

ORIGINAL ARTICLE

MUC1 is a novel regulator of ErbB1 receptor traffickingMR Pochampalli¹, RM el Bejjani¹ and JA Schroeder*Department of Molecular and Cellular Biology, Arizona Cancer Center and Bio5 Institute, University of Arizona, Tucson, AZ, USA*

ErbB receptors are key regulators of cell survival and growth in normal and transformed tissues. The oncogenic glycoprotein MUC1 is a binding partner and substrate for erbB1 and MUC1 expression can potentiate erbB-dependent signal transduction. After receptor activation, erbB1 is typically downregulated via an endocytic pathway that results in receptor degradation or recycling. We report here that MUC1 expression inhibits the degradation of ligand-activated erbB1. Through the use of both RNAi-mediated knock down and overexpression constructs of MUC1, we show that MUC1 expression inhibits erbB1 degradation after ligand treatment in breast epithelial cells. This MUC1-mediated protection against erbB1 degradation can increase total cellular pools of erbB1 over time. Biotinylation of surface proteins demonstrates that cell-surface associated erbB1 receptor is protected by MUC1 against ligand-induced degradation, although this is accompanied by an increase in erbB1 internalization. The MUC1-mediated protection against degradation occurs with a decrease in EGF-stimulated ubiquitination of erbB1, and an increase in erbB1 recycling. These data indicate that MUC1 expression is a potent regulator of erbB1 receptor stability upon activation and may promote transformation through the inhibition of erbB1 degradation.

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Introduction

The erbB receptor family of tyrosine kinases are frequently deregulated in cancer, and commonly amplified and/or overexpressed in invasive carcinoma (reviewed in Schroeder and Lee (1997)). The family is comprised of four homologous receptors and multiple related ligands. The receptors are type 1 tyrosine kinase transmembrane glycoproteins and include erbB1

(epidermal growth factor receptor/HER1), erbB2 (HER2/neu), erbB3 (HER3) and erbB4 (HER4). Ligands for the family include epidermal growth factor (EGF), transforming growth factor alpha (TGF α), amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin (BTC), epiregulin (EPR) and epigen (reviewed in Schroeder and Lee (1997) and Strachan *et al.* (2001)). Ligand-induced receptor homo- or heterodimerization results in tyrosine kinase activation and transphosphorylation of tyrosine residues in the cytoplasmic domain. This leads to the recruitment of a variety of effector proteins including Src, PI 3-kinase, Shc, PLC γ , STATs, Grb2 and cbl, resulting in proliferation, inhibition of apoptosis, differentiation or degradation of endocytosed receptors (Alroy and Yarden, 1997; Olayioye *et al.*, 1998, 1999; Carpenter, 2000).

In addition to activation of signaling cascades at the cell surface, erbB1 also maintains signaling complexes during endocytosis (Waterman and Yarden, 2001). Upon ligand binding, erbB1 becomes bound by the ubiquitin ligase, cbl, and is recruited into clathrin-coated vesicles and internalized (Levkowitz *et al.*, 1999; Waterman and Yarden, 2001). These early endosomes traffic through the cell, eventually maturing into late endosomes and finally delivering their cargo to the lysosome, where the activated receptor is degraded. ErbB1 continues to activate signaling pathways during endosomal trafficking, including Ras and Akt (Wang *et al.*, 2002; Bivona and Philips, 2003). Receptors not targeted for lysosomal degradation are sent to the recycling endosome after releasing their ligand, where they return to the cell surface. It is important to note that degradation of activated receptor is critical to normal regulation of erbB1, and the loss of erbB1 degradation machinery results in transformation (Thien *et al.*, 2001; Shtiegman and Yarden, 2003).

The function of erbB receptors can be modulated by the coexpression of non-erbB transmembrane proteins. One protein shown to modulate the function of erbB1 is the proto-oncogene MUC1 (DF3, CD227, episialin, PEM) (Schroeder *et al.*, 2001). MUC1 is a heavily *O*-glycosylated heterodimeric protein of >300 kDa, normally expressed abundantly on the apical surface of glandular epithelia. In greater than 90% of human breast carcinomas and metastases, apical localization is lost and MUC1 is overexpressed (by greater than 10-fold) and underglycosylated (Zotter *et al.*, 1988; Hilken *et al.*, 1995). *O*-linked glycosylation of MUC1 occurs through repeated rounds of endocytic recycling via the *trans* Golgi network (Litvinov and Hilken,

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1993). Fully glycosylated MUC1 is maintained through constant endocytosis and recycling (Hanisch and Muller, 2000).

MUC1 expression has been shown to induce transformation in a number of systems, including MMTV-MUC1 transgenic mice and MUC1 transfected-3Y1 rat fibroblasts (Li *et al.*, 2003; Schroeder *et al.*, 2004). In MMTV-MUC1 transgenic mice, tumorigenesis is accompanied by a failure of the mammary gland to undergo complete postlactational regression via apoptosis (Schroeder *et al.*, 2004). Transfection of MUC1 constructs into colon cancer cells demonstrates that MUC1 overexpression inhibits drug-induced apoptosis as well (Ren *et al.*, 2004).

It has been established in both human breast cancer cell lines and transgenic mice overexpressing MUC1 (MMTV-MUC1) that MUC1 and the erbB family of receptor tyrosine kinases biochemically interact (Schroeder *et al.*, 2001; Li *et al.*, 2001b). Importantly, experiments in the MMTV-MUC1 transgenic model have shown that this interaction results in the potentiation of EGF-dependent signaling pathways. Examination of the Ras/MAP kinase pathway in these transgenic mice demonstrated that overexpression of MUC1 vastly increases EGF-dependent p42/44 ERK activation during lactation (Schroeder *et al.*, 2001). In the present study, we have evaluated the ability of MUC1 expression to inhibit erbB1 degradation as a mechanism of modulating erbB signaling. These studies demonstrate that MUC1 expression inhibits the ligand-mediated ubiquitination and degradation of erbB1 while enhancing its internalization and recycling.

Results

MUC1 expression prolongs EGF-dependent erbB1 phosphorylation

Regulation of erbB1 receptor signaling and its role in transformation are dependent upon the expression levels and duration of activation. Therefore, we sought to determine if MUC1 plays a role in either or both of these events by altering the levels of MUC1 expression in breast epithelial cell lines. Three cell lines were chosen for our study, two breast cancer cell lines, BT20 and MDA-MB-231, and one immortalized breast epithelial line, MCF10A. Both BT20 and MCF10A cells express significant levels of MUC1 and erbB1 and were used to analyse the effects of MUC1 knock down on erbB1 expression in transformed and immortalized conditions, respectively. Parental MDA-MB-231 cells express low levels of MUC1, but similar levels of erbB1 to BT20 cells, and were used to determine the effects of MUC1 overexpression on erbB1 expression and function.

Using RNAi (siRNA), we transiently reduced the expression of MUC1 in BT20 cells (Figure 1a, panel 3). We verified the specificity of our siRNA by demonstrating that the expression of an unrelated protein, β -actin, was unaffected (Figure 1a, panel 4). Furthermore, we verified that these effects were specific to the MUC1 siRNA by using an unrelated control siRNA that had

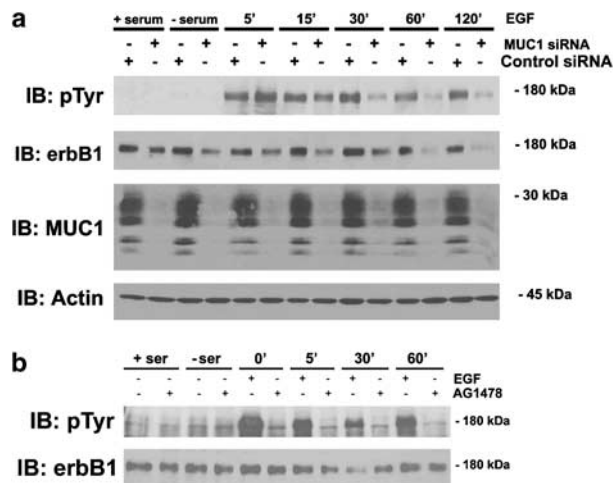


Figure 1 MUC1 inhibits the degradation of EGF-stimulated erbB1. (a) BT20 cells were transfected with either MUC1 siRNA or control (nonsilencing) siRNA, and either left in normal growth conditions (+ serum), serum starved overnight (-serum), or serum starved overnight and treated with 20 ng/ml EGF for the indicated times before lysis. Protein lysates (25 μg) were separated and immunoblotted with antibodies to detect phosphotyrosine (PY99), erbB1 (1005), MUC1 (CT2) or β -actin. (b) BT20 cells were serum-starved overnight, treated with 10 μM AG1478 for 2 h before treatment with 20 ng/ml EGF for 10 min on ice. Cells were then washed with PBS and incubated at 37°C for the indicated times and lysed. Proteins were then immunoblotted with either anti-erbB1 (1005) or anti-phosphotyrosine (PY99) antibodies. Note: Human MUC1 separates into multiple, differentially glycosylated and phosphorylated sizes (and the cytoplasmic tail that reacts to CT2 is ~14–35 kDa). Molecular weights are indicated on the right.

no effect on MUC1 protein expression (Figure 1a, panel 3). The control siRNA sequence was chosen based on its known nonhomology to any mammalian genes, and did not result in the alteration in the expression of MUC1, erbB1 or β -actin (see Figures 1–4). Finally, we also used four additional MUC1 siRNA oligos that gave similar results to those described here to verify specificity (data not shown).

Ligand-bound erbB1 typically dimerises, becomes internalized into early, then late endosomes, and is either degraded or recycled to the cell surface (Kuwada *et al.*, 1998; Levkowitz *et al.*, 1998, 1999; Waterman and Yarden, 2001). To induce ligand-dependent degradation of erbB receptors, cells transfected with siRNA to MUC1 (BT20-M) or a nonsilencing control (BT20-C) were treated with EGF, and endocytosis was allowed to proceed. At times corresponding to receptor activation and internalization (5 min) and receptor trafficking (15 min) and degradation (30 min or greater), cells were lysed and levels of phosphorylated receptor were analysed. Cells that were grown in the presence or absence of serum (but not treated with EGF) were used as controls to distinguish the effects of the growth factors present in serum.

We found that while the total levels of tyrosine-phosphorylated receptor (Figure 1a, top panel) are unchanged in BT20-M at early timepoints (5 and 15 min), the same cells showed a significant decrease in

phosphorylated erbB receptor at 30 min. We continued to monitor the effect of MUC1 expression on erbB stability for 60 and 120 min and found that the presence of MUC1 prevented the degradation of phosphorylated erbB receptor for these extended time points. Additionally, we observed decreased levels of total erbB receptor in the same treatment groups indicating total protein loss occurred, instead of loss of receptor phosphorylation (Figure 1a, panel 2).

To determine if this effect was dependent upon active erbB1 kinase, BT20 cells (not treated with RNAi) were treated with the erbB1 kinase inhibitor AG1478 (10 μ M) 2 h before EGF treatment (Pai *et al.*, 2002; Lee *et al.*, 2005). In these cells, the detection of phosphorylated erbB receptor was ablated by treatment with the kinase inhibitor (Figure 1b). This demonstrated that the observed phosphorylation was dependent upon the kinase activity of erbB1 receptor.

As cells transfected with MUC1 siRNA showed a loss of erbB1 over time, we next determined the long-term effect of changes in MUC1 expression through the transfection and stable selection of either a RNAi hairpin loop vector (pSuper or pSuper-MUC1) or a CMV-MUC1 overexpression construct. To perform these experiments, we utilized either the MCF10A cell line (to knock down MUC1 expression) or the MDA-MB-231 cell line (to overexpress MUC1). The MDA-MB-231 cells endogenously express very low levels of MUC1 compared to MCF10A cells, but similar levels of erbB1 (Figure 2b).

We first examined if overexpression of MUC1 would stabilize erbB1 expression upon EGF treatment (have the opposite effect to MUC1 knock down observed in Figure 1a). We stably transfected the MDA-MB-231 cell line with either a CMV-MUC1 (CM) or CMV (C) expression construct (McDermott *et al.*, 2001), which resulted in increased expression of MUC1 (Figure 2a, panel 3). We found that overexpression of MUC1 in these cells stabilized phospho-erbB1 expression in response to EGF treatment as early as 5 min after ligand treatment, when phospho-erbB1 begins to be lost in these cells (Figure 2a, panel 1). This inhibition of degradation continued on and became more pronounced at 30 min. Furthermore, after EGF treatment we observed a decrease in the degradation of total erbB1 levels in the presence of MUC1, similar to our observations with the BT20 RNAi experiment (compare Figure 2a, panel 2 with Figure 1a, panel 2). Note that in the absence of serum total levels of erbB1 were unaffected by MUC1 overexpression (Figure 2a).

Finally, we found that stable knock down of MUC1 expression altered the EGF-induced degradation of phosphorylated erbB1 in MCF10A breast epithelial cells. We used the commercially available pSuper vectors to express either MUC1 or control sequences that form hairpin loops upon transcription (Brummelkamp *et al.*, 2002). These hairpins are then processed by the cells endogenous machinery to create dsRNA, inducing RNAi-mediated degradation of target sequences. MCF10A cells were transfected with either the pSuper-MUC1 (pSM) or pSuper-Control (pS) vector and

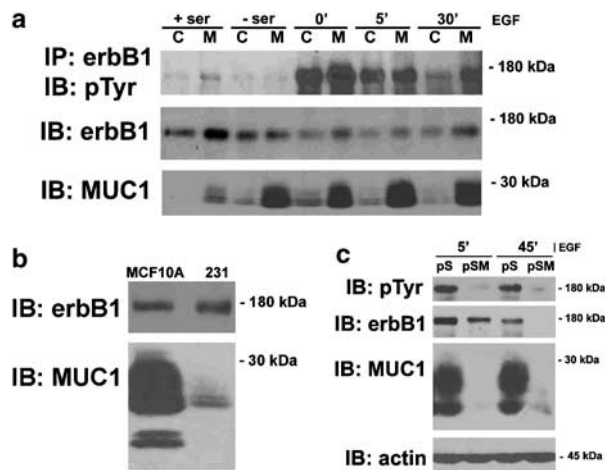


Figure 2 Stable alteration of MUC1 expression affects erbB1 degradation. (a) MDA-MB-231 cells were transfected with either CMV-MUC1 (CM) or parental CMV (C) driven vectors, then stably selected with neomycin. Cell lines were serum starved (-ser) overnight, then treated with 20 ng/ml EGF for 10 min at 4°C and washed with PBS to remove unbound ligand. Cells were then incubated for the indicated times at 37°C and lysed. Protein lysates were immunoprecipitated (IP) and separated by SDS-PAGE and immunoblotted (IB) with either anti-phosphotyrosine (PY99), anti-erbB1 (1005) or anti-MUC1 (CT2) antibodies. (b) Protein lysates from parental MCF10A and MDA-MB-231 were made, and 40 μ g were separated by SDS-PAGE. Relative levels of erbB1 and MUC1 expression in each cell line were determined by immunoblotting with anti-erbB1 (1005) and anti-MUC1 (CT2) antibodies, respectively. (c) MCF10A cells were transfected with either pSuper-Control (pS) or pSuper-MUC1 (pSM) RNAi constructs, then stably selected with neomycin. Cells were then treated and immunoblotted as described above in (a). Molecular weights are indicated on the right.

selected with neomycin. Stable selection resulted in a significant loss of MUC1 expression in the pSM, while not affecting MUC1 expression in the pS cells (Figure 2c, panel 3). This loss of MUC1 correlates with a significant loss of erbB phosphorylation in response to EGF treatment at the 5 min time point, although total erbB1 expression is minimally affected at this time. After 45 min of treatment with EGF, total erbB1 expression is lost in the absence of MUC1 expression, while erbB1 is still detected in MUC1 expressing cells.

MUC1 expression affects levels of plasma membrane localized erbB1

To determine if the pool of erbB1 that is affected by MUC1 was surface or cytoplasmically derived, we biotinylated cell surface proteins (with cell-impermeable biotin) and performed endocytosis assays to determine the fate of surface-associated erbB1. BT20 cells (either control or treated with MUC1 siRNA) were treated with biotin to label all surface proteins, the biotin reaction was quenched, cells were treated with EGF to induce endocytosis, and biotinylated protein was precipitated with streptavidin beads (Figure 3a, top panel). We found that a reduction of MUC1 expression by RNAi resulted in reduced detection of surface biotinylated erbB1 at the 30 and 60 min time points.

Alternatively, surface biotinylated erbB1 continued to be detected at these time points in the presence of MUC1 expression. Densitometry analysis of three separate experiments demonstrated an average of ~50% reduction in erbB1 receptor expression in cells treated with MUC1 RNAi compared to controls after 30 min, which increased to ~80% after 60 min (Figure 3b). Importantly, these data demonstrate that the pool of erbB1 receptors that is affected by MUC1 expression resides at the membrane. Additionally, as we detected total surface erbB1 and its degradation, these data clarify that we detected the degradation of erbB1 protein, and not merely a loss of erbB1 phosphorylation.

We next determined where MUC1 and erbB1 localized in the cell before and after treatment with EGF. To do this, we examined localization of MUC1 and erbB1 in serum starved or EGF-treated BT20 cells (without siRNA treatment). As expected, MUC1 and

erbB1 colocalized at the cell surface before treatment with ligand (Figure 3c, left panel, arrow).

MUC1 and erbB1 colocalized in internal compartments upon treatment with EGF for 30 min, accompanied by an accumulation at distinct intracellular vesicles (Figure 3c, right panel, arrow). These results demonstrate that while MUC1 interacts with erbB1 at the cell surface, the two proteins can be observed in cytoplasmic compartments upon ligand treatment.

MUC1 promotes internalization of erbB1 in response to EGF

As MUC1 expression inhibits the degradation of plasma membrane-localized erbB1, we next examined if MUC1 expression alters erbB1 retention at the membrane. Stably transfected MDA-MB-231 C and CM cells (described in Figure 2) were serum-starved then treated with EGF to induce internalization (Figure 4a). At 5, 30, 60 and 120 min after internalization, cell surface proteins were biotinylated, and precipitated with streptavidin. It is important to emphasize that in this experiment, we biotinylated surface proteins after EGF treatment and internalization, a method that would indicate levels of receptor remaining on the surface after ligand-induced internalization. Therefore, only those proteins that remained on the cell surface after ligand treatment and internalization (or had returned to the surface through recycling) would be detected. We observed significantly less erbB1 on the cell surface in the presence of MUC1 (after ligand treatment and endocytosis proceeded; Figure 4a), indicating that MUC1 promotes the loss of erbB1 from the cell surface. Increased internalization of erbB1 was strongly enhanced by MUC1 expression at 5 and 30 min, with total loss of surface erbB1 observed at 60 min regardless of MUC1 expression. Densitometry

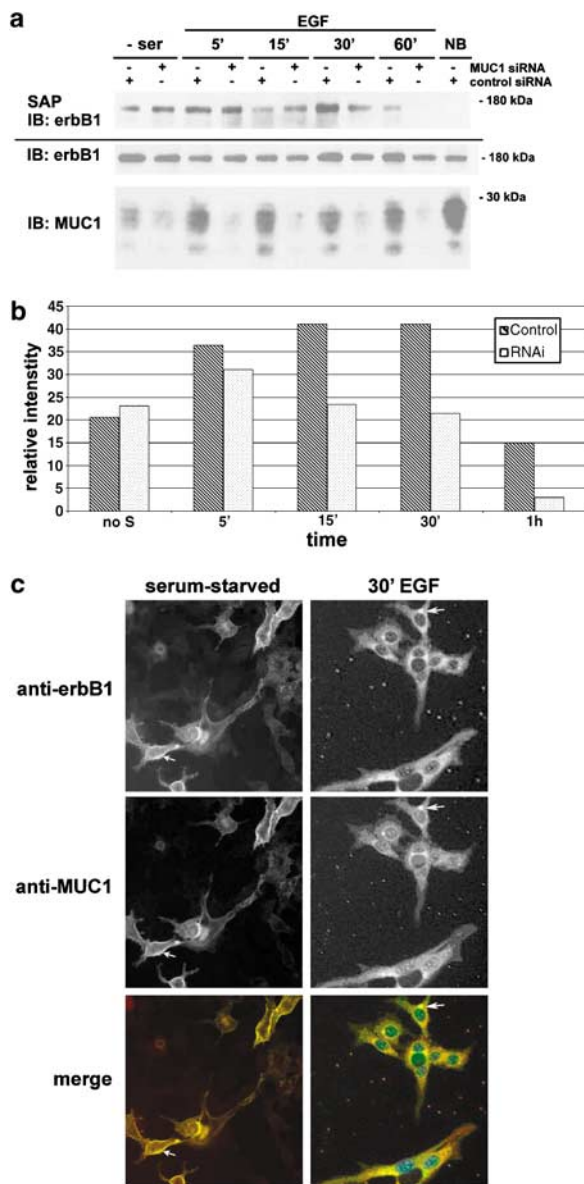


Figure 3 MUC1 expression alters cell-surface erbB1. (a) BT20 cells were transfected with either MUC1 siRNA or control (nonsilencing) siRNA. After knock down, cells were biotinylated at 4°C to label cell surface receptors, and the reaction was quenched with a Tris buffer. After washing, cells were treated with EGF for the indicated time points, lysed and protein was precipitated using streptavidin beads (SAP). Proteins were then immunoblotted with anti-erbB1 (1005). Total cell lysates were also separated to demonstrate total cellular levels of protein and immunoblotted with anti-erbB1 (1005) or anti-MUC1 (CT2), bottom two panels. NB = Non-biotinylated control. Molecular weights are indicated on the right. (b) Quantification of the differences observed in a. Densitometry from three separate experiments was obtained using the ScionImage software, corrected against background and averaged. Striped bars represent control-RNAi treated cells and dotted bars represent MUC1-RNAi-treated cells (c). Left panel: to analyse surface colocalization, BT20 cells were serum-starved overnight. Cells were then labeled with anti-erbB1 (Ab-21) and anti-MUC1 (DF3) for the primary antibodies and anti-rabbit Alexa 594/red and anti-mouse Alexa 488 for the secondaries. (MUC1 = green, erbB1 = red) Right panel: to analyse internal colocalization during EGF treatment, BT20 cells were serum-starved overnight, then incubated with 20 ng/ml EGF for 5 min, washed and incubated at 37°C for 30 min. Cells were labeled with anti-erbB1 (Ab-1) and anti-MUC1 (CT2) for the primary antibodies and anti-rabbit Alexa 594/red and anti-american hamster FITC for the secondaries. Magnification is × 400. Arrows indicate areas of colocalization.

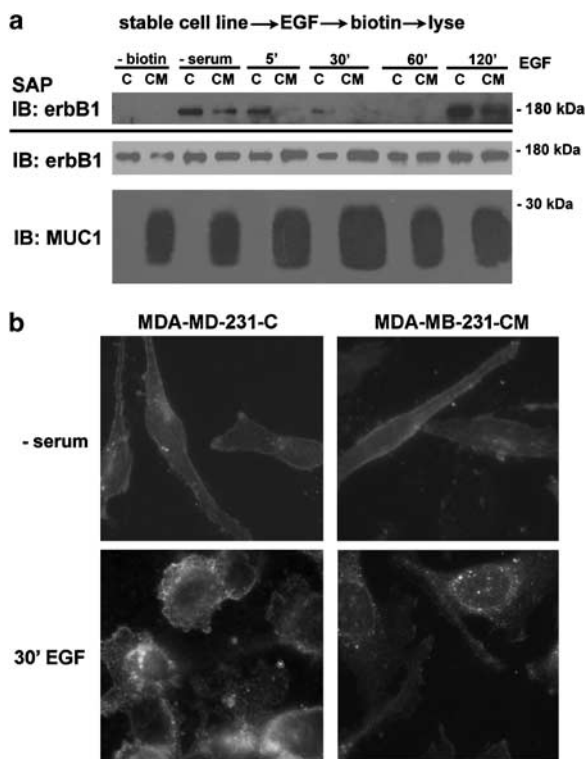


Figure 4 MUC1 expression promotes EGF-dependent internalization. **(a)** To assess receptor internalization, MDA-MB-231 breast cancer cells stably transfected with MUC1 (CM) or with vector alone (C) were treated with 100 ng/ml EGF at 4°C (to saturate erbB1 receptors) and incubated for the indicated time point before being surface biotinylated and then extracted. Biotinylated proteins were then precipitated with streptavidin (SAP) and immunoblotted with anti-erbB1 (1005). A separate set of protein lysates were treated and prepared as described and analysed for MUC1 expression using anti-MUC1 (CT2), bottom panel, under separation line. **(b)** Immunofluorescence of erbB1 in MDA-MB-231 cells expressing either the CMV vector (C) or CMV-MUC1 (CM), serum starved (top panels) or serum starved and treated with 10 ng/ml EGF on ice, washed, then incubated for 30 min at 37°C (bottom panels). Cells were then fixed with methanol/acetone and stained with antibodies against erbB1 (Ab-1), magnification = × 630.

analysis of three separate experiments showed a decrease of surface erbB1 in the presence of MUC1 of ~40% after 5 min, with total levels of surface erbB1 returning to approximately equal after 2 h (relative intensity = 16 (C) vs 10 (CM) at 5 min; and 16 (C) vs 14 (CM) at 2 h). Note that we are comparing relative levels of C to CM at these time points.

Analysis of erbB1 localization by immunofluorescence in stably transfected MDA-MB-231 cells (both CM and C) visually recapitulated this observation. In the absence of serum, erbB1 is found at the plasma membrane regardless of MUC1 expression (Figure 4b, top panels). The MUC1 overexpressing cells (CM) display significant internalized erbB1, with very little surface erbB1 visible upon 30 min of EGF treatment (Figure 4b, right panel). Alternatively, while the control (C) cells demonstrated internalized erbB1 in response to EGF treatment, there were still significant levels of erbB1 observed on the surface (Figure 4b, left panel).

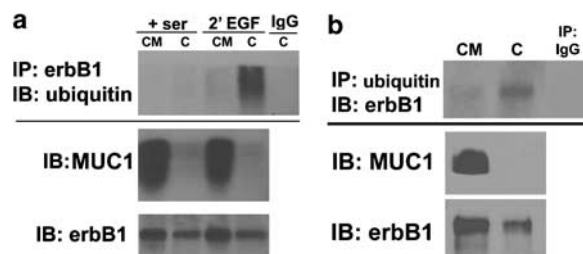


Figure 5 MUC1 expression inhibits the ubiquitination of erbB1. **(a)** MDA-MB-231 cells overexpressing CMV-MUC1 (CM) or the CMV vector (C) were serum starved overnight and treated with 20 ng/ml EGF at 37°C for 2 min or grown in serum and lysed (+ ser). Protein lysates (500 µg) were immunoprecipitated with anti-erbB1 antibody (Ab-1) or control mouse IgG and immunoblotted with an anti-ubiquitin antibody (Santa Cruz). Protein lysates (50 µg) from these samples were also immunoblotted with anti-MUC1 (CT2) or anti-erbB1 (Santa Cruz, 1005) to show total levels of protein (bottom two panels). **(b)** MDA-MB-231 cells overexpressing CMV-MUC1 (CM) or the CMV vector (C) were grown in serum and lysed. Protein lysates (300 µg) were immunoprecipitated with anti-ubiquitin antibody (NCL-ubiq) or control rabbit IgG and immunoblotted with anti-erbB1 antibody (Santa Cruz, 1005). Protein lysates (50 µg) from these samples were also immunoblotted with anti-MUC1 (CT2) or anti-erbB1 (Santa Cruz, 1005) to show total levels of protein (bottom two panels). Note that detection of ubiquitinated erbB1 in **(a)** was obtained with an exposure time of 30 s (using DuraSignal, Pierce Chemical Co.), while the detection of ubiquitinated erbB1 in **(b)** was obtained with an overnight exposure (using DuraSignal, Pierce Chemical Co.).

This data again indicates that in those cells expressing high levels of MUC1, erbB1 internalization was enhanced. Together, these results demonstrate that, although MUC1 inhibits the degradation of ligand-activated erbB1, it also promotes the internalization of the receptor.

MUC1 expression inhibits the ubiquitination of erbB1

To determine the mechanism of MUC1-mediated inhibition of erbB1 degradation, we examined the ubiquitination of erbB1 in response to EGF treatment. Ligand-bound erbB1 is normally ubiquitinated (ub-erbB1) upon internalization and vesicular trafficking then proceeds (reviewed by Waterman and Yarden (2001)). We treated either MDA-MB-231C or CM cells with serum alone or EGF for 2 min at 37°C to induce internalization and ubiquitination. While ub-erbB1 was observed with this treatment in control cells (C), ub-erbB1 was markedly reduced in CM cells (Figure 5a). Also, while ub-erbB1 was not observed in the presence of serum at the exposure times required to detect EGF-induced ub-erbB1, we did observe ub-erbB1 in the presence of serum at longer exposure times (Figure 5b). This ub-erbB1 was also reduced in the presence of MUC1 expression, indicating that the growth factors present in serum can also induce (albeit much less) ubiquitination of erbB1. These data demonstrate that MUC1 expression alters the ubiquitination of erbB1, indicating a mechanism for the decrease in erbB1 degradation.

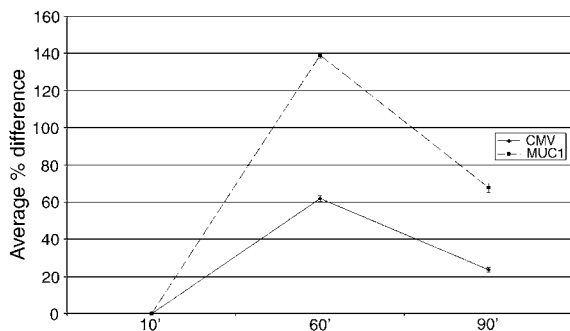


Figure 6 MUC1 overexpression promotes the recycling of erbB1. MDA-MB-231 cells expressing CMV-MUC1 (MUC1/dashed line/squares) or CMV (CMV/solid line/diamond) were pulsed with 10 ng/ml EGF for 10 min on ice and then incubated at 37°C to induce internalization, and cells were harvested at either 60 or 90 min. The mean fluorescence intensity (MFI) was measured by flow cytometry, and the MFI at 10 min after EGF stimulation was considered a reference point at which the activated receptors are internalized but not yet recycled. Recycling is represented as the percentage difference between the MFI at the indicated time point and the MFI at the 10 min reference time point. Percentage recycling = $((TP - T10 \text{ min}) / T10 \text{ min} \times 100)$.

MUC1 expression increases erbB1 recycling

To determine if the decrease in erbB1 ubiquitination was due to altered erbB1 trafficking, we examined erbB1 recycling to the plasma membrane. MDA-MB-231C and CM cells were pulsed with EGF for 10 min on ice and unbound EGF was then washed off. Treated cells were then chased by incubating at 37°C for 10, 60 and 90 min before being harvested and incubated with an erbB1-PE antibody (Kowanetz *et al.*, 2004; Reinheckel *et al.*, 2005; Yan *et al.*, 2005).

We detected an ~80% increase in the amount of recycled erbB1 in CM cells compared to C cells 60 min after EGF stimulation, and an approximate 40% increase in recycling 90 min after stimulation (Figure 6). Recycling is shown as the percentage difference between the average corrected geometric mean at each experimental time point (TP) minus the starting (10 min) time point $((TP - T10 \text{ min}) / T10 \text{ min} \times 100)$, and represents the amount of erbB1 that is recycled to the cell surface after each time analysed. These results indicate that MUC1 overexpression promotes the recycling of erbB1 to the cell surface, providing a mechanism by which MUC1 inhibits erbB1 degradation and promotes erbB1 signaling.

Discussion

We report here that MUC1 expression inhibits the degradation of ligand-activated erbB1 receptor. We show through RNAi-mediated loss of MUC1 expression that MUC1 inhibits the degradation of phosphorylated erbB1 after ligand binding in both BT20 breast cancer cells and MCF10A breast epithelial cells. Overexpression of MUC1 in MDA-MB-231 cells recapitulates this effect by stabilizing ligand-activated erbB receptor.

Biotinylation of surface proteins demonstrates that MUC1 promotes the internalization of cell-surface associated erbB1 while protecting it against ligand-activated degradation. Importantly, MUC1 expression inhibits the ubiquitination of erbB1 and enhances its recycling to the plasma membrane.

MUC1 has been previously shown to both interact with erbB receptors (Schroeder *et al.*, 2001; Li *et al.*, 2001a) and affect EGF-dependent activation of MAP kinase pathways in lactating mammary glands (Schroeder *et al.*, 2001). The mechanism by which MUC1 accomplishes this task, although, was undetermined. ErbB receptors can be regulated both by their activation (i.e. through ligand binding or as a substrate for src kinase (Ishizawa and Parsons, 2004)) and through downregulation of the receptor. Work involving the ubiquitin ligase cbl has shown that downregulation of erbB1 is a critical component of its normal function, and degradation of erbB1 is a key component in preventing it to act in an oncogenic manner (Thien *et al.*, 2001; Shtiegman and Yarden, 2003). Our current data now defines MUC1 as another important regulator of erbB1 receptor degradation. By examining both overexpression constructs and RNAi knockdowns of MUC1, we have demonstrated a functional role for MUC1 as a regulator of erbB1 signaling. We found that CM cells mimicked pS cells (both expressing high MUC1 and displaying stable erbB1 expression) and C cells, in turn, mimicked pSM cells (both expressing very low MUC1 and displaying accelerated erbB1 degradation in response to ligand). These experiments show that both overexpression of MUC1 in low-MUC1-expressing cells or loss of expression in high-MUC1-expressing cells can modulate the stability of phosphorylated erbB1 expression. While a previous study reported that MUC1 expression affects the transcription of erbB1 (Li *et al.*, 2005), we were unable to detect alterations of the steady state level of erbB1 levels in response to MUC1 loss or overexpression. It will be interesting in future work to evaluate the effect of MUC1 expression on the ligand-induced degradation of all members of the erbB family. Given that MUC1 and erbB receptors are commonly overexpressed in breast cancer (reviewed in Schroeder and Lee (1997) and Gendler (2001)), the interactions between these proteins may be potent inducers of carcinoma in the appropriate molecular setting.

Our data demonstrate that although MUC1 inhibits ligand-induced degradation of erbB1, it also promotes its internalization. This apparent paradox may be explained by one of two non-exclusive hypotheses. The first is that MUC1 promotes the entry of erbB1 into a recycling pathway instead of an ubiquitination pathway. Our data demonstrate that MUC1 expression does, in fact, inhibit the ubiquitination of erbB1 concurrently with an increase in the recycling of erbB1 to the plasma membrane. Additionally, MUC1 expression could also be driving erbB1 into an alternate internal trafficking pathway, one that does not lead to protein degradation. This could potentially involve trafficking to the ER or Trans Golgi Network, both of which have been shown to alter the degradation of ligand-activated erbB1

(Haj *et al.*, 2002; Puertollano and Bonifacino, 2004). We are currently examining potential mechanisms of alternate trafficking.

Our data indicate that MUC1 alters ligand-induced internalization and degradation of erbB1. While MUC1 promotes internalization of erbB1, MUC1 also inhibits the degradation of erbB1 that would normally follow. Importantly, we have found that MUC1 is altering the normal ubiquitination of internalized erbB1 and promoting the recycling of erbB1 to the cell surface. This study implicates MUC1 as a potentially critical mediator of erbB1 stability and function, one that may have dramatic implications for erbB1-mediated breast cancer progression.

Materials and methods

Cell lines

BT20 and MDA-MB-231 human breast cancer cells and MCF10A immortalized human mammary epithelial cells were purchased from ATCC and maintained in RPMI (Gibco, Carlsbad, CA, USA) with 10% FBS (Biomed, Foster City, CA, USA) and 1.0% Penicillin-streptomycin (Gibco) in 5.0% CO₂ at 37°C. Growth medium for MCF10A cells was supplemented with 10 ng/ml cholera toxin (Sigma), 0.5 µg/ml hydrocortisone (Sigma, St Louis, MO, USA) and 5 ng/ml EGF (Invitrogen, Carlsbad, CA, USA). Cells were grown to 80% confluency for use.

Antibodies, growth factors and cDNA constructs

CT2 (MUC1 cytoplasmic domain) and erbB1 Ab-1 and Ab-21 were purchased from Neomarkers Inc. (Fremont, CA, USA), erbB1 1005 and PY99 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The DF3 antibody (MUC1 extracellular domain) was purchased from DAKO Corporation. Ubiquitin (p4d1) was from Santa Cruz Biotechnologies and NCL-Ubiq was from Novocastra (New Castle, UK). Antibodies to detect β-actin is from Sigma Chemical Company. Secondary antibodies conjugated to HRP were purchased from Pierce and the HRP-conjugated Hamster antibody was purchased from Jackson Laboratories (Bar Harbor, ME, USA). All Alexa-conjugated antibodies were purchased from Molecular Probes (Carlsbad, CA, USA) (Invitrogen). ErbB1 kinase inhibitor (AG1478) was obtained from Sigma. The MUC1 cDNA was a kind gift from MA Hollingsworth at the Eppley Cancer Institute, University of Nebraska Medical Center. The MUC1 cDNA was subcloned into the pCMV-DNA3.1 vector (Invitrogen) using standard techniques and constructs were transfected into MDA-MB-231 cells using Lipofectamine 2000, and selected with 1 mg/ml G418.

RNAi

RNAi was performed by either transient siRNA treatment (Qiagen, Valencia, CA, USA or Dharmacon, Chicago, IL, USA) or stable selection of pSUPER hairpin loop vectors (OligoEngine, Seattle, WA, USA).

siRNA. MUC1-specific siRNA was generated to the following target sequence of the MUC1 extracellular domain: ³¹⁰⁴AAGACTGATGCCAGTAGCACT (Lan *et al.*, 1990), and a non-silencing siRNA was designed to the following target sequence, which lacks homology to any known

mammalian gene: AATTCTCCGAACGTGTACCGT (Qiagen). Transfections were performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's suggestions. For MCF10A cells, the transfection was performed once and the cells were lysed on day 3 post-transfection. BT20 cells required a double transfection for complete knock down. The cells were transfected on day 1 then transfected again on day 3. BT20 lysates were collected on day 5. Knock down was verified by immunoblotting for protein expression.

Additional controls were utilized to verify the effects of the Qiagen manufactured siRNA by testing MUC1 siRNA oligos from a second company (Dharmacon, SMARTpool of four siRNA oligos) on these assays. These target sequences were as follows (and lie in the extracellular (#1–3) and cytoplasmic domain (#4)): (#1)³²⁷⁰ACCAAGAGCTGCAGAGAGA, (#2)³³⁶³GATCTGTGGTGGTACAATT, (#3)³⁴⁶⁵GATATAACCTGACGATCTC, (#4)³⁷⁴⁶GATCGTAGCCCCATGAGA (Lan *et al.*, 1990). Those studies were performed following the manufacturer's instructions.

pSuper. pSuper vectors were purchased from (OligoEngine), using the companies software to generate both MUC1 and control RNAi hairpin loops. The following extracellular target sequence was used for the MUC1 hairpin loop: ³⁰⁷³TACTCC TACCACCCTTGCC (Lan *et al.*, 1990). Cells were transfected using Lipofectamine 2000 (Invitrogen), selected using G418 (Invitrogen) and were continually grown under 250 µg/ml during experiments.

Endocytosis assays

Cells were serum starved overnight, then incubated with 20 ng/ml receptor grade EGF (Invitrogen) for 10 min on ice. Unbound EGF was then removed by washing 2 × with PBS at 4°C. Cells in serum-free media were incubated at 37°C for the indicated time points and lysed (20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA pH 8.0, 2 mM EGTA pH 8.0 and 1.0% Triton X-100, 2.0 mM sodium orthovanadate, 50.0 µM ammonium molybdate and 10.0 mM sodium fluoride and Complete protease inhibitors (Roche, Indianapolis, IN, USA)). Cell lysates were vortexed briefly, centrifuged and the supernatant stored at –80°C. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL, USA).

Biotinylation assays

BT20 or MDA-MB-231 (C or CM) cells were grown serum-free overnight, then incubated at 4°C with 4 ml of 0.3 mg/ml Sulfo-NHS-SS-biotin (Pierce) for 30 min to analyse receptor internalization (Figure 3a). The biotinylation reaction was stopped by washing 3 × with ice cold quenching buffer (10 mM Tris pH 7.4, 154 mM NaCl). Cells were then washed with ice cold PBS and treated with EGF as described in the endocytosis assay. For analysis of surface retention (Figure 4a), cells were first treated with EGF and incubated for 5 min at 37°C to promote internalization, then treated with biotin and lysed as described above. Lysates were precipitated with streptavidin coated agarose beads, washed and resuspended in sodium dodecyl sulfate–PolyAcrylamide Gel Electrophoresis (SDS–PAGE) buffer (0.4 M Tris, 0.2 mM EDTA, 25% glycerol, 10% β-mercaptoethanol and 0.4% bromophenol blue) for immunoblotting.

Immunofluorescence analysis

Cells were grown on glass coverslips and serum starved overnight. Cells were treated with EGF as described for the endocytosis experiment, washed with 0.02% NaN₃/PBS, then fixed with a 1:1 mixture of ice cold methanol–acetone. Cells

were blocked with 20% FBS/0.02% NaN₃/PBS, incubated with the indicated antibody overnight at 4°C, washed and then incubated with Alexa fluor-conjugated secondary antibodies. Cells were then mounted with Slowfade Antifade reagent with DAPI (Molecular Probes) and visualized using a Leica DMBL 100s system with MagnaFire software.

Immunoprecipitation and immunoblotting

Protein lysates were incubated with their respective antibodies in TNEN buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0 and 0.5% Igepal CA 630, 2.0 mM Sodium orthovanadate, 50.0 μM ammonium molybdate and 10.0 mM sodium fluoride and Complete protease inhibitors) and rProtein G agarose beads (Invitrogen). Precipitates were washed with TNEN buffer 3 ×, and proteins were resuspended in 2 × SDS-PAGE buffer. Proteins were then separated by SDS-PAGE and transferred onto PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with either 5% nonfat milk in PBS (0.1% Tween20) or 3% BSA in TBS (0.1% Tween20) and immunoblotted. The membrane was then treated with Super Signal West Pico Chemiluminescent Substrate (Pierce), visualized on Imagetech-B film (American X-ray supply Inc., Louisville, TN, USA), and developed with a Konica SRX-101C.

For ubiquitination experiments, TNEN buffer also contained 10 mM *N*-ethyl-maleimide (Pierce) to inhibit deubiquitination.

Recycling assay

MDA-MB-231 cells transfected with MUC1 or with pcDNA3 alone were plated in triplicates in presence of serum and

treated with 10 ng/ml EGF at 4°C for 10 min. Unbound EGF was removed by washing (2 ×) with cold PBS, cells were incubated at 37°C for 10, 60, or 90 min, trypsinized and blocked with 5% BSA for 1 h on ice. The cells were then incubated with R-phycoerythrin (R-PE) conjugated erbB1 antibody (BD Pharmingen, San Diego, CA, USA) on ice. Fluorescence intensity was then immediately determined on a FACScan flow cytometer (BD biosciences, San Jose, CA, USA) and the results were analysed using a CellQuestPro 4.0 software (BD biosciences, San Jose, CA, USA) using standard methodology followed by the Arizona Cancer Center Flow Cytometry Shared Service (FCSS). The geometric mean of stained cells was corrected against nonstained cells to eliminate any autofluorescence and the results were plotted as a percentage difference where the 10 min time point was considered the time point of origin at which no recycling occurs. ((TP–T10 min)/T10 min × 100).

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