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Muc4 is required for activation of ErbB2 in signet ring carcinoma cell lines

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Abstract

Signet-ring cell carcinoma is one of the most malignant tumors, classified histologically as a poorly differentiated adenocarcinoma. The ErbB2/ErbB3 complex is often constitutively activated, which suggests that the ErbB2/ErbB3 signaling pathway may be important for malignancy of this tumor. However, the mechanism underlying this activation has not been understood. Here, we show that ErbB2 and Muc4 bind in signet ring carcinoma cells, which was not seen in highly differentiated adenocarcinoma cell lines. ErbB3 was suggested to be a substrate of ErbB2 because knockdown of ErbB2 resulted in less phosphorylation of ErbB3. Inhibition of expression of Muc4 at the cell surface by the treatment of the cells with benzyl-GalNac, an inhibitor of mucin secretion, blocked phosphorylation of ErbB3, suggesting that activity of ErbB2 depends on the expression of Muc4. These results supply the biochemical backgrounds in recent studies suggesting the contribution of Muc4 in the tumorigenesis.

Keywords: PI 3-kinase; Muc4; ErbB2; ErbB3; Adenocarcinomas

Signet-ring cell carcinoma is one of the most malignant tumors often found in stomach, classified histologically as a poorly differentiated adenocarcinoma [1,2]. The cells are characterized by loss of cell to cell interaction and formation of large vacuoles, which may reflect enhanced secretion of the proteins such as mucins. In previous studies, we reported that phosphoinositide (PI) 3-kinase was activated by binding to ErbB3 phosphorylated on tyrosine in native signet ring cell carcinoma cell lines [3,4]. These signet ring cell carcinoma cell lines express high levels of ErbB2 as well, which are also constitutively phosphorylated on tyrosine. It is likely that the ErbB2/ErbB3 complex is activated in these cells [4].

ErbB2 is a member of the ErbB receptor tyrosine kinase family [5]. Unlike other ErbB family members, ErbB2 does not have its high-affinity ligands but forms complexes with other ErbB family members such as ErbB3 to contribute to signal transduction. ErbB2 and Muc4 have suggested to form a ligand–receptor-like complex although this complex has been detected only when these proteins were overexpressed in the cultured cells [6]. Muc4 is composed of two noncovalently associated subunits, ASGP-1 and ASGP-2 [7], which arise from proteolytic processing of a single gene product [8]. Subunit ASGP-2 (120 kDa) [9] has been suggested to be an intramembrane ligand for ErbB2 via an EGF-like domain [10]. This interaction induces phosphorylation of ErbB2 in the absence of a soluble ligand and potentiates the phosphorylation of the ErbB2–ErbB3 heterodimer in the presence of the ErbB3-soluble ligands, such as heregulin [6].

In this paper, we demonstrate that ErbB2 is bound to Muc4 in signet ring carcinoma cell lines derived native tumors, which may contribute to constitutive activation of the ErbB2–ErbB3 pathway.

Materials and methods

Cell culture. PLAT-A and 293T cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 5% calf

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serum [11,12]. PLAT-A cells were maintained in the presence of puromycin (1 μg/ml) and blastocydin S (10 μg/ml). NUGC4, KATOIII, Sch, MKN74, and HCC2998 cells were cultured in RPMI1640 medium containing 5% fetal bovine serum [4].

Preparation of expression vectors for sh-RNAs. After testing several oligonucleotides, 5′-aagaacaaccagctggctctc-3′ was selected to suppress the expression of ErbB2. The oligonucleotide was introduced between *Hin*dIII and *Bgl*II sites of pSUPER, an expression of sh-RNA, to produce pSUPER-sh-ErbB2 (OligoEngine, Seattle, WA).

Introduction of DNA into the cells and establishment of NUGC4 cells expressing sh-RNAs. Transfection of 293T and PLAT-A cells was done by calcium phosphate method as described before [13]. Introduction of DNA into NUGC4 cells was done by a retrovirus mediated method [12]. Briefly, culture supernatant of PLAT-A cells transfected with the expression vectors for the sh-RNA were added to NUGC4 cells in the presence of 8 μg/ml of polybrene for 12 h. Then the medium was replaced with fresh one containing 1 μg/ml puromycin to select the infected cells. More than 80% of the cells survived indicating that the infection of the cells with the viruses was efficiently done.

Antibodies used in this study. Rabbit anti-ErbB3 antibody (C17) was purchased from Santa Cruz. Rabbit polyclonal anti-ErbB2 and anti-Muc4 antibodies were raised against bacterially expressed full length ErbB2 and Muc4 tagged with glutathione-*S*-transferase (GST), respectively. Mouse monoclonal anti-ErbB2 antibody CB11 was from Progen Biotechnik (Heidelberg). Mouse monoclonal anti-Muc4 antibody was from Invitrogen (Carlsbad, CA). Anti-phosphotyrosine antibody, PY20, was as described before [14].

Immunoprecipitation. Immunoprecipitation was done in RIPA buffer with antibodies bound to protein A agarose beads (EMD Biosciences, Darmstadt, Germany). After washing three times with RIPA buffer, the proteins bound to the beads were analyzed by Western blotting.

Staining of the cells. NUGC4 cells were fixed with 3.7% formaldehyde and stained with rabbit polyclonal anti-Muc4 antibody. After washing with phosphate-buffered saline (PBS), the signal of Muc4 was visualized with a second antibody conjugated with Alexa-488 (Invitrogen). Cells were analyzed with a Fluoview laser-scanning confocal microscope (Olympus, Tokyo).

Biotinization of the cells. After washing the intact cells in the culture dish with PBS, cells were treated with 0.4 mg/ml Sulfo-NHS-biotin on ice for 30 min. After stopping the reaction with PBS containing 50 mM NH_4Cl, the cells were lysed with RIPA buffer. The biotinated proteins were precipitated by Avidin agarose beads (Sigma–Aldrich, St. Louis, MO).

Results

ErbB2 may be activated to phosphorylate ErbB3 in signet ring carcinoma cell lines

It has been shown that ErbB3 is often heavily phosphorylated on tyrosine in signet ring carcinoma cell lines, most likely by ErbB2, which may form a complex with ErbB3. To confirm this, we knocked down ErbB2 by sh-RNA in NUGC4 cells, a signet ring cell line. As shown in Fig. 1A, the level of ErbB2 was decreased by the sh-RNA compared to the wild-type NUGC4 while that of p85α a subunit of phosphatidylinositol 3-kinase (PI3K), as a control remained unchanged. It has been shown that ErbB2 and ErbB3 form a complex and ErbB2 may phosphorylate ErbB3. ErbB3 was immunoprecipitated with anti-ErbB3 antibody and its phosphoylation on tyrosine was examined by Western blotting with anti-phosphotyrosine antibody. As shown in Fig. 1B, phosphorylation of ErbB3 on tyrosine was decreased when comparable amount of ErbB3 was immunoprecipitated. These results confirm that ErbB3 may be a substrate of ErbB2 in NUGC4 cells and that constant phosphorylation of ErbB3 may be the result of constitutive activation of ErbB2.

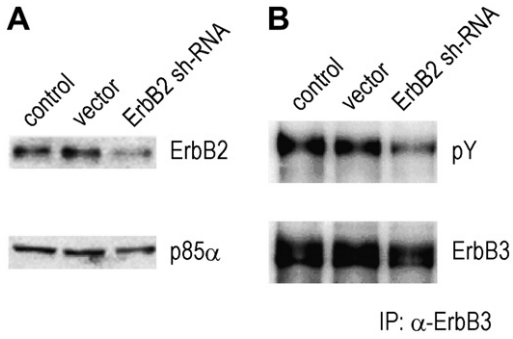

Fig. 1. Expression of sh-RNA for ErbB2 inhibits phosphorylation of ErbB3 on tyrosine. (A) Expression level of ErbB2 in NUGC4 cells expressing sh-RNA for ErbB2 was examined by Western blotting with anti-ErbB2 antibody, CB11. Western blotting with anti p85 antibody, AB6, was also done with the same samples for the controls. (B) ErbB3 was immunoprecipitated from the cells and the level of phosphorylation on tyrosine was analyzed by Western blotting with anti-phospohotyrosine antibody, PY20. The filter was reprobed with anti-ErbB3 antibody to confirm that the similar amounts of ErbB3 were present in each immunoprecipitate. Control, wild type NUGC4 cell; vector, NUGC4 cells harboring pSUPER; ErbB2 sh-RNA, NUGC4 cells expressing containing pSUPER-sh-ErbB2.

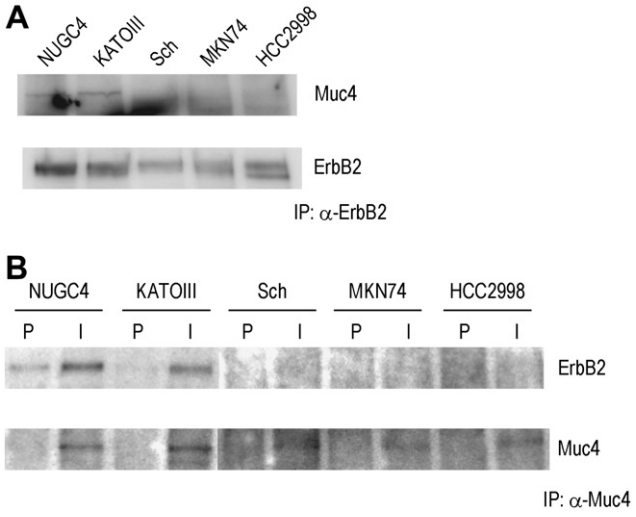

Fig. 2. Muc4 binds to ErbB2 in signet ring cell carcinoma lines. (A) ErbB2 was immunoprecipitated with a rabbit anti-ErbB2 antibody from the cell lysates containing approximately 500 μg protein and Muc4 present in the immunoprecipitates was detected with a monoclonal anti-Muc4 antibody. The lower panel shows the amounts of ErbB2 in the immunoprecipitates detected by a monoclonal anti-ErbB2 antibody, CB11. (B) ErbB2 was immunoprecipitated with a rabbit anti-Muc4 antibody or with preimmune serum and ErbB2 present in the immunoprecipitates was detected with a monoclonal anti-ErbB2 antibody, CB11. The lower panel shows the amounts of Muc4 in the immunoprecipitates detected by a monoclonal anti-Muc4 antibody.

ErbB2 and Muc4 co-immuoprecipitates in signet ring cell carcinoma cell lines

It has been suggested that secretion and expression at the plasma membrane of mucins including Muc1 and Muc4 are enhanced in signet ring cell carcinoma cell lines [4]. It has been suggested that ASGP-2 may bind to ErbB2 to activate it as a ligand on the plasma membrane. We therefore tested whether or not Muc4 bound to ErbB2 in these cells. Another signet ring cell carcinoma cell line KATOIII and highly differentiated adenocarcinoma cell lines Sch, MKN74, and HCC2998 were analyzed as well. As shown in Fig. 2, the band of ASGP-2 was detected in the immunoprecipitates of ErbB2 from the signet ring cell carcinomas but not in those from highly differentiated adenocarcinomas. This was confirmed by the immunoprecipitation with anti-Muc4 antibody. Although a nonspecific band was detected at the position of ErbB2 even in the immunoprecipitate of pre-immune serum, the signals of ErbB2 were readily detectable in the immunoprecipitates of Muc4 from NUGC4 or KATOIII cells, which was not found in those of other cell lines. These results suggest that ErbB2 and Muc4 form a complex in signet ring carcinoma cells but not in other cell lines.

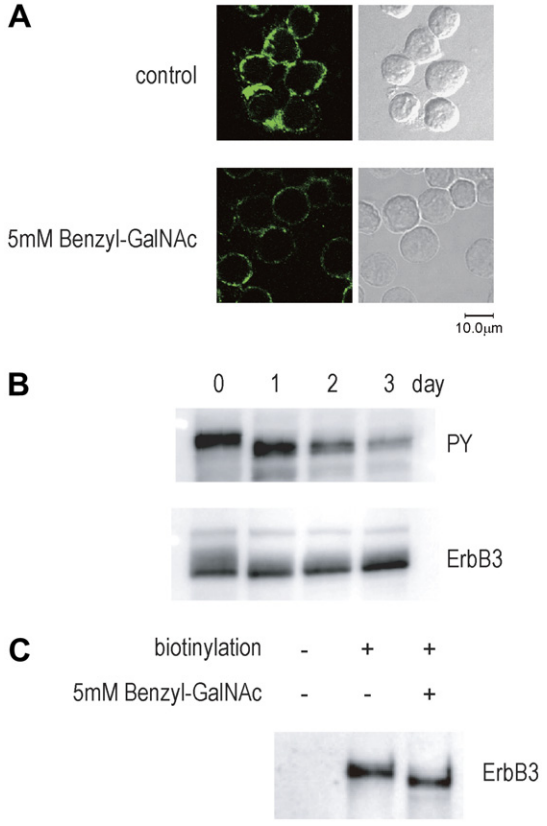

Fig. 3. Inhibition of mucin expression at the membrane blocks activation of ErbB2. (A) NUGC4 cells were cultured for 2 days in the presence or absence of 5 mM benzyl-GalNac. Muc4 at the cell surface was stained with polyclonal anti-Muc4 antibody. (B) ErbB3 was immunoprecipitated with an anti-ErbB3 antibody. Phosphorylation of ErbB3 on tyrosine was analyzed by Western blotting with anti-phosphotyrosine antibody, PY20. Amounts of ErbB3 in the immunoprecipitates were analyzed by reprobing the membrane with anti-ErbB3 antibody (lower panel). (C) NUGC4 cells cultured in the presence or absence of 5 mM benzyl-GalNac were biotinized. After lysing the cells, the biotinyzed proteins were precipitated with avidin beads and ErbB3 bound to the beads was analyzed by Western blotting with anti-ErbB3 antibody.

Inhibition of mucin secretion blocks phosphorylation of ErbB3

To test whether or not membrane expression of ASGP-2 induces activation of ErbB2, the effect of benzyl-GalNac, a reagent that blocks membrane expression of mucins, was tested [15]. Upon treatment with benzyl-GalNac, presence of Muc4 at the plasma membrane was examined by immunostaining with anti-Muc4 antibody without permeabilization. As shown in Fig. 3A, membrane expression of Muc4 was markedly inhibited by treatment with 5 mM benzyl-GalNac. Under this condition, phosphorylation of ErbB3, which may reflect the activity of ErbB2, was inhibited (Fig. 3B). The expression level of ErbB3 remained unchanged by the treatment with benzyl-GalNac although ErbB3 migrated slightly faster in the SDS–PAGE, suggesting that glycosylation of ErbB3 was affected as well (Fig. 3C). These results suggest that presence of ASGP-2 at the plasma membrane is important for phosphorylation of ErbB3 by ErbB2, although we cannot rule out the possibility that inhibition of glycosylation of ErbB3 contributes to the phosphorylation of ErbB3.

Discussion

In this paper, we demonstrated that ErbB2 is bound to ASGP-2 in signet ring cell carcinoma lines. Judging from tyrosyl phosphorylation of ErbB3 as a substrate of ErbB2, this binding correlates tightly with activation of ErbB2. Sch, MKN74, and HCC2998 cells have been shown to express ErbB2 and ErbB3 but activation of ErbB2 was not observed [4]. We detect presence of ASGP-2 in the cells, however, its binding to ErbB2 was not detectable in these cells (see Fig. 2). Why only signet ring cell carcinoma cell lines exhibited the binding remains unknown. Probably, some unknown additional factors may be required for the binding.

Membrane expression of ASGP-2 may depend on the activity of PI 3-kinase [3,4,16]. Indeed, inhibition of the PI 3-kinase pathway by knockdown of ErbB2 or ErbB3, which inhibits activation of PI 3-kinase by binding to ErbB3, or by treatment of the cells with ZSTK474, a potent inhibitor of PI 3-kinase, resulted in retarded membrane expression of Muc4 (data not shown) [17]. These results suggest that there may be an autocrine loop of ErbB2/ErbB3/PI 3-kinase/Muc4/ErbB2 in signet ring cell carcinoma cells, which may explain constitutive activation of ErbB2 in these cells.

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