

# 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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Joyce A. Schroeder, Melissa C. Thompson, Melissa Mockensturm Gardner,  
and Sandra J. Gendler‡

From the Mayo Clinic Scottsdale, S.C. Johnson Research Building, Scottsdale, Arizona 85259

**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 ( $\Delta$ CT), or tandem repeat deleted ( $\Delta$ TR)-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 hematopoietic 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<sup>1</sup>-

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  $\beta$ -catenin and GSK3 $\beta$ , through motifs in the cytoplasmic tail similar to those found in the adenomatous polyposis coli protein, leading to a reduction in the binding of  $\beta$ -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

toplasmic tail; PCNA, proliferating cell nuclear antigen; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; ERCT, EGFR cytoplasmic tail; MMF, MMTV-MUC1FLA6; NRG, neuregulin.

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‡ To whom all correspondence should be addressed: Mayo Clinic, S.C. Johnson Research Bldg., 13400 E. Shea Blvd., Scottsdale, AZ 85259. Tel.: 480-301-7062; Fax: 480-301-7017; E-mail: gendler.sandra@mayo.edu.

<sup>1</sup> The abbreviations used are: MMTV mouse mammary tumor virus; EGFR, epidermal growth factor receptor; TR, tandem repeat; CT, cy-

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 EGF-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, Shc, phospholipase C $\gamma$ , 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 ( $\Delta$ TR) were cloned behind the MMTV LTR promoter (5) via *Hind*III and *Eco*RI sites. The FLAG epitope tag was engineered into all constructs, with the tag in the full-length and  $\Delta$ TR construct inserted in the *Bsm*I site (a gift from M. A. Hollingsworth (27)). The FLAG epitope in the cytoplasmic tail deleted construct ( $\Delta$ CT) was generated by polymerase chain reaction using the following primer pairs and used to replace the *Aat*II (forward primer A, beginning at base pairs 1143 (8)) to *Eco*RI cassette (reverse primer B) in the CT3 MUC1 clone (28): Primer A, 5'-TCAGACGTCAGCGTGAGTGATGTCCCA-3' (cloning site bolded); Primer B, 5'-GCCCTTTTGAATTCGTCGTCGT-CATCCTTGTAATCGGCGGCACT-3' (cloning site bolded, FLAG epitope tag italicized). Constructs were excised using *Sa*I and *Spe*I (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 *Bam*HI/*Spe*I that hybridizes to a segment of the MUC1 construct (the size varies with the construct) and the SV40 3'-untranslated region of the cDNA. Expression of the transgene was confirmed in the founder lines by Western blot analysis using antibodies to the FLAG epitope (M2, Sigma), and MUC1 (B27.29, Biomira, (29, 30) HMFG-2 (29, 31), and CT2 (see below).

**Animals and Cell Lines**—All studies were performed on the FVB strain of mice with wild-type Muc1, transgenic hMUC1, or Muc1-null (6). Human MUC1 is designated MUC1 while the mouse homologue is designated Muc1. For EGF injection, 1  $\mu$ g/g body weight receptor grade mouse EGF (Sigma and Collaborative Biosciences) was injected intraperitoneally. After 20 min, animals were sacrificed and the mammary glands were harvested. T47D and MDA-MB-468 cell lines were from ATCC and cultured as suggested.

**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 which 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

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

**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  $\mu$ M 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  $-80^{\circ}$ C.

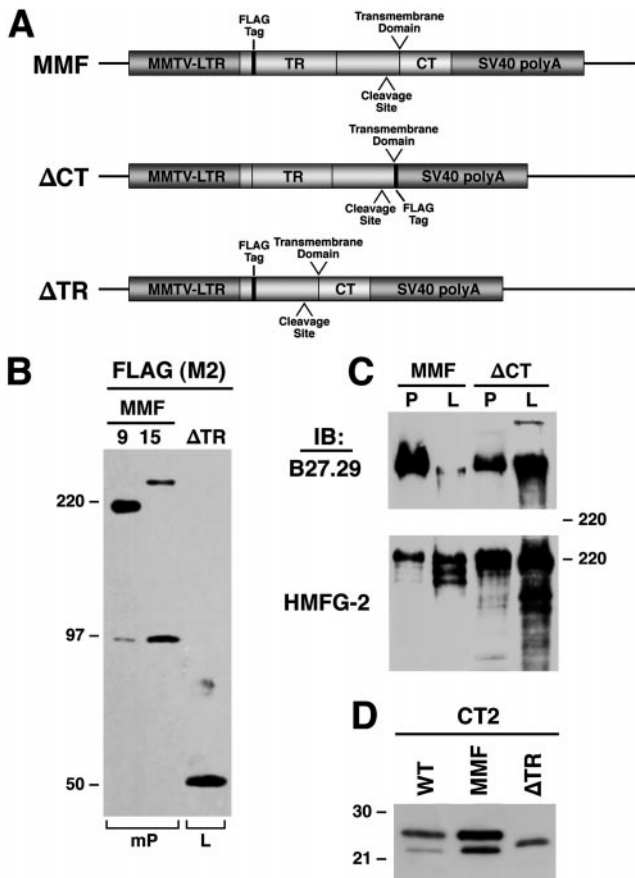
Immunoprecipitations were performed with 0.5–4.0 mg of protein lysate, using Protein A/G-agarose conjugate (Santa Cruz). Western blots were performed using 200  $\mu$ g of protein lysate per sample. Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon) for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

**In Vitro Kinase Assay**—A GST fusion protein (generated in the pGEX-2TK expression vector (Amersham Pharmacia Biotech)) containing the 72-amino acid cytoplasmic tail of MUC1 (GST-CT) was purified using glutathione-Sepharose and used as the substrate. The kinase reaction contained 25 mM HEPES, pH 7.5, 120  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (3000–5000 cpm/pmol), 50  $\mu$ M sodium vanadate, 2.2  $\mu$ M GST-CT, and 0.5  $\mu$ g/ml EGFR kinase domain (Stratagene). Reactions were incubated at 22  $^{\circ}$ C for 10 min, the proteins resolved by SDS-PAGE, and exposed to film. Negative control was 3.1  $\mu$ M pGEX-2TK protein (GST).

**Immunofluorescence**—Tissues were fixed in methacarn, paraffin embedded (Mayo Clinic Scottsdale Histology core), and either 5  $\mu$ m (brightfield) or 20  $\mu$ m (confocal) sections were cut. Slides were deparaffinized in xylene, rehydrated, preincubated in enhancing wash buffer (Immunex), blocked in normal goat serum and incubated with primary antibodies overnight at 4  $^{\circ}$ C. Slides were washed in enhancing wash buffer, incubated with either HRP- or fluorescent-conjugated secondary antibodies, washed in enhancing wash buffer, and for immunohistochemistry, developed with 3',3'-diaminobenzidine (Santa Cruz Biotechnology) and counterstained with Meyers hematoxylin (Sigma). For confocal microscopy, slides were coverslipped (1.5  $\mu$ m) in antifade solution (Molecular Probes) and visualized with a Zeiss laser scanning microscope 510, and analyzed using LSM 510 software version 2.5. Negative controls included antibody-specific peptide blocking and Muc1 knockout tissues. Dilutions for the antibodies are as follows: B27.29-HRP, 1:100, CT2, 1:200, EGFR, 1:250, dpERK, 1:400, PCNA, 1:100.

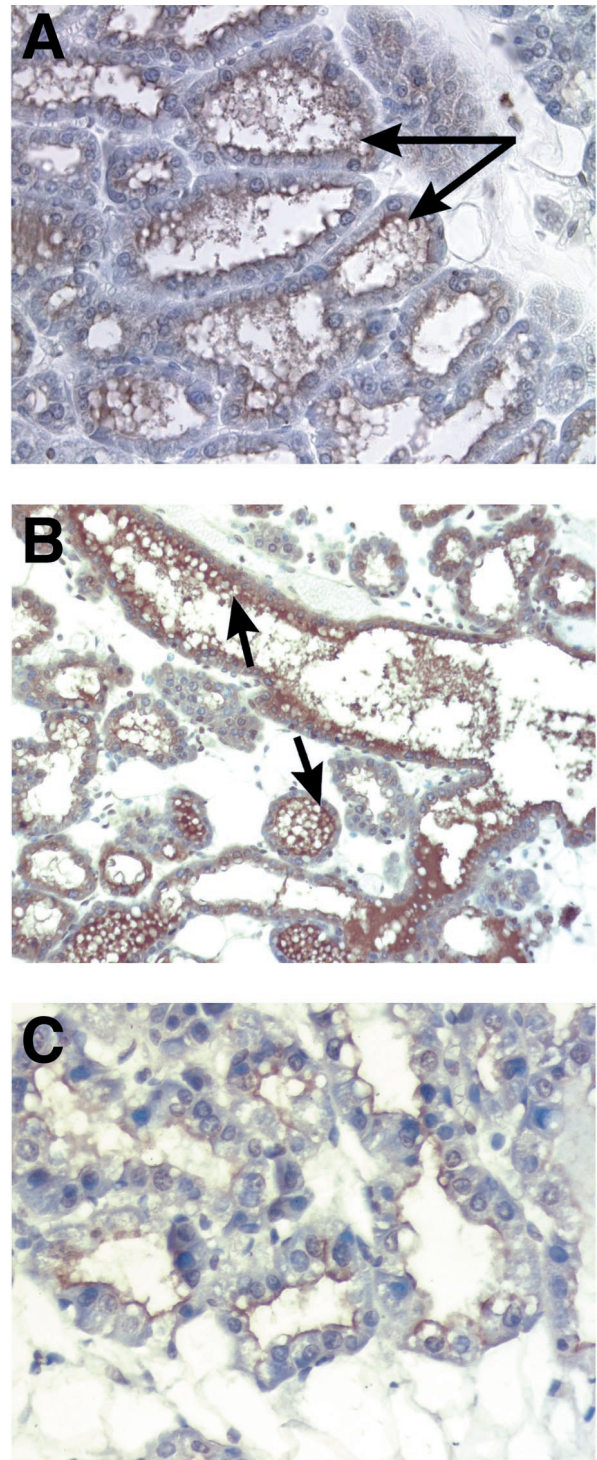
#### RESULTS

**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 (MMF), cytoplasmic tail deleted human MUC1 ( $\Delta$ CT), both on the wild-type background, or tandem-repeat domain-deleted human MUC1 ( $\Delta$ TR), on the Muc1 null (Muc1 $^{-/-}$ ) background (Fig. 1A). MMF 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 relative expression of transgenic MUC1 compared with wild-type Muc1 in the pregnant gland is shown in Fig. 1D. Note that  $\Delta$ TR is on the Muc1 $^{-/-}$  background and displays levels comparable to the wild-type. This is in contrast to MMF (wild-type background) where expression levels are substantially higher than in the nontransgenic counterpart. Analyses using B27.29 and HMFG-2 antibodies to the tandem repeat domain, CT2



**FIG. 1. Transgenic constructs and protein expression patterns in the mouse mammary gland.** A, diagram showing the MMTV-long terminal repeat (*LTR*) promoter driving expression of the various MUC1 cDNAs, including the 42 tandem repeat containing full-length cDNA (MMF), the cytoplasmic tail-deleted cDNA ( $\Delta$ CT), and tandem repeat domain-deleted cDNA ( $\Delta$ TR) constructs (not to scale). Note the placement of the FLAG epitope tag is in the extracellular region in both the MMF and  $\Delta$ TR, and in the intracellular region of the  $\Delta$ CT transgenic. B, immunoblot detection of transgenic proteins from day 10 pregnant (*mP*, MMF) or lactating ( $\Delta$ TR) glands. Lysates (600  $\mu$ g) were immunoprecipitated (*IP*) and immunoblotted (*IB*) using antibodies against the FLAG epitope tag (M2). The results from two separate founders (numbers 9 and 15) are shown for MMF. C, immunoblot detection of MMF and  $\Delta$ CT transgenic proteins from pregnant or lactating glands (200  $\mu$ g) using monoclonal antibodies against the tandem repeat region (B27.29 and HMFG-2). D, immunoblot of pregnant mammary gland lysates (200  $\mu$ g) from wild-type, MMF, and  $\Delta$ TR animals using an antibody to the cytoplasmic tail domain of MUC1 (CT2). P, pregnant; *mP*, midpregnant, L, lactating; V, 3–5-month-old virgin animals.

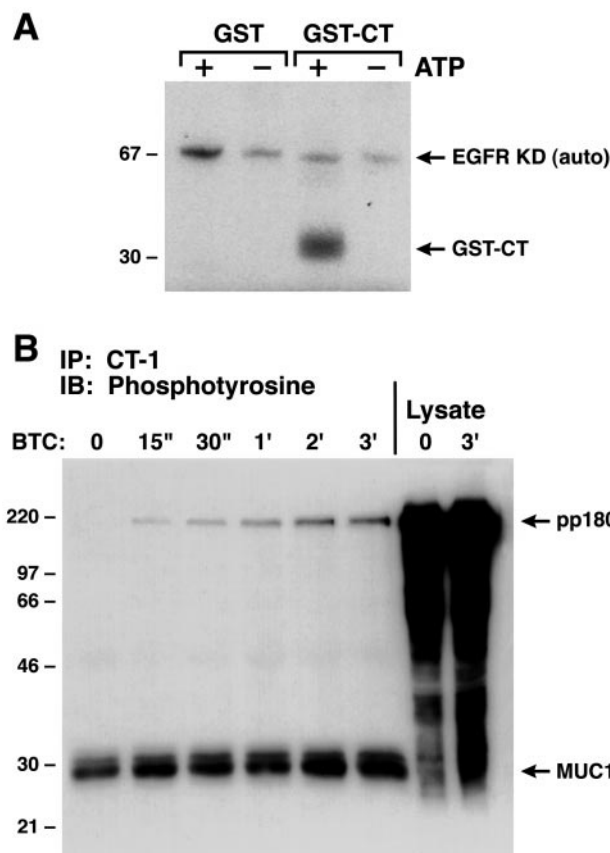
antibody to the cytoplasmic domain or antibodies to the FLAG epitope demonstrated a variety of glycosylation forms. CT2 detects a doublet that represents the cytoplasmic tail and transmembrane domain, as well as 58 amino acids of the extracellular region, up to the cleavage site at Ser-Val-Val-Val.<sup>2</sup> 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 occa-



**FIG. 2. Transgenic MUC1 is expressed on the apical side of ductal and alveolar epithelium.** Immunohistochemical detection of lactating mammary gland using B27.29-HRP for MMF and  $\Delta$ CT (A and B, respectively),  $\times 200$  magnification, and using CT2 for  $\Delta$ TR/Muc1<sup>-/-</sup> (C),  $\times 400$  magnification. Also note the staining of the luminal contents in both MMF and  $\Delta$ CT (arrows).

sionally 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).

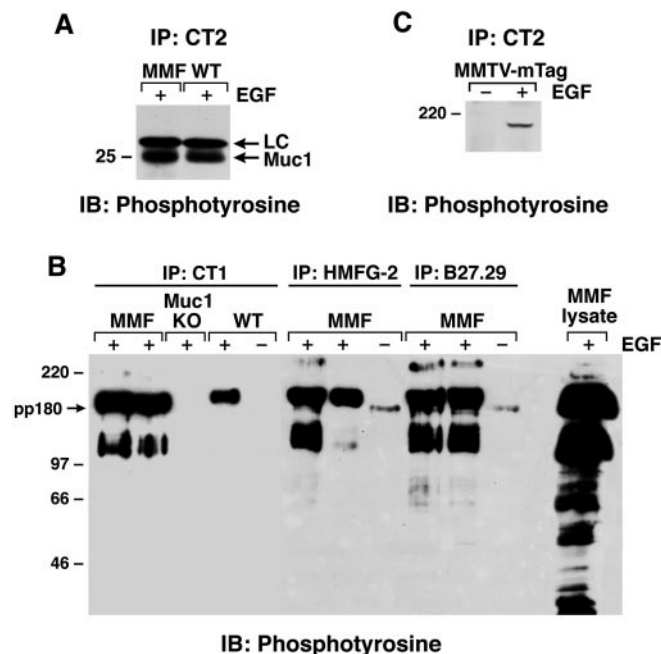
<sup>2</sup> A. Harris, personal communication.



**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.

The  $\Delta$ CT 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  $\Delta$ CT transgenic animals (Fig. 1C). The  $\Delta$ TR transgenic was generated on the *Muc1*<sup>-/-</sup> 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,  $\Delta$ TR is detected using the FLAG or CT2 antibodies (Fig. 1, *B* and *D*). The apparent molecular mass of  $\Delta$ TR extracellular domain is ~50 kDa, indicating that the transgenic protein is *N*- and *O*-glycosylated.<sup>3</sup> Also, relative expression of the transgene in this founder line is lower than that observed for MMF or  $\Delta$ CT (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  $\Delta$ CT (Fig. 2B) are detected in

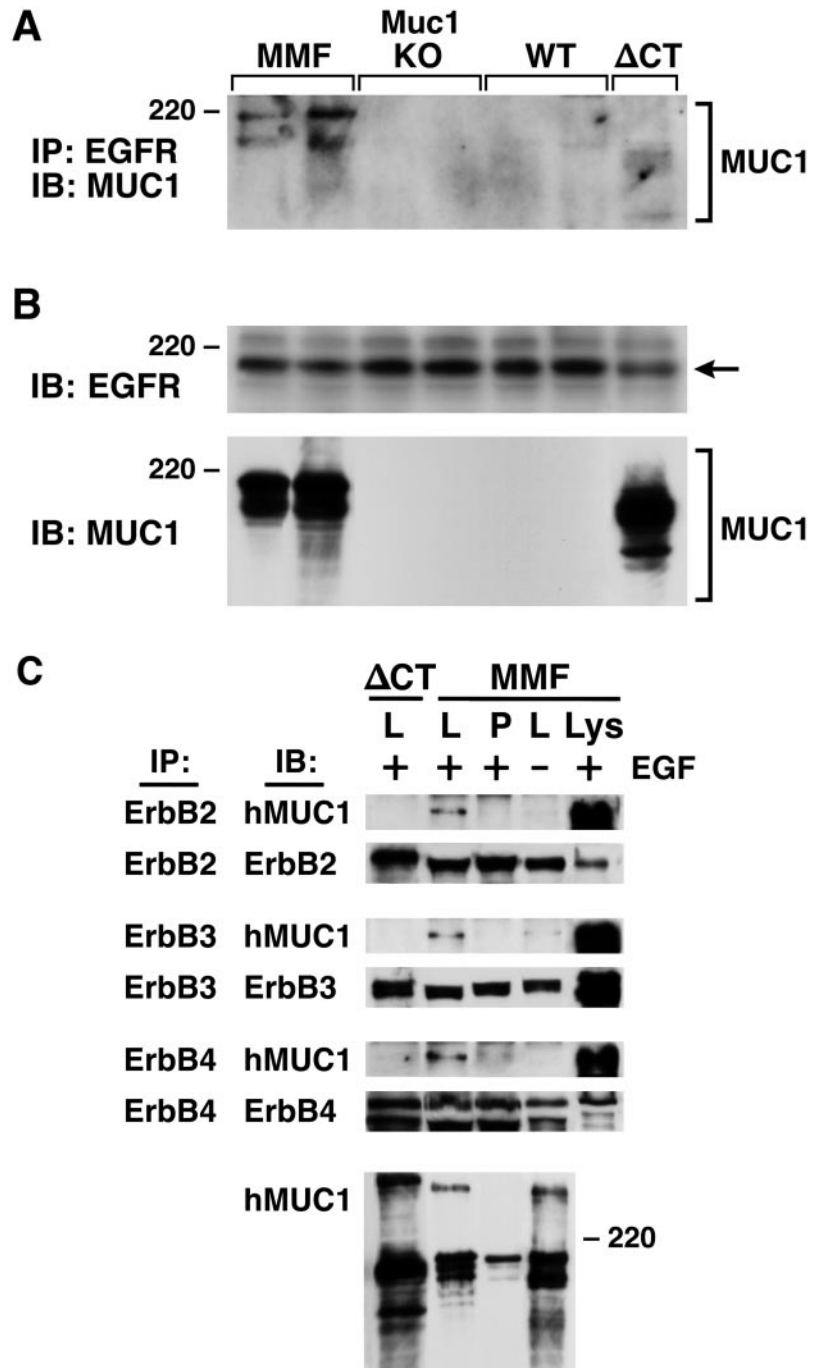


**FIG. 4. MUC1 cytoplasmic tail is tyrosine phosphorylated and associates with a pp180.** *A*, in each lane, 2 mg of lysate were immunoprecipitated with anti-MUC1 (*CT2*) and immunoblotted with anti-phosphotyrosine (*RC20-HRP*). Lanes identified as (+) represent animals injected with 1  $\mu$ g/g body weight EGF, while (-) represents endogenous EGF. *B*, mammary gland lysates (4 mg) were immunoprecipitated with anti-MUC1 antibodies (*CT2*, *B27.29*, or *HMFG-2*) and immunoblotted with antiphosphotyrosine (*RC20-HRP*). The *lysate only* lane represents 200  $\mu$ g 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 mg of lysate were immunoprecipitated with anti-MUC1 (*CT1*) and immunoblotted with anti-phosphotyrosine (*RC20-HRP*).

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  $\Delta$ TR 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- $\alpha$ , but not NRG $\alpha$  in MDA-MB-468 cells (data not shown). Treatment with any of these ligands (except NRG $\alpha$ ) 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

<sup>3</sup> W. Xie and S. J. Gendler, unpublished data.



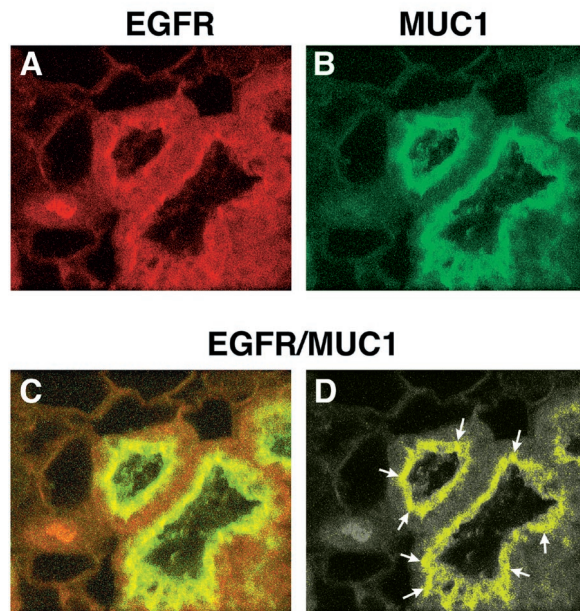
**FIG. 5. MUC1 associates with EGFR, erbB2, erbB3, and erbB4 in the mouse mammary gland.** *A*, mammary gland lysate from day 2/3 lactating females (2 mg) was immunoprecipitated with anti-EGFR (SC 1005) and blotted with anti-MUC1 (HMFG-2). Note that HMFG-2 does not react with mouse Muc1. *B*, lysates (200  $\mu$ g) from the same mammary gland samples shown in the *top panel* were blotted with anti-EGFR (ERCT) or anti-MUC1 (HMFG-2). *C*, mammary gland lysates (2 mg) were immunoprecipitated with anti-erbB2, anti-erbB3, or anti-erbB4 and blotted with anti-MUC1 (B27.29-HRP). Membranes were then reprobed with the same antibody used to perform the immunoprecipitation. The *bottom panel* shows levels of MUC1 (200  $\mu$ g) in the same mammary gland samples used in the erbB2, -3, and -4 immunoprecipitations. MUC1 immunoblotting was performed using both HMFG-2 and B27.29 antibodies.

or the cytoplasmic tail (Fig. 4*B*). Additionally, a pp  $\sim$ 120 kDa and pp  $\sim$ 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. 4*C*). 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-MD-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. 5*A* and data not shown). While co-immunoprecipitation experiments demonstrate an interaction between full-length MUC1 and EGFR, this interaction is markedly reduced in the  $\Delta$ CT transgenic (Fig. 5*A*). Complexes between MUC1 and the remaining 3 erbB receptors could also be identified in pregnant and lactating mammary glands (Fig. 5*C*). Again, little to no  $\Delta$ CT MUC1 protein could be found precipitating with erbB2, erbB3, or erbB4 antibodies. Importantly, equal if not more of the  $\Delta$ CT MUC1 protein is present in the mammary glands where little to no co-immunoprecipitation was observed (Fig. 5*C*, *bottom*

## Day 2 Lactating Mammary Gland



**FIG. 6. MUC1 and EGFR colocalize in the lactating mammary gland.** Paraffin sections (20  $\mu\text{m}$ ) were probed with anti-EGFR (SC, 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  $\times 400$  magnification using a 510 laser scanning microscope. Arrows (D) indicate areas of intense co-localization.

panel). Therefore, as MMF and  $\Delta\text{CT}$  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 EGFR signaling. To determine whether the presence or absence of MUC1 effected the ability of EGFR 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 EGFR 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<sup>-/-</sup> 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.

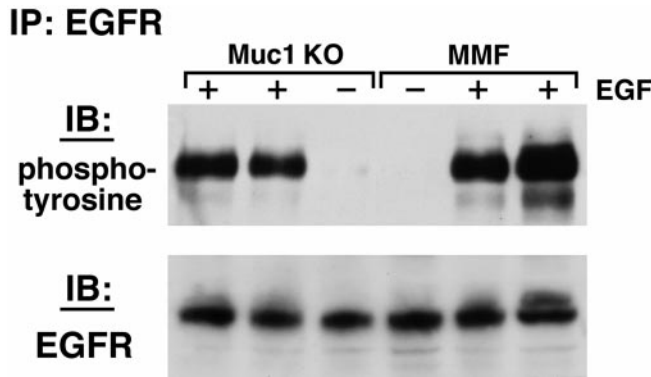
We also examined potential EGFR effector proteins for involvement in MUC1 signal transduction to ERK1/2 activation. It has been previously reported that Grb2/SOS associates with MUC1 in breast carcinoma cell lines (15). We detected MUC1 co-immunoprecipitating with both Grb2 and SOS in wild-type, MMF, and  $\Delta\text{TR}$  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).

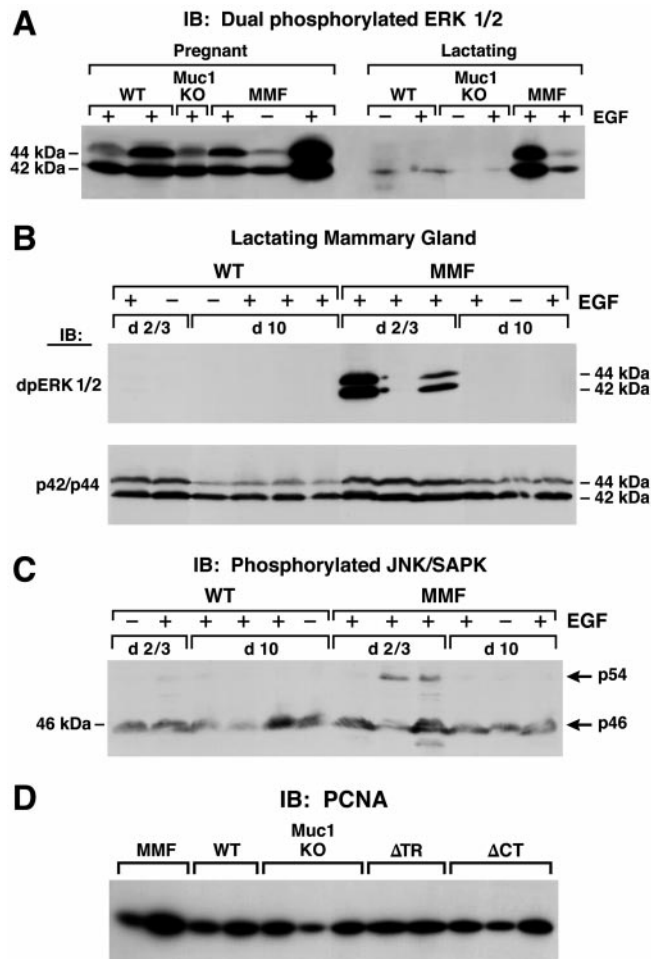
## DISCUSSION

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.

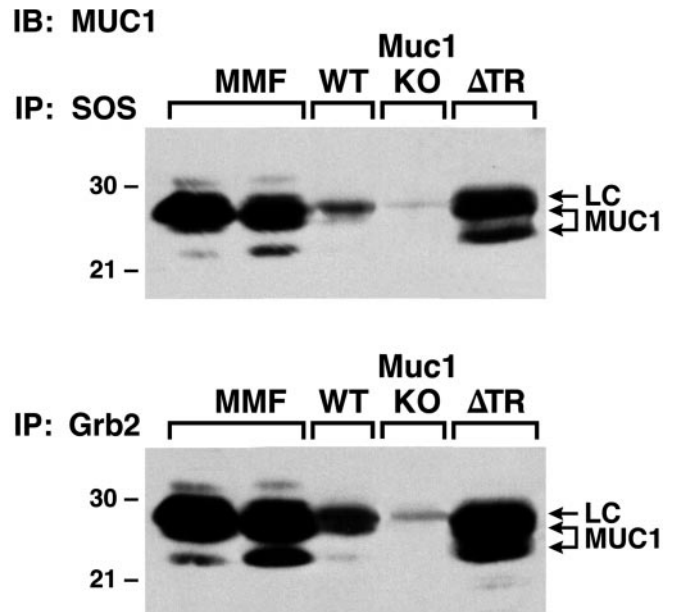
The interaction between transmembrane mucins and members of the erbB family has been demonstrated previously. Caraway *et al.* (34) demonstrated a co-immunoprecipitation between erbB2 and MUC4 (ASGP1 and -2) in both the metastatic ascites 13762 rat mammary carcinoma cell line as well the pregnant rat. 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



**FIG. 7. EGFR kinase activity is largely unaffected by MUC1 expression.** Mammary gland lysates (2 mg) were immunoprecipitated with anti-EGFR antibody (SC, 1005) and immunoblotted with an anti-phosphotyrosine antibody (RC20-HRP). Lysates (200  $\mu$ g) were then immunoblotted with anti-EGFR antibody (ERCT) (bottom panel). EGF represents either endogenous EGF (-) or exogenous treatment with 1  $\mu$ g/g body weight EGF (+).



**FIG. 8. Overexpression of full-length MUC1 activates p42/p44 ERK.** A, samples from mammary gland lysates were immunoblotted with anti-dpERK1/2. B, lysates were immunoblotted with anti-dpERK (top panel) or anti-ERK 1/2 (bottom panel). C, JNK/SAPK immunoblots are from lactational samples only, during early (d 2/3) or late (d 10) lactation. D, MMF (full-length MUC1 transgenic), wild-type (WT), Muc1 knockout (KO),  $\Delta$ TR and  $\Delta$ CT lysates were immunoblotted with an anti-PCNA antibody. In A-D, 200  $\mu$ g of protein lysate was separated on a 10% SDS-PAGE for each sample and EGF represents either endogenous EGF (-) or exogenous treatment with 1  $\mu$ g/g body weight EGF (+).



**FIG. 9. MUC1 associates with Grb2 and SOS.** Mammary gland protein lysates (2 mg) were immunoprecipitated with either anti-Grb2 or anti-SOS antibodies and immunoblotted with an anti-MUC1 antibody (CT2). MMF, full-length MUC1 transgenic; WT, wild-type; KO, knockout; LC, immunoglobulin light chain.

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 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. 2D), 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-

ble interpretation of these data is that EGFR phosphorylation of MUC1 allows recruitment of Grb2 into a complex that includes both EGFR and MUC1. Preliminary experiments have shown a significant increase in EGFR binding to SOS in the presence of full-length MUC1, but not the cytoplasmic tail-deleted transgenic mammary glands. Interestingly, when the remaining erbB receptors were examined for changes in SOS-binding, no modulation was observed in relation to the presence or absence of MUC1, indicating a specific relation between EGFR and MUC1. The presence of MUC1 in immunoprecipitations of erbB2, erbB3, and erbB4 is likely due to heterodimerization complexes between the four erbB receptors and MUC1. It has been previously demonstrated that all erbB receptors are capable of being transphosphorylated by EGF in the lactating mammary gland, indicating that these complexes do form during this stage of development (16).

EGFR and MUC1 have previously been localized in the mammary epithelium in the pregnant and lactating mammary gland, MUC1 to the apical side and EGFR throughout the cell. We have co-localized EGFR and MUC1 mainly to apical-lateral regions between cells, leading us to ask if these proteins may be interacting at tight junctions. To investigate this observation, we stained sections for MUC1 and ZO-1 (zona occludens 1), a cytoplasmic protein found in the tight junctions of polarized epithelial cells (38). In preliminary experiments, we observed co-localization of these two proteins at the apical-lateral region of the epithelium of lactating epithelium through confocal microscopy, indicating that MUC1 is indeed found at the tight junctions, and is not merely an apically localized protein. Interestingly, Chen *et al.* (39) recently demonstrated that increased MAPK activity negatively regulates tight junction formation. As MUC1 overexpression (as is seen in the neoplastic state as well as these transgenics) increases MAPK activity and decreases cellular adhesion *in vitro* (2, 3), we might speculate a role for normal (wild-type expression levels) MUC1/EGFR signaling in the preservation of the tight junction complex.

There are numerous 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 mitogenesis. Alternately, we have examined the possibility of driving the cells into a state of G<sub>0</sub> arrest by activating p21. This would potentially provide a population of cells that do not apoptose in response to the postlactational 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.

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. This novel data points to a potential mechanism, that of potentiating the signaling of the tyrosine kinase receptor EGFR, a protein whose increased expression is correlated to aggressive breast cancer (46, 47).

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