rodents (17, 18). Overexpression of either the receptors or ligands in this family generally occurs in advanced, metastatic disease, resulting in poor overall patient outcomes (17). Ligands of the epidermal growth factor family (including EGFr-like members and neuregulin family members) induce dimerization of these receptors, leading to the activation of a variety of effector proteins including Src, phosphatidylinositol 3-kinase, She, phospholipase Cγ, STAT, Grb2/SOS, and others (19–22). The activation of many of these proteins results in the phosphorylation of nuclear translocating kinases, including SAPK/JNK and the MAP kinases, p38 and ERK1/2 (23–25). One mechanism of MAP kinase activation is through the recruitment of the Grb2/SOS complex to the phosphorylated receptor, resulting in Ras activation and phosphorylation of Raf, MEK, and ERK1/2. Upon activation, ERK1/2 can translocate to the nucleus and induce transcription of a variety of genes involved in mitogenesis, differentiation, apoptosis, and quiescence (17, 19, 26).

To explore signaling roles of MUC1 in the mammary gland, we have generated a number of transgenic animals overexpressing full-length and deletion constructs of human MUC1 in the mouse mammary gland using the mouse mammary tumor virus (MMTV) promoter. We have demonstrated that treatment with exogenous betacellulin, in addition to other EGFr ligands, can induce tyrosine phosphorylation of MUC1 in culture. Immunoprecipitation and co-localization experiments have revealed a physical interaction between MUC1 and EGFr that occurs through the cytoplasmic tail of MUC1. Furthermore, we demonstrate that EGF-dependent activation of ERK1/2 MAPK is strongly induced in the presence of high levels of MUC1 in the mouse mammary gland.

MATERIALS AND METHODS

Transgenic Constructs—Muc1 knockout animals have been described previously (6). The 42 tandem repeat human MUC1 (27), human MUC1 lacking the cytoplasmic tail (28), or human MUC1 lacking the tandem repeat domain (ΔTR) were cloned behind the MMTV LTR promoter (5) via HindIII and EcoRI sites. The FLAG epitope tag was engineered into all constructs, with the tag in the full-length and ΔTR construct inserted in the BsmI site (a gift from M. A. Hollingsworth (27)). The FLAG epitope in the cytoplasmic tail deleted construct (ΔCT) was generated by polymerase chain reaction using the following primer pairs and used to replace the AatII (forward primer A, beginning at base pair 928) and BsmI (reverse primer B) in the mouse MUC1 clone (28): Primer A, 5′-TCAGACTGCTAGCAGTGTGATCGCCGA-3′ (cloning site bolded); Primer B, 5′-GCCCCTTTCGAATTCGCTGCTG-3′ (cloning site bolded, FLAG epitope tag italicized). Constructs were excised using SauI and SpeI (New England Biolabs), purified using QiaQuick (Qiagen), and injected into FVB-fertilized oocytes (Mayo Clinic Scottsdale Transgenic Core Facility). Potential founders were screened by Southern blot using a probe generated using BsmI/HindIII that hybridizes to a segment of the MUC1 construct (the size varies with the construct) and the SV40 untranslated region. Three different lines of MUC1 transgenic mice were created, expressing either full-length or tandem-MUC1 overexpression and the contributions of the various tissues. Dilutions for the antibodies are as follows: B27.29-HRP, 1:100, CT2, 1:200, EGFR, 1:250, dERK, 1:400, PCNA, 1:100.

RESULTS

MMTV-MUC1 Transgenics—To investigate the effects of MUC1 overexpression and the contribution of the various MUC1 subdomains to signaling, transgenic animals were created. Transgenic constructs were derived by inserting the MUC1 cDNA (see below) into the construct designed by Guy et al. (5) which uses the MMTV long terminal repeat promoter and SV40 3′-untranslated region. Three different lines of MUC1 transgenic mice were created, expressing either full-length human MUC1 (MFM), cytoplasmic tail deleted human MUC1 (ΔCT), both on the wild-type background, or tandem-repeat domain-deleted human MUC1 (ΔTR), on the Muc1 null (Muc1−/−) background (Fig. 1A). MFM was created using the FLAG epitope-tagged 42-tandem repeat human MUC1 described by Burdick et al. (27). Expression was detected in the virgin (data not shown), pregnant (Fig. 1, B–D), lactating (Fig. 1C), and post-lactational (data not shown) mammary gland by Western blot and immunohistochemical analysis (Fig. 2). The expression of transgenic MUC1 compared with wild-type Muc1 in the pregnant gland is shown in Fig. 1D. Note that ΔTR is on the Muc1−/− background and displays levels comparable to the wild-type. This is in contrast to MFM (wild-type background) where expression levels are substantially higher than in the nontransgenic counterparts. Analyses using B27.29 and HMFG-2 antibodies to the tandem repeat domain, CT2

against mouse and human MUC1 appears similar to CT1 in immuno-
precipitation, immunoblot, and immunohistochemistry (32, 33). Anti-
bodies to erbB1, erbB2, erbB3, and erbB4 as well as Grb2, SOS, and 
FCNA-HRP were all from Santa Cruz, and ERCT was a kind gift from 
H. S. Earp (University of North Carolina, Chapel Hill, NC). The phos-
lipase Cγ antibody (RC20-HRP) was from Transduction Laborato-
ries. HRP-conjugated secondary antibodies for Western blot analysis were from Pierce and Jackson Laboratories and Alexa-conjugated sec-
ondary antibodies for confocal imaging were from Molecular Probes. Dual-phosphorylated ERK antibody is from Sigma and p42/44, phos-
pho-p38, and phospho-SAPK/JNK are from New England Biolabs Cell 
Signalting.

Protein Analysis—Glands were homogenized in Triton X-100 lysis buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM sodium orthovanadate, 50 mM ammonium molybdate, 10 mM sodium fluoride, and Complete inhibitor mixture (Sigma). BCA assays (Pierce) were performed to determine protein concentration and samples were stored frozen at −80 °C.

Immunoprecipitations were performed with 0.5–4.0 mg of protein 
lysat, using Protein A/G-agarose conjugate (Santa Cruz). Western 
blots were performed using 200 μg of protein lystate per sample. 
Samples were separated by SDS-PAGE and transferred to polyvinylidene 
difluoride membrane (Mobiilon) for Western blot analysis.

In Vitro Kinase Assay—A GST fusion protein (generated in the 
plasmid pGEX2TK expression vector (Amersham Pharmacia Biotech)) contain-
ing the 72-amino acid cytoplasmic tail of MUC1 (GST-CT) was purified 
with glutathione-Sepharose and used as the substrate. The kinase 
reaction contained 25 mM HEPES, pH 7.5, 120 μM γ-[32P]ATP (3000– 
5000 cpn/ml), 50 mM sodium vanadate, 2.2 mM GST-CT, and 0.5 μg/ml 
EGFR kinase domain (Stratagene). Reactions were incubated at 22 °C 
for 10 min, the proteins resolved by SDS-PAGE, and exposed to film. 
Negative control was 3.1 μg pGEX-2TK protein (GST).

Immunofluorescence—Tissues were fixed in methacarn, paraffin em-
bedded (Mayo Clinic Scottsdale Histology core), and either 5 μm (brightfield) or 20 μm (confocal) sections were cut. Slides were depara-
inized in xylene, rehydrated, preincubated in washing buffer (Immunex), 
blocked in normal goat serum and incubated with primary antibodies 
on overnight at 4 °C. Slides were washed in washing buffer, incubated with either HRP- or fluorescent-conjugated secondary antibodies, 
washing in enhanced washing buffer, and for immunohisto-
chemistry, developed with 3,3'-diaminobenzidine (Santa Cruz Biotech-
nology) and counterstained with Mayer's hematoxylin (Sigma). For 
cellular microscopy, slides were coverslipped (1.5 μm) in antifade solution 
(Molecular Probes) and visualized with a Zeiss laser scanning micro-
scope 510, and analyzed using LSM 510 software version 2.5. Negative 
controls included antibody-specific peptide blocking and Muc1 knock-
out tissues. Dilutions for the antibodies are as follows: B27.29-HRP, 1:100, 
CT2, 1:200, EGFR, 1:250, dERK, 1:400, PCNA, 1:100.

Animals and Cell Lines—Mice were bred as described by Burdick et 
al. (5) and maintained under specific pathogenfree conditions. Cell 
lines were derived from tumors from the Muc1−/− mice on a C57BL6 
background (34). MDA-MB-321, a cell line derived from a breast tumor 
from a Muc1−/− mouse, was used as a positive control. All cell lines 
were tested and found free of mycoplasma contamination.

Antibodies—Antibodies to MUC1 included HMFG-2 (kindly provided 
by J. Taylor-Papadimitriou, ICRF, London, United Kingdom) and 
B27.29 (kindly provided by Biomira), both mouse monoclonals 
that react with the human tandem repeat domain, and CT1 (32) and CT2. 
CT2 is an Armenian hamster monoclonal antibody generated to the last 
17 amino acids of the cytoplasmic domain of MUC1. Its reactivity

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antibody to the cytoplasmic domain or antibodies to the FLAG epitope demonstrated a variety of glycosylation forms. CT2 detects a doublet that represents the cytoplasmic tail and transmembrane domain, as well as 58 amino acids of the extracellular region, up to the cleavage site at Ser-Val-Val-Val. These 58 extracellular amino acids contain 13 potential glycosylation sites (12 Ser/Thr and 1 Asn site), resulting in a 20–25-kDa cytoplasmic tail-containing species at all stages of development (Fig. 1D and data not shown). An ~200-kDa form was detected during pregnancy and lactation with HMFG-2 (Fig. 1C), whereas a >300-kDa form was apparent most often during late pregnancy and lactation with B27.29 (Fig. 1C). Note that B27.29 and HMFG-2 detect only human MUC1 whereas CT2 detects both mouse and human Muc1. HMFG-2 reacts most strongly with the ~200-kDa species, while occasionally reacting with the >300-kDa form. B27.29, on the other hand, reacts most strongly with the largest form (while also recognizing the ~200-kDa form), and has been previously characterized as binding better to highly glycosylated MUC1 (30). Importantly, we found that the FLAG epitope was seemingly masked by glycosylation in the mammary gland, as we were unable to detect the >300-kDa form with anti-FLAG reagents (Fig. 1B).
A type Muc1, MMF (Fig. 2) and physiologically relevant sites, immunohistochemistry was performed with a pp180 in cell culture. A, in vitro phosphorylation reactions on the fusion protein of the MUC1 cytoplasmic tail with glutathione S-transferase (GST-CT) and GST alone. EGFR KD (auto) represents autophosphorylation that occurs in the absence of excess cold ATP. B, MDA-MB-468 cells treated with increasing concentrations of betacellulin (BTC) immunoprecipitated (IP) with anti-MUC1 (CT1) and immunoblotted with anti-phosphotyrosine (RC20-HRP). Arrows indicate the tyrosine-phosphorylated protein ~180 kDa (top) and the cytoplasmic tail of MUC1 (bottom). IB, immunoblotted.

The ΔCT transgenic was derived from the cytoplasmic tail deleted clone generated by Pemberton et al. (28). This clone contains the putative stop transfer sequence, Arg-Arg-Lys, of the cytoplasmic domain, followed by the FLAG epitope tag on its C-terminal end. Consistently high expression was detected with both B27.29 and HMFG-2 antibodies in the pregnant and lactational mammary glands of the ΔCT transgenic animals (Fig. 1C). The ΔTR transgenic was generated on the Muc1+/− background and contains 3 N-glycosylation sites (1 on the CT domain region and 2 on the extracellular domain). Additionally 30% of the amino acids contained in the extracellular domain are potential sites for O-glycosylation. As the tandem repeat domain is missing, ΔTR is detected using the FLAG or CT2 antibodies (Fig. 1, B and D). The apparent molecular mass of ΔTR extracellular domain is ~50 kDa, indicating that the transgenic protein is N- and O-glycosylated. Also, relative expression of the transgene in this founder line is lower than that observed for MMF or ΔCT (Figs. 1D and 2).

To determine whether the transgenic proteins trafficked to physiologically relevant sites, immunohistochemistry was performed. Pregnant and lactating glands displayed predominantly apical staining for all constructs, although cytoplasmic staining was also observed (Fig. 2). Note that similar to wild-type Muc1, MMF (Fig. 2A) and ΔCT (Fig. 2B) are detected in the lumen of the lactating alveoli, presumably either shed or present on the plasma membrane during the release of milk proteins and fat into the lumen. Although not quantitative, the lower level of expression in the ΔTR transgenic is also apparent here (Fig. 2C).

MUC1 Co-immunoprecipitates a pp180 in Response to EGF Family Ligand Treatment—The cytoplasmic domain of MUC1 is tyrosine-phosphorylated both in vitro (Fig. 3B, lower arrow) and in vivo (Fig. 4A). To determine the mechanism of this phosphorylation, a panel of potential kinases were analyzed for activity with MUC1, and phosphorylation was observed with EGFR kinase, among others (Fig. 3A). To examine phosphorylation of MUC1 by the EGFR kinase in culture, multiple EGF family ligands were used to treat MDA-MB-468 and T47D mammary carcinoma cells. Phosphorylation of MUC1 could be induced in a dose-dependent manner with betacellulin in MDA-MB-468 but not T47D cells (Fig. 3B and data not shown). Additionally, phosphorylation was induced with EGF, amphiregulin, and transforming growth factor-α, but not NGFs in MDA-MB-468 cells (data not shown). Treatment with any of these ligands (except NGFs) resulted in the co-immunoprecipitation of a tyrosine-phosphorylated protein of ~180 kDa (pp180) with MUC1 in MDA-MB-468 cells (Fig. 3B, top arrow, and data not shown). To determine whether this interaction was physiologically relevant to the intact mammary gland, pregnant and lactating glands from both wild-type and transgenic mice were injected intraperitoneally with receptor grade EGF, and mammary gland lysates prepared. The pp180 could also be readily identified in vivo as co-immunoprecipitating with MUC1 using antibodies to both the tandem repeat region.

Fig. 3. EGFR kinase phosphorylates MUC1 and MUC1 associates with a pp180 in cell culture. A, in vitro phosphorylation reactions on the fusion protein of the MUC1 cytoplasmic tail with glutathione S-transferase (GST-CT) and GST alone. EGFR KD (auto) represents autophosphorylation that occurs in the absence of excess cold ATP. B, MDA-MB-468 cells treated with increasing concentrations of betacellulin (BTC) immunoprecipitated (IP) with anti-MUC1 (CT1) and immunoblotted with anti-phosphotyrosine (RC20-HRP). Arrows indicate the tyrosine-phosphorylated protein ~180 kDa (top) and the cytoplasmic tail of MUC1 (bottom). IB, immunoblotted.

Fig. 4. MUC1 cytoplasmic tail is tyrosine phosphorylated and associates with a pp180. A, in each lane, 2 μg of lysate were immunoprecipitated with anti-MUC1 (CT2) and immunoblotted with anti-phosphotyrosine (RC20-HRP). Lanes identified as (+) represent animals injected with 1 μg/g body weight EGF, while (−) represents endogenous EGF. B, mammary gland lysates (4 μg) were immunoprecipitated with anti-MUC1 antibodies (CT2, B27.29, or HMFG-2) and immunoblotted with anti-phosphotyrosine (RC20-HRP). The lysate only lane represents 200 μg of protein, and the pp180 in the no EGF lane is due to endogenous phosphorylation. C, MUC1 in mammary gland tumors from MMTV-mTag transgenic animals also co-immunoprecipitates pp180. Lanes represent individual animals, either treated or untreated with EGF before sacrifice, and 2 μg of lysate were immunoprecipitated with anti-MUC1 (CT1) and immunoblotted with anti-phosphotyrosine (RC20-HRP).

3 W. Xie and S. J. Gendler, unpublished data.
or the cytoplasmic tail (Fig. 4B). Additionally, a pp \( \approx 120 \) kDa and pp \( \approx 250 \) kDa also co-immunoprecipitated in the MMF samples, but not in the wild-type. On lighter exposure, the designated pp180 band is not a single protein species in the mammary gland, indicating it either represents multiple forms of one protein or multiple proteins of the same apparent size. As MUC1 is commonly overexpressed in breast cancer, we next examined if the interaction with the pp180 was detectable in a mouse tumor model. We observed the co-immunoprecipitation of a pp180 with MUC1 using tumor protein lysates derived from MMTV-mTag transgenic mice (5) treated with exogenous EGF (Fig. 4C). This interaction in mammary tumors indicates that this Muc1-pp180 association is not unique to the normal mammary gland, as is indicated by the data from the MDA-MB-468 cell line (Fig. 3).

**EGFR Physically Associates with MUC1**—Members of the erbB receptor tyrosine kinase family range in size from 170 to 190 kDa. To determine whether the co-immunoprecipitating pp180 was one or more of the erbB receptors, mammary gland lysates were immunoprecipitated with an antibody to the erbB proteins and blotted with MUC1 antibodies. EGFR and MUC1 complexes were observed in lysates from both wild-type animals and MMF transgenics using antibodies to both the tandem repeat region and the cytoplasmic domain (Fig. 5A and data not shown). While co-immunoprecipitation experiments demonstrate an interaction between full-length MUC1 and EGFR, this interaction is markedly reduced in the ΔCT transgenic (Fig. 5A). Complexes between MUC1 and the remaining 3 erbB receptors could also be identified in pregnant and lactating mammary glands (Fig. 5C). Again, little to no ΔCT MUC1 protein could be found precipitating with erbB2, erbB3, or erbB4 antibodies. Importantly, equal if not more of the ΔCT MUC1 protein is present in the mammary glands where little to no co-immunoprecipitation was observed (Fig. 5C, bottom panel).
**Day 2 Lactating Mammary Gland**

**EGFR**  
**MUC1**  
**EGFR/MUC1**

**Fig. 6. MUC1 and EGFR colocalize in the lactating mammary gland.** Paraffin sections (20 µm) were probed with anti-EGFR (ISC, 1005) and anti-MUC1 (CT2) primary antibodies and Alexa 594 streptavidin/biotin-anti-rabbit and fluorescein isothiocyanate anti-Armenian hamster secondary antibodies. These were examined at x 400 magnification using a 510 laser scanning microscope. Arrows (D) indicate areas of intense co-localization.

Therefore, as MMF and ΔCT transgenic MUC1 proteins are both present in the same cellular location at high levels (Fig. 2, A and B), these results suggest a requirement for the MUC1 cytoplasmic tail in this interaction with the erbB receptors.

**MUC1 and EGFR Co-localize to the Apical Membrane**—To give insight to the localization of this complex formation in the gland, we used confocal microscopy to analyze MUC1/EGFR co-localization. We have localized MUC1 to the apical membrane during pregnancy and lactation, and observed it also in the alveolar lumen during lactation (Figs. 2 and 6B). Using antibodies to EGFR, we detected protein throughout the alveolar epithelium during both pregnancy and lactation (Fig. 6A), as has been previously reported (16). Dual staining for EGFR and MUC1 revealed that they are co-localized mainly in the apical membrane proximal region (Fig. 6C). Furthermore, by removing all but the most intensely dual-staining colors through computer enhancement, we determined that the co-localization appears to be concentrated at points of cell-cell contact (Fig. 6D).

**MUC1 Effects EGF-dependent Signaling**—We next investigated the potential effects of MUC1 overexpression on EGF signaling. To determine whether the presence or absence of MUC1 affected the ability of EGF to autophosphorylate, transgenic and Muc1-null animals were injected intraperitoneally with receptor-grade EGF, and mammary gland lysates prepared. We detected similar levels of phosphorylation of the EGFR in both transgenic and knockout animals in response to EGF treatment (Fig. 7). Multiple kinase pathways lie downstream of EGF activation, and we next explored whether MUC1 overexpression promotes signaling through these molecules in the mammary gland. Using antibodies directed against the phosphorylated forms of p38, p42/44 ERK1/2 (dpERK), and p46/54 SAPK/JNK, we observed a striking pattern of activation. MMF, Muc1 knockout, and wild-type animals were injected intraperitoneally with receptor-grade EGF to stimulate signaling in the mammary gland. Upon stimulation, phosphorylated ERK1/2 was strongly induced in MMF lactating mammary gland, while it was detectable in comparably low amounts in the wild-type and Muc1−/− lactating mammary gland (Fig. 8A). ERK1/2 is activated in the wild-type pregnant gland, making phosphorylation increases in the transgenic lysates difficult to detect. Given this, we do observe an increase in phospho-ERK1/2 in some MMF pregnant lysates compared with the pregnant gland of wild-type mice (Fig. 8B). We observed that this activation of ERK is limited to early lactation (day 2/3), as by day 10 lactation, dpERK levels in transgenic glands resembled that of the wild-type (Fig. 8B). The overall levels of ERK1/2 are similar in both wild-type and transgenic mammary glands (Fig. 8B, bottom panel). Note that lysates from some MMF transgenic mammary glands do not show ERK1/2 activation. This may be due to reduced amounts of EGF reaching the gland in that experiment, different physiological makeup of that particular gland, or simply missing the kinetic window of kinase activation with that animal. Importantly, activation of ERK1/2 was consistently and repeatedly demonstrated in mammary gland lysates from EGF-injected MMF transgenics. Phospho-p46 appeared similar in all genotypes and conditions examined (Fig. 8C), and while p54 shows a modest increase of phosphorylation in some samples (Fig. 8C), this increase was not duplicated in subsequent experiments. Phosphorylated p38 was undetectable by these methods. These results indicate that only one of the kinase pathways analyzed, ERK1/2, is selectively activated in response to heightened levels of full-length MUC1 in the lactating mammary gland.

We also examined potential EGF effector proteins for involvement in MUC1 signal transduction to ERK1/2 activation. It has been previously reported that Grb2/SOS associates with MUC1 in breast carcinoma cell lines (15). We detected MUC1 co-immunoprecipitating with both Grb2 and SOS in wild-type, MMF, and ΔTR mammary gland lysates (Fig. 9). Collectively, these results further implicate the presence of Muc1 in a complex with EGFR in the mammary gland.

To examine potential effects of activated MAPK, we have investigated the possibility of increased mitogenesis by comparing nuclear staining (data not shown) and immunoblot detection of PCNA (proliferating cell nuclear antigen). We compared levels of PCNA in wild-type, knockout, and transgenic animals, and observed no significant difference between the groups over multiple samples (Fig. 8D).

**DISCUSSION**

In an effort to recapitulate the overexpression of MUC1 observed in human breast cancer, we have generated transgenic animals that overexpress both full-length and domain-deleted human MUC1 in the mouse mammary gland. The mammary glands of these transgenics appear developmentally and functionally normal, and transgene expression is localized to the apical border of both ducts and alveoli of the mammary gland. We have demonstrated that MUC1 co-localizes with and physically interacts with members of the erbB receptor kinase family. Finally, we have demonstrated a strong activation of dual-phosphorylated p42/44 ERK in the presence of transgenic, full-length MUC1 in the lactating mammary gland.

The interaction between transmembrane mucins and members of the erbB family has been demonstrated previously. Caraway et al. (34) demonstrated a co-immunoprecipitation between erbB2 and MUC4 (ASGP1 and -2) in both the metastatic ascites 13762 rat mammary carcinoma cell line as well the pregnant rat. Unlike MUC1 and EGFR, this interaction occurs in the extracellular domain of the proteins. Furthermore, increased proliferation rates and a potentiation of NRG signaling correlates with MUC4 expression. Interestingly, they
have recently shown that regulation of MUC4 expression is dependent upon activation of the ERK pathway in 13762 cells (35). MUC4 is also a transmembrane member of the mucin family, with a processed protein core that is heavily \( \text{O} \)-glycosylated, similar to MUC1 (36). Unlike MUC1, MUC4 contains EGF-like repeats in the extracellular portion of its membrane-spanning domain, which appear to be responsible for its interactions with erbB2. While MUC4 appears to interact only with erbB2, MUC1-erbB associations appear to be much more permissive, although modulation of Grb2/SOS interactions with the erbBs is restricted only to EGFR (see below). Interestingly, ligand-independent activation of EGFR and subsequent downstream MAPK activation has been recently described by Pece and Gutkind (37) through interactions with E-cadherin. This is further evidence that the activity of erbB family of transmembrane receptors can be modulated by unique mechanisms in addition to activation by cognate ligands.

It is important to note that while MUC1 could be detected in erbB immunoprecipitations by a variety of MUC1 antibodies, the erbB receptors could not be identified in MUC1 immunoprecipitations. This may be due to the extremely high levels of MUC1 being expressed and released into the alveolar lumen compared with the relatively modest levels of the erbB receptors present in the apical epithelium. Proportionately, only a very small fraction of the total MUC1 being expressed may be complexing with the erbB receptors while the opposite may be true for the erbBs at that cellular location. Additionally, as the pp180 that is identified in immunoprecipitates appears to be multiple bands of the approximate same size (Fig. 2), it is possible that the phosphotyrosine immunoblot is in fact detecting all four erbB receptors complexing with MUC1. If this is the case, the detection of a single erbB from the complex becomes increasingly difficult.

Pandey et al. (15) report interactions between MUC1 (DF3) and Grb2/SOS in MCF7 breast carcinoma cell lines through the Src homology domain 2 domain of Grb2, although no downstream signaling was reported. We were able to observe MUC1 directly interacting with Grb2 and SOS in mammary glands from both the full-length and tandem repeat-deleted MUC1 transgenic. As MUC1 has no intrinsic kinase domain, a possi-
MUC1 Associates with erbB Receptors

It is tempting to speculate that the modulation of EGFR signaling and MAP kinase activation are a component of the mechanism of MUC1-associated tumorigenesis. While aberrant MUC1 expression has been linked with a high percentage of breast carcinomas, the role of this overexpression is undefined. Indeed, there are several potential consequences for activation of the p42/p44 MAP kinase proteins including induction of proliferation, quiescence, apoptosis, and differentiation (40–42). We have investigated levels of PCNA in transgenic animals, and found no increase that would correlate to increased mitogene- sis. Alternately, we have examined the possibility of driving the cells into a state of G0 arrest by activating p21. This would potentially provide a population of cells that do not apoptose in response to the postapoptotic stimuli, and remain in the gland as potential targets of transformation (26, 43–45). While we have been unable to detect increased levels of p21 in preliminary experiments, we have observed a trend toward delayed regression in the postapoptotic glands of some MMF transgenic animals (data not shown). These possibilities are being explored in subsequent experiments.

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