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MUC4 Involvement in ErbB2/ErbB3 Phosphorylation and Signaling in Response to Airway Cell Mechanical Injury

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ABSTRACT

The receptor tyrosine kinases ErbB2 and ErbB3 are phosphorylated in response to injury of the airway epithelium. Since we have shown that the membrane mucin MUC4 can act as a ligand/modulator for ErbB2, affecting its localization in polarized epithelial cells and its phosphorylation, we questioned whether Muc4 was involved, along with ErbB2 and ErbB3, in the damage response of airway epithelia. To test this hypothesis, we first examined the localization of MUC4 in human airway samples. Both immunocytochemistry and immunofluorescence showed a co-localization of MUC4 and ErbB2 at the airway luminal surface. Sequential immunoprecipitation and immunoblotting from airway cells demonstrated that the MUC4 and ErbB2 are present as a complex in airway epithelial cells. To assess the participation of MUC4 in the damage response, cultures of NCI-H292 or airway cells were scratch-wounded, then analyzed for association of phospho-ErbB2 and -ErbB3 with MUC4 by sequential immunoprecipitation and immunoblotting. Wounded cultures exhibited increased phosphorylation of both receptors in complex with MUC4. Scratch wounding also increased activation of the downstream pathway through Akt, as predicted from our previous studies on Muc4 effects on ErbB2 and ErbB3. The participation of MUC4 in the phosphorylation response was also indicated by siRNA repression of MUC4 expression, which resulted in diminution of the phosphorylation of ErbB2 and ErbB3. These studies provide a new model for the airway epithelial damage response, in which the MUC4–ErbB2 complex is a key element in the sensor mechanism and phosphorylation of the receptors. J. Cell. Biochem. 107: 112–122, 2009.

KEY WORDS: MUC4; ErbB2; ErbB3; PHOSPHORYLATION; AIRWAY INJURY

Membrane mucins are large, highly glycosylated proteins that are expressed at the luminal surfaces of most wet-surfaced epithelia [Carraway, 2000]. Although they are primarily recognized for their steric protection of cell surfaces [Komatsu et al., 1999], recent studies have indicated an additional role in cell signaling functions that regulate cell proliferation, differentiation and death [Carraway et al., 2003]. For example, MUC1 is phosphorylated on tyrosines on its cytoplasmic tail to provide docking sites for initiating signaling pathways [Carraway et al., 2003]. Muc4 modulates signaling by acting as a ligand for the receptor tyrosine kinase ErbB2 to influence both its cellular localization [Ramsauer et al., 2003; Funes et al., 2006] and its phosphorylation [Carraway et al., 1999; Ramsauer et al., 2006]. In this way Muc4 can potentially influence all of the signaling pathways driven by ErbB2.

Rat Muc4 is a heterodimeric glycoprotein originally identified and isolated from a mammary ascites tumor [Sherblom and Carraway, 1980] and is composed of a mucin subunit ASGP-1 [Sherblom et al., 1980] attached to the membrane by a transmembrane subunit ASGP-2 [Hull et al., 1990]. The human homologue MUC4 similarly contains two subunits, called MUC4α and MUC4β, respectively [Moniaux et al., 1999]. The two subunits are products of a single gene, which is transcribed as a single mRNA of 9 kb in the rat [Sheng et al., 1992] (18–27 kb polymorphic transcript in the human), which is translated into a polypeptide of approx. 300 kDa with extensive *N*-glycosylation in the C-terminal third of the precursor protein [Sheng et al., 1990]. This precursor is then cleaved to its two subunits prior to the extensive *O*-glycosylation which occurs in the ASGP-1 subunit (N-terminal two-thirds of precursor) [Sheng et al., 1990]. Shortly after

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completion of the synthesis of the Muc4, it forms a complex with ErbB2, which then transits to the cell surface [Ramsauer et al., 2003]. In polarized, cultured epithelial cells, the complex is primarily found at the apical surface [Ramsauer et al., 2003] because the apically directed Muc4 overrides signals which would otherwise direct ErbB2 to the lateral surface [Borg et al., 2000; Dillon et al., 2002].

The ErbB family of receptor tyrosine kinases has been implicated in proliferation, differentiation, cell death and development [Klapper et al., 2000]. Signaling by these four receptors is activated through binding of ligands of the epidermal growth factor family to stimulate receptor phosphorylation via the kinase activity of their cytoplasmic domains [Ullrich and Schlessinger, 1990]. However, ErbB2 has no soluble, high affinity ligand, and ErbB3 has no kinase activity [Klapper et al., 2000]. Instead, these two receptors act as a heterodimeric couple to produce a potent signal for cell proliferation and cell survival [Carraway and Cantley, 1994]. This signaling capability can contribute substantially in proliferative diseases such as cancer [Klapper et al., 2000]. Thus, it is very important in differentiated cells to control activation of this pair of receptors. We have proposed that Muc4 contributes to this regulation [Carraway et al., 2002]. In differentiated, polarized cells, Muc4 association with ErbB2 causes the ErbB2 to transit to the apical surface [Ramsauer et al., 2003]. In contrast, ErbB3 transits to the lateral surface, co-localized with E-cadherin [Ramsauer et al., 2006]. This mechanism effectively segregates the two receptors [Ramsauer et al., 2006; Carraway and Carraway, 2007], preventing their heterodimerization and the downstream signaling that promotes proliferation.

Both Muc4 and the ErbBs have been implicated in the airway. In the rat airway Muc4 is found as a membrane form at the luminal surface of the epithelial cells and as a soluble form in the liquid associated with the luminal surface [McNeer et al., 1998]. ErbB2 and ErbB3 are also found in the luminal cells and have been proposed to act in a sensor and repair mechanism on the damaged airway [Vermeer et al., 2003; Carraway and Carraway, 2007]. A key observation is that the ErbB3 ligand neuregulin is found in the luminal fluid of the airway epithelium [Vermeer et al., 2003]. Thus, it is segregated from its receptor ErbB3 in the lateral cell surface by the epithelial tight junction barrier. Damage to the epithelium and the barrier allow the ligand to bind its receptor, induce ErbB2–ErbB3 heterodimerization, activate receptor phosphorylation and initiate downstream signaling [Vermeer et al., 2003; Carraway and Carraway, 2007]. Based on our previous work, described above, the question then arises whether Muc4 participates in this signaling mechanism, since it was not considered in the previous work on the mechanism for ErbB activation in airway damage [Vermeer et al., 2003]. We have addressed this question by examining the localization of Muc4 and ErbB2 in the airway, by establishing their presence together in a complex and by analyzing the participation of Muc4 in the activation of ErbB2 and ErbB3 phosphorylation and downstream signaling in response to damage in an airway epithelial cell model.

MATERIALS AND METHODS

CELL CULTURE

NCI-H292 cells were obtained from ATCC, and maintained according to ATCC specifications (RPMI 1640 with 2 mM L-glutamine, and 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum). Normal human tracheobronchial epithelia cells (NHBE) (Cambrex Corp.), and air–liquid interface cells (ALI) (Mattek Corp.) were maintained according to the providers' specifications. Briefly NHBE were grown on plastic in bronchial/tracheal cell basal medium (BEBM), with the following additives: bovine pituitary extract, human epidermal growth factor, hydrocortisone, epinephrine, transferrin, insulin, retinoic acid, thiodothyronine, gentamicin, and amphotericin. ALI cells were previously grown 25 days by the manufacturer and maintained on collagen-coated Millipore filters. In our laboratory, serum free DMEM with various hormones and growth factors (provided by Mattek Corp.) was changed each day in the basal compartment of these ALI cells. Primary rat tracheal epithelia (RTE) were isolated from Fischer 344 rats and were grown as previously described [Kaartinen et al., 1993].

CELL LYSATE PREPARATION

Cells were rinsed twice with PBS and lysed in the plate with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl, pH 8.0) containing protease and phosphatase inhibitors (Sigma, St. Louis, MO). Cells were scraped from the culture dishes to generate total cell lysates, which were then cleared at 4°C in a microcentrifuge at 14,000 rpm for 10 min. The protein concentration of each cell lysate was determined using the Advanced Protein Assay Reagent (Cytoskeleton, Denver, CO). These lysates were used in immunoprecipitation and/or immunoblotting analyses.

IMMUNOPRECIPITATION

Cell lysates in RIPA buffer, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, were utilized (150–200 μg per immunoprecipitation) in the immunoprecipitation with the IP Catch and Release 2.0 (Upstate Biosciences) according to the protocol provided. Briefly, columns were cleared and equal concentrations of protein lysates were added, as was the specific immunoprecipitating antibody: 2 μl of human c-pep anti-MUC4 rabbit polyclonal antibody (from laboratory stocks), 2 μl of Zymed 1G8 Ab (provided at 0.5 mg/ml), or of Neomarkers Ab 17 (provided at 200 μg/ml) with 10 μl of affinity ligand provided by the manufacturer, and wash buffer to a total volume of 0.5 ml. Columns were rotated overnight at 4°C, and the immunoprecipitate was collected for immunoblotting. For Figure 3B,C an alternative immunoprecipitation method was utilized. Cell lysates in RIPA buffer, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40 (150–200 μg lysate protein per IP) were used with Protein A/G agarose beads (Calbiochem). Equal concentrations of protein lysates were added. Immunoprecipitating antibodies were added at 10 μl each: anti-MUC4 4F12 (laboratory stocks of 400 μg/ml), Dako A0485 (provided at 0.5 g/L) or Neomarkers Ab 17, and Ab 21 (provided at 200 μg/ml), and wash buffer to a total volume of 0.5 ml. Beads were rotated overnight at 4°C, and the immunoprecipitate was collected for immunoblotting.

IMMUNOBLOTTING

Cell lysates and immunoprecipitates were collected as described above. Laemili buffer containing β-mercaptoethanol (Biorad, Hercules, CA) was added, and the samples were boiled for 5 min. For the separation of membranous and hydrophilic proteins the Mem-PER Eukaryotic Protein Extraction Kit (Pierce Corporation) was used as per the manufacturer's instructions. Equal amounts of proteins in cell lysates were loaded and separated by 8% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at 120 V for 1 h. Proteins were transferred to a nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI) for 1 h at 100 V or overnight at 15 V, or to a nitrocellulose membrane (Hybond, Amersham) for 2 h at 40 V. Immunoblotting procedures were performed according to the protocol for each antibody. Membranes were probed with primary antibodies against phospho-Akt 473 (Cell Signaling, Beverly, MA), β-Actin (Sigma), ErbB2 Ab 3 (Calbiochem), phospho-Tyr 1248 ErbB2 (Upstate, Lake Placid, NY), anti-ErbB2 Ab 17 (Neomarkers), phospho-Tyr 1248 ErbB2 (Ab 18, Neomarkers), human c-ErbB2 (DAKO Cytomation, Carpinteria, CA), ErbB3 (1B2E) and phospho-ErbB3 Tyr 1289 (21D3) (Cell Signaling, Beverly, MA), E-cadherin (BD Biosciences, San Jose, CA), phospho-Thr 202/Tyr 204 p44/p42 MAP Kinase (Cell Signaling, Beverly, MA), Muc4 4F12 [Rossi et al., 1996] or 1G8 [Zhang et al., 2005] and phospho-p70s6K (Cell Signaling, Beverly, MA). The secondary antibodies used were goat anti-mouse or anti-rabbit (Promega, Madison, WI) immunoglobulins coupled to peroxidase. Membranes were developed using the Supersignal West Pico Chemiluminescence substrate (Pierce Biotechnology, Rockford, IL) and exposed on X-ray film. The intensity of the bands was quantified by digitizing the image (Scion Image, Scion Corporation) from X-ray film.

IMMUNOHISTOCHEMISTRY

Paraffin-embedded, consecutive 5 μm sections of normal human airway were immunostained for MUC4 (1G8 mAb, Zymed) and ErbB2 (Calbiochem Ab 3) as described previously [Weed et al., 2004; Zhang et al., 2006]. Briefly, slides were dried for 1 h in a horizontal position at 58°C in an oven. Tissues were then dewaxed in xylene and rehydrated through an ethanol gradient to water series. After washing in water, the endogenous peroxidases were blocked with a freshly made 3% H_2O_2 solution for 5 min. Slides were then treated in a preheated target retrieval solution (DAKO S1699) for 20 min at 97°C and cooled for 20 min at room temperature. After washing, tissues were stained with the antibodies in an antibody diluent solution with background reduction at 37°C in a humidified chamber for 30 min. A biotinylated secondary antibody was applied to the slides and incubated for 30 min, and conjugated streptavidin-peroxidase (DAKO K1500) was added for 30 min at room temperature. Peroxidase activity was detected by incubation with DAKO Liquid DAB (3′3-diaminobenzidine dehydrate) in substrate-chromogen solution for about 2 min. Slides were rinsed in water, counterstained with hematoxylin for 25 s, then rinsed in running water. Slides were then dehydrated in ethanol and cleared with xylene before mounting (VWR mounting medium). Images were acquired with an Olympus BX40 microscope (Olympus, Japan). Negative controls had no primary antibody added.

IMMUNOFLUORESENCE

Consecutive 5 μm sections of paraffin-embedded normal human trachea were analyzed using tyramide signal amplification immunofluorescence (Molecular Probes) with 1G8 primary mAb against MUC4β or Ab 3 primary mAb against ErbB2. Paraffin was removed, and sections were hydrated with three 3 min baths in xylene, xylene/ethanol 1:1, ethanol, 95% ethanol, 80% ethanol and water. Sections were then bathed four times, for 10 min each, in a freshly made 5 mg/ml solution of sodium borohydride in phosphate-buffered saline (PBS), then rinsed with PBS. Cells were permeabilized in acetone for 15 min, then rinsed again with PBS. The sections were quenched in a 3% H_2O_2 solution for 2 h and further rinsed in PBS. Slides were blocked with 1% blocking reagent (TSA kit, Molecular Probes) for 1.5 h and rinsed with PBS. Biotin was blocked by incubating with reagent A (Molecular Probes) for 15 min, rinsing with PBS, then incubating with reagent B for 15 min, and rinsing with PBS. Primary antibodies were diluted in Blocking Solution (Molecular Probes), and sections were incubated at 4°C overnight. After rinsing with PBS, sections were incubated with peroxidase-conjugated secondary antibodies (Molecular Probes) diluted in blocking solution for 45 min. The manufacturer's instructions were followed for Tyramide amplification. Sections were mounted and laser confocal microscopy was performed with a Zeiss LSM 510 microscope (Carl Zeiss, GmbH, Germany) using a 40× objective in the Imaging Core at the Diabetes Research Institute, University of Miami School of Medicine.

Immunofluoresence immunostaining was used to localize MUC4 protein expression in ALI cells. Media were removed and cells were washed with PBS, and fixed with 3% paraformaldehyde. Cells were then permeabilized in 0.2% Triton X-100, and blocked with 5% bovine serum albumin. Then primary antibodies were added overnight, and washed three times. Secondary antibody (goat-anti-rabbit-Texas Red, Molecular Probes) was added, and then washed. DAPI was included in the subsequent wash, and slides were mounted (Prolong gold medium, Invitrogen Corp.). Laser microscopy was performed as described above.

MECHANICAL WOUNDING

NCI-H292 cells were cultured as indicated above, and wounding was done by scratching cell layers with a pipette tip multiple times in each well, using a cross-hatch pattern of four vertical and four horizontal scratches. Three and 24 h post-injury, cell lysates were collected as described above. Cell lysates from three wells were pooled for immunoprecipitation and immunoblotting.

MUC4 siRNA INHIBITION

NCIH292 cells were transfected with 100 nM MUC4 siRNA (Dharmacon, Lafayette, CO) or non-targeted siRNA using transfection reagent 2 (Dharmacon, Lafayette, CO) as per the manufacturer's instructions 48 h before wounding was performed. Triplicates for each condition were pooled.

AG825 TREATMENTS

NCI-H292 cells were grown as described above. Cells were treated with AG825 (Biosource)/tyrophostin as done previously [Ramsauer et al., 2006], or treated with AG825 and injured as described above.

Total cell lysates were collected 3 h post-injury and analyzed by immunoblot. Triplicates for each condition were pooled.

RESULTS

Muc4/MUC4 AND ErbB2 CO-EXPRESSION AND CO-LOCALIZATION IN AIRWAY EPITHELIAL CELLS

Our previous work has shown that Muc4 is expressed at the apical surface of the rat trachea [McNeer et al., 1998]. However, Muc4 expression is sometimes lost when cells are placed in culture [Komatsu et al., 1997]. Therefore, we have examined the expression of Muc4 and ErbB2 in four different types of airway cell cultures. Figure 1A shows strong expression of both Muc4/MUC4 and ErbB2 in rat tracheal epithelial cells, normal human bronchial epithelial cells and air–liquid interface cultures. We have never encountered a time during which MUC4 protein expression was lost in these cell lines. NCI-H292 cells are frequently used as a model culture system for the airway epithelium [Kim et al., 2005] and undergo contact growth inhibition [van Schilfgaarde et al., 1995]. This culture system forms tight junctions between cells [van Schilfgaarde et al., 1995] and expresses all of the catenins, E-cadherin, ICAM-1 [Molock and Lillehoj, 2006], and actin. These cells undergo differentiation as they approach confluence, with increased amounts of E-cadherin (Fig. 1B) and the formation of adherens junctions. This differentiation is accompanied by increased expression of both MUC4 and ErbB2 (Fig. 1B).

Our previous results on cultured epithelial cells predict that Muc4 and ErbB2 will be co-localized at airway luminal surfaces [Ramsauer et al., 2003, 2006]. To examine this question in the airway, we used both immunohistochemical and immunofluorescence analyses in airway tissues. As shown by immunohistochemistry of human airway sections, MUC4 is located at the apical surfaces of the cells of all three layers of the pseudostratified epithelium (Fig. 2A). ErbB2 is co-localized with the MUC4 in the cells exposed to the luminal surface. The co-localization is further shown by merged immunofluorescence images of Muc4 and ErbB2 in human tissue (Fig. 2B). In this case the extracellular Muc4 in the ciliary layer can clearly be seen above the apical surface of the epithelium, where the Muc4 and ErbB2 are co-localized in the apical membrane. These results raise the question whether the MUC4 observed in the ciliary layer is actually associated with the cilia or only with the periciliary fluid. To address this question, we used fluorescent staining of ALI cultures. As shown in Figure 2C, MUC4 staining of ALI clearly shows the presence of the MUC4 in the cilia and apical membrane. ErbB2 and ErbB3 staining were not observed in the cilia and were limited to the cell membrane. This staining pattern was similar to that seen in

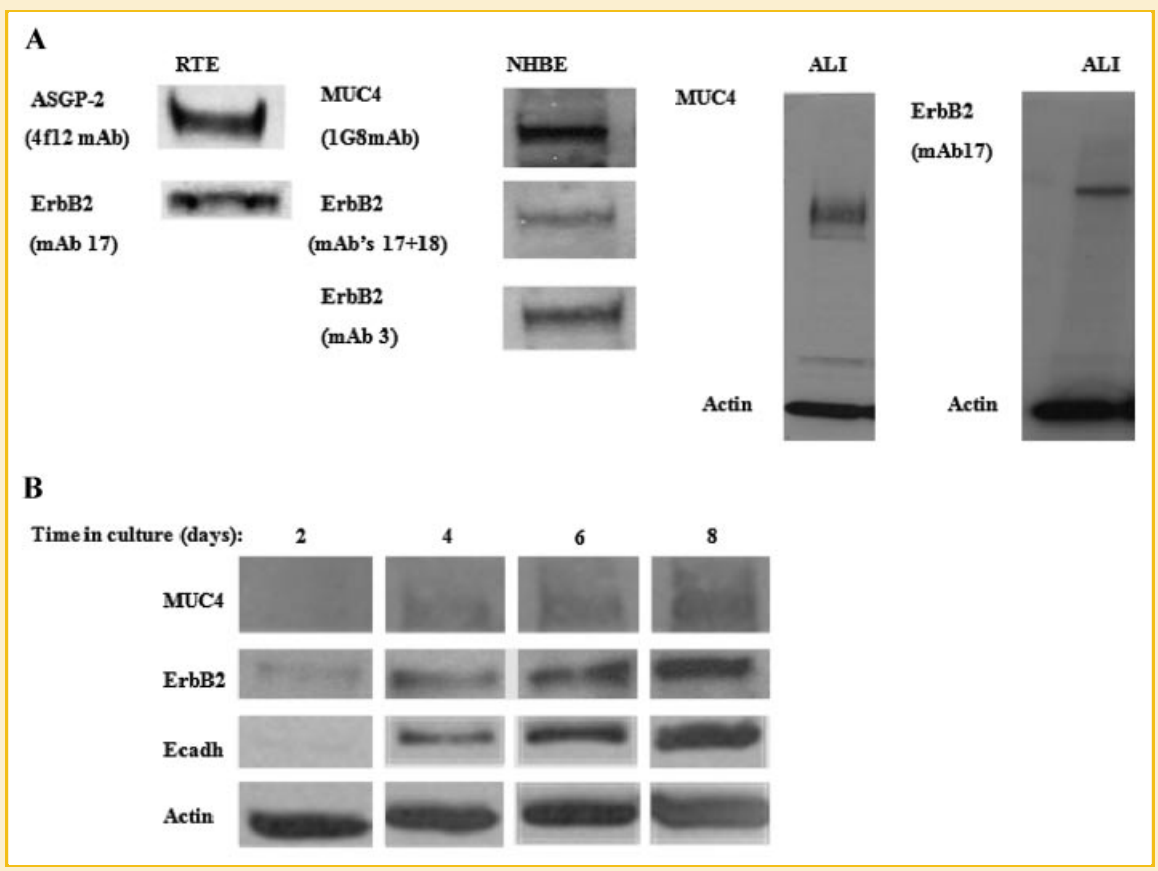

Fig. 1. Expression of Muc4/MUC4 and ErbB2 in airway epithelial cells. A: Immunoblots of Muc4/MUC4 and ErbB2 in RTE, NHBE and ALI cell cultures. B: Time course of expression of MUC4 and ErbB2 compared to E-cadherin in NCI-H292 cell cultures growing to confluence and undergoing differentiation.

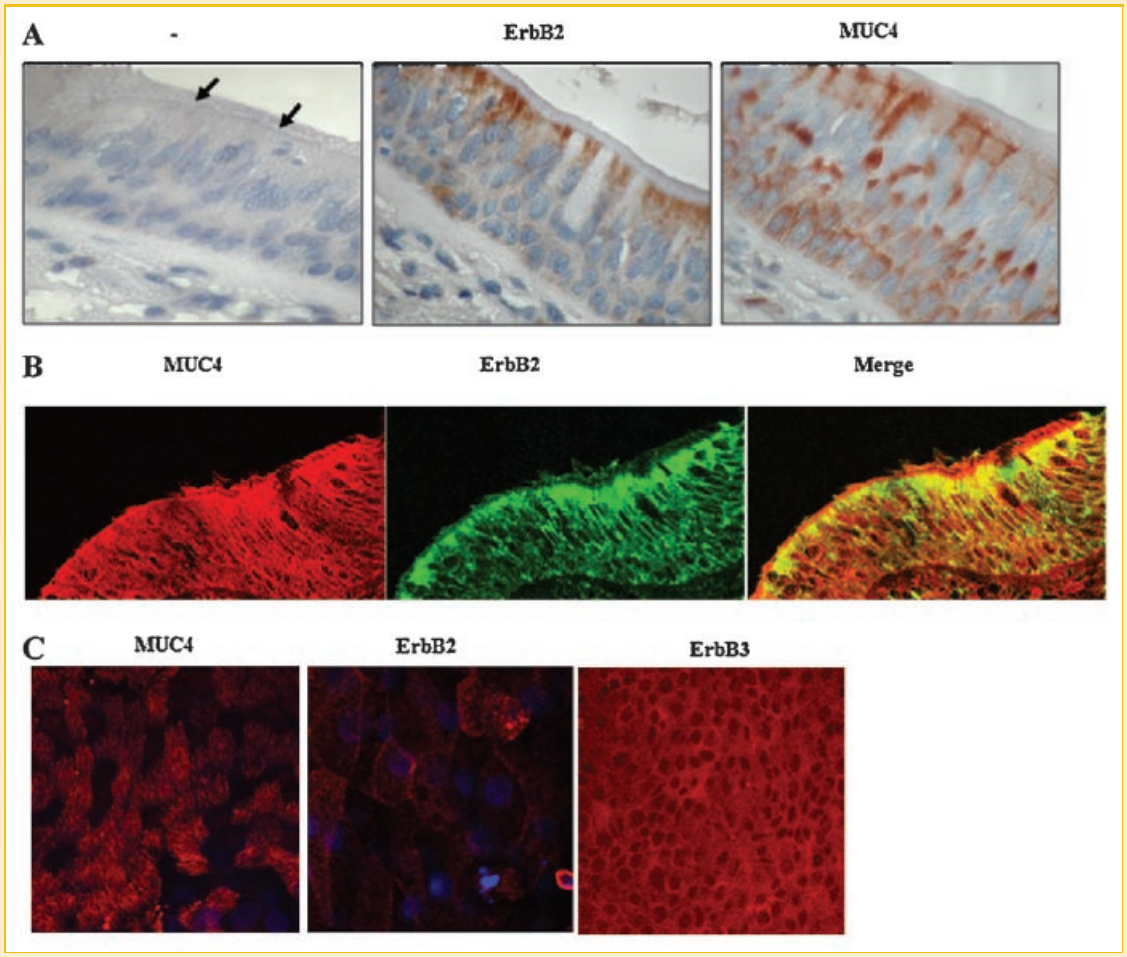

Fig. 2. Co-localization of MUC4 and ErbB2 in human airway epithelium. A: Immunohistochemical staining of MUC4 and ErbB2 versus control in human airway tissue. Arrows in control note luminal cell surface below ciliary layer. B: Immunofluorescence staining of MUC4 (red) and ErbB2 (green) in human airway tissue. In merged panel, note soluble form of MUC4 in periciliary layer and co-localization of MUC4 and ErbB2 at luminal surface (yellow color). C: Confocal microscopy of ALI cell apical surfaces permeabilized and stained with C-pep (anti-MUC4). Cilia and membranous staining is visible (400×). ALI cells permeabilized and stained with Dako pAb (antiErbB2). Membranous staining is visible. Cilia are not stained (400×). ALI cells were permeabilized and satined with anti-ErbB3 mAb (Cell Signaling). Membranous staining is visible.

human airway tissue (Fig. 2B). Importantly, ErbB2 was associated with the apical surface, while ErbB3 was associated with the lateral surfaces of the cells (Fig. 2C), as we have shown in previous studies. These findings were corroborated by immunohistochemical localization of MUC4 to the apical membrane of ALI cell cultures (data not shown). These combined results also show, in contrast to previous studies [Vermeer et al., 2003], that ErbB2 is present at the luminal surface of the airway. As we have demonstrated previously, the localization of ErbB2 is highly dependent on the antibody used for its detection [Idris et al., 2001]. Thus, we have used multiple antibodies to confirm the presence of ErbB2 at the luminal surface. Our studies do not, of course, rule out the possibility that some ErbB2 is also localized to lateral surfaces, as we have shown in mammary epithelia [Price-Schiavi et al., 2005], only that it is present at the apical surface with Muc4.

Our previous studies have also shown that a complex of Muc4 and ErbB2 can be demonstrated in multiple tissues [Carraway et al., 2002]. To test for the complex, we immunoprecipitated lysates of rat tracheal epithelial cells with anti-Muc4. MUC4 binding may alter epitopes on ErbB2 [Idris et al., 2001] due to phosphorylation and binding of signaling complexes. Hence for immunoprecipitations of the complex, we used both extracellular and intracellular domain-oriented antibodies to the ErbB2 and MUC4 to improve immunoprecipitation efficacy. Immunoblotting of the immunoprecipitates for ErbB2 demonstrated the precipitation of a complex of Muc4 and ErbB2 (Fig. 3A), as we had previously shown for other tissues [Price-Schiavi et al., 2005] and cell types [Carraway et al., 1999]. The MUC4–ErbB2 complex was similarly demonstrated in human ALI cultures. In this case the lysates were immunoprecipitated with either anti-ErbB2 or anti-MUC4 and the immunoprecipitates were immunoblotted for ErbB2 (Fig. 3B, left panel). In ALI cells, we also immunoblotted for phospho-ErbB2 (1248) and found the phosphorylated receptor in complex with MUC4 (Fig. 3B, center panel, Ab 18). The presence of ErbB2 in the cell lysate was confirmed via immunoblot as a positive control (Fig. 3B, right panel). We previously showed the specificity of the Ham1/HCpep anti-MUC4

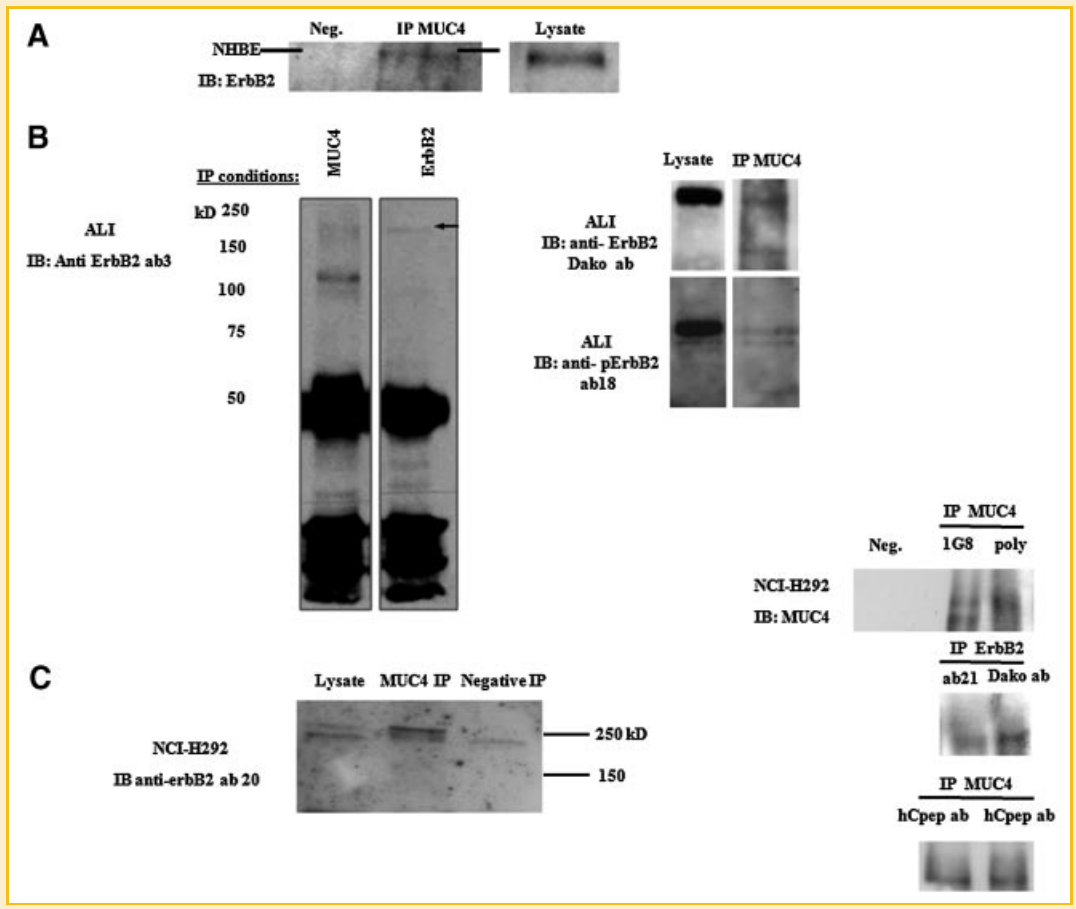

Fig. 3. Association of MUC4 and ErbB2 in complex in three types of human airway epithelial cells. A: Lysates of NHBE cells were immunoprecipitated (IP) with anti-MUC4 (IP/IB) or control (neg) antibody. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-ErbB2 Ab. B: Left: Lysates of ALI cell cultures were immunoprecipitated with anti-ErbB2 Dako or anti-MUC4 4F12. The immunoprecipitates were analyzed by immunoblotting with anti-ErbB2 antibody. Arrows show ErbB2 bands. Right: Lysates of ALI cells were immunoprecipitated with the anti-MUC4 antibody Hcep/Ham1. Immunoprecipitated samples and cell lysates were then immunoblotted with anti-ErbB2 antibody (top), and anti-phospho ErbB2 antibody (bottom). C: Lysates of NCI-H292 cells were immunoprecipitated for; left: MUC4 with anti-MUC4 or control antibody (negative IP). Those immunoprecipitates were immunoblotted for ErbB2, as was a total cell lysate control right: (top) MUC4 with the 1G8 mAb, and the polyclonal anti-MUC4 Ab (center) ErbB2 with Neomarkers Ab 21, and Dako c-erbB2 Ab (bottom) MUC4 in two reactions with human c-pep Ab.

antibody for membranous MUC4 via immunoblot (data not shown). MUC4–ErbB2 complex was also seen in NCI-H292 cells that were immunoprecipitated for MUC4, followed by immunoblotting for the extracellular domain of ErbB2 (Fig. 3C, left (Ab 20)). Next we immunoprecipitated MUC4 and ErbB2 and immunoblotted for MUC4 (Fig. 3C, right). Thus, immunoprecipitation in all three model cultures detected the MUC4/ErbB2 complex. Taken together, these results in Figures 2 and 3 clearly show that the MUC4/ErbB2 complex is present in the apical membrane of airway epithelial cells. Our data support the hypothesis of a functional complex in which MUC4 may mediate ErbB2 phosphorylation.

PARTICIPATION OF MUC4 IN ErbB2/ErbB3 PHOSPHORYLATION RESPONSE TO NCI-H292 CELL CULTURE DAMAGE

As model systems for airway epithelial cell damage, we used the NCI-H292 cells, a widely used cell model for wounding experiments [Shaykhiev et al., 2008], and ALI cells. Differentiated cultures of NCI-H292 cells (Fig. 1B) were subjected to scratch wounding. After a 3 or 24 h period the cells were lysed and immunoprecipitated with anti-MUC4. The immunoprecipitates were analyzed by immunoblotting with anti-phospho-ErbB2 and anti-phospho-ErbB3 to determine whether the two phosphorylated receptors are associated with MUC4. As shown in Figure 4A, phosphorylated ErbB2 and ErbB3 in complex with MUC4 was observed at both the 3 and 24 h periods. In immunoblots utilizing two different anti-MUC4 antibodies, MUC4 was not detectable in the "flow-through" material from these immunoprecipitates (data not shown). These results indicate that HAM1 pAb is efficient in the immunoprecipitation of MUC4. Next, the specificity of the HAM1 Ab for membranous MUC4 was studied. The anti-MUC4 HAM-1pAb was used to detect MUC4 in NCIH292 cellular lysate preparations. Soluble and membrane preparations were subjected to SDS–PAGE electrophoresis and immunoblotted (Fig. 4B, left). HAM1 bound to the membrane preparation and not the soluble preparation. In addition, MUC4 siRNA inhibition was used to demonstrate the effect of MUC4 on ErbB3 phosphorylation (Fig. 4B, right). Upon siRNA inhibition of MUC4, HAM1 immunoprecipitation of MUC4 and a subsequent

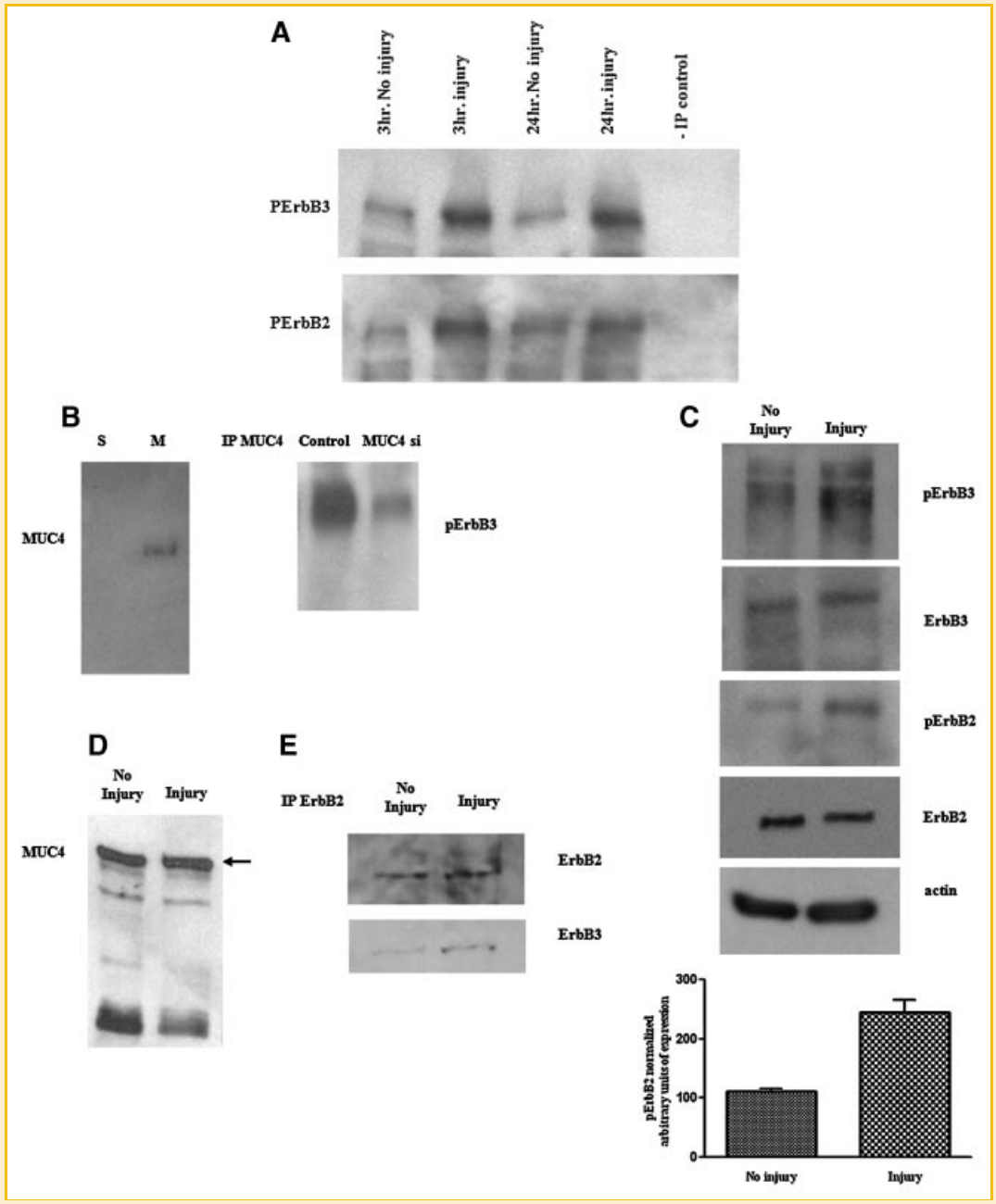

Fig. 4. Effect of scratch wounding of airway cell cultures on ErbB phosphorylation in complexes with MUC4. A: NCI-H292 cultures with or without scratch-wounding were kept for 3 or 24 h in culture, then lysed for immunoprecipitation with anti-MUC4 or a control antibody. The immunoprecipitates were then immunoblotted with anti-phospho-ErbB2 or anti-phospho-ErbB3. B: Right: Soluble (S) and membrane (M) NCI-H292 cell lysates were immunoblotted for MUC4 with HCpep Ab left: NCI-H292 lysates from control and MUC4 siRNA treated cells was immunoblotted for pErbB3. C: ALI cells with or without scratch wounding were kept for 3 h in culture and then were immunoblotted with the listed antibodies. Quantitation of pErbB2 is provided below. D: NCI-H292 cultures with or without scratch-wounding were kept 3 h, then lysed for immunoblotting with anti-MUC4 Ab. E: ALI cells with or without scratch wounding were immunoprecipitated with anti-ErbB2 Ab (Neomarkers Ab 17) and then immunoblotted with anti-ErbB2 Ab (Dako) and anti-ErbB3 Ab (Cell Signaling).

immunoblot for phospho-ErbB3 showed a decrease of detectable phospho-ErbB3.

These experiments indicated the presence of a complex of MUC4 with the phosphorylated ErbBs in wounded cultures and suggested that wounding increases ErbB2 and ErbB3 phosphorylation. We then performed the same wounding experiment on ALI cells. After a 3 h period, wounding of ALI cells increased ErbB3 and ErbB2 phosphorylation but did not alter total levels of these molecules, as detected by immunoblotting. Therefore, the same pathway was activated by wounding as in the NCI-H292 cells (Fig. 4C). Three

hours post-injury, ErbB2 phosphorylation was increased more than two and half times relative to uninjured cells (Fig. 4C, bottom). These combined observations agree with previous studies showing increased phosphorylation of these receptors during airway damage [Vermeer et al., 2003], but also indicate that MUC4 is associated with the complex of receptors in the cell cultures subjected to damage. In contrast to the change in receptor phosphorylation, no change was observed in the levels of MUC4 in the wounded cultures (Fig. 4D). In ALI, we immunoprecipitated total ErbB2 and then immunoblotted for total ErbB2 and total ErbB3 (Fig. 4E). As was demonstrated in Figure 4B via immunoblot, total ErbB2 levels are not altered when comparing pre- to post-injury although phospho-ErbB2 levels were increased post-injury. However, we do see an increase in ErbB3 in complex with ErbB2. These immunoprecipitates demonstrate that the precipitation is specific for MUC4, and show an enhanced association between ErbB2 and ErbB3 post-injury as compared to pre-injury in ALI cells.

As further evidence for the importance of ErbB phosphorylation in the damage response, we examined effects of culture wounding on pathways downstream of the ErbB2/ErbB3 complex, specifically Erk phosphorylation and the Akt pathway [Jepson et al., 2002; Funes et al., 2006]. No changes were observed in Erk phosphorylation due to the epithelial damage (Fig. 5A). However, phosphorylation of both Akt and p70rs6k (Rsk) from the PI3K/Akt and PI3K/rsk pathways were increased in the wounded cultures (Fig. 5B). The levels of pAkt after injury were two times greater than in non-injured cells (Fig. 5C). When these experiments were repeated in the ALI system, the results were similar to data from NCI-H292 cells (Fig. 5D). These results are in accord with our previous studies of the effects of Muc4 on downstream signaling [Jepson et al., 2002].

Work from our laboratory has shown that formation of the MUC4–ErbB2 complex in epithelial cells enhances ErbB2 phosphorylation at position 1248 [Ramsauer et al., 2006]. To further test the involvement of MUC4 in the phosphorylation response of the receptors, we knocked down the MUC4 in the NCI-H292 cells with siRNA before inducing damage to the cultures. Using this method, we were able to achieve 80–90% decreases in the levels of MUC4 (Fig. 6A). This MUC4 inhibition did not subsequently decrease the total levels of endogenous ErbB2 receptor (Fig. 6A). As noted in Figure 6B, loss of MUC4 results in a diminution of the phosphorylation of both ErbB2 and ErbB3 in the NCI-H292 cultures in response to wounding. These findings are consistent with our previous work demonstrating that MUC4 expression upregulates ErbB2 phosphorylation without impacting total expression of the tyrosine kinase [Ramsauer et al., 2003; Funes et al., 2006]. The mean relative decrease in ErbB2 phosphorylation upon MUC4 siRNA inhibition was nearly fivefold compared to un-inhibited cells (Fig. 6C). We previously showed that although ErbB phosphorylation is altered post-injury, total ErbB receptor levels are not affected. To further investigate the role of ErbB2 during the post-injury

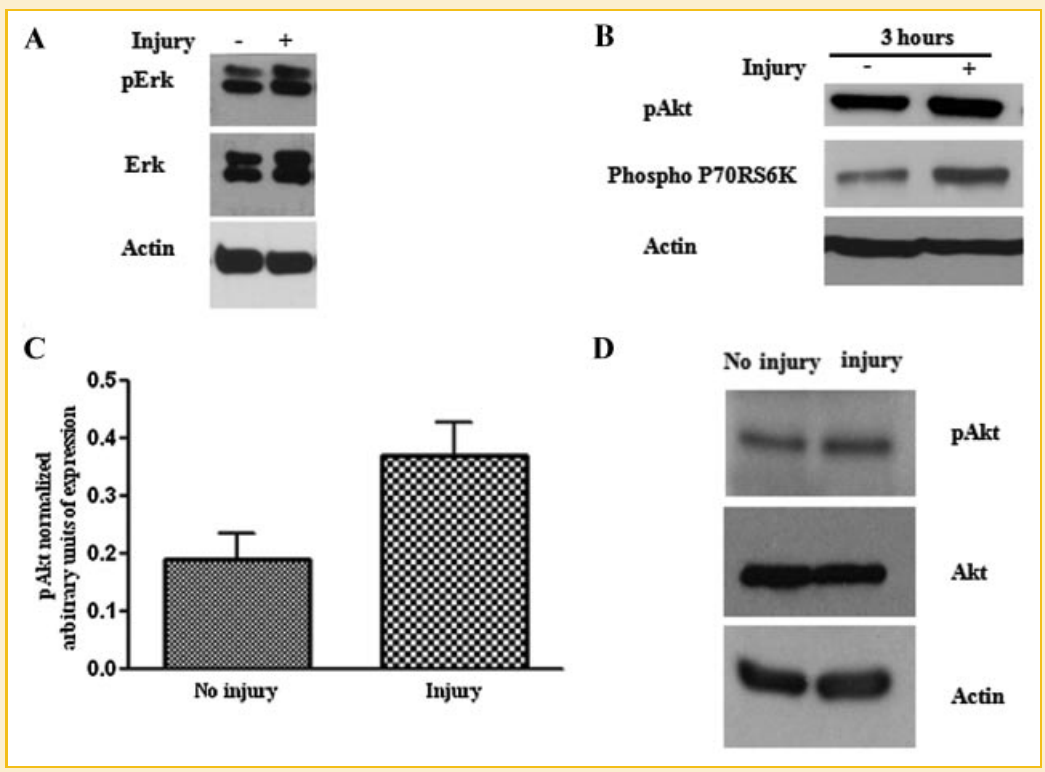

Fig. 5. Downstream pathways affected by scratch wounding of airway cell cultures. A: NCI-H292 cell cultures with or without scratch-wounding were kept for 3 h, then lysed for immunoblotting with anti-phospho-Erk. B: NCI-H292 cultures with or without scratch wounding were kept for 3 h, then lysed for immunoblotting with anti-phospho-Akt and anti-phospho-p70rsk. C: Quantitation of normalized pAkt with and without scratch wounding. D: ALI cultures with or without scratch wounding were kept for 3 h, then lysed for immunoblotting with anti-phospho-Akt, Akt, and actin.

period, ErBb2 phosphorylation was partially inhibited by the specific inhibitor AG825 (Fig. 6D). We find that inhibition of ErBb2 phosphorylation diminishes ErbB3 and Akt phosphorylation. Together, these results indicate that scratch wounding activates the Akt signaling path through ErbB2. Next NCI-H292 cells were injured and treated with the inhibitor (Fig. 6E). Phosphorylation of both Akt and Rsk were diminished upon AG825 treatment compared to controls in both wounded and unwounded cultures. These results suggest that Akt and Rsk activation upon mechanical injury was mediated by ErbB2 kinase activity in airway epithelia, although they do not rule out participation by other kinases inhibited by this agent.

DISCUSSION

Previous studies by others introduced a novel model for the response of the airway epithelium to damage via phosphorylation of the receptors ErbB2 and ErbB3 [Vermeer et al., 2003]. Damage breaks the tight junction barrier segregating ErbB3 from its ligand neuregulin and induces formation of the ErbB2/ErbB3 receptor heterodimer with its concomitant activation and phosphorylation. However, this model neglects a possible contribution from the ErbB2 ligand MUC4. We have previously shown that MUC4 modulates the ErbB2/ErbB3 complex in polarized epithelial cells by regulating the localization and phosphorylation of ErbB2 [Ramsauer et al., 2006]. In the present studies we provide evidence that MUC4 also participates in the response to airway damage. Three aspects are particularly important. (1) MUC4 and ErbB2 are co-localized and present in a complex at the luminal surface of the airway epithelium. One particularly interesting aspect of that localization is the observation of MUC4 without ErbB2 in the cilia of the airway cells. This result implies that there is a mechanism for transport of the MUC4, but not the MUC4–ErbB2 complex into the ciliary membrane. What function the MUC4 serves at the ciliary surface is unknown, but its anti-adhesive properties might suggest a role in preventing interactions between cilia which might hinder ciliary movement. (2) Damage to airway cell cultures increases the phosphorylated ErbB2 and ErbB3 associated with MUC4. The presence of phosphorylated ErbB3 in this complex is particularly important because phosphorylated sites on ErbB3 are involved in activating the phosphatidyl-inositol 3-kinase pathway upstream of the Akt and Rsk pathways. Similar effects of Muc4 have been observed in other

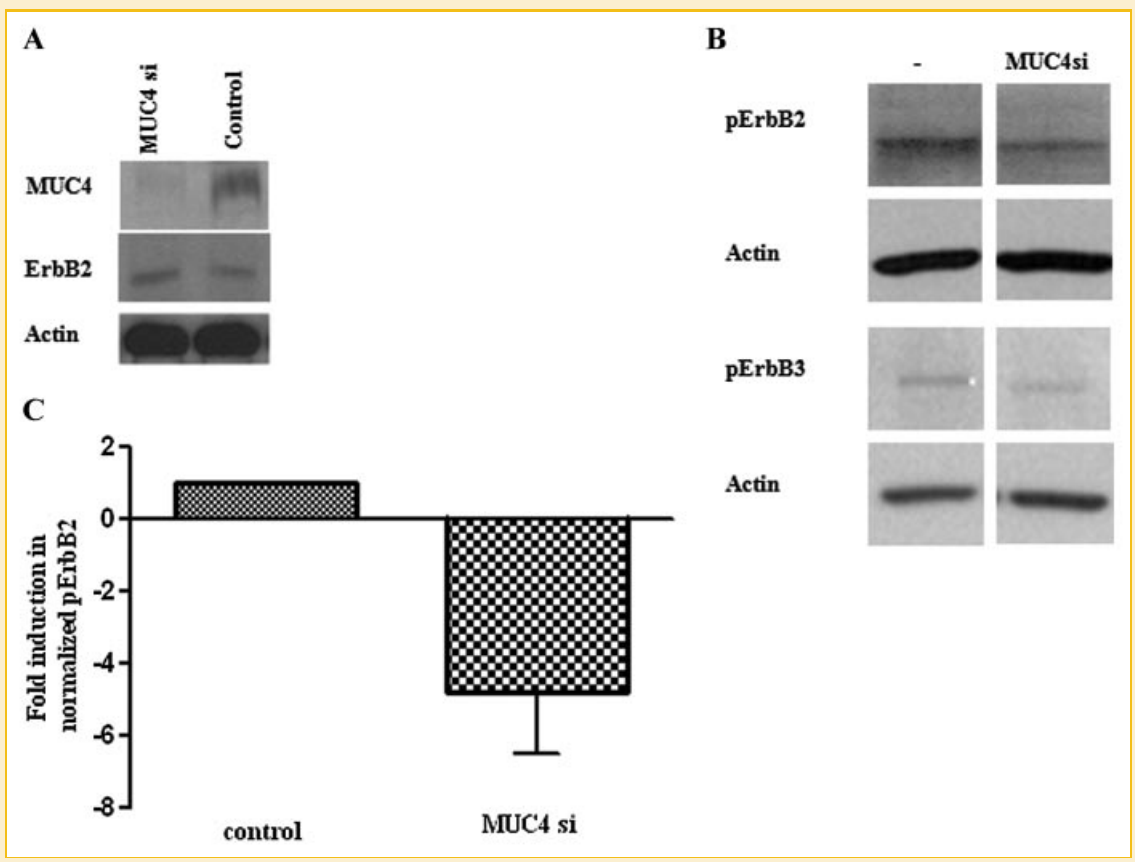

Fig. 6. Effect of MUC4 knockdown on scratch-wounding induction of ErbB2 and ErbB3 phosphorylation. A: NCI-H292 cells were treated with MUC4 siRNA or control RNA and analyzed for MUC4 and ErbB2 (Ab 3) by immunoblotting. B: NCI-H292 cultures treated with MUC4 siRNA or control RNA were scratch-wounded as in Figure 5 and analyzed for phospho-ErbB2 and phospho-ErbB3 by immunoblotting. C: Quantitation of normalized pErbB2 expression with and without MUC4siRNA inhibition. Results are shown as fold induction over pErbB2 expression without MUC4siRNA inhibition. D: NCI-H292 cells were exposed to multiple mechanical wounds, with or without treatment with ErbB2 inhibitor AG825 (3.5 μM), and cell lysates were immunoblotted for phospho-ErbB2 and -ErbB3. E: NCI-H292 cells with or without wounding and the ErbB2 inhibitor AG825 (7.0 μM/AG 7.0) were immunoblotted for phospho-Akt and -Rs6k (Rsk).

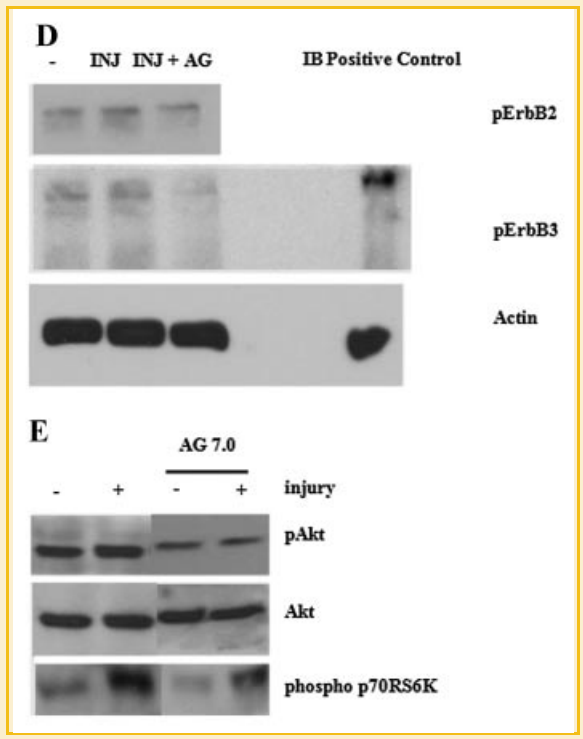

Fig. 6. (*Continued*)

cell types [Jepson et al., 2002]. Furthermore, we show that ErbB2 is present on the membrane of ALI cells apically prior to injury (Fig. 2C) although ErbB3 is present on the membrane basolaterally. Although total ErbB2 levels do not change upon injury, the amount of ErbB3 in complex with ErbB2 increases. These changes occur upon the disruption of cell–cell contacts post-injury. We do not believe that this increase in ErbB2/3 complex can be attributed to new ErbB3 protein synthesis since the samples are collected only 3 h post-injury. (3) Downregulation of MUC4 demonstrates its involvement in the effects on ErbB phosphorylation. Moreover, the effects of the ErbB2 inhibitor establish the role of ErbB2 in the MUC4 activation of the downstream pathways.

These results suggest a new model for the airway damage response (Fig. 7). This model is based on two previous studies, the work by Vermeer et al. [2003] showing ErbB2/3 phosphorylation in response to airway damage and our own work showing ErbB2 and ErbB3 segregation promoted by Muc4 in polarized epithelial cells [Ramsauer et al., 2006]. The model is further supported by our present work demonstrating the presence of ErbB2 with MUC4 at the apical surface of airway epithelia, the presence of ErbB3 at the lateral surface and the increased phosphorylation of both ErbB2 and ErbB3 in complex with MUC4 after damage to an airway epithelial cell culture. In this model damage to the epithelium not only allows access of ErbB3 to its ligand, but also breaks the segregation barrier between the MUC4–ErbB2 on the apical surface and ErbB3 on the lateral surface of the polarized airway epithelial cells, as described in our previous work [Ramsauer et al., 2006]. Thus the damage promotes formation of the MUC4–ErbB2–ErbB3 complex, its consequent phosphorylation and activation of the downstream Akt and Rsk pathways.

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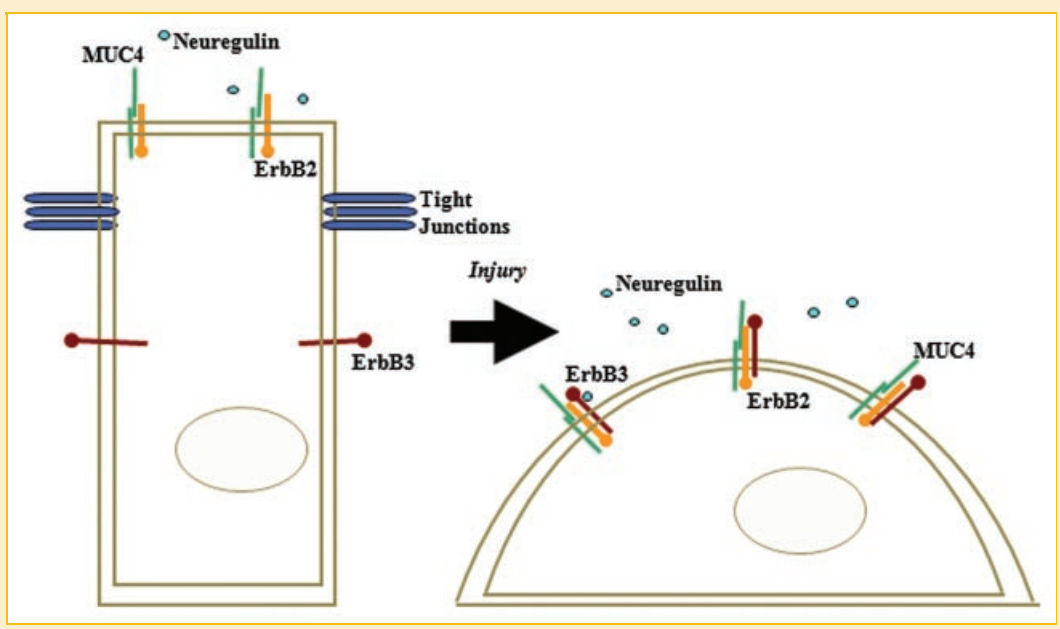

Fig. 7. Model for participation of MUC4 in phosphorylation response of ErbB2 and ErbB3 to airway epithelial injury.

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