The Epidermal Growth Factor Receptor Regulates Interaction of the Human DF3/MUC1 Carcinoma Antigen with c-Src and β-Catenin*

The DF3/MUC1 mucin-like, transmembrane glycoprotein is aberrantly overexpressed in most human carcinomas. The MUC1 cytoplasmic domain interacts with the c-Src tyrosine kinase and thereby increases binding of MUC1 and β-catenin. In the present work, coimmunoprecipitation studies demonstrate that MUC1 associates constitutively with the epidermal growth factor receptor (EGF-R) in human ZR-75-1 breast carcinoma cells. Immunofluorescence studies show that EGF-R and MUC1 associate at the cell membrane. We also show that the activated EGF-R phosphorylates the MUC1 cytoplasmic tail on tyrosine at a YEK motif that functions as a binding site for the c-Src SH2 domain. The results demonstrate that EGF-R-mediated phosphorylation of MUC1 induces binding of MUC1 to c-Src in cells. Moreover, in vitro and in vivo studies demonstrate that EGF-R increases binding of MUC1 and β-catenin. These findings support a novel role for EGF-R in regulating interactions of MUC1 with c-Src and β-catenin.

The epidermal growth factor receptor (EGF-R, HER1) is a member of a family of transmembrane receptor tyrosine kinases that includes HER2/neu, HER3, and HER4. EGF-R is activated by EGF, transforming growth factor-α, amphiregulin, and betacellulin. Following ligand binding, inactive monomeric EGF-R undergoes homodimerization or heterodimerization with other members of the HER family (2). Activation of EGF-R is associated with phosphorylation of specific tyrosine residues in the cytoplasmic region and thereby the recruitment of effector proteins that contain SH2 domains. For example, interaction of EGF-R with the Shc and Grb2 adaptor proteins links receptor activation to the Ras signaling pathway (3–5). Activation of EGF-R is also associated with the formation of complexes with the c-Src nonreceptor tyrosine kinase (6, 7). The finding that overexpression of EGF-R in fibroblasts confers growth in soft agar and induces tumorigenicity in nude mice has indicated that EGF-R can function as an oncogene (8, 9). Other studies in cells overexpressing both EGF-R and c-Src have shown that c-Src potentiates EGF-R-mediated tumorigenesis (7). The interaction between EGF-R and c-Src is further supported by the demonstration that c-Src is required for EGF-R-dependent mitogenesis (10).

The human DF3/MUC1 mucin-like glycoprotein is highly overexpressed by human carcinomas (11). Whereas MUC1 expression is restricted to the apical borders of normal secretory epithelium, this transmembrane protein is aberrantly expressed by carcinoma cells at high levels over the entire cell surface (11). The MUC1 protein consists of an N-terminal ectodomain with variable numbers of conserved 20 amino acid tandem repeats that are subject to O-glycosylation (12, 13). The C-terminal region includes a transmembrane domain and a 72-amino acid cytoplasmic tail that contains seven sites for tyrosine phosphorylation. The >250-kDa ectodomain associates with the ~25 kDa C-terminal region as a heterodimer at the cell surface. β-Catenin, a component of the adherens junction of mammalian epithelial cells, binds directly to MUC1 at a SAGNGGSSL motif in the cytoplasmic domain (14). Similar motifs are responsible for interactions of β-catenin with E-cadherin and the adenomatous polyposis coli tumor suppressor (15–17). Glycogen synthase kinase 3β (GSK3β) also binds to MUC1 and phosphorylates serine in a SPYEKV site adjacent to the YEK motif in the cytoplasmic tail. These findings support a novel role for EGF-R in regulating interactions of MUC1 with c-Src and β-catenin.

* This work was supported by Grant CA87421 awarded by the NCI. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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Received for publication, June 26, 2001, and in revised form, July 30, 2001.

Published, JBC Papers in Press, August 1, 2001.

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Printed in U.S.A.
EFG-R regulates the human DF3/MUC1 carcinoma antigen

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EGF-R and MUC1 (Fig. 1C). Similar results were obtained when anti-EGF-R immunoprecipitates were analyzed by immunoblotting with anti-MUC1 (Fig. 1D). These findings demonstrate that MUC1 constitutively associates with EGF-R.

Colocalization of EGF-R and MUC1 by Immunofluorescence Microscopy—To assess the subcellular localization of MUC1 and EGF-R, confocal microscopy was performed with rabbit anti-EGF-R and mouse anti-MUC1 antibodies. In control ZR-75-1 cells, EGF-R was distributed uniformly over the cell membrane (Fig. 2A, left). Similar findings were obtained for the distribution of MUC1 (Fig. 2A, middle). Overlay of the EGF-R (green) and MUC1 (red) signals supported colocalization (red + green → yellow) (Fig. 2A, right). Following EGF stimulation, the EGF-R signals were clustered in patches at the cell membrane (Fig. 2B, left). An identical pattern was observed for MUC1 (Fig. 2B, middle). Moreover, overlay of the signals showed that EGF-R and MUC1 colocalize in clusters at the cell membrane (Fig. 2B, right). Analysis of the control and EGF-stimulated cells by coimmunoprecipitation studies demonstrated no detectable difference in the association between EGF-R and MUC1 (data not shown). These findings and those obtained in coprecipitation studies demonstrate that MUC1 and EGF-R associate constitutively at the cell membrane.

**EGF-R Phosphorylates MUC1 in Vitro and in Vivo**—To determine whether EGF-R phosphorylates MUC1, anti-MUC1 immunoprecipitates from control and EGF-stimulated ZR-75-1 cells were analyzed by immunoblotting with anti-Tyr(P). The results demonstrate a detectable level of tyrosine-phosphorylated MUC1 in control cells (Fig. 3A). Moreover, EGFR stimulation was associated with an increase in phosphorylation of MUC1 on tyrosine (Fig. 3A). EGF-induced tyrosine phosphorylation of MUC1 was also observed in 293 cells transfected to express EGF-R and MUC1 (Fig. 3B). The 72-amino acid MUC1 cytoplasmic domain (MUC1/CD) contains 7 tyrosines (see schema in Fig. 4D). To define potential sites of EGF-R phosphorylation, we incubated the MUC1 cytoplasmic domain (MUC1/CD) with EGF-R and γ-[32P]ATP. Analysis of the reaction products demonstrated that EGF-R phosphorylates MUC1/CD (Fig. 3C). Mutation of the Tyr26 site to Phe had no detectable effect on EGF-R-mediated phosphorylation of MUC1/CD (Fig. 3C). There was also no apparent effect when the Tyr20 or Tyr35 sites were mutated to Phe (Fig. 3C). By contrast, incubation of MUC1/CD(Y46F) with EGF-R was associated with a marked decrease in phosphorylation as compared with that found with wild-type MUC1/CD (Fig. 3C). Mutation of Tyr26 also resulted in decreased phosphorylation, but to a lesser extent than that obtained with Y46F (Fig. 3C). To determine whether the Tyr46 site is phosphorylated in vivo, human HCT116 cells, which express EGF-R and not MUC1, were stably transfected to express the empty vector, wild-type MUC1, or the MUC1(Y46F) mutant. Analysis of anti-MUC1 immunoprecipitates with anti-Tyr(P) demonstrated that EGF-mediated phosphorylation of MUC1(Y46F) is decreased compared with that obtained with wild-type MUC1 (Fig. 3D). Relative intensities of the anti-Tyr(P) signals were determined by densitometric scanning (Fig. 3D). Similar results were obtained in three separate experiments (legend to Fig. 3D). The findings that the MUC1(Y46F) mutation decreases tyrosine phosphorylation only in part is in concert with additional tyrosine sites in the MUC1/CD, which can function as substrates for other tyrosine kinases. These results thus demonstrate that EGF-R phosphorylates MUC1 on Tyr46 in vitro and in cells.

**EGF-R Regulates Interaction of MUC1 with c-Src and β-Catenin**—To determine whether EGF-R-mediated phosphorylation regulates the interaction of MUC1 with c-Src and β-catenin, we incubated MUC1/CD with EGF-R and ATP and then assessed binding to GST-Src-SH2 and GST-β-catenin. Immunoblot analysis of adsorbates to glutathione beads with anti-MUC1/CD showed that GST-Src SH2 binds to MUC1/CD following EGF-R phosphorylation (Fig. 4A). In addition, compared with MUC1/CD, there was substantially less binding of GST-Src-SH2 to the MUC1/CD(Y46F) mutant that had been incubated with EGF-R and ATP (Fig. 4A). Similar findings were obtained for binding of GST-β-catenin (Fig. 4A). To assess whether EGF-R-mediated phosphorylation of MUC1 induces binding of MUC1 to c-Src and β-catenin, we incubated MUC1/CD with c-Src and β-catenin. Analysis of lysates from control ZR-75-1 cells demonstrated a low but detectable interaction of MUC1 with c-Src and β-catenin (Fig. 4B). In contrast with the in vitro results, stimulation of ZR-75-1 cells with EGF induced the interaction of MUC1 with c-Src and β-catenin (Fig. 4B). To confirm involvement of the MUC1 Tyr46 site, HCT116 cells stably expressing wild-type MUC1 or MUC1(Y46F) were stimulated with EGF. Immunoblot analysis of anti-MUC1 immunoprecipitates with anti-c-Src demonstrated that, compared with wild-type MUC1, there was less EGF-induced binding.
of MUC1(Y46F) to c-Src (Fig. 4C). Similar findings were obtained for β-catenin (Fig. 4C). These results show that EGF-R-mediated phosphorylation of MUC1 Y46 induces the interaction of MUC1 with c-Src and β-catenin.

**MUC1 Integrates EGF-R, c-Src, and β-Catenin Signaling**

The present findings and those recently reported for the mouse mammary gland (20) demonstrate that MUC1 interacts with EGF-R. The present results further show that EGF-R phosphorylates the MUC1 cytoplasmic tail on the YEKV motif. These findings and the recent demonstration that c-Src phosphorylates Tyr46 (19) have supported regulation of the YEKV site by GSK3β. The present results further show that EGF-R phosphorylation of MUC1 on Tyr46 functions as a binding site for both EGF-R and c-Src. The available evidence indicates that Tyr46 is functionally important in regulating the interactions of MUC1 with multiple signaling pathways. EGF-R-mediated phosphorylation of MUC1 Tyr46 functions as a binding site for the c-Src SH2 domain. Moreover, EGF stimulation is associated with increased binding of MUC1 and c-Src in vitro. EGF-R-mediated phosphorylation of MUC1 Tyr46 also induces binding of MUC1 with β-catenin in vitro and in cells. In addition, phosphorylation of MUC1 Tyr46 by EGF-R or c-Src down-regulates the interaction of MUC1 and GSK3β (Ref. 19 and data not shown). In concert with these findings, the YEKV motif resides between the GSK3β binding and phosphorylation site (STDRSP) (18) and the β-catenin binding site (SAGNGGSSLS) (14) (Fig. 4D). Taken together, the present results and those of previous studies (14, 18–20) support a model in which EGF stimulation induces phosphorylation of MUC1 on Tyr46 and thereby integrates signaling among the c-Src, β-catenin, and GSK3β pathways. The aberrant overexpression of MUC1 in human carcinoma cells could thus contribute to the transformed phenotype by dysregulation of EGF-R, c-Src, β-catenin, and/or GSK3β signaling.

**Acknowledgment**—We are grateful to Kamal Chauhan for his excellent technical support.

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