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A Novel Role for Carcinoembryonic Antigen-Related Cell Adhesion Molecule 6 as a Determinant of Gemcitabine Chemoresistance in Pancreatic Adenocarcinoma Cells

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ABSTRACT

Most patients with pancreatic adenocarcinoma present with surgically incurable disease. Gemcitabine, the principal agent used to treat such patients, has little impact on outcome. Overexpression of carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 6, a feature of this malignancy, is associated with resistance to anoikis and increased metastasis. The purpose of this study was to determine the role of CEACAM6 in cellular chemoresistance to gemcitabine. CEACAM6 was stably overexpressed in Capan2 cells, which inherently express very low levels of the protein. Suppression of CEACAM6 expression was achieved in BxPC3 cells, which inherently overexpress CEACAM6, by stable transfection of a CEACAM6 small interfering RNA-generating vector. The effects of modulating CEACAM6 expression on gemcitabine-induced cytotoxicity were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay, flow cytometric apoptosis quantification, caspase profiling, and Western analysis of cytoplasmic cytochrome *c* release. The roles of Akt and c-Src kinases as downstream targets of CEACAM6 signaling were examined. Stable overexpression of CEACAM6 in Capan2 increased gemcitabine chemoresistance, whereas CEACAM6 gene silencing in BxPC3 markedly increased the sensitivity of these cells to gemcitabine. Differential expression of CEACAM6 modulates Akt activity in a c-Src-dependent manner, and CEACAM6 overexpression appears to protect cells from cytochrome *c*-induced caspase 3 activation and apoptosis.

INTRODUCTION

Highly aggressive locoregional invasion and early metastasis are characteristic of pancreatic adenocarcinoma, such that the majority of patients with this malignancy present with advanced, surgically unresectable disease (1, 2). The nucleoside analog gemcitabine is one of the few agents shown to affect survival and quality of life in such patients. However, despite being the current standard of care for advanced pancreatic cancer, the clinical impact of gemcitabine remains modest (3, 4) due to a high degree of inherent and acquired chemoresistance.

Carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 6 is a glycosylphosphatidylinositol (GPI)-linked immunoglobulin superfamily member that is overexpressed in a variety of gastrointestinal malignancies (5, 6). Despite lacking an intracellular domain, CEACAM6 is able to influence intracellular signaling events and represents an important determinant of gastrointestinal cancer progression (6–9). Previously, we have demonstrated that pancreatic adenocarcinoma cells differentially express CEACAM6 and that overexpression of CEACAM6 is associated with a higher tolerance of anchorage-independent culture and greater *in vivo* metastatic ability (10, 11). Furthermore, we have shown that posttranscriptional inhibition of CEACAM6 expression inhibits the ability of pancreatic adenocarcinoma cells to form experimental liver metastases *in vivo* (11). However, the role of CEACAM family members in chemoresistance has not yet been investigated.

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The purpose of this study was to characterize the contribution of CEACAM6 to pancreatic adenocarcinoma cellular chemoresistance to gemcitabine. Our results indicate that levels of CEACAM6 expression can modulate gemcitabine chemoresistance.

MATERIALS AND METHODS

Cells and Cell Culture. Capan2 and BxPC3 human pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM containing 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) and incubated in a humidified (37°C, 5% CO_2) incubator, grown in 75-cm^2 culture flasks, and passaged on reaching 80% confluence.

Expression Vector Construction and Transfection. Polyadenylated RNA was reverse transcribed using an anchored oligo(dT) primer with a *Xho*I restriction site. Double-stranded CEACAM6 cDNA was prepared and ligated into *Eco*RI adaptors, digested with *Xho*I, ligated into the pOTB7 vector, and transferred to the pIRES-eGFP expression plasmid (termed pIRES-CEACAM6). Cells were transfected with pIRES-CEACAM6 or pIRES-eGFP, which acted as a control, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. Stable clones were selected by exposure to incrementally increasing concentrations of G418 (Gibco), isolated using cloning cylinders, and maintained in medium containing 0.8 mg/ml G418. Clones pIRES-CEACAM6.1 and pIRES-CEACAM6, which expressed the highest levels of CEACAM6, were studied. Constitutively active (myristoylated) Akt (myr-Akt) and dominant negative Src [Src (K296R/Y528F)] expression constructs were obtained from Upstate (Waltham, MA). Transient transfection was performed using LipofectAMINE 2000.

Stable CEACAM6 RNA Interference. CEACAM6-specific and single base mismatch small interfering RNA expression constructs were generated by ligation of inserts (containing *Xho*I and *Xba*I overhang sites) targeting the following sequences into the pSuppressorNeo expression vector in accordance with the manufacturer's instructions (Imgenex, San Diego, CA): CEACAM6 (psiCEACAM6), 5′-CCGGACAGTTCCATGTATA-3′; and control (psiControl), 5′-CCCGACAGTTCCATGTATA-3′. Correct insert ligation was confirmed by absence of linearization by *Sal*I. Cells were transfected as described. Stably transfected clones were established using G418 selection. Clones stably transfected with empty vector, psi(−), and psiControl served as controls.

Cytotoxicity Assay. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Trevigen Inc., Gaithersburg, MD) in accordance with the manufacturer's instructions. Results of the MTT assay have been shown to correlate well with [^{3}H]thymidine incorporation in pancreatic cancer cell lines (12). Logarithmically growing cells were plated at 5×10^3 cells/well in 96-well plates, allowed to adhere overnight, and cultured in the presence or absence of 0–10 μM gemcitabine. Gemcitabine-induced cytotoxicity was determined after 48 h of exposure. Plates were read using a V_{max} microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm corrected to 650 nm and normalized to controls. Each independent experiment was performed 3 times, with 10 determinations for each condition tested. The concentration of gemcitabine required to inhibit proliferation by 50% (IC_{50}) was calculated from these results. At identical time points, cells were trypsinized to form a single cell suspension. Intact cells, determined by trypan blue exclusion, were counted using a Neubauer hemocytometer (Hausser Scientific, Horsham, PA). Cell counts were used to confirm MTT results.

Apoptosis Staining. After gemcitabine treatment, cells were washed and resuspended in 0.5 ml of PBS, and 1 μl/ml YO-PRO-1 and propidium iodide were added (Vybrant Apoptosis Assay Kit #4; Molecular Probes, Eugene, OR). Cells were incubated for 30 min on ice and then analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ), measuring fluorescence

emission at 530 and 575 nm. Cells stained with the green fluorescent dye YO-PRO-1 were counted as apoptotic; necrotic cells were stained with propidium iodide. The number of apoptotic cells was divided by the total number of cells (minimum of 10^4 cells), resulting in the apoptotic fraction. Data were analyzed using CellQuest software (Becton Dickinson). All observations were reproduced at least three times in independent experiments.

Formalin-fixed, paraffin-embedded tumor xenograft specimens were stained using a fluorescent terminal deoxynucleotidyl transferase-mediated nick end labeling-based apoptosis detection kit (*In Situ* Cell Death Kit; Roche Applied Science, Penzberg, Germany) in accordance with the manufacturer's instructions. Fluorescence microscopy was performed using a ×40 objective (Zeiss Plan-Neofluar) on a Nikon Eclipse TE2000-S inverted phase microscope (Nikon, Melville, NY). Images were analyzed using ImagePro Plus software version 4.0. The number of apoptotic cells counted in five random high power fields was used to calculate a mean apoptotic fraction.

Caspase Profiling and Cell-Free Assay. Whole cell lysates were assayed for caspase 3 activity using the BD ApoAlert fluorometric Caspase Assay Plate (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. Plates were read (excitation, 360 nm; emission, 480 nm) using a CytoFluor 4000 multiwell fluorescence plate reader (Applied Biosystems, Foster City, CA). All measurements were performed in triplicate, each with four determinations for each condition. Caspase 3 activation by cytochrome *c* was quantified as described previously (13). Briefly, cell-free extracts were generated as described by Slee *et al.* (14), with some modifications. Cells were pelleted and washed twice with PBS (pH 7.2), followed by washing once with 5 ml of ice-cold cell extract buffer [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 μM okadaic acid, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotinin]. The cell pellet was resuspended in 2 volumes of ice-cold extraction buffer and incubated on ice for 15 min. Cells in extract buffer were disrupted by sonication. Lysates were transferred to Eppendorf tubes and centrifuged at 15,000 × *g* for 15 min at 4°C. The resulting supernatants were obtained and frozen in aliquots at −80°C until required. Cell-free reactions were set up in a 120-μl reaction volume. Cell extracts were brought to a final volume of 120 μl in extract buffer with protein concentration of 2.5 mg/ml. Caspase 3 was activated by addition of bovine heart cytochrome *c* (or BSA negative control) and dATP to extracts at a final concentration of 50 μg/ml and 1 mM, respectively. The reactions were allowed to proceed at 37°C for 15 min, and caspase 3 activation was quantified using the fluorometric assay described.

Western Blotting. Cells (2×10^6) were harvested and rinsed twice with PBS. Cell extracts were prepared with lysis buffer [20 mM Tris (pH 7.5), 0.1% Triton X, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin] and cleared by centrifugation at 12,000 × *g* at 4°C. Total protein concentration was measured using the BCA assay kit (Sigma, St. Louis, MO) with BSA as a standard, according to the manufacturer's instructions. Cell extracts containing 30 μg of total protein were subjected to 10% SDS-PAGE, and the resolved proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen). Equal protein loading was confirmed by Coomassie Blue (Bio-Rad, Hercules, CA) staining of the gel. After blocking with PBS containing 0.2% casein for 1 h at room temperature, membranes were incubated with 3–5 μg/ml antibody in PBS containing 0.1% Tween 20 overnight at 4°C. Anti-CEACAM6 monoclonal antibody was obtained from InnoGenex (San Ramon, CA), and anti-c-Src monoclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Chemiluminescent detection (Upstate Biotechnology) was performed in accordance with the manufacturer's instructions. The CEACAM6 signal was quantified using ImagePro Plus software version 4.0 and normalized to that of actin. Blots were performed in triplicate.

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 μg of total protein) were prepared using anti-c-Src monoclonal antibody immobilized onto protein G-Sepharose beads (Zymed Laboratories Inc., San Francisco, CA). 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). Phosphorylated substrate was quantified by chromogenic detection using horseradish peroxidase-conjugated anti-phosphotyrosine antibody. Optical densities were determined at 492 nm using a V_{max} microplate spectrophotometer. c-Src kinase activity was compared with an epidermal growth factor receptor standard. Kinase assays were performed in triplicate with four determinations per condition.

Akt Kinase Assay. Akt activity was quantified using a commercially available nonradioactive *in vitro* kinase assay, in accordance with the manufacturer's instructions (Cell Signaling Technology, Beverly, MA). Akt was immunoprecipitated from 200 μl of cell lysates containing equal total protein. The resulting immunoprecipitates were incubated with glycogen synthase kinase-3 (GSK-3) fusion protein in the presence of ATP and kinase buffer. Phosphorylation of GSK-3, a physiological target of Akt, was measured by Western blot using anti-phospho-GSK-3α/β (Ser 21/9) antibody. Densitometric analysis was performed using ImagePro Plus software. Mean values from three independent experiments with 3 samples/group are shown.

Nude Mouse Xenograft Model. Male athymic nu/nu mice (5 weeks of age, weighing 20–22 g, and specific pathogen free) were obtained from

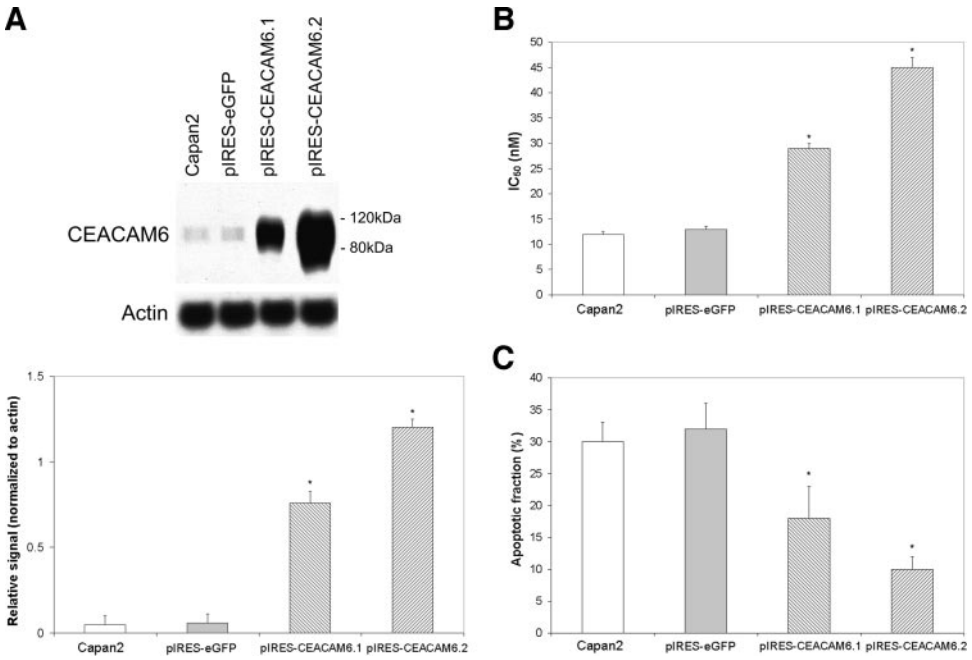

Fig. 1. *A*, Western blot analysis of parental Capan2 cells, pIRES-eGFP, pIRES-CEACAM6.1, and pIRES-CEACAM6.2 transfectants confirmed stable overexpression of CEACAM6 in both pIRES-CEACAM6.1 and pIRES-CEACAM6.2. Representative blot (*top panel*) and mean ± SD densitometric values (normalized to actin; *bottom panel*) from three independent experiments are shown. *, $P < 0.05$ *versus* Capan2 and pIRES-eGFP. *B*, effect of CEACAM6 overexpression on gemcitabine chemosensitivity, quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay and expressed as a gemcitabine IC_{50}. Both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 exhibited greater gemcitabine chemoresistance than Capan2 and pIRES-eGFP. Mean values from triplicate experiments with 10 determinations/condition are shown. *, $P < 0.05$ *versus* Capan2 and pIRES-eGFP. *C*, increased gemcitabine chemoresistance was associated with a lower apoptotic fraction in pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants after exposure to 1 μM gemcitabine for 48 h. Values are means ± SD from triplicate experiments. *, $P < 0.05$ *versus* Capan2 and pIRES-eGFP.

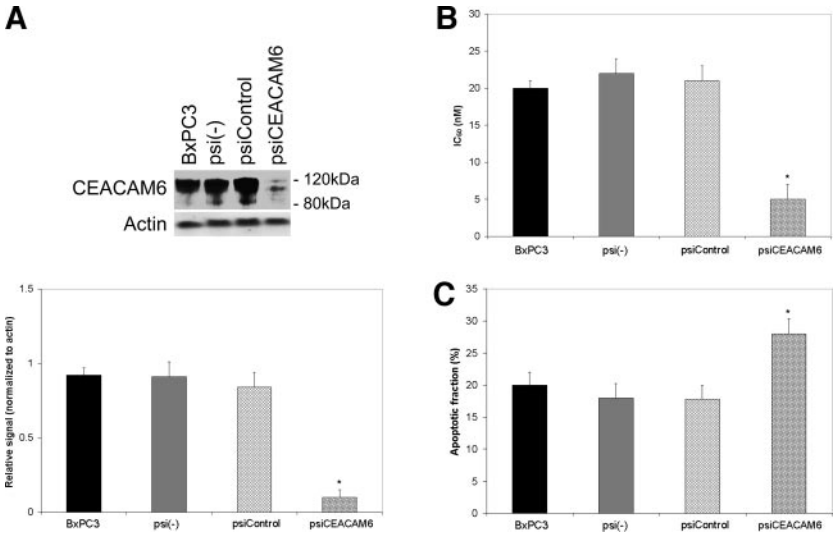

Fig. 2. *A*, Western blot analysis of CEACAM6 expression in parental BxPC3 cells and psiControl and psiCEACAM6 transfectants confirmed stable suppression of CEACAM6 protein in psiCEACAM6, but not psiControl transfectants, relative to both untreated BxPC3 cells and psi(−) transfectants. Representative blot (*top panel*) and mean ± SD densitometric values (normalized to actin; *bottom panel*) from three independent experiments are shown. ∗, $P < 0.05$ *versus* BxPC3 and psiControl. *B*, gemcitabine chemoresistance was significantly impaired in psiCEACAM6 transfectants. Gemcitabine chemoresistance was unchanged in psiControl transfectants, relative to both untreated parental BxPC3 cells and psi(−) transfectants. Mean values from triplicate experiments with 10 determinations/condition are shown. ∗, $P < 0.05$ *versus* BxPC3 and psiControl transfectants. *C*, this chemosensitization was associated with an increased apoptotic fraction in psiCEACAM6 transfectants after exposure to 1 μM gemcitabine for 48 h. Values are the means ± SD from triplicate experiments. ∗, $P < 0.05$ *versus* Capan2 and pIRES-eGFP.

Charles River Laboratories (Wilmington, MA). Mice were housed in microisolator cages with autoclaved bedding in a specific pathogen-free facility with 12-h light/dark cycles. Animals received water and food *ad libitum* and were observed for signs of tumor growth, activity, feeding, and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals. Mice were anesthetized with i.p. ketamine (200 mg/kg) and xylazine (10 mg/kg) and inoculated with 10^6 pancreatic adenocarcinoma cells in 75 μl of PBS by s.c. implantation. Treatment commenced 20 days after implantation, when tumors were approximately 50 mm^3 in volume. Mice received gemcitabine (150 mg/kg) in 100 μl of PBS vehicle by twice-weekly i.p. injection. Tumor dimensions were measured weekly using micrometer calipers. Tumor volumes were calculated using the following formula: Volume = ½ $a \times b^2$, where *a* and *b* represent the larger and smaller tumor diameters, respectively. After 6 weeks of treatment and 4 days after the final gemcitabine injection, necropsy was performed, and primary tumors were excised, formalin fixed, paraffin embedded, and stained by terminal deoxynucleotidyl transferase-mediated nick end labeling.

Statistical Analysis. Differences between groups were analyzed using Student's *t* test, multifactorial ANOVA of initial measurements, and Mann-Whitney *U* test for nonparametric data, as appropriate, using Statistica 5.5 software (StatSoft, Inc., Tulsa, OK). In cases in which averages were normalized to controls, the SDs of each nominator and denominator were taken into account in calculating the final SD. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of CEACAM6 Overexpression on Gemcitabine Chemosensitivity. We have reported previously that pancreatic adenocarcinoma cells differentially express CEACAM6 (11). BxPC3 cells markedly overexpress CEACAM6, relative to Capan2 cells (11, 15), and exhibit greater chemoresistance to gemcitabine (16). We directly determined the effect of CEACAM6 overexpression on gemcitabine chemoresistance by establishing clones of Capan2 cells that stably overexpressed CEACAM6, after transfection with the pIRES-CEACAM6 expression vector. Capan2 cells stably transfected with pIRES-eGFP served as controls. The gemcitabine IC_{50} was determined for each clone by MTT cytotoxicity assay. Stable overexpression of CEACAM6 by the clones pIRES-CEACAM6.1 and pIRES-CEACAM6.2 was confirmed by Western blot analysis (Fig. 1*A*). Both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants demonstrated increased gemcitabine chemoresistance, compared with parental Capan2 cells and pIRES-eGFP transfectants (Fig. 1*B*). Exposure to 1 μM gemcitabine for 48 h resulted in a lower apoptotic fraction in pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants, relative to control cells (Fig. 1*C*).

The effect of CEACAM6 overexpression on cellular proliferation in the absence of gemcitabine was determined and, consistent with our previously reported observation that CEACAM6 gene silencing does not affect cellular proliferation (11), we observed no difference in the doubling time of pIRES-CEACAM6.1, pIRES-CEACAM6.2, and pIRES-eGFP transfectants and Capan2 cells.

Effect of CEACAM6 Gene Silencing on Gemcitabine Chemosensitivity. Next, we sought to determine whether suppression of CEACAM6 expression would alter the sensitivity of BxPC3 pancreatic adenocarcinoma cells to gemcitabine. To induce stable suppression of CEACAM6 expression, BxPC3 cells were stably transfected

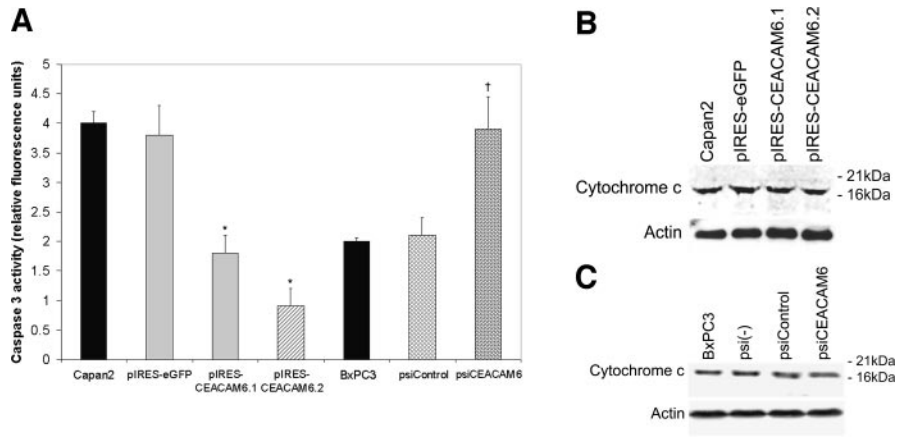

Fig. 3. Targeted suppression of CEACAM6 expression increases caspase 3 activity but does not affect gemcitabine-induced cytochrome *c* release. *A*, caspase 3 activity was quantified by fluorometric assay after exposure to 1 μM gemcitabine for 48 h. Both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants exhibited lower levels of gemcitabine-induced caspase 3 activation than pIRES-eGFP transfectants or parental Capan2 cells. Conversely, psiCEACAM6 transfectants exhibited greater caspase 3 activation than psiControl or BxPC3 cells. Values are means ± SD of three experiments, with four determinations performed for each condition. ∗, $P < 0.05$ *versus* pIRES-eGFP transfectants and parental Capan2 cells; †, $P < 0.05$ *versus* psiControl and BxPC3. *B*, Western blot analysis of cytoplasmic cytochrome *c* release revealed no significant difference between Capan2 cells and pIRES-eGFP, pIRS-CEACAM6.1, or pIRES-CEACAM6.2 transfectants. *C*, cytochrome *c* release was also not significantly different between BxPC3 cells and psi(−), psiControl, or psiCEACAM6 transfectants.

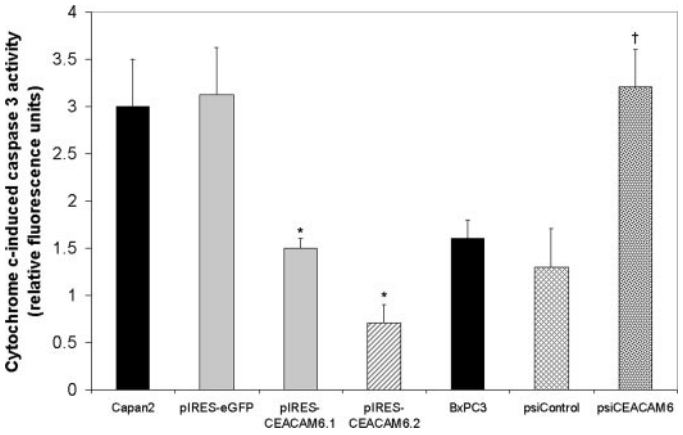

Fig. 4. Cytosolic extracts from pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants demonstrated significantly lower levels of cytochrome *c*-induced caspase 3 activation than those from pIRES-eGFP transfectants or Capan2 cells. BxPC3 psiCEACAM6 transfectants exhibited higher levels of cytochrome *c*-induced caspase 3 activation. Taken together, this suggests that CEACAM6 overexpression may exert a cytoprotective effect by inhibiting cytochrome *c*-induced caspase 3 activation. Experiments were performed in triplicate, with four determinations/condition. ∗, $P < 0.05$ *versus* pIRES-eGFP transfectants and parental Capan2 cells; †, $P < 0.05$ *versus* psiControl and BxPC3.

with the CEACAM6-specific small interfering RNA expression vector (psiCEACAM6) and selected on the basis of their resistance to G418. Cells stably transfected with the empty vector [psi(−)] and a vector encoding a single base mismatch small interfering RNA (psiControl) served as controls. Stable suppression of CEACAM6 expression (up to 90%) was confirmed by Western blot analysis (Fig. 2*A*). Consistent with our previous observations, stable suppression of CEACAM6 did not affect cellular proliferation in the absence of gemcitabine. However, psiCEACAM6 transfectants exhibited a significant decrease in their chemoresistance to gemcitabine, compared with both psi(−) and psiControl transfectants (Fig. 2*B*). Relative to control cells, psiCEACAM6 transfectants exposed to 1 μM gemcitabine for 48 h exhibited a higher apoptotic fraction (Fig. 2*C*).

Modulation of CEACAM6 Expression Affects Gemcitabine-Induced Caspase 3 Activation, but Not Cytochrome *c* Release. Mitochondrial damage with release of cytochrome *c* is implicated in cell death signaling pathways and occurs in pancreatic adenocarcinoma cells after exposure to gemcitabine (17). Gemcitabine-induced cytochrome *c* release is associated with caspase 3 activation in human adenocarcinoma cells (18, 19). The apoptosome, a multiprotein complex comprising Apaf-1, cytochrome *c*, and initiator caspase 9, functions to activate executioner caspase 3 downstream of mitochondria in response to apoptotic signals (20, 21). Gemcitabine-induced activation of caspase 3 was significantly reduced in pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants, relative to pIRES-eGFP and parental Capan2 cells. Similarly, suppression of CEACAM6 expression in BxPC3 pIRES-CEACAM6 transfectants was associated with increased gemcitabine-induced caspase 3 activation, compared with BxPC3 cells and psi(−) and psiControl transfectants. (Fig. 3*A*). The psiControl vector had no effect on gemcitabine-induced caspase 3 activity. Interestingly, there was no significant difference in the degree of gemcitabine-induced cytochrome *c* release in pIRES-CEACAM6.1, pIRES-CEACAM6.2, or pIRES-eGFP transfectants (Fig. 3*B*). Similarly, there was no significant difference in gemcitabine-induced cytochrome *c* release among BxPC3 cells and psi(−), psiControl, and psiCEACAM6 transfectants (Fig. 3*C*).

In view of these findings, we performed cell-free *in vitro* assessment of the effect of CEACAM6 overexpression on cytochrome *c*-induced activation of caspase 3. Addition of cytochrome *c* and dATP to cytosolic extracts results in caspase 3 activation (22). Cell extracts prepared from pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants demonstrated significantly lower levels of caspase 3 activity after addition of cytochrome *c* and dATP than those from pIRES-eGFP transfectants or Capan2 cells. Conversely, BxPC3 psiCEACAM6 transfectants exhibited higher levels of caspase 3 activity after addition of cytochrome *c* and dATP (Fig. 4). We confirmed the importance of caspase activation in mediating the enhanced gemcitabine-induced apoptosis observed in psiCEACAM6 by exposing cells to the caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk; 100 μM), which reduced the mean apoptotic fraction of psiCEACAM6 cells exposed to gemcitabine from 28% to 9% ($P < 0.05$). This finding, taken together with the previous observations, suggests that CEACAM6 overexpression may exert a cytoprotective effect, at least in part, by inducing inhibition of caspase 3 activation in response to cytochrome *c* release, an effect that appears to occur downstream of cytochrome *c* release.

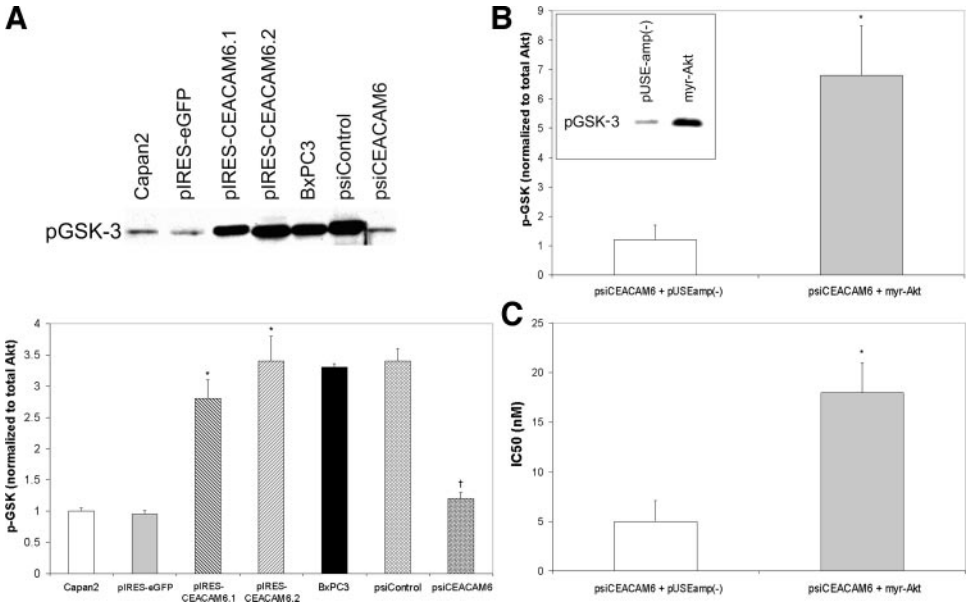

Fig. 5. *A, top panel,* Akt activity was quantified by glycogen synthase kinase 3 phosphorylation (*p-GSK-3*). *Bottom panel,* both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 exhibited significantly greater Akt activity than parental Capan2 cells and pIRES-eGFP transfectants. Values are the means of three independent experiments. Suppression of CEACAM6 expression is associated with decreased Akt activity. psiCEACAM6 transfectants exhibited lower Akt activity than BxPC3 and psiControl cells. Values are the means of three independent experiments. ∗, $P < 0.05$ *versus* Capan2 cells and pIRES-eGFP. †, $P < 0.05$ *versus* BxPC3 and psiControl. *B,* BxPC3 cells stably transfected with psiCEACAM6 were transiently transfected with the constitutively active (myristoylated) Akt expression construct (myr-Akt). Increased Akt activity was confirmed by assaying GSK-3 phosphorylation (p-GSK, representative blot shown, *inset*). *C,* whereas the empty vector, p-USE-amp(−), had no effect on the gemcitabine IC_{50}, transfection of myr-Akt increased Akt activity and significantly increased the gemcitabine IC_{50} in psiCEACAM6 transfectants. Values are the means of triplicate experiments with 10 determinations/condition. ∗, $P < 0.05$ *versus* p-USE-amp(−) in each case.

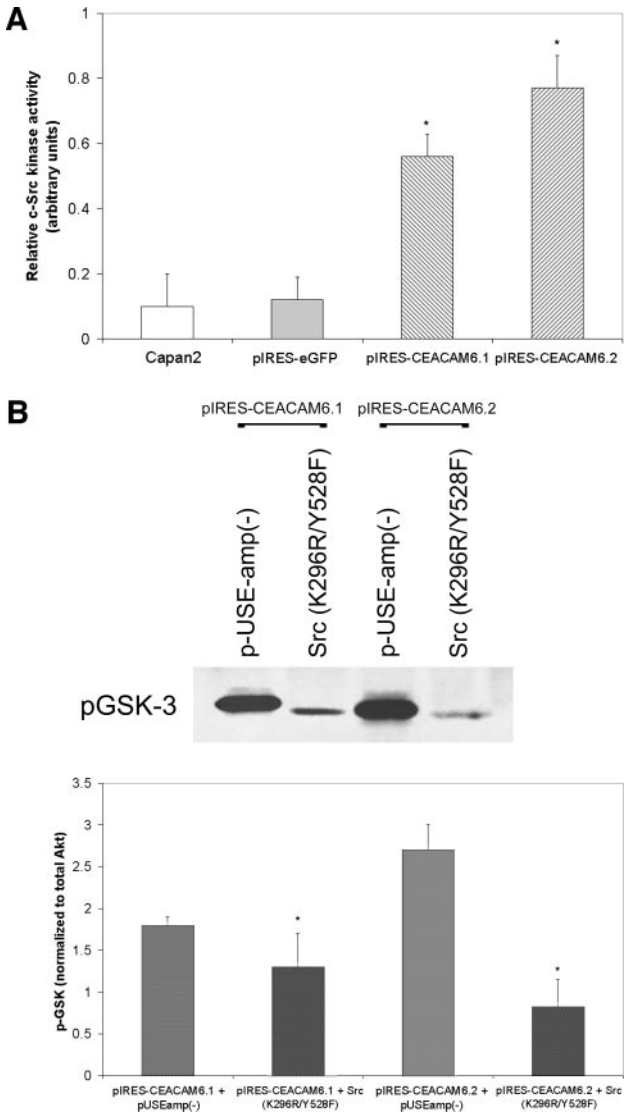

Fig. 6. Increased c-Src activity is associated with and required for elevated Akt activity in pIRES-CEACAM6.1 and pIRES-CEACAM6.2. *A*, pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants exhibit increased c-Src kinase activity, as determined by *in vitro* phosphorylation of poly-Glu-Tyr, compared with untreated Capan2 cells and pIRES-eGFP transfectant controls. *B*, pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants were transiently transfected with dominant negative Src [*Src (K296R/Y528F)*]. Suppression of c-Src kinase activity was confirmed by *in vitro* kinase assay. *Top panel*, Akt activity was quantified by glycogen synthase kinase phosphorylation assay. *Bottom panel*, dominant negative Src markedly decreased Akt activity in both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants, suggesting that c-Src is required for the increased Akt activity exhibited by CEACAM6-overexpressing transfectants. Values are the means of three independent experiments. ∗, $P < 0.05$ *versus* empty vector, p-USE-amp(−).

Effect of CEACAM6 Overexpression on Akt Kinase Activity. Akt represents an important determinant of gemcitabine chemoresistance in pancreatic adenocarcinoma (23) and is a central regulator of cellular proliferation in pancreatic adenocarcinoma cells, including BxPC3 (24). Akt is also of functional importance in the regulation of caspase activity (13, 25). Previously, we have reported that CEACAM6 gene silencing suppresses Akt Ser-473 phosphorylation, a marker of Akt activation (11). We therefore assessed the effect of CEACAM6 overexpression on Akt kinase activity because this may account for the increased chemoresistance of cells overexpressing CEACAM6. In addition, we assessed the effect of CEACAM6 gene silencing on Akt activity in BxPC3 cells. Akt activity was quantified using an *in vitro* kinase assay that detects phosphorylation of GSK-3, a physiological substrate of Akt. BxPC3 cells demonstrate greater *in vitro* Akt activity than Capan2 cells. Furthermore, overexpression of CEACAM6 significantly increased Akt activity in Capan2 cells. Conversely, psiCEACAM6 BxPC3 transfectants demonstrated a lower level of Akt activity than psiControl BxPC3 transfectants (Fig. 5*A*).

To determine whether Akt was able to compensate for the effect of suppressing CEACAM6 expression on gemcitabine chemoresistance, we introduced a constitutively active (myristoylated) p-USE-amp-based Akt expression construct (myr-Akt) into psiCEACAM6 BxPC3 transfectants. Cells transfected with the empty vector [p-USE-amp(−)] served as controls. Transfection of myr-Akt increased Akt activity and returned the gemcitabine IC_{50} to values close to those of psiControl transfectants (Fig. 5, *B* and *C*). Together, these observations indicate that levels of CEACAM6 modulate Akt and that this contributes to the chemoprotective effect of CEACAM6 overexpression.

CEACAM6-Dependent Akt Activation Is Mediated by c-Src. It has recently been recognized that c-Src directly activates Akt through an interaction between its SH3 domain and a conserved proline-rich motif in the COOH-terminal regulatory region of Akt (26). Furthermore, Src family kinases have been reported to associate with CEACAM family members (27). Increased c-Src activity, detected by

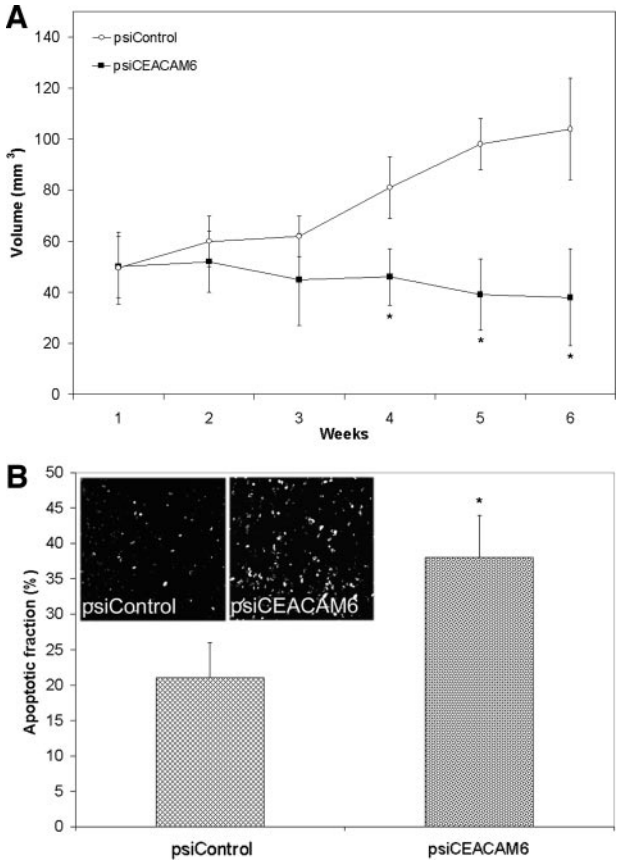

Fig. 7. Suppression of CEACAM6 increases gemcitabine chemosensitivity in the nude mouse s.c. xenograft model. Mice ($n = 10$ mice/group) were allocated to one of two groups. Group 1 received 10^6 BxPC3 psiCEACAM6 transfectant cells. Group 2 received 10^6 BxPC3 psiControl transfectant cells. Once tumors reached approximately 50 mm^3, treatment with 150 mg/kg gemcitabine twice weekly, by i.p. injection, was commenced. Tumor volumes were recorded over the 6-week treatment period. Mean values ± SD are shown (*A*). ∗, $P < 0.05$ *versus* psiControl transfectants. After necropsy, tumor sections were stained by terminal deoxynucleotidyl transferase-mediated nick end labeling, and apoptotic cells were quantified in five random fields for each tumor. Mean values ± SD for psiCEACAM6 and psiControl transfectants tumors are shown (*B*). ∗, $P < 0.05$ *versus* psiControl tumors. *Inset* shows representative fields from terminal deoxynucleotidyl transferase-mediated nick end labeling-stained tumors derived from psiControl and psiCEACAM6 transfectant cells.

in vitro kinase assay, was observed in lysates from both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants, compared with control cells (Fig. 6*A*). We therefore assessed the effect of transfection of dominant negative c-Src on Akt activity after CEACAM6 overexpression. Dominant negative c-Src almost completely abolished the increase in Akt activity in both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 (Fig. 6*B*), suggesting that CEACAM6 overexpression increases Akt activity in a c-Src-dependent manner.

***In Vivo* Chemoresistance.** Mice implanted with either BxPC3 psiCEACAM6 or psiControl transfectants were treated with gemcitabine over a 6-week period. Tumor volume was measured weekly. Mice implanted with psiCEACAM6 transfectant xenografts exhibited slower tumor growth than those implanted with psiControl transfectants cells (Fig. 7*A*). Terminal deoxynucleotidyl transferase-mediated nick end labeling staining of the tumor specimens revealed a 1.8-fold higher level of apoptosis in psiCEACAM6 transfectant tumors than in psiControl transfectant tumors (Fig. 7*B*).

DISCUSSION

Pancreatic adenocarcinoma is among the most chemoresistant of malignancies, and there exists an urgent need to establish new therapeutic targets and approaches. In this study, we have identified the GPI-anchored protein CEACAM6 as a determinant of pancreatic adenocarcinoma cellular chemoresistance to gemcitabine. Despite lacking transmembrane and intracellular domains, CEACAM6 is able to influence intracellular events critical to the regulation of apoptosis. Our study is the first to demonstrate a role for CEACAM6 in gemcitabine chemoresistance and provides evidence that Akt may convey the chemoprotective effect of CEACAM6 overexpression in a c-Src-dependent manner.

Growing evidence implicates CEACAM6 in gastrointestinal cancer progression (6, 8, 9, 11). We have shown previously that modulation of CEACAM6 expression influences cellular susceptibility to the proapoptotic stimulus of anchorage-independent culture (11). In the current study, overexpression of CEACAM6 resulted in increased gemcitabine chemoresistance, and this, together with our observation that gemcitabine-induced apoptosis is attenuated by knockdown of CEACAM6 expression, is consistent with a model in which CEACAM6 determines cellular susceptibility to apoptosis. CEACAM6 is not alone among GPI-anchored proteins in having importance in the regulation of apoptosis. Bounhar *et al.* (28) reported that the GPI-linked prion protein potently inhibits Bax-induced apoptotic cell death in human primary neurons. GPI-defective Jurkat T cells are reportedly more susceptible to Fas-mediated apoptosis (29), and antibody-mediated targeting of the GPI-linked CAMPATH-1H (CD52) antigen has been shown to result in apoptosis of human lymphoma cells (30). Although it appears increasingly likely that GPI-linked proteins play a part in regulating apoptosis, the mechanisms through which this family of proteins influences intracellular apoptotic pathways have received relatively little attention.

Considerable evidence derived from work performed in a variety of malignancies, including pancreatic adenocarcinoma, indicates that Akt plays an important role in the regulation of cellular apoptosis (13, 23, 31–33). Active Akt appears to protect cells from a variety of apoptotic stimuli, including exposure to gemcitabine (13, 23, 32, 33). The caspase cascade of proteolytic enzymes comprises initiator and executioner elements, which, after activation, lead to degradation of intracellular targets resulting in apoptotic cell death. Active Akt has been shown to phosphorylate initiator caspase 9 directly, preventing its activation (25). Inhibition of this initiator caspase may interfere with apoptosome function and impair activation of effector caspase 3. Akt has also been reported to inhibit caspases by posttranslational modification of an as yet unidentified cytosolic factor located downstream of cytochrome *c* release and upstream of caspase 9 activation (13). The results of our study are consistent with this paradigm of Akt-mediated caspase regulation. Firstly, we have shown that Akt activity is increased by CEACAM6 overexpression and decreased by CEACAM6 gene silencing. Secondly, the effects of modulating CEACAM6 expression appear to influence the apoptotic pathway downstream of cytochrome *c* release because altering CEACAM6 expression influences cytochrome *c*-induced caspase activity but does not affect cytoplasmic cytochrome *c* release. The mechanisms underlying these observations will require further investigation, but Akt would appear to be a candidate mediator of CEACAM6-dependent apoptotic regulation.

The tyrosine kinase c-Src has recently been shown to induce activating phosphorylation of Akt through a direct interaction with the c-Src SH3 domain and a conserved proline-rich motif in the COOH-terminal regulatory region of Akt (26). The previously reported association of Src family kinases with CEACAM6 is consistent with the increased c-Src activity we detect in lysates from both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants. The ability of a dominant negative Src expression construct to impair the increased Akt activity seen in CEACAM6-overexpressing clones further supports the role of c-Src in mediating CEACAM6-dependent Akt activation. In a recent study, we illustrated the functional importance of c-Src-Akt signaling in gemcitabine chemoresistance. Treatment of pancreatic adenocarcinoma cells with the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and transfection of a dominant negative Src expression construct were each sufficient to impair gemcitabine chemoresistance. Conversely, overexpression of active Src was chemoprotective in pancreatic adenocarcinoma cells. Src inhibition was also associated decreased Akt (Ser-473) phosphorylation status, consistent with results of the present study (34).

Although RNA interference induced by transfection of small interfering RNA oligonucleotide is emerging as a powerful tool, this approach is limited by its transient nature (35). By stably transfecting cells with a short hairpin expression vector, as was used in this study, this limitation can be overcome (36). The ability to sustain a cell line that stably underexpresses CEACAM6 demonstrates that this molecule is not essential for cell survival under standard culture conditions and is consistent with our previously reported observations that suppression of CEACAM6 expression does not affect cellular proliferation or apoptosis in monolayer culture (11).

In summary, our findings indicate that overexpression of CEACAM6 can protect pancreatic adenocarcinoma cells from gemcitabine-induced cytotoxicity. Conversely, knockdown of CEACAM6 expression has a chemosensitizing effect. In addition to functioning as an intercellular adhesion molecule, CEACAM6 may constitute part of an antiapoptotic survival pathway in these cells. This cytoprotective pathway may contribute to gemcitabine chemoresistance, and targeting this pathway may be a potential therapeutic strategy in this highly treatment-resistant disease.

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