The c-Src Tyrosine Kinase Regulates Signaling of the Human DF3/MUC1 Carcinoma-associated Antigen with GSK3β and β-Catenin*

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The DF3/MUC1 mucin-like glycoprotein is aberrantly overexpressed in most human carcinomas. The cytoplasmic domain of MUC1 interacts with glycoprotein 3β (GSK3β) and thereby decreases binding of MUC1 and β-catenin. The present studies demonstrate that MUC1 associates with the c-Src tyrosine kinase. c-Src phosphorylates the MUC1 cytoplasmic domain at a YEKV motif located between sites involved in interactions with GSK3β and β-catenin. The results demonstrate that the c-Src SH2 domain binds directly to pYEKV and inhibits the interaction between MUC1 and GSK3β. Moreover and in contrast to GSK3β, in vitro and in vivo studies demonstrate that c-Src-mediated phosphorylation of MUC1 increases binding of MUC1 and β-catenin. The findings support a novel role for c-Src in regulating interactions of MUC1 with GSK3β and β-catenin.

β-catenin, a component of the adherens junctions of mammalian epithelial cells, binds directly to the cytoplasmic domain of the transmembrane E-cadherin protein that functions in Ca2+-dependent epithelial cell-cell interactions (1). In turn, α-catenin binds to β-catenin and thereby links the complex to the actin cytoskeleton (2). Formation of the cadherin-catenin complex is essential for adherens junction function (3). In the cytosol, β-catenin binds directly to the adenomatous polyposis coli (APC)1 tumor suppressor (4–6). GSK3β also binds directly to MUC1 and phosphorylates serine in a DRSPY site adjacent to that for the β-catenin interaction (17). GSK3β-mediated phosphorylation of MUC1 decreases the association of MUC1 and β-catenin (17).

The present studies demonstrate that the c-Src tyrosine kinase interacts directly with MUC1. A YEKV motif in the MUC1 cytoplasmic domain (CD) has been identified as a site for c-Src phosphorylation. The results demonstrate that c-Src regulates the interactions of MUC1 with GSK3β and β-catenin.

MATERIALS AND METHODS

Cell Culture—Human ZR-75-1 breast carcinoma cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 10 units/ml penicillin, and 2 mM L-glutamine. 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin.

Lysate Preparation—Subconfluent cells were disrupted on ice in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 100 μg/ml leupeptin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) for 30 min. Lysates were cleared by centrifugation at 14,000 × g for 20 min.

Immunoprecipitation and Immunoblotting—Equal amounts of protein from cell lysates were incubated with normal mouse IgG, MAb DF3 (anti-MUC1) (18), anti-c-Src (Upstate Biotechnology, Lake Placid, NY), or the rabbit anti-DF3-E antibody prepared against a peptide derived from the MUC1 extracellular domain (HDVQTQFYKITEAAS). After incubation for 2 h at 4 °C, the immune complexes were precipitated with protein G-agarose. The immunoprecipitates were washed with lysis buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The immunoblots were probed with 500 ng/ml anti-MUC1 or 1 μg/ml anti-c-Src. Reactivity was detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (ECL, Amersham Pharmacia Biotech).

Preparation of MUC1 and c-Src Mutants—The MUC1/CD(Y46F) and MUC1(Y46F) mutants were generated using site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA) to change Tyr-46 to Phe. Kinase-inactive c-Src was similarly generated by mutation of Lys-295 to Arg (K295R) (24).

In Vitro Phosphorylation—Purified wild-type and mutant MUC1/CD proteins were incubated with 1.5 units of purified c-Src (Oncogene Research Products, Cambridge, MA) in 20 μl of kinase buffer (20 mM

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at 30 °C, the reaction was stopped by addition of sample buffer and GST-MUC1/CD were incubated with c-Src and . (upper panel) and anti-P-Tyr immunoblot analysis with anti-MUC1/CD (lower panel). The gel was stained with Coomassie Blue to assess loading of the wild-type and mutant SH3 domains (right panel).

FIG. 1. Interaction of MUC1 with c-Src. A, lysates from ZR-75-1 cells were subjected to immunoprecipitation with anti-MUC1 (Mab DF3; left panel) or anti-c-Src (right panel). Mouse IgG was used as a control. The immunoprecipitates and lysates not subjected to immunoprecipitation were analyzed by immunoblotting with anti-c-Src (left panel) and anti-MUC1 (right panel). B, purified MUC1/CD was incubated with GST, GST-Src-SH2, or GST-Src-SH3 for 1 h at 4 °C. Proteins precipitated with glutathione-Sepharose 4B beads were subjected to SDS-PAGE and immunoblot analysis with anti-MUC1/CD. C, purified MUC1/CD was incubated with GST, GST-Src-SH3, or GST-Src-SH3De90/92 (deletion of amino acids 90/92). Adsorbors to glutathione beads were subjected to immunoblot analysis with anti-MUC1/CD (left panel). The gel was stained with Coomassie Blue to assess loading of the wild-type and mutant SH3 domains (right panel).

Binding Studies—Purified wild-type and mutant MUC1/CD proteins were incubated with 1.5 units of c-Src in the presence or absence of 200 μM ATP for 30 min at 30 °C. GST, GST-Src-SH3, GST-Src-SH3De90/92 (Ref. 25, provided by Dr. J. Brugge, Harvard Medical School), GST-Src-SH2, or GST-β-catenin bound to glutathione beads was then added, and the reaction was incubated for 1 h at 4 °C. After washing, the proteins were subjected to SDS-PAGE and immunoblot analysis with the anti-MUC1/CD antibody that was generated against the cytoplasmic domain (17). In other studies, GST-MUC1/CD bound to glutathione beads was incubated with 1.5 units of c-Src in the presence and absence of 200 μM ATP for 30 min at 30 °C before adding 0.1 mg of purified GSK3β (New England BioLabs) for an additional 1 h. Precipitated proteins were analyzed by immunoblotting with anti-c-Src and anti-GSK3β.

Transient Transfection Studies—ZR-75-1 or 293 cells were transiently transfected with pcMV, pcMV-MUC1, pcMV-c-Src (provided by Dr. R. Rickles, ARIAD Pharmaceuticals, Inc., Cambridge, MA) or pcMV-c-Src(R295R) using electroporation methods. Efficiency of transient transfections ranged from 40–50% of ZR-75-1 cells and 70–80% of 293 cells. Cell lysates were prepared at 48 h after transfection.

RESULTS AND DISCUSSION

To determine whether DF3/MUC1 forms a complex with c-Src, anti-MUC1 immunoprecipitates from lysates of human ZR-75-1 cells were analyzed by immunoblotting with anti-c-Src. The results demonstrate that c-Src coprecipitates with MUC1 (Fig. 1A, left). In the reciprocal experiment, analysis of anti-c-Src immunoprecipitates by immunoblotting with anti-MUC1 confirmed the association of MUC1 and c-Src (Fig. 1A, right). Similar results have been obtained in human HeLa cells (data not shown). To assess whether the binding is direct, we incubated purified His-tagged MUC1 cytoplasmic domain (His-MUC1/CD) with a GST fusion protein that contains the c-Src SH2 domain. The gel was stained with Coomassie Blue to assess loading of the wild-type and mutant SH3 domains (right panel).

Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol). The reaction was initiated by addition of 10 μCi [γ-³²P]ATP. After incubation for 15 min at 30 °C, the reaction was stopped by addition of sample buffer and boiling for 5 min. Phosphorylated proteins were separated by SDS-PAGE and analyzed by autoradiography.

Binding Studies—Purified wild-type and mutant MUC1/CD proteins were incubated with 1.5 units of c-Src in the presence or absence of 200 μM ATP for 30 min at 30 °C. GST, GST-Src-SH3, GST-Src-SH3De90/92 (Ref. 25, provided by Dr. J. Brugge, Harvard Medical School), GST-Src-SH2, or GST-β-catenin bound to glutathione beads was then added, and the reaction was incubated for 1 h at 4 °C. After washing, the proteins were subjected to SDS-PAGE and immunoblot analysis with the anti-MUC1/CD antibody that was generated against the cytoplasmic domain (17). In other studies, GST-MUC1/CD bound to glutathione beads was incubated with 1.5 units of c-Src in the presence and absence of 200 μM ATP for 30 min at 30 °C before adding 0.1 mg of purified GSK3β (New England BioLabs) for an additional 1 h. Precipitated proteins were analyzed by immunoblotting with anti-c-Src and anti-GSK3β.

FIG. 2. Phosphorylation of MUC1 by c-Src in vitro. A, GST or GST-MUC1/CD were incubated with c-Src and [γ-³²P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. B, schematic representation of wild-type and mutant forms of MUC1/CD. TR, tandem repeat; TM, transmembrane; CD, cytoplasmic domain. Numbers (1–72) reflect amino acids in the CD. Underlined codons and amino acids are those that differ from the wild type. C, purified MUC1/CD and the MUC1/CD(Y46F) mutant were incubated with purified c-Src and [γ-³²P]ATP. As a control, MUC1/CD was incubated with only [γ-³²P]ATP. After incubation for 15 min at 30 °C before adding GSK3β for an additional 1 h.
MUC1 Integrates c-Src, GSK3β, and β-Catenin Signaling

FIG. 4. c-Src-mediated phosphorylation of the MUC1 YEKV site increases binding of MUC1 to β-catenin. A, purified MUC1/CD and MUC1/CD(Y46F) were incubated with (+) or without (−) purified c-Src and ATP for 30 min at 30 °C. The MUC1/CD and MUC1/CD(Y46F) proteins were then incubated with GST or β-catenin for 1 h at 4 °C. Proteins precipitated with glutathione beads were subjected to immunoblot analysis with anti-MUC1/CD (upper panel) and anti-β-catenin (lower panel). B, ZR-75-1 cells were transiently transfected with a His-tagged c-Src (left) or a His-tagged c-Src(Y46F) (right). The cells were then incubated with MUC1/CD and the immunoprecipitates were analyzed by immunoblotting with anti-c-Src (upper panel), anti-P-Tyr (middle panel), and anti-β-catenin (lower panel). C, 293 cells were transiently transfected with MUC1, MUC1+c-Src, or MUC1(Y46F)+c-Src. After 48 h, the cells were harvested, and lysates were subjected to immunoprecipitation with IgG or anti-MUC1. Left, the immunoprecipitates were analyzed by immunoblotting with anti-c-Src (upper panel), and lysates not subjected to immunoprecipitation were analyzed with anti-MUC1 (lower panel). Middle, the immunoprecipitates were analyzed by immunoblotting with anti-c-Src (upper panel), anti-P-Tyr (middle panel), and anti-β-catenin (lower panel). Right, lysates not subjected to immunoprecipitation were assayed by immunoblotting with anti-MUC1 or anti-c-Src (upper and lower panels).

MUC1/CD to GST-Src SH3, and not GST or a GST-Src SH2 fusion protein (Fig. 1B). As an additional control, His-MUC1/CD was incubated with a GST fusion protein containing a mutated c-Src SH3 domain (GST-Src SH3De90/92) (26). The finding that MUC1/CD binds to wild-type c-Src SH3 but not the mutant supported a direct interaction between MUC1 and c-Src (Fig. 1C).

To determine whether MUC1/CD is a substrate for c-Src, we incubated MUC1/CD with purified c-Src and [γ-32P]ATP. Analysis of the reaction products by SDS-PAGE and autoradiography demonstrated c-Src-mediated phosphorylation of MUC1/CD (Fig. 2A). Previous studies have demonstrated that GSK3β phosphorylates MUC1/CD on Ser at a DRSPYEKV site (17). As the adjacent YEKV sequence represents a consensus for c-Src phosphorylation, MUC1/CD was generated with a FEKV mutation (Fig. 2B). Incubation of MUC1/CD(Y46F) with c-Src demonstrated a decrease in phosphorylation as compared with that found with wild-type MUC1/CD (Fig. 2C). These findings indicate that c-Src phosphorylates MUC1/CD predominantly but not exclusively at the YEKV site. As the c-Src SH2 domain interacts with a preferred yEYEEI sequence (27), c-Src-mediated phosphorylation of YEKV in MUC1/CD provides a potential site for c-Src SH2 binding. To determine whether the c-Src SH2 domain binds to phosphorylated MUC1/CD, we incubated MUC1/CD with c-Src and ATP and then assessed binding to GST-Src SH2. The results demonstrate that GST-Src SH2 associates with phosphorylated but not unphosphorylated MUC1/CD (Fig. 2D). Moreover, compared with MUC1/CD, there was substantially less binding of GST-Src SH2 to the MUC1/CD(Y46F) mutant that had been incubated with c-Src and ATP (Fig. 2D). These results support c-Src-mediated phosphorylation of MUC1/CD and thereby a direct interaction of phosphorylated MUC1/CD with the c-Src SH2 domain.

As the c-Src phosphorylation site on MUC1/CD resides next to the binding and phosphorylation site for GSK3β (17), we asked if the interaction of MUC1/CD with c-Src affects that with GSK3β. GST-MUC1/CD was incubated with c-Src and ATP before addition of GSK3β. Analysis of proteins precipitated with glutathione beads demonstrated that c-Src-mediated phosphorylation of MUC1/CD is associated with a decrease in binding of MUC1/CD and GSK3β (Fig. 3). To assess the effects of c-Src on the interaction of MUC1/CD and GSK3β in vivo, ZR-75-1 cells were transfected to express the empty vector or c-Src. Anti-MUC1 immunoprecipitates were analyzed by immunoblotting with anti-GSK3β. The results demonstrate that c-Src also decreases the interaction of MUC1 and GSK3β in vivo (Fig. 3B). These findings indicate that GSK3β interacts with MUC1/CD by a c-Src-dependent mechanism.

Phosphorylation of MUC1 by GSK3β decreases binding of MUC1 to β-catenin in vitro and in cells (17). To determine whether c-Src-mediated phosphorylation of MUC1 affects the interaction of MUC1 with β-catenin, we incubated MUC1/CD with c-Src and ATP. Phosphorylated and unphosphorylated MUC1/CD were then incubated with GST or GST-β-catenin. Similar studies were performed with the MUC1/CD(Y46F) mutant. Analysis of proteins bound to glutathione beads by immunoblotting with anti-MUC1/CD demonstrated that c-Src-mediated phosphorylation of MUC1/CD increases binding of c-Src to MUC1/CD (Fig. 3C). As an additional control, His-MUC1/CD was incubated with a GST fusion protein containing a mutated c-Src SH3 domain (GST-Src SH3De90/92) (26). The finding that MUC1/CD binds to wild-type c-Src SH3 but not the mutant supported a direct interaction between MUC1 and c-Src (Fig. 1C).
phorylation of the MUC1 C-terminal subunit and increased
analysis of the precipitates demonstrated c-Src-mediated phos-
extracellular region of the C-terminal subunit. Immunoblot
with the anti-DF3-E antibody that was generated against the
these findings, we performed immunoprecipitation studies
interaction between MUC1/CD and
kinases (32). The available evidence indicates that c-Src phos-
the epidermal growth factor receptor (EGF-R), platelet-derived
protein-coupled receptors (28) and neuronal ion channels (29–
pared with the control (Fig. 4B, middle panel). To extend these findings, MUC1-negative 293 cells (17) were transfected to express MUC1 or MUC1(Y46F) in which the CD YEKV site has been mutated to FEKV. There was a low but detectable level of MUC1 binding to endogenous c-Src (Fig. 4C, left panel). Moreover, cotransfection of MUC1 and c-Src was associated with increased tyrosine phosphorylation of MUC1(CD) and binding of MUC1 and c-Src (Fig. 4C, middle panel). By contrast, cotransfection of MUC1(Y46F) and c-Src resulted in little binding of these proteins (Fig. 4C, middle panel). Moreover, there was little if any tyrosine phosphorylation of MUC1(Y46F) (Fig. 4C, middle panel). Importantly, cotransfection of MUC1 but not MUC1(Y46F) with c-Src induced the binding of MUC1 and β-catenin (Fig. 4C, middle panel). These findings demonstrate that c-Src-mediated phosphorylation of the MUC1 YEKV site increases the interaction of MUC1 and β-catenin in cells.

The present findings thus demonstrate that signaling of β-catenin and the MUC1 carcinoma-associated protein is reg-
ulated by the c-Src tyrosine kinase. Previous studies have shown that β-catenin interacts with the cytoplasmic domain of MUC1 and that GSK3β inhibits the formation of MUC1/CD-β-
catenin complexes (17). By contrast, the present work supports a model in which c-Src phosphorylates MUC1/CD and promotes the interaction of MUC1/CD and β-catenin. The c-Src kinase functions in signaling pathways activated by heterotrimeric G protein-coupled receptors (28) and neuronal ion channels (29–31). c-Src also participates in the transduction of signals from the epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGF-R) and other receptor tyrosine kinases (32). The available evidence indicates that c-Src phos-
phorylates the EGF-R and thereby contributes to mitogenesis
and transformation (33). Mitogenesis induced by PDGF is also positively regulated by c-Src-mediated phosphorylation of the PDGF-R (34). Other substrates of c-Src that include focal adhesion kinase, p130Cas, and cortactin have functional associations with the actin cytoskeleton (32). These findings have collectively provided support for the involvement of c-Src in the integration of mitogenic, cell adhesion, and cytoskeletal responses. The present studies extend these findings by demonstr-
ating that the MUC1 carcinoma-associated antigen is also a substrate for c-Src and that interaction of MUC1 with GSK3β and β-catenin are regulated by c-Src-dependent signals.

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