CEACAM6 Cross-linking Induces Caveolin-1-dependent, Src-mediated Focal Adhesion Kinase Phosphorylation in BxPC3 Pancreatic Adenocarcinoma Cells*

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Despite lacking transmembrane or intracellular domains, glycosylphosphatidylinositol-anchored proteins can modulate intracellular signaling events, in many cases through aggregation within membrane “lipid raft” microdomains. CEACAM6 is a glycosylphosphatidylinositol-linked cell surface protein of importance in the anchorage-independent survival and metastasis of pancreatic adenocarcinoma cells. We examined the effects of antibody-mediated cross-linking of CEACAM6 on intracellular signaling events and anchorage-independent survival of the CEACAM6-overexpressing pancreatic ductal adenocarcinoma cell line, BxPC3. CEACAM6 cross-linking increased c-Src activation and induced tyrosine phosphorylation of p125FAK focal adhesion kinase. Focal adhesion kinase phosphorylation was dependent on c-Src kinase activation, for which caveolin-1 was required. CEACAM6 cross-linking induced a significant increase in cellular resistance to anoikis. These observations represent the first characterization of the mechanism through which this important cell surface oncprotein influences intracellular signaling events and hence malignant cellular behavior.

CEACAM6 is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein that is overexpressed in a variety of human malignancies, including pancreatic adenocarcinoma (1, 2). This immunoglobulin superfamily member is emerging as an important determinant of a variety of aspects of the malignant cellular phenotype (1–3). We reported previously that levels of CEACAM6 expression modulate the susceptibility of pancreatic adenocarcinoma cells to anoikis (2). Normal epithelial cells require anchorage to an extracellular matrix for growth, survival, and differentiation. Resistance to anoikis, a subset of apoptosis induced by inadequate or inappropriate cell-substrate contact in normal cells, is a property of transformed cells that is associated with enhanced tumorigenesis and metastatic ability. Despite clear indications of its important role in cancer cell biology, the mechanisms through which CEACAM6 influences intracellular signal transduction remain poorly understood.

Despite lacking transmembrane and intracellular domains, several GPI-anchored proteins, including CEACAM family members, are able to influence intracellular events and have been implicated in transmembrane signaling via tyrosine kinases (4–7). GPI-anchored proteins are known to exist in clusters and form microdomains at the surface of the plasma membrane, referred to as “lipid rafts” (8). These microdomains appear to be important for GPI-anchored protein signal transduction. A number of signal transduction components, including Src family tyrosine kinases, are associated with lipid rafts, and cross-linking of cell surface molecules by a variety of techniques has proven to be a useful tool for exploring the functional roles of raft-associated proteins (9–14).

Here, we examine the effects of antibody-mediated cross-linking of CEACAM6 in pancreatic adenocarcinoma cells. We determine the effects of CEACAM6 cross-linking on the activity of c-Src and on the phosphorylation status of its substrate p125FAK focal adhesion kinase (FAK), a tyrosine kinase of key importance in pancreatic adenocarcinoma cellular resistance to anoikis (15) and among the main targets for tyrosine phosphorylation in v-Src-transformed cells (16). We also provide evidence that modulation of c-Src tyrosine kinase activity by CEACAM6 is a caveolin-1-dependent process and illustrate the functional effects of CEACAM6 cross-linking on anchorage-independent cell survival, which correlates with tumorigenic, invasive, and metastatic potential in a range of malignancies, including pancreatic adenocarcinoma (17–21).

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—BxPC3 human pancreatic ductal adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Invitrogen), incubated in a humidified (37 °C, 5% CO₂) incubator, grown in 75-cm² culture flasks, and passaged upon reaching 80% confluence.

CEACAM6 Cross-linking—The By114 mouse monoclonal antibody was used to cross-link CEACAM6. This antibody is highly specific for CEACAM6 and is non-cross-reactive with closely related CEACAM family molecules (22, 23). Cross-linking was performed in a manner similar to that described previously (12). Briefly, BxPC3 cell cultures, 3 days post-seeded in 35-mm well plates, were incubated with 50 μg/ml By114 or irrelevant (control) isotype-matched mouse IgG on ice for 30 min in bovine serum albumin medium (DMEM) without fetal bovine serum containing 1% bovine serum albumin and 20 mM HepES, pH 7.4. After washing with cold bovine serum albumin medium, the cells were incubated with 50 μg/ml anti-IgG affinity-purified polyclonal antibody (secondary antibody) in bovine serum albumin medium containing 1% bovine serum albumin and 20 mM HepES, pH 7.4. After washing with ice-cold phosphate-buffered saline, lysates were prepared in 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride at 4 °C. After centrifugation at 14,000 × g for 3 min, the supernatants were used for further analysis. The cells exposed to pri-
mary antibody only served as additional controls. To disrupt GPI anchor assembly, cells were cultured for 24 h in medium containing 10 mM mannosamine (2-amino-2-deoxy-d-mannose, Sigma) prior to performing antibody cross-linking (24).

**Immunoprecipitation**—Cells were washed extensively and lysed in buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 2.5 mM MgCl$_2$, 0.5% Triton X-100, 10 mM dithiothreitol) supplemented with a protease and phosphatase inhibitor mixture (Sigma). After standing on ice for 30 min with brief intermittent vortexing, the cell lysates were centrifuged at 23,000 $\times$ g for 15 min at 4 $^\circ$C. Protein concentrations were determined and normalized, and crude lysates were precleared with protein-A-Sepharose (Zymed Laboratories Inc., San Francisco, CA). The lysates were subjected to immunoprecipitation assay using 25 $\mu$l of an appropriate monoclonal antibody. Beads were washed with 60 $\mu$l of protein A-Sepharose beads for 6 h at 4 $^\circ$C followed by sequential washes in immunoprecipitation buffer containing 500 mM, 300 mM, and 150 mM NaCl (twice) supplemented with 1 $\times$ radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, and 0.1% w/v SDS). After the final wash, the pellet was resuspended in Laemmli sample buffer, and the proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis followed by immunodetection by Western blotting or subjecting to *in vitro* tyrosine kinase assay, as described below.

**Western Blotting**—Cells were harvested and rinsed twice with phosphate-buffered saline. The cell extracts were prepared with lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotonin, 10 $\mu$g/ml leupeptin) and cleared by centrifugation at 12,000 $\times$ g, 4 $^\circ$C. Total protein concentration was measured using the BCA assay kit (Sigma) with bovine kinase buffer (final solution containing 0.3 mM ATP) and incubated for 30 min in 96-well plates coated with tyrosine kinase substrate solution (Frederick, MD). siRNAs were dissolved in buffer (100 mM potassium phosphate buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, and 0.1% w/v SDS). After the final wash, the pellet was resuspended in Laemmli sample buffer, and the proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis followed by immunodetection by Western blotting or subjecting to *in vitro* tyrosine kinase assay, as described below.

**c-Src Tyrosine Kinase Assay**—c-Src tyrosine kinase activity was determined using a commercially available kinase assay kit (Sigma) according to the manufacturer’s instructions. c-Src immunoprecipitates (20 $\mu$l of total protein) were prepared using anti-c-Src monoclonal antibody immobilized onto protein G-Sepharose beads (Zymed Laboratories Inc.). Immunoprecipitates were washed and dissolved in tyrosine kinase buffer (final solution containing 0.3 mM ATP) and incubated for 30 min in 96-well plates coated with tyrosine kinase substrate solution (poly-Glu-Tyr). The phosphorylated substrate was quantified by chromatographic detection using horseradish peroxidase-conjugated anti-phosphotyrosine antibody. Optical densities were determined at 492 nm using a Vmax microplate spectrophotometer. c-Src kinase activity was compared with an epidermal growth factor-receptor standard. Kinase assays were performed in triplicate with four determinations/condition. *p* values were compared using Student’s t test, multifactorial analysis of initial measurements, and Mann Whitney U test for nonparametric data (as appropriate) using Statistica version 5.5 software (StatSoft, Inc., Tulsa, OK).

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In cases where averages were normalized to controls, the standard deviations of each nominator and denominator were taken into account in calculating the final standard deviation. $p < 0.05$ was considered statistically significant.

RESULTS

**CEACAM6 Cross-linking Increases Cellular c-Src Tyrosine Kinase Activity and Decreases Tyr-527 Phosphorylation**—Tyrosine kinases play a central role in the malignant phenotype of cancer cells (25–27), and GPI-anchored proteins, including CEACAM family members, modulate intracellular signaling events via tyrosine kinases (28–30). We examined the effect of CEACAM6 cross-linking on the tyrosine kinase activity of the prototype Src family protein tyrosine kinase, c-Src. BxPC3 cells were chosen for this study as these cells markedly overexpress CEACAM6 (2, 31). Following treatment with either anti-CEACAM6 antibody or control IgG (alone or followed by secondary antibody cross-linking), cell lysates were prepared, and c-Src kinase activities in these lysates were quantified by *in vitro* kinase assay. CEACAM6 cross-linking resulted in a marked increase in c-Src kinase activity, whereas the control antibody did not affect c-Src activity (Fig. 1A). Total levels of c-Src did not differ among groups. Disruption of GPI assembly by pretreatment with mannosamine (24) almost completely abolished activation of c-Src by CEACAM6 cross-linking, indicating a requirement for the GPI anchor.

The activity of c-Src is modulated by the phosphorylation status of residue Tyr-527. Csk exerts important negative regulatory control on c-Src by phosphorylating this tyrosine residue (32), which impairs the ability of c-Src to phosphorylate tyrosine in downstream targets, including FAK (33). We performed phospho-specific c-Src (Tyr-527) immunoblotting on cell lysates following exposure to anti-CEACAM6 or control (irrelevant) isotype-matched IgG, alone or followed by the cross-linking secondary antibody. Lower levels of c-Src Tyr-527 phosphorylation were observed in lysates from CEACAM6 cross-linked cells, consistent with the higher levels of c-Src activity observed in these cell lysates relative to those derived from control non-cross-linked cells. Mannosamine pretreatment preserved c-Src Tyr-527 phosphorylation at a higher level following CEACAM6 cross-linking, consistent with the GPI dependence of the increased c-Src kinase activity following CEACAM6 cross-linking (Fig. 1B).

**CEACAM6 Cross-linking Induces Dephosphorylation of Caveolin-1 and Reduces Its Association with Csk**—Targeting of GPI-anchored protein to the exoplasmic leaflet of caveolar membranes disrupts caveolin-1 phosphorylation (34), and decreased caveolin-1 phosphorylation reduces recruitment of Csk to the membrane, leading to disinhibition of c-Src (33, 35). These variations in c-Src Tyr-527 phosphorylation are consistent with a decreased level of Csk-mediated c-Src inhibition.
following CEACAM6 cross-linking, and we therefore hypothesized that cross-linking CEACAM6 may impair Tyr-527 phosphorylation by reducing phosphorylation of caveolin-1, in turn leading to decreased membrane recruitment of Csk and disinhibition of c-Src. We tested this hypothesis by examining the effect of CEACAM6 cross-linking on the phosphorylation status of caveolin-1 using phospho-caveolin-1 (Tyr-14)-specific immunoblotting. We observed that CEACAM6 cross-linking significantly decreases caveolin-1 phosphorylation (Fig. 2A). Furthermore, when caveolin-1 immunoprecipitates were immunoblotted for Csk, we observed a lower level of Csk immunoreactivity in immunoprecipitates derived from CEACAM6-cross-linked cells compared with those from control cells (Fig. 3), supporting the hypothesis that CEACAM6 cross-linking reduces Csk recruitment through modulation of the phosphorylation status of caveolin-1. Although levels of c-Src association with CEACAM6 were relatively unchanged by CEACAM6 cross-linking, Csk co-precipitation with CEACAM6 decreased (Fig. 2B). These observations may account for the decreased c-Src Tyr-527 phosphorylation and increased c-Src kinase activity detected in lysates derived from CEACAM6-cross-linked cells. Mannosamine inhibited caveolin-1 dephosphorylation and preserved levels of Csk associated with caveolin-1, confirming the dependence of these effects on the GPI anchor.

**Caveolin-1 Is Required for CEACAM6 Cross-linking-induced c-Src Activation**—In view of the effects of CEACAM6 cross-linking on caveolin-1 phosphorylation, we speculated that caveolin-1 might act as an adaptor between the GPI anchor of CEACAM6 and c-Src, together with its regulator, Csk. We determined whether caveolin-1 is required for the increase in c-Src activity induced by CEACAM6 cross-linking by suppressing caveolin-1 expression using RNA interference. Control (scramble) siRNA had no effect on caveolin-1 expression. The blots were performed in triplicate. Values are means (±S.D.) from three independent blots normalized to caveolin-1. *, p < 0.05 versus cells treated with control IgG and secondary antibody and those pretreated with mannose prior to cross-linking.

**CEACAM6 Antibody Cross-linking Induces c-Src-dependent Tyrosine Phosphorylation of p125FAK**—FAK is an important substrate of c-Src and forms a central point in a variety of signaling cascades of importance in determining the malignant phenotype (37, 38). Following treatment
CEACAM6 cross-linking induces c-Src-dependent tyrosine phosphorylation of FAK. FAK was immunoprecipitated following treatment with control (irrelevant) isotype-matched IgG or anti-CEACAM6 antibody either alone or in combination with secondary cross-linking antibody. Mannosamine (MM) was used to disrupt GPI assembly. CEACAM6 cross-linking increased FAK phosphorylation at Tyr-397. This effect was attenuated by prior mannosamine treatment. Dominant negative c-Src (Src(K296R/Y528F)) transfection inhibited FAK phosphorylation. Lane 1, IgG; lane 2, By114; lane 3, IgG + secondary IgG; lane 4, By114 + secondary IgG; lane 5, By114 + secondary IgG + mannosamine; lane 6, By114 + secondary IgG + pUSEamp(−); lane 7, By114 + secondary + Src(K296R/Y528F), dominant negative (DN) Src. Values are means (±S.D.) from triplicate blots. *, p < 0.05 versus cells treated with control IgG and secondary antibody and those pretreated with mannosamine; †, p < 0.05 versus pUSEamp(−).

**Antibody-mediated CEACAM6 Cross-linking Inhibits Anoikis**—To define the functional implications of CEACAM6 cross-linking, we determined its effect on cellular resistance to anoikis (40). Resistance to this subset of apoptosis induced by anchorage-independent conditions is a feature of transformed cells associated with tumorigenesis, invasion, and metastasis (18–20). Following exposure to anti-CEACAM6 or irrelevant control isotype-matched IgG (either alone or followed by cross-linking secondary antibody), BxPC3 cells were exposed to poly-HEMA culture for 18 h, and anoikis was quantified by flow cytometry. CEACAM6 cross-linking increased cellular resistance to anoikis by 66%, whereas the control antibody had no effect on the anoikis fraction. Mannosamine (MM) treatment markedly attenuated the effect of CEACAM6 cross-linking on anoikis resistance, confirming the GPI-dependence of this effect. Transfection of cells with a Src(K296R/Y528F) dominant negative construct (DN Src), but not the pUSEamp(−) empty vector, almost completely abolished the protective effect of CEACAM6 cross-linking, consistent with the c-Src dependence of CEACAM6 cross-linking-induced events in vitro. Anoikis fractions are mean values (±S.D.) from triplicate experiments, each using three samples for each condition and scoring no less than 10,000 cells. *, p < 0.05 versus cells treated with control IgG and secondary antibody and those pretreated with mannosamine; †, p < 0.05 versus pUSEamp(−).

**DISCUSSION**

CEACAM6 is a cell surface glycoprotein that is overexpressed in a range of gastrointestinal neoplasms (1, 3, 41). We have recently shown that modulation of CEACAM6 expression markedly alters the malignant phenotype of pancreatic adenocarcinoma cells (2). The purpose of this study was to examine how CEACAM6, a GPI-linked molecule lacking transmembrane and intracellular domains, is able to modulate intracellular signaling events and influence cellular behavior. Using the CEACAM6-overexpressing cell line, BxPC3, as a model system, we have demonstrated that CEACAM6 cross-linking promotes anoikis resistance and activates c-Src in a caveolin-1-dependent manner. Furthermore, we have shown that c-Src activation is associated with attenuated membrane recruitment of Cek, a negative regulator of c-Src. We have also demonstrated that activation of c-Src by CEACAM6 cross-linking is associated with c-Src-dependent tyrosine phosphorylation of its substrate FAK. Taken together, these observations indicate a potential mechanism through which CEACAM6 could influence important intracellular oncogenic pathways and hence the malignant phenotype.

Despite the absence of transmembrane or cytoplasmic domains, a large number of GPI-anchored proteins have the ability to transduce cellular activation signals (4). Cross-linking of GPI-anchored molecules has been shown to phosphorylate tyrosine residues on a number of intracellular substrates, leading to signal transduction (42). However, the mechanisms mediating signal transduction from an outer leaflet-associated GPI anchor and intracellular targets remain poorly understood. Cross-linking of GPI-linked molecules is a strategy commonly used to examine the effects of GPI-linked protein aggregation, an event that often affects downstream signaling targets via proteins association within lipid rafts. We used the highly specific By144 monoclonal antibody (22, 23) followed by a cross-linking secondary antibody to induce aggregation of CEACAM6. Our observation that CEACAM6 cross-linking decreases caveolin-1 phosphorylation is consistent with work conducted by Lee et al. (34) who showed that targeting GPI-linked protein to the exoplasmic leaflet of caveolar membranes disrupts caveolin-1 phosphorylation. Caveolin-1 may act as an
adaptor for the GPI anchor of CEACAM6 as it appears to do in other systems (43, 44). Csk is recruited to the membrane by phosphorylated caveolin-1 (35), and Csk negatively regulates c-Src through phosphorylation of tyrosine residue 527, resulting in decreased tyrosine phosphorylation of a variety of downstream targets, including FAK (33). Our observations support this model of c-Src inhibition leading to FAK activation.

In neutrophils, a range of GPI-anchored proteins interact with and signal via tyrosine kinases (14, 30, 45). TheSrc family of nonreceptor tyrosine kinases, most notably (although not exclusively) c-Src, is frequently overexpressed or aberrantly activated in epithelial and nonepithelial cancers and has functions that are critical in primary tumor progression and metastasis (27, 46, 47). Via interactions with targets such as FAK, c-Src has an important influence on the malignant phenotype (48). FAK plays an important role in the anchorage-dependent behavior of cells, and the functional consequences of caveolin phosphorylation remain poorly understood, increased phosphorylation of caveolin-1 has been shown to be tyrosine-phosphorylated and activated by Src kinases (49–52).

Levels of FAK activity, which depend on FAK tyrosine phosphorylation status, correlate with a variety of aspects of proliferative adenocarcinoma malignant cellular behavior (37, 38). FAK Tyr-397 is a potential high affinity binding site for the subunit of v-Src, and is found to co-precipitate with and signal via tyrosine kinases (14, 30, 45). The Src family of tyrosine kinases are activated (54, 55) and promotes FAK phosphorylation induced by CEACAM6 cross-linking. Our observations illustrate a potential mechanism through which CEACAM6 may influence intracellular events and cellular behavior.

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