

# CUB Domain-Containing Protein 1 Is a Novel Regulator of Anoikis Resistance in Lung Adenocarcinoma<sup>∇†</sup>

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**Malignant tumor cells frequently achieve resistance to anoikis, a form of apoptosis induced by detachment from the basement membrane, which results in the anchorage-independent growth of these cells. Although the involvement of Src family kinases (SFKs) in this alteration has been reported, little is known about the signaling pathways involved in the regulation of anoikis under the control of SFKs. In this study, we identified a membrane protein, CUB-domain-containing protein 1 (CDCP1), as an SFK-binding phosphoprotein associated with the anchorage independence of human lung adenocarcinoma. Using RNA interference suppression and overexpression of CDCP1 mutants in lung cancer cells, we found that tyrosine-phosphorylated CDCP1 is required to overcome anoikis in lung cancer cells. An apoptosis-related molecule, protein kinase C $\delta$ , was found to be phosphorylated by the CDCP1-SFK complex and was essential for anoikis resistance downstream of CDCP1. Loss of CDCP1 also inhibited the metastatic potential of the A549 cells in vivo. Our findings indicate that CDCP1 is a novel target for treating cancer-specific disorders, such as metastasis, by regulating anoikis in lung adenocarcinoma.**

Src family kinases (SFKs) play important roles in various cell functions, including cell proliferation, cell adhesion, and cell migration, under the control of extracellular stimuli (26). Many studies have shown elevated activity of SFKs or increased protein expression in a variety of human cancers (31). The activities of SFKs often correlate with the malignant potential of cancer and poor prognosis (36). SFKs may contribute to various aspects of tumor progression, including uncontrolled proliferation and migration, disruption of cell-cell contacts, invasiveness, angiogenesis, and resistance to apoptosis.

Anoikis is a form of apoptosis triggered by the loss of cell survival signals generated from interaction with the extracellular matrix (10). Anoikis is considered to be physiologically important in the maintenance of homeostasis and tissue architecture (24). On the other hand, the resistance to anoikis acquired during carcinogenesis has been described as a core aspect of cancer cells for tumor progression and metastasis (12). This property indicates the existence of survival signals in tumor cells, which compensate for similar signals supported by cell-matrix interactions. Since they were originally described by Frisch and Francis (9), several previous reports have shown the crucial role of SFKs in the anoikis resistance of tumor cells. Viral Src oncoprotein abrogates anoikis in epithelial cells (13). Src activation is also important for resistance to anoikis in various cancers, such as colon tumor and lung adenocarcinoma cells (33, 35). However, the exact mechanism that is responsible for the anoikis resistance mediated by SFKs in human cancer cells has not been clearly elucidated.

The purpose of this study, therefore, was to identify the key

molecules of anoikis resistance, which mediate signals from activated SFKs in human cancer cells. For that purpose, we analyzed proteins binding to SFKs with and without cell attachment in a number of human lung cancer cell lines. We found that tyrosine phosphorylation of a 135-kDa SFK-binding protein is associated with elevated anchorage independence in a group of lung cancer cell lines, especially in a cell suspension condition. This 135-kDa phosphoprotein was purified and identified as CUB-domain-containing protein 1 (CDCP1) by mass spectrometry. The protein CDCP1 is a type I transmembrane protein that has possible roles in cell-cell and cell-matrix adhesion (3, 5). The molecule has been reported to be highly expressed in lung, breast, and colon cancers (6, 28). Using an RNA interference (RNAi) technique, it was determined that CDCP1 is required for the survival of lung cancer cells both in suspension culture and in soft agar. This study identifies a novel modulator that sustains anoikis resistance under the control of SFKs in lung cancer cells.

## MATERIALS AND METHODS

**Plasmids, antibodies, and reagents.** Full-length cDNA of human CDCP1 with a FLAG tag at the C terminus (wild type [WT]) was obtained by reverse transcription-PCR amplification from the mRNA of A549 human lung adenocarcinoma cells and cloned into pcDNA3.1 (Invitrogen). The cytoplasmic-domain mutants of CDCP1, Y734F (Tyr734 to Phe), Y762F (Tyr762 to Phe), and Y2F (Y734 and Y762 double mutant), were generated by PCR using the overlap extension method of Ho et al. (14). The C2 domain of protein kinase C $\delta$  (PKC $\delta$ ) corresponding to amino acids (1 to 160) with a hemagglutinin (HA) tag at the C terminus was obtained by PCR and cloned into pcDNA3.1 (Invitrogen). To express the Fyn Src homology 2 (SH2) domain fused with glutathione S-transferase (GST) protein (GST-FynSH2), a cDNA fragment of the Fyn SH2 domain corresponding to nucleotides 1018 to 1299 of the reported sequence (GenBank accession number NM 002037) was amplified by PCR and cloned into pGEX4T2 (Amersham Pharmacia).

Antiphosphotyrosine antibody (4G10) and anti-c-Src antibody (clone GD11) were purchased from Upstate Biotechnology. Anti-Akt antibody, anti-phospho-Akt (Ser473) antibody, anti-ERK1/2 antibody (p44/p42 mitogen-activated protein kinase [MAPK] antibody), anti-phospho-ERK1/2 antibody (phospho-p44/p42 MAPK [Thr202/Tyr204] antibody), anti-p38MAPK antibody, anti-phospho-

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p38MAPK (Thr180/Tyr182) antibody, and anti-phospho-PKC $\delta$  (Tyr311) antibody were purchased from Cell Signaling. Anti-HA (Y-11), anti-PKC $\delta$  (C-20), and anti-Fyn (FYN3) antibodies were purchased from Santa Cruz Biotechnology. Anti-FLAG antibody (Anti-FLAG M2 peroxidase conjugate specific antibody) and antitubulin antibody (clone B-5-1-2) were purchased from Sigma. The anti-c-Yes antibody was purchased from Transduction Laboratories. An anti-CDCP1 antibody (ab1377) was purchased from Abcam Ltd. To generate the CDCP1 antibody, anti-CDCP1 and the anti-phospho-CDCP1 (Tyr734) antibody were obtained by rabbit immunization using the cytoplasmic domain of CDCP1 fused to GST, and the amino peptides NDSHV(pY<sup>734</sup>)AVIEC of CDCP1 were obtained from MBL Co., Ltd. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were purchased from Amersham Pharmacia. The HRP-conjugated anti-goat immunoglobulin G (IgG) antibody was purchased from ZYMED. Mouse, rabbit, and goat IgGs were purchased from DakoCytomation. The SFK inhibitor PP2 and the structural analog PP3 were purchased from Calbiochem-Novabiochem Ltd. Etoposide and Rottlerin were purchased from Sigma-Aldrich.

**Cell culture and transfection.** The human lung adenocarcinoma cell lines A549, PC14, and H322 and human lung squamous carcinoma cell lines H520 and H157 were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C with 5% CO<sub>2</sub>. For transfection, cells were seeded on a cell culture plate or a 2-methacryloyloxyethyl phosphorylcholine (MPC)-coated plate (Nunc) at 1.5 × 10<sup>5</sup> cells per six wells or 9.0 × 10<sup>5</sup> cells/10-cm plate, and transfection was performed after 14 h. Expression plasmids were transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To investigate the effect of PP2 treatment, cells were treated with 10 μM of PP2 or 10 μM of PP3.

**Construction of dicer, stealth siRNAs, and miR RNAi vectors.** Dicer small interfering RNAs (siRNAs) of human c-Src, Fyn, and c-Yes were generated using the BLOCK-iT RNAi TOPO transcription kit and BLOCK-iT complete dicer RNAi kit (Invitrogen) according to the manufacturer's instructions. In the generation of siRNA for Src, a 726-bp fragment from the initiation codon of human c-Src was chosen as the target sequence and amplified by PCR using the primers forward, 5'-ATGGGTAGCAACAAGAGCAAG-3', and reverse, 5'-GTGGCACAGGCCATCGGCGTG-3'. As for Fyn and c-Yes, 999-bp and 857-bp fragments were chosen as the target sequences, respectively, and amplified with the following primers: Fyn forward, 5'-ATGGGCTGTGTGCAATGTAGG-3', and reverse, 5'-CACCACTGCATAGAGCTGGAC-3'; c-Yes forward, 5'-CTGAA AATACTCCAGAGCCTG-3', and reverse, 5'-CTTTGCTCTAGTTTAACTCTAG-3'. Dicer siRNA of LacZ was generated by the same procedure as dicer siRNAs of c-Src, Fyn, and c-Yes and was used as a negative control. The stealth siRNAs of human CDCP1, PKC $\delta$ , and the negative control were ordered from Invitrogen. Specific primers were as follows: CDCP1 forward, 5'-GCUCUGCCACGAGAA GCAACAUUA-3', and reverse, 5'-UAAUGUUGCUUUCUGUGGCAGAGC-3'; PKC $\delta$  forward, 5'-GGUGCAGAAGACGGACCAUGUAU-3', and reverse, 5'-AUACAUGGUCGGUUCUUCUGCACC-3'. Transfection of both dicer and stealth siRNAs was performed with Lipofectamine 2000 (Invitrogen), and the effect was analyzed less than 48 h after the transfection.

A system stably expressing siRNA was generated using the BLOCK-iT Pol II miR RNAi expression vector kit (Invitrogen) according to the manufacturer's instructions. In the generation of the miR RNAi vector for humans, CDCP1 was chosen as the target sequence, using the forward primer 5'-TG CTGAATGTTGCTTTCTCGTGGCAGGTTTTGGCCACTGACTGACCT GCCACGAAAGCAACATT-3' and the reverse primer 5'-CCTGAATGTT GCTTTGCTGGCAGGTCAGTGGCCAAAACCTGCCACGAGAA AGCAACATTC-3'. Cells stably expressing the miR RNAi vector for CDCP1 and LacZ were established and cultured in medium containing blasticidin (Invivogen) at a concentration of 10 μg/ml for 3 weeks. Two clones expressing the CDCP1 RNAi vector (miCDCP1-1 and -2) were selected by significant suppression of the CDCP1 protein (<10%), and two clones from the control LacZ vector were also selected (miLacZ-1 and -2).

**Soft-agar colony assay.** Six-well tissue culture plates were coated with a layer of RPMI 1640-10% FBS containing 0.5% ultrapure agarose (Invitrogen). Subconfluent A549 cells transfected with the dicer siRNA or miR RNAi vector-expressed clone were treated with EDTA, washed in phosphate-buffered saline twice, and resuspended in RPMI 1640-10% FBS at 6 × 10<sup>3</sup> cells/ml. Then, a 500-μl cell sample was added to 1 ml of RPMI 1640-10% FBS containing 0.5% ultrapure agarose (final concentration, 0.33%). The cells were plated on the coated tissue culture plates, allowed to solidify, and then placed in a 37°C incubator. After 30 days, colonies were scanned using a GS-800 calibrated densitometer (Bio-Rad), and the numbers of colonies per well were determined. Soft-agar assays were performed three times.

**Immunoprecipitation and Western blotting.** Cell lysates were prepared with protease inhibitors in PLC buffer (10 mM Tris-HCl, pH 7.5, 5 mM EGTA, 150

mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 1 mM sodium orthovanadate [Na<sub>2</sub>VO<sub>4</sub>], and 100 μg/ml leupeptin). The protein concentration was measured by BCA protein assay (Pierce). For purification, 1 μg of monoclonal or affinity-purified polyclonal antibody was added to the proteins, which were then incubated with 500 μl (2 mg/ml) of cell lysate for 2 h at 4°C. Next, they were precipitated with protein A- or protein G-agarose for 1 h at 4°C. The immunoprecipitates were extensively washed with PLC buffer and prepared for Western blotting.

For Western blotting, samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). After blocking of the membrane with blocking buffer (Blocking One; Nakarai Tesque), the membrane was probed with antibodies for detection. The membrane was further probed with HRP-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (1:4,000) to visualize the reacted antibody.

Images were captured by a molecular imager (GS-800; Bio-Rad), and the density of each smear was quantified using Quantity One software (Bio-Rad).

**Identification of CDCP1.** Isolated GST-FynSH2 protein coupled with cyanogen bromide (CNBr) was used to purify the 135-kDa and 70-kDa proteins from the A549 cells cultured for 48 h on MPC-coated plates in growth medium. Briefly, ~3 × 10<sup>7</sup> suspended A549 cells in a total of 400 dishes (10-cm dish; 30 ml culture medium) were collected and lysed in PLC buffer. The cell lysate was rotated for 8 h at 4°C with GST-FynSH2 protein and washed four times using PLC buffer before being eluted with GST-FynSH2-coupled proteins using 8 M urea buffer (8 M urea, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100). Secondly, the eluted sample was dialyzed three times against a 100-fold volume of dialysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>), and then the sample was affinity purified with antiphosphotyrosine monoclonal antibody (4G10) coupled with CNBr. 4G10-coupled proteins were washed four times using PLC buffer and once using heptyl-glucoside buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% heptyl-glucoside). Next, the samples were eluted using 0.1 M phenylphosphate in heptyl-glucoside buffer. The purified 135-kDa and 70-kDa proteins were concentrated, electrophoresed, and blotted onto a ProBlott membrane (Applied Biosystems). After visualization with colloidal gold total-protein stain (Bio-Rad), the isolated 135-kDa and 70-kDa bands were analyzed by mass spectrometry. Four sets of amino acid sequences determined from the 135-kDa band and an amino acid sequence determined from the 70-kDa band indicated that both the 135-kDa and 70-kDa proteins were CDCP1.

**Apoptosis assay.** Each cell was treated with EDTA, and 1 × 10<sup>4</sup> cells were reseeded onto normal or MPC-coated 96-well plates. After 24 h, the cells were lysed and used for the detection of apoptosis. Apoptosis levels were determined using a cell death ELISA kit (Roche Molecular Biochemicals), which detects the presence of nucleosomes in the cytoplasm of apoptotic cells. The absorbance of the samples was measured at a wavelength of 405 nm using a microplate reader model 550 (Bio-Rad).

**BrdU incorporation assay.** Cell proliferation was analyzed with the cell proliferation ELISA BrdU kit according to the manufacturer's instructions (Roche Molecular Biochemicals) based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis of proliferating cells. Briefly, A549 cells were cultured in triplicate for 24 h in 96-well plates (1.0 × 10<sup>4</sup> cells/well) with or without cell attachment. The cells were subjected to BrdU incorporation for 6 h. The colorimetric change was measured at 450 nm on a microplate reader model 550 (Bio-Rad).

**Infection of retroviral constructs.** The retroviral vectors PQCXIN (Clontech) and pCMSCVbsd were used to express human CDCP1 (WT) and Y734F with a FLAG tag at the C terminus and full-length cDNA of Fyn kinase with a double HA tag at the C terminus (FynHA), respectively. pCMSCVbsd contains the blasticidin resistance gene in place of the puromycin resistance gene of pCMSCVpuro (20). These retroviral vectors were converted into the destination vectors with a vector conversion kit (Invitrogen). The cDNA segments were first cloned into pDONR221 and then into the destination vector, pDEST-PQCXIN or pDEST-CMSCVbsd, according to the manufacturer's instructions (Invitrogen). The production of recombinant retroviruses was performed as described previously (25). Briefly, the retroviral vector and the packaging construct pCL-10A1 were cotransfected into 293T cells using TransIT-293 (Mirus Co., Madison, WI) according to the manufacturer's instructions, and culture fluid was harvested 48 to 72 h posttransfection. H322 cells were infected with the viral fluid in the presence of 4 mg/ml Polybrene, and the infected cells were selected in the presence of 800 μg/ml G418 or 5 μg/ml blasticidin. For combinations of retroviral infections, cells were first transduced with CDCP1 and then with Fyn kinase.

**Experimental metastasis assay.** Female BALB/cA/Jc1-nu nude mice were purchased from CLEA Japan Inc. All of the mice used in these experiments were

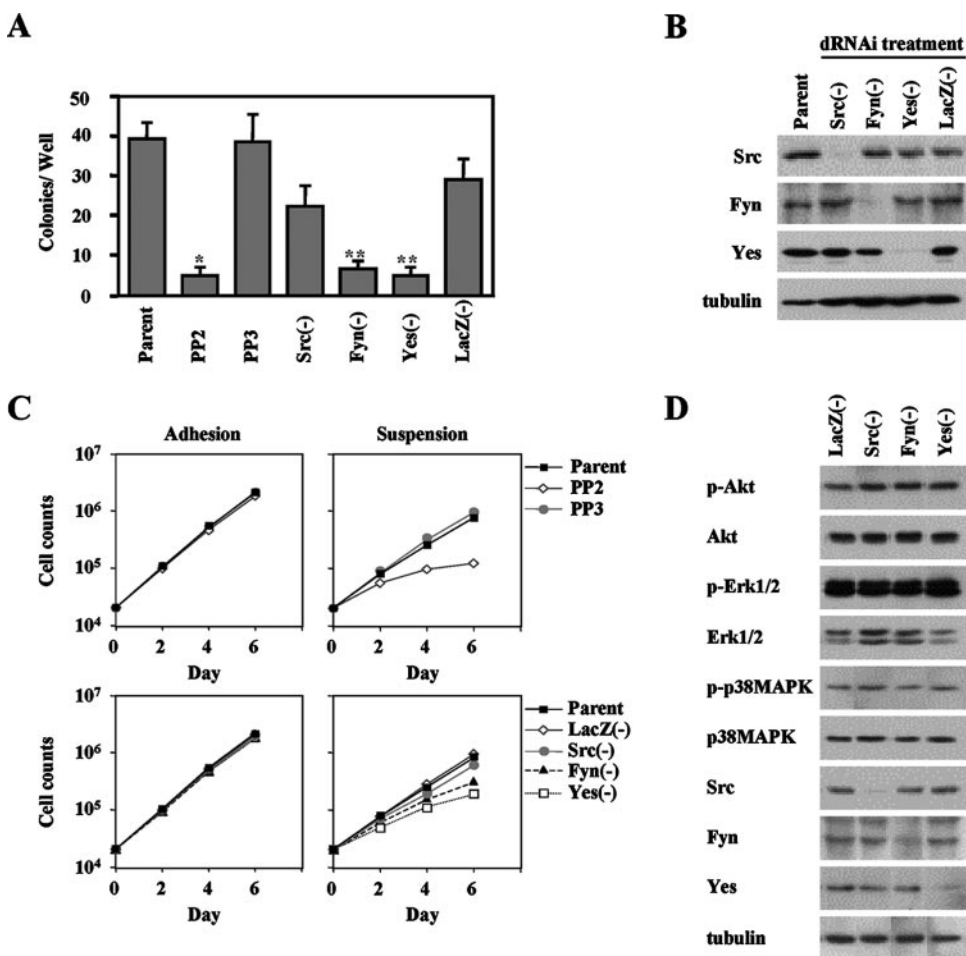


FIG. 1. Anchorage independence of lung adenocarcinoma cells requires SFK. (A) The effect of SFKs on anchorage independence was determined by soft-agar assay. A549 cells were treated with the SFK inhibitor PP2 (10  $\mu$ M) and SFK dicer RNAi [bars Src(-), Fyn(-), and Yes(-)], and controls [bars Parent, PP3, and LacZ(-)] were seeded onto each soft-agar plate ( $3 \times 10^3$  cells). Colonies equal to and larger than 0.5 mm in diameter were counted after 30 days. The error bars represent standard deviations, and the asterisk indicates statistically significant differences ( $P < 0.01$ ) between the parent and PP2 treatment cells, while the double asterisk indicates statistically significant differences ( $P < 0.01$ ) between LacZ(-) and each of the SFK RNAi treatment cells. (B) A549 cells transiently transfected with c-Src, Fyn, c-Yes, or LacZ dicer RNAi (dRNAi) were incubated for 48 h in culture plates. The cells were lysed and subjected to immunoblotting with the indicated antibodies. (C) Cell growth in A549 cells was subjected to a determination of the number of cells, as described for panel A. Approximately  $2 \times 10^4$  cells were seeded onto normal (Adhesion) or MPC-coated (Suspension) culture plates with medium. The growth medium was changed every 2 days. The total cell number on each plate was determined every 2 days by a Coulter particle counter z1 (Beckman). (D) SFKs did not affect the phosphorylation of Akt, Erk1/2, or p38MAPK. The lysate of suspended A549 cells transiently transfected with dicer RNAi for each of the SFKs was prepared and subjected to immunoblotting with the indicated antibodies.

6 to 8 weeks old. A549 clones generated by the miR RNAi system and H322 cells were evaluated by experimental metastasis assay, as described by Fidler et al. (8). Briefly, cells ( $5 \times 10^5$  cells/0.2 ml of medium without serum;  $n = 6$ ) were injected into the tail veins of mice. The mice were sacrificed 100 days after cell inoculation for the counting of metastatic nodules. The numbers of lung metastases and nodule formations were determined.

To determine the effect of CDCP1 on tumor growth in nude mice, A549 clones ( $3 \times 10^6$  cells/0.2 ml of medium without serum) were subcutaneously injected into the right flanks of mice. The mice were killed at 30 days. The results are expressed as the mean weight of tumors from three mice  $\pm$  standard error.

## RESULTS

**The anchorage independence of lung cancer cells involves SFKs.** We first examined the involvement of SFKs in the anchorage independence of A549 lung adenocarcinoma cells using the colony formation assay on soft agar with or

without PP2, an SFK inhibitor (Fig. 1A). A549 cells and cells treated with PP3, an inactive derivative of PP2, formed a similar number of colonies in soft agar, while the addition of PP2 caused a significant decrease in the numbers of colonies. A similar effect of PP2 was observed in most lung cancer cells, such as the PC3, PC14, H520, and LK2 cell lines (data not shown).

To determine which member of the SFKs mainly contributes to the anchorage independence of A549 cells, individual expression of c-Src, Fyn, and c-Yes was downregulated using RNAi technology (Fig. 1B), and colony formation assays were performed on soft agar (Fig. 1A). A549 cells treated with Fyn or c-Yes siRNAs formed significantly fewer colonies than the control cells treated with LacZ siRNA, while cells treated with c-Src siRNA formed slightly fewer colonies. We also observed



a similar suppressive effect of Fyn and c-Yes RNAi in PC14 and H520 lung cancer cells (data not shown).

To further assess the significance of SFKs during the anchorage-independent growth, the effects of PP2 or RNAi for each SFK on cell growth were examined with or without cell attachment using a normal culture dish or MPC-coated dish, respectively. In adherent culture, no significant effect on cell numbers was observed by treatment with PP2 or by any siRNA for the SFKs (Fig. 1C, Adhesion). In suspension culture, however, A549 cells treated with PP2 or with siRNAs for Fyn or c-Yes showed a significant reduction of cell counts compared with untreated cells, while cells treated with c-Src siRNA showed only a slight reduction in cell numbers (Fig. 1C, Suspension). Neither control siRNA nor PP3 had a detectable effect on cell growth in suspension culture.

The phosphatidylinositol (PI) 3-kinase/Akt pathway and the MAPK pathway are the most significant pathways mediating growth factor signals during cell survival and cell proliferation. We therefore examined whether downstream signals of SFKs, which lead to anchorage independence of A549 cells, involve the activation of these two pathways. Suppression of the expression of c-Src, Fyn, or c-Yes by each RNAi had no significant effects on the phosphorylation of Akt and ERK in the suspension culture of A549 cells. These sets of siRNAs also had no effect on the phosphorylation of p38MAPK (Fig. 1D).

Taken together, these data suggest that Fyn and c-Yes are required for anchorage independence in A549 cells, and the cellular signals mediated by these kinases are independent of the Akt, ERK, and p38MAPK pathways.

**Purification of 135-kDa and 70-kDa phosphotyrosine proteins associating with SFKs in suspension culture.** To identify which molecules mediate the SFK signals specific for anchorage independence, we analyzed the phosphotyrosine-containing proteins that bind to SFKs. Among the proteins associated with SFKs, two proteins with molecular masses of 135 kDa and 70 kDa were prominently phosphorylated, even in suspension culture (Fig. 2A), and these proteins were also phosphorylated in PC14 and H520 cell lines, which also exhibit a high level of anchorage independence (Fig. 2A, High). In contrast, the H322 and H157 cell lines, which formed a small number of colonies in soft agar (Fig. 2A, Low), displayed rather lower levels of phosphorylation of these two proteins (Fig. 2A). Using these results, we sought to identify proteins that function as downstream mediators of SFKs in anchorage-independent growth.

As several antibodies against the known phosphoproteins failed to recognize the 135-kDa and 70-kDa proteins, we applied affinity purification. Using A549 cells cultured for 48 h with and without cell attachment, these 135-kDa and 70-kDa proteins were pulled down with the Fyn SH2 domain more efficiently under suspension conditions (Fig. 2B). A total of  $\sim 1.2 \times 10^{10}$  cells in suspension culture were first purified with the Fyn SH2 domain. After purification by a second affinity column using a 4G10 antiphosphotyrosine antibody, samples were analyzed by Western blotting (Fig. 2C, WB) and colloidal gold total-protein stain (Fig. 2C, G). Bands corresponding to the two proteins were cut out and analyzed by mass spectrometry. Four peptides from the 135-kDa band and one peptide from the 70-kDa band were determined by mass spectrometry to be the recently identified membrane protein CDCP1 (Fig.

2C). The proteins at molecular masses of both 135 kDa and 70 kDa in the suspension culture appeared to contain the single protein CDCP1. The 70-kDa protein was estimated to be a cleaved product of 135-kDa CDCP1, as previously reported (5).

**Identification of the major phosphoprotein of the 135-kDa and 70-kDa proteins as CDCP1 and its association with anchorage independence.** Anti-CDCP1 antibody recognized proteins of exactly the same molecular masses as the 135-kDa and 70-kDa proteins both in the whole-cell lysate and in the sample pulled down by the Fyn SH2 domain (Fig. 3A, WCL and PD). CDCP1 was also clearly coimmunoprecipitated with each SFK molecule originally expressed in A549 cells, especially with Fyn and c-Yes, which is consistent with the original 135-kDa protein (Fig. 3A, IP). On the other hand, immunoprecipitation with anti-CDCP1 antibody revealed that CDCP1 was strongly associated with Fyn and c-Yes and very weakly with c-Src (Fig. 3B).

To confirm that the phosphorylation of CDCP1 in each lung cancer cell line is associated with high anchorage independence, we generated a phosphospecific antibody (p-CDCP1 [Tyr734]) against tyrosine 734 of CDCP1, which is reported to be a major phosphorylation site for SFK (1, 5), and analyzed the phosphorylation of CDCP1 in each cell line (Fig. 3C). Prominent phosphorylation of CDCP1 was observed in the A549, PC14, and H520 lung cancer cells with high anchorage independence (Fig. 3C, High), while the H322 and H157 cells, which have low anchorage independence (Fig. 3C, Low), exhibited rather low levels of phosphorylation of CDCP1. From these results, we concluded that the 135-kDa phosphoprotein detected in lung cancer cells is CDCP1, and its phosphorylation status appears to be associated with anchorage independence.

We examined whether the phosphorylation of CDCP1 is altered with or without cell attachment in the culture. After detachment of A549 cells with EDTA, the cells were either plated on a normal dish to cause readhesion or plated on an MPC-coated dish to grow in suspension for 48 h. Under either plating condition, the phosphorylation level of CDCP1 was continuously increased until 24 h; however, it exhibited a sudden decrease at 48 h in adhesion culture, while it increased further in suspension culture (Fig. 3D). Notably, these dynamic changes of phosphorylation during cell suspension and readhesion appear to partially reflect the change in the expression level of CDCP1.

**CDCP1 is a regulator of anoikis resistance in lung adenocarcinoma.** Next, we checked whether CDCP1 is involved in the regulation of the anchorage independence of A549 cells under the control of SFK activity. For this purpose, we obtained the stable A549 cell clones miCDCP1-1 and miCDCP1-2, which showed suppressed expression of the CDCP1 protein, by using the siRNA for CDCP1 with a BLOCK-iT Pol II miR RNAi expression vector system (Fig. 4A). Both of the clones formed significantly fewer colonies in the soft-agar assay than the LacZ clones (Fig. 4B), suggesting that CDCP1 is actually required for the anchorage independence of A549 lung adenocarcinoma.

Anchorage independence may reflect the persistence of growth and/or survival of cancer cells in suspension; therefore, the effects of CDCP1 expression in suspended cells on cell

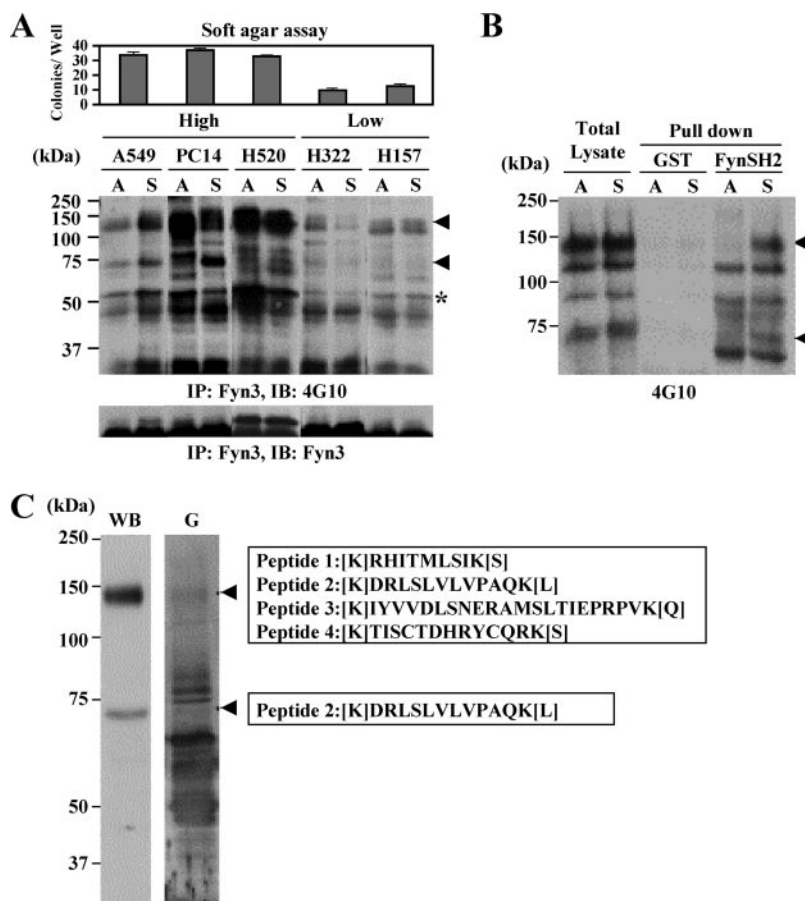


FIG. 2. Purification of phosphotyrosine-containing 135-kDa and 70-kDa protein-forming complexes with SFKs in suspension culture. (A) Anchorage independence in a series of lung cancer cell lines was examined by soft-agar assay (top). The large number of colonies formed in the lung cancer cell lines A549, PC14, and H520 (High) and the small number of colonies formed in the H322 and H157 cell lines (Low) cultured for 48 h under both adhesion and suspension conditions were collected and subjected to immunoprecipitation (IP) with anti-Fyn (Fyn3) antibody and immunoblotting (IB) with antiphosphotyrosine (4G10) antibody. Phosphotyrosine-containing proteins coimmunoprecipitated with Fyn at the molecular masses of 135 kDa and 70 kDa are indicated by arrowheads. The asterisk indicates phosphorylated Fyn. The expression of Fyn in each cell lysate was confirmed by immunoblotting (bottom). A, adhesion; S, suspension. The error bars represent standard deviations. (B) GST-FynSH2 protein generated by *Escherichia coli* was used to pull down the lysate of A549 cells cultured under adhesion or suspension conditions. The isolated samples were immunoblotted with antiphosphotyrosine (4G10) antibody. The arrowheads indicate the phosphotyrosine-containing 135-kDa and 70-kDa proteins. (C) Phosphotyrosine-containing proteins (135 kDa and 70 kDa) were purified according to the protocol described in Materials and Methods. Aliquots of the purified 135-kDa and 70-kDa phosphotyrosine-containing proteins were examined by Western blotting (WB) using antiphosphotyrosine (4G10) antibody, and the remaining samples were stained with colloidal gold total-protein stain (G). Four peptides determined by mass spectrometry (peptides 1 to 4) were identified within the sequence of CDCP1.

proliferation and on cell apoptosis were individually examined. Each miCDCP1 clone in suspension culture showed an increased level of apoptosis compared with miLacZ clones (Fig. 4C). In contrast, no significant change in the cell growth level was observed in each of the miCDCP1 and miLacZ clones compared with the parental A549 cells in suspension culture (Fig. 4D). Importantly, there was no significant change in either cell growth or apoptosis in the adhesion culture (Fig. 4C and D).

We also examined the effect of the expression of phosphorylated CDCP1 on cell proliferation and cell apoptosis using H322 lung adenocarcinoma cells with low anchorage independence. CDCP1 (WT) and/or Fyn kinase with double HA tags at the C terminus (FynHA) were expressed in H322 cells by retroviral vectors, and the expression was checked by Western blotting (Fig. 5A). Additionally, a CDCP1 mutant lacking a putative SFK-binding site (Y734F) was also expressed

(2). An increased level of phosphorylation of CDCP1 was observed in H322 cells overexpressing both WT and Fyn kinase (Fig. 5A, WT+FynHA), which caused an inhibition of apoptosis in suspension culture (Fig. 5B). These changes were not observed with either Fyn kinase or WT CDCP1 alone. On the other hand, expression of Y734F alone increased the level of apoptosis in suspension culture, suggesting a dominant-negative effect of this CDCP1 mutant (Fig. 5B, Y734F). A slight enhancement of cell proliferation in suspension culture was observed by expressing Fyn kinase and either WT or mutant CDCP1, but this change was not significant (Fig. 5C).

These results suggest that phosphorylation of CDCP1 confers anchorage independence through the inhibition of apoptosis. In other words, phosphorylation of CDCP1 regulates resistance to anoikis in lung cancer cells.

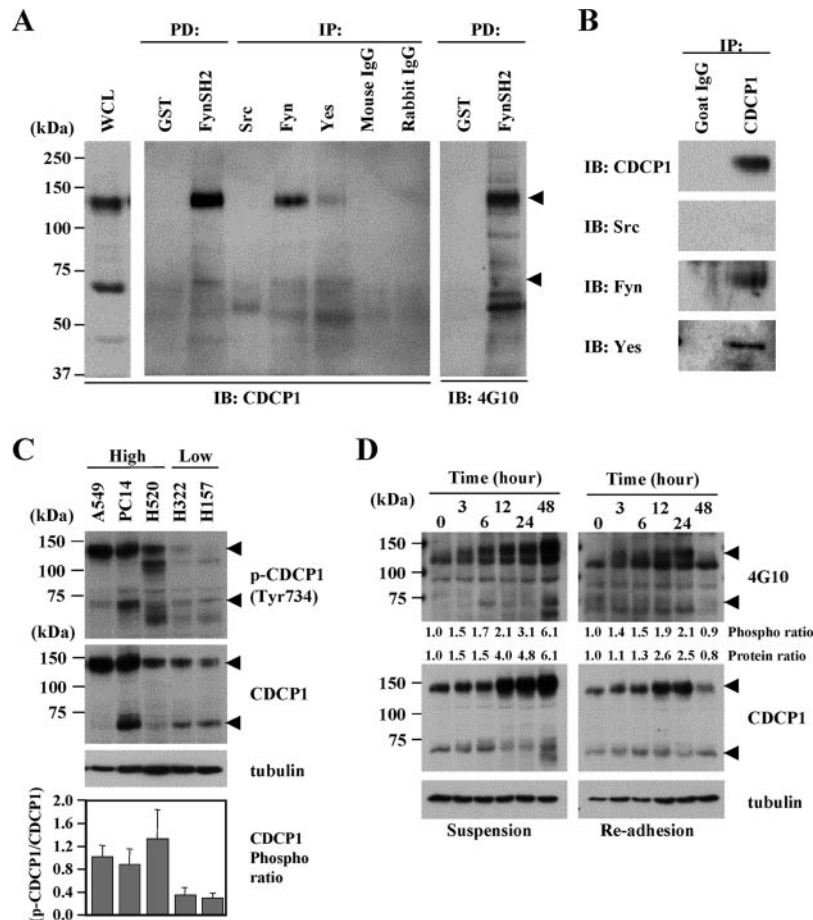


FIG. 3. Identification of the 135-kDa and 70-kDa proteins as CDCP1 and its phosphorylation associated with anchorage independence. (A) The lysate of A549 cells was subjected to whole-cell lysate (WCL) or pull-down assay with GST-FynSH2 protein (PD) or immunoprecipitated with anti-c-Src, anti-Fyn, and anti-c-Yes antibodies (IP) and immunoblotted (IB) with anti-CDCP1 antibody. The same blot was rehybridized with antiphosphotyrosine (4G10) antibody. (B) The lysate of A549 cells was immunoprecipitated with anti-CDCP1 antibody (ab1377) or goat IgG as indicated. The precipitates were subjected to immunoblotting with anti-c-Src, anti-Fyn, anti-c-Yes, and anti-CDCP1 antibodies. (C) The large number of colonies formed by the lung cancer cell lines A549, PC14, and H520 (High) and the small number of colonies formed by the H322 and H157 cell lines (Low) cultured for 48 h in the suspension condition were collected and subjected to immunoblotting with anti-phospho-CDCP1 (Tyr734) and CDCP1 antibodies. This experiment was performed three times. The ratio of the phosphorylation level in each lung adenocarcinoma cell was measured as described in Materials and Methods. The error bars represent standard deviations. (D) Time course analysis of CDCP1 expression and phosphorylation with or without cell attachment. A549 cells were reseeded on normal cell culture plates and an MPC-coated plate at a density of  $1.5 \times 10^5$  cells per plate with complete medium. For the preparation of the reseeded cells, 2 mM EDTA/Hanks' balanced salt solution was used to detach the cells. For each time point, cells were collected and subjected to immunoblotting with the indicated antibody. The same membrane rehybridized with antitubulin antibody confirmed the concentration of total proteins in each lysate (tubulin). The arrowheads indicate CDCP1.

**PKC $\delta$  is a signal molecule downstream of CDCP1 during anoikis resistance in lung adenocarcinoma.** CDCP1 protein has been shown to bind PKC $\delta$  in a phosphorylation-dependent manner (2). PKC $\delta$  is a regulator of apoptosis, and it has been reported that the phosphorylation of PKC $\delta$  requires the activity of SFKs (37). By treatment with the SFK inhibitor PP2, both the association of PKC $\delta$  with CDCP1 and the phosphorylation of PKC $\delta$  at Tyr311 were clearly inhibited (Fig. 6A). CDCP1 (WT) and the CDCP1 protein with a point mutation at Tyr734 (Y734F) were C-terminally FLAG tagged and expressed in COS7 cells. After transfection with each plasmid, the association of the Fyn SH2 domain with WT and Y734F mutants was examined. The Fyn SH2 domain was able to pull down the WT but not the Y734F mutants (Fig. 6B, PD: Fyn SH2). The levels of tyrosine phosphorylation of Y734F mu-

mutants was much lower than that of the WT in A549 cells (Fig. 6B, IB: 4G10), suggesting that Tyr734 of CDCP1 directly binds to Fyn and that the association is essential for the phosphorylation of CDCP1. The association between CDCP1 and PKC $\delta$  was also impaired in the Y734F mutant compared with the WT, indicating that the phosphorylation of CDCP1 is required for the association.

Overexpression of Y734F in A549 cells also blocked the association between PKC $\delta$  and CDCP1 and the phosphorylation of PKC $\delta$  at Tyr311 (Fig. 6B). Moreover, treatment with CDCP1 siRNA also decreased the phosphorylation level of PKC $\delta$  (Fig. 6C). In addition, the phosphorylation level of PKC $\delta$  at Tyr311 was elevated in H322 cells by overexpressing both WT CDCP1 and Fyn kinase but not significantly with either the WT, the Y734F mutant, or Fyn kinase alone (see the

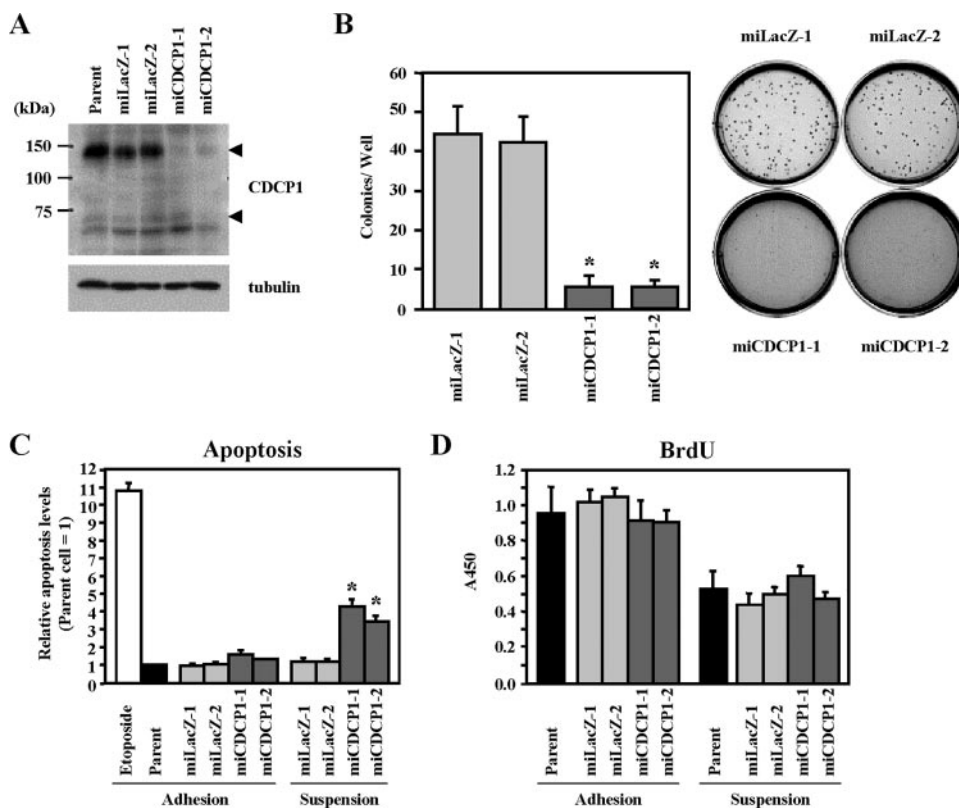


FIG. 4. CDCP1 confers anchorage independence by inhibiting apoptosis in suspended lung adenocarcinoma. (A) CDCP1-defective A549 cell clones (miCDCP1-1 and miCDCP1-2) were generated by an miR RNAi expression vector kit (Invitrogen). miLacZ-1 and miLacZ-2 were control clones. The expression of CDCP1 in each clone ( $1.5 \times 10^5$  cells) cultured for 24 h in an MPC-coated plate was examined by Western blotting using CDCP1 antibody. The concentration of total protein in each clone was confirmed by the same membrane rehybridized with antitubulin antibody (bottom). The arrowheads indicate CDCP1. (B) Each CDCP1-defective clone and control clone was seeded onto soft-agar plates ( $3 \times 10^3$  cells) (right). Colonies equal to and larger than 0.5 mm in diameter were counted after 30 days. The error bars represent standard deviations, and the asterisks indicate statistically significant differences ( $P < 0.01$ ) (left). (C) CDCP1-defective A549 cell clones (miCDCP1-1 and -2) and control miLacZ clones ( $1.0 \times 10^4$  cells) were cultured in normal and MPC-coated 96-well plates. After 24 h, the cells were lysed and apoptosis was examined using a cell death ELISA kit (Roche). The total apoptotic level of A549 cells was examined by treatment with etoposide (25  $\mu$ M). The relative apoptosis levels are shown as the levels of apoptosis in each clone compared with those of parental cells. In suspension culture, miCDCP1 clones exhibited an increased level of apoptosis compared with that of miLacZ clones. The error bars represent standard deviations, and the asterisks indicate statistically significant differences ( $P < 0.01$ ). (D) Cell proliferation was determined with a cell proliferation ELISA BrdU kit (Roche). Each clone ( $1.0 \times 10^4$  cells) was cultured on normal and MPC-coated 96-well plates. No significant change in cell proliferation was observed in the miCDCP1 or in miLacZ clones compared with parental A549 cells with or without cell attachment. The error bars represent standard deviations.

supplemental material). Therefore, CDCP1 might be required for the phosphorylation of PKC $\delta$  by linking PKC $\delta$  to SFKs in a phosphorylation-dependent manner.

To check whether PKC $\delta$  can regulate anoikis in lung adenocarcinoma cells, cell apoptosis caused by the suspension of miCDCP1 and miLacZ clones was examined with or without PKC $\delta$  RNAi. As shown in Fig. 6D, PKC $\delta$  RNAi increased the level of apoptosis in the control A549 cells (miLacZ) to a degree similar to that achieved by the suppression of CDCP1 expression (miCDCP1); however, no additive effect on cell apoptosis was observed by the suppression of both CDCP1 and PKC $\delta$ . Similar results were obtained from two other independent sets of siRNAs for PKC $\delta$  (data not shown). Moreover, treatment with the PKC inhibitor Rottlerin increased the level of apoptosis compared with the parental A549 cells (Fig. 6E). We also examined whether the blocking of the CDCP1-PKC $\delta$  signal pathway affects anoikis resistance in A549 cells by overexpressing the C2 domain of PKC $\delta$ , which has been shown to

be responsible for the association with tyrosine-phosphorylated CDCP1 (2). The HA-tagged C2 domain of PKC $\delta$  (C2HA) expressed in A549 cells was actually associated with phosphorylated CDCP1 (Fig. 6F, upper panel) and suppressed the tyrosine phosphorylation levels of PKC $\delta$  (Fig. 6F, bottom). At the same time, overexpression of C2HA resulted in a significant increase in the level of apoptosis in suspension culture compared with a mock-transfected control, while it had no significant effect on adherent culture (Fig. 6G).

These results suggest that the CDCP1-SFK complex is required for the phosphorylation of PKC $\delta$  under suspension conditions and that PKC $\delta$  is a signal molecule for regulating anoikis resistance downstream of CDCP1 signaling.

**CDCP1 affects the metastatic potential of A549 lung adenocarcinoma in vivo.** Anchorage independence is thought to be an important characteristic of cancer cells that acquire metastatic potential. In order to determine the effect of CDCP1 for in vivo metastasis, miCDCP1 and miLacZ cells were injected



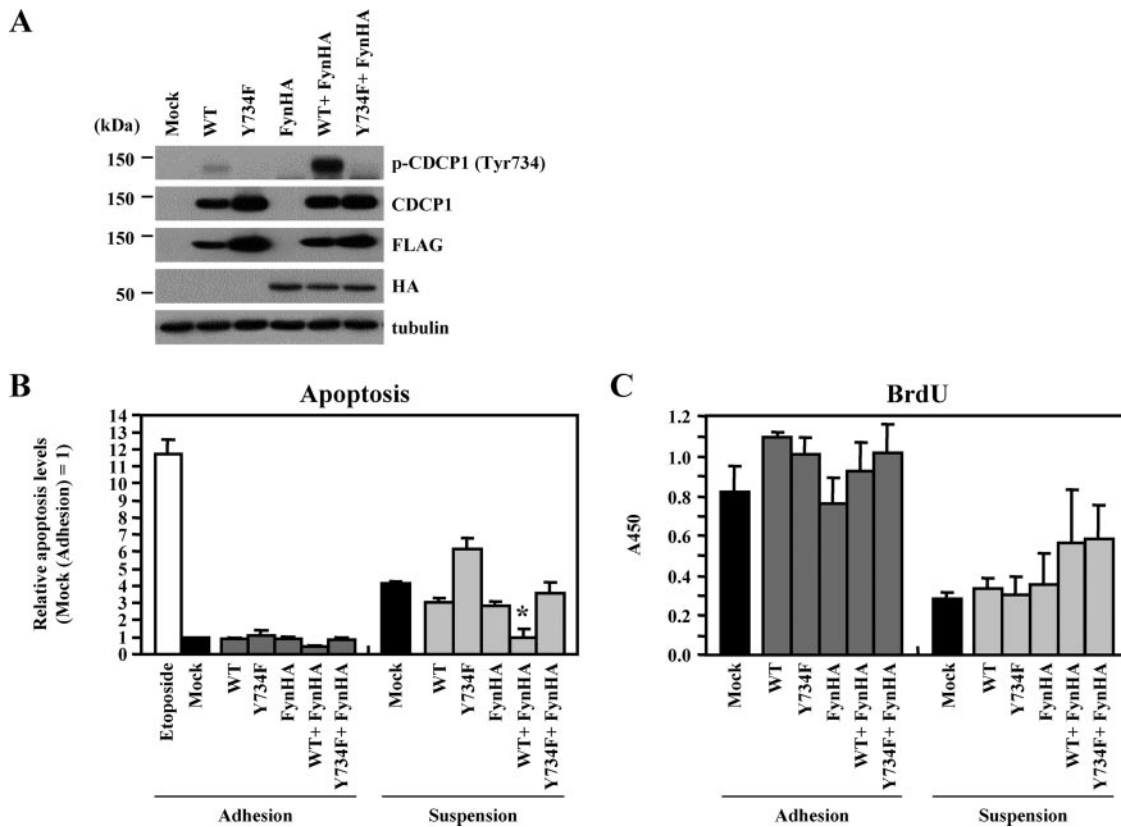


FIG. 5. Anoikis resistance was recovered by phosphorylated CDCP1 in H322 cells with low anchorage independence. (A) H322 cells that overexpressed CDCP1 (WT), a CDCP1 mutant (Y734F), and/or Fyn kinase tagged with HA (FynHA) was incubated for 24 h in MPC-coated plates. The cells were lysed and subjected to immunoblotting with the indicated antibodies. (B) Cells, as indicated ( $1.0 \times 10^4$  cells), were cultured in normal and MPC-coated 96-well plates. After 24 h, the cells were lysed and apoptosis was examined using a cell death ELISA kit (Roche). The total apoptotic level of mock-infected cells was examined by treatment with etoposide ( $25 \mu\text{M}$ ). The relative apoptosis levels are shown as the levels of apoptosis in each of the cells compared with mock-infected cells in adhesion culture. The error bars represent standard deviations, and the asterisk indicates a statistically significant difference ( $P < 0.05$ ) between mock-transfected cells and other cells in suspension culture. (C) Cell proliferation was determined with a cell proliferation ELISA BrdU kit (Roche). Each of the cells ( $1.0 \times 10^4$  cells) was cultured on normal and MPC-coated 96-well plates. No significant change in cell proliferation was observed in each of the cells compared with mock-infected cells with or without cell attachment (BrdU). The error bars represent standard deviations.

into the tail veins of mice and raised for 100 days. The metastatic capacity was assessed from the number of metastatic cell nodules in mouse lungs. The frequency and number of the metastatic nodules observed in the lungs of each miCDCP1 clone were much less than those found in A549 miLacZ (Fig. 7B). Additionally, H322 cells that belong to the group with low anchorage independence displayed metastasis in only one out of six mice. The average of each of the metastatic nodules and the results of metastasis for each mouse are shown in Table 1. Interestingly, no significant change in tumor growth in nude mice was observed in the miCDCP1-1 clone compared with the A549 miLacZ-1 clone (Fig. 7A). Since the metastatic assay mimics only the middle and late processes of metastasis, these results indicate that CDCP1 affects the later process in the metastasis of lung adenocarcinoma in vivo, possibly through the regulation of anchorage independence.

## DISCUSSION

This study has identified CDCP1 as a crucial regulatory molecule of anoikis resistance in lung cancer cells. The signal

mediated by the CDCP1-SFK complex appears to play the principal role in overcoming anoikis. CDCP1 has previously been identified as a novel epithelial tumor antigen (28) and as a tumor-associated protein preferentially expressed by highly metastatic epidermoid carcinoma (15), although little is known about the function of CDCP1 in tumor cells. Some putative functions have been suggested, such as the hypothesis that CDCP1 is a mitotic substrate of SFKs under cell cycle regulation in MDA-468 breast cancer cells (3). In this study, we found a distinct novel function of CDCP1 in tumor cells that occurs through phosphorylation by SFKs.

We found that the disruption of CDCP1 expression in A549 cells resulted in defective colony formation in soft agar, suggesting that CDCP1 affects anchorage independence (Fig. 4B). Anchorage independence is an outstanding characteristic of tumor cells, which confers the ability to grow without attachment to the extracellular matrix. Anchorage independence may come from either persistent cell growth or resistance to apoptosis in a suspension condition. As found in this study, CDCP1 does not significantly affect cell growth. A key finding here is that the loss of CDCP1 induces the apoptosis of lung



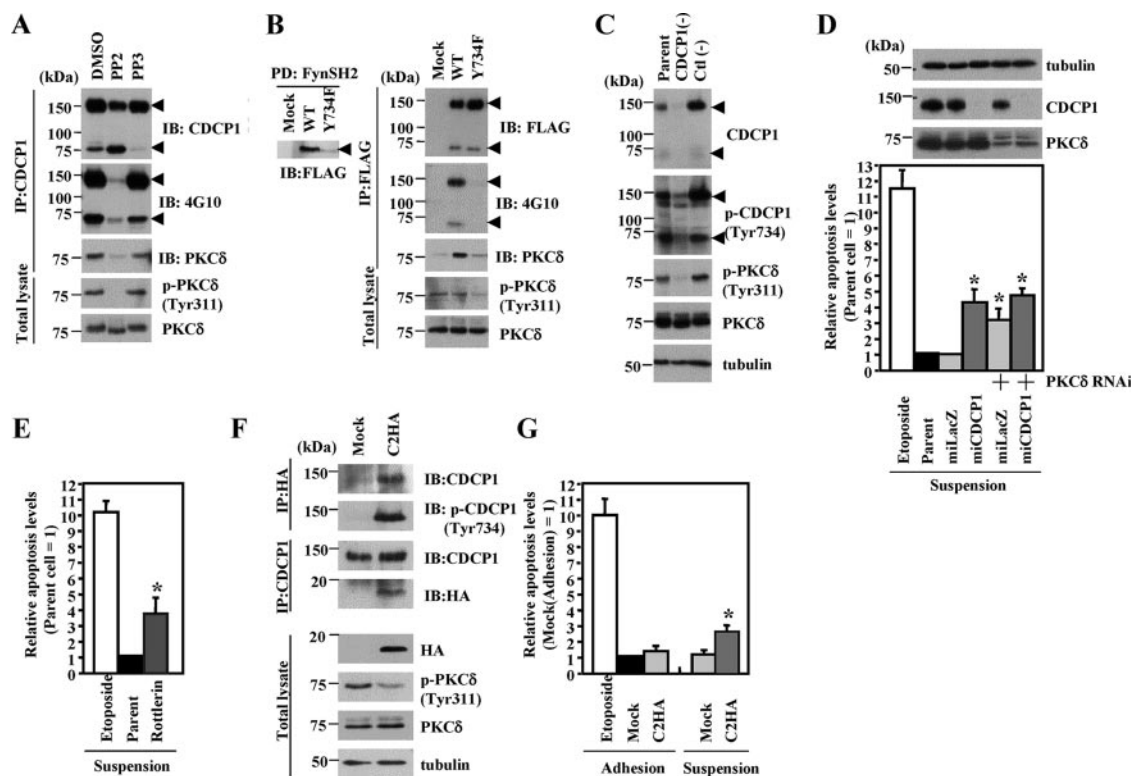


FIG. 6. PKC $\delta$  is a signaling molecule downstream of CDCP1 during anoikis resistance. (A) Treatment with the SFK inhibitor PP2 blocked the physical association between PKC $\delta$  and CDCP1 and at the same time suppressed phosphorylation of PKC $\delta$  at Tyr311. A549 cells treated with 10  $\mu$ M of PP2 and 10  $\mu$ M of PP3 in suspension culture were collected and subjected to immunoprecipitation with anti-CDCP1 antibody (ab1377) and immunoblotting (IB) with the indicated antibodies. The phospho-specific antibody against PKC $\delta$  (p-PKC $\delta$  [Tyr311]) total cell lysate was used to detect the phosphorylation of PKC $\delta$ , and the expression of PKC $\delta$  was also confirmed. (B) CDCP1 mutants were expressed in COS7 cells and pulled down (PD) with GST-FynSH2 protein. The samples pulled down were immunoblotted with FLAGM2 antibody (left). CDCP1 mutants were transiently transfected in A549 cells. After 24 h, cells were collected and subjected to immunoprecipitation (IP) with anti-FLAGM2 antibody. The immunoprecipitates were subjected to immunoblotting with the indicated antibodies. Each total cell lysate was used to detect the phosphorylation and the expression of PKC $\delta$ . (C) A549 cells treated with CDCP1 stealth siRNA and control siRNA were collected and subjected to immunoblotting with the indicated antibodies. (D) The effect of PKC $\delta$  on apoptosis was determined by apoptosis assay. PKC $\delta$  stealth siRNA was transiently transfected into CDCP1-defective A549 cell clones and control miLacZ clones. After 48 h, each cell clone ( $1.0 \times 10^4$  cells) was reseeded onto MPC-coated 96-well plates and cultured for 24 h. The cells were lysed and examined for apoptosis using a cell death ELISA kit (Roche). The total apoptotic level of A549 cells was examined by treatment with etoposide (25  $\mu$ M). The relative apoptosis levels are shown as the level of apoptosis compared with the parent cells. The error bars represent standard deviations, and the asterisks indicate statistically significant differences ( $P < 0.01$ ) between the parent and each of the other cells. Expression of CDCP1 and PKC $\delta$  was determined by Western blotting with the indicated antibodies (top). (E) The effect of PKC $\delta$  activation on apoptosis was determined by apoptosis assay. A549 cells ( $1.0 \times 10^4$  cells) were seeded onto MPC-coated 96-well plates and treated or not with Rottlerin (5  $\mu$ M). The relative apoptosis levels after culture for 24 h are shown as the level of apoptosis compared with parent cells. The error bars represent standard deviations, and the asterisk indicates a statistically significant difference ( $P < 0.01$ ) between the parent and Rottlerin-treated cells. (F) The C2 domain of PKC $\delta$  with the HA tag (C2HA) was expressed in A549 cells. After 24 h, cells were collected and subjected to immunoprecipitation with anti-CDCP1 (ab1377) or anti-HA antibody. Immunoprecipitates were subjected to immunoblotting with the indicated antibodies. Total cell lysate was used to detect the expression of C2HA and the phosphorylation level of endogenous PKC $\delta$  in A549 cells. (G) The cells transiently transfected with C2HA or mock vector, as indicated ( $1.0 \times 10^4$  cells), were cultured in normal and MPC-coated 96-well plates. After 24 h, the cells were lysed and apoptosis was examined using a cell death ELISA kit (Roche). The relative apoptosis levels are shown as the level of apoptosis in each of the cells compared with the control mock cells in adhesion culture. The error bars represent standard deviations, and the asterisk indicates a statistically significant difference ( $P < 0.05$ ) between the mock cells and each of the other cells in suspension culture.

adenocarcinoma cells in a suspended condition but not in an adherent condition (Fig. 4C). This phenomenon strongly suggests that CDCP1 is involved in the suppression of anoikis, a form of apoptosis triggered by disruption of cell-matrix interactions.

The molecules and signaling pathways in the anoikis resistance of human cancer cells are not sufficiently understood. Previous reports have shown that oncogenes encoding, e.g., Ras, Src, and their downstream signaling molecules, such as PI 3-kinase/Akt and MAPK, are critical players in compensating

for the cell survival signals derived from matrix attachment via integrins (9, 16). Inhibition of PI 3-kinase/Akt and Erk1/2 does not induce apoptosis in lung cancer cells, while SFK inhibitor causes apoptosis in these cells (32, 33). This study has revealed that the inhibition of SFKs blocked anchorage independence in lung cancer cells without affecting the phosphorylation state of PI 3-kinase/Akt, Erk1/2, or p38MAPK (Fig. 1D). These results suggest that SFKs are critical regulators of anoikis in cancer cells. On the other hand, the inhibition of SFKs was effected independently of the PI 3-kinase/Akt pathway.

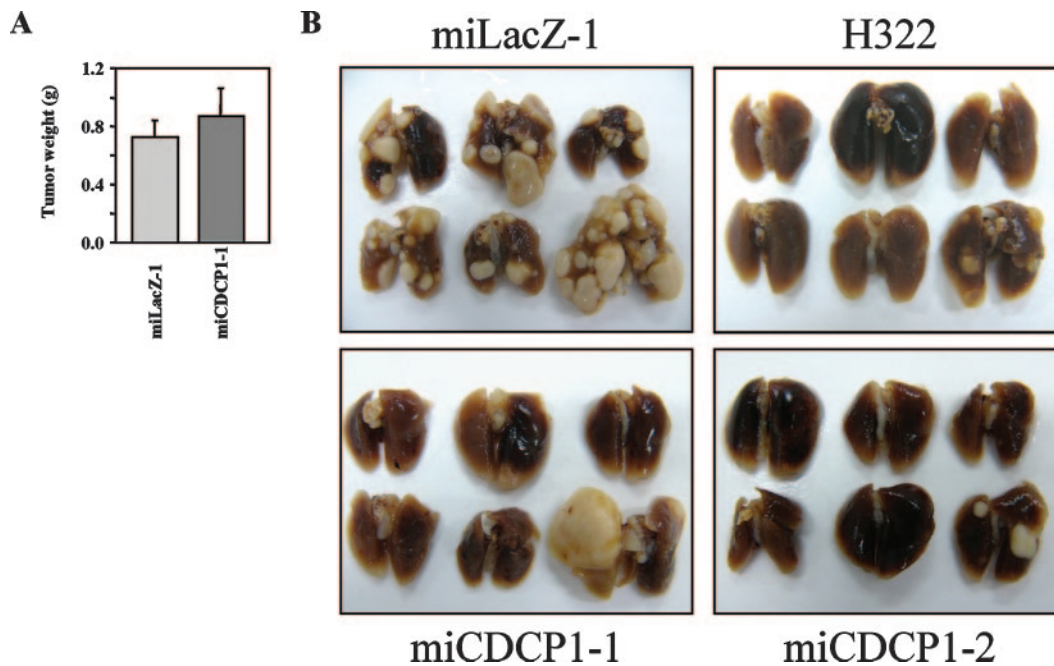


FIG. 7. Metastatic capacity of CDCP1-defective lung adenocarcinoma cells. (A) The effect of CDCP1 on tumor growth in nude mice was determined as described in Materials and Methods. The data represent the weights of tumors from the miCDCP1-1 clone or the miLacZ-1 clone ( $n = 3$ ). The error bars indicate standard deviations. (B) The metastatic potential was evaluated from the number of metastatic cell nodules in mouse lungs after injection of tumor cells from the tail vein ( $n = 6$ ). Lung tissues were fixed with 10% formaldehyde solution. Many metastatic nodules were observed in the control A549 miLacZ-1 clone, while fewer nodules were observed in the miCDCP1-1 and miCDCP1-2 clones and H322 cells. The number of mice with obvious lung metastasis and the average number of metastatic nodules per mouse for each cell clone are shown in Table 1.

CDCP1 is a potent substrate of SFKs within cells, and its function is likely modulated by phosphorylation of the tyrosine residues in the cytoplasmic domain (2, 3, 5). In our study, the SFK inhibitor PP2 inhibited phosphorylation of CDCP1, and at the same time, soft-agar colony formation of A549 cells was also inhibited (Fig. 1A, PP2). In fact, the level of tyrosine phosphorylation of CDCP1 is associated with the capacity for anchorage independence in lung cancer cells (Fig. 3C). Together with the observation that apoptosis of H322 cells in suspension culture was inhibited by overexpression of CDCP1 and Fyn kinase together but not CDCP1 or Fyn kinase alone, or by the Y734F mutant of CDCP1, this suggested that active SFKs confer anoikis resistance through tyrosine phosphorylation of CDCP1.

Among the SFKs, the expression of c-Src, Fyn, and c-Yes is

TABLE 1. Effects of CDCP1 downregulation on lung cancer metastasis in vivo<sup>a</sup>

Cells	Metastasis <sup>b</sup>	No. of nodules in lung <sup>c</sup>
A549 miLacZ	6/6	12.8
H322	1/6	1.3
A549 miCDCP1-1	1/6	0.2
A549 miCDCP1-2	1/6	0.5

<sup>a</sup> Mice were sacrificed 100 days after inoculation.

<sup>b</sup> Data are shown as the number of mice bearing tumors in the lung/total number of mice.

<sup>c</sup> Average number of metastatic tumor nodules larger than 2 mm in the lung per mouse.

commonly observed in human solid tumors (31). In this study, we detected the expression of c-Src, Fyn, and c-Yes in the suspension culture of A549 cells (Fig. 1B, Parent). Among these kinases, Fyn and c-Yes may regulate CDCP1-mediated cell survival in A549 cells, since these kinases are associated with CDCP1 (Fig. 3A and B), and downregulation of Fyn or c-Yes inhibits soft-agar colony formation in A549 cells (Fig. 1A). On the other hand, the amount of phosphorylated CDCP1 was either partially or remarkably reduced by Fyn or c-Yes dicer siRNA, respectively (data not shown), supporting the claim that these two members of the SFKs have a considerable effect on the phosphorylation of CDCP1. A dynamic balance of active SFK and protein tyrosine phosphatase activities regulates the phosphorylation of CDCP1 during cell attachment (5). This balance may shift when integrin signaling is shut off by cell detachment. As shown in Fig. 3D, dynamic changes in the amount of tyrosine-phosphorylated CDCP1 were also caused by changes in the expression level of CDCP1, although it is not yet clearly understood how the expression of CDCP1 is regulated by the cell detachment/attachment signal.

Benes et al. (2) recently reported that the C2 domain of PKC $\delta$  associates with phosphorylated CDCP1. Several studies have also reported on the phosphorylation of PKC $\delta$  by SFKs (19, 30), but the regulatory mechanism of PKC $\delta$  phosphorylation remains unclear. Our study found that PKC $\delta$  was remarkably phosphorylated in suspended A549 cells and also confirmed a physical association through the regulation of the phosphorylation state of CDCP1 in A549 lung adenocarcinoma cells (Fig. 6A, B, and C). Both the expression of CDCP1

and the association of CDCP1 with SFKs are required for the phosphorylation of PKC $\delta$ , which suggests that CDCP1 mediates the phosphorylation of PKC $\delta$  by SFKs. We found that an increased level of apoptosis was observed with the treatment of siRNA for PKC $\delta$  or with the PKC inhibitor Rottlerin in A549 cells in a suspension condition (Fig. 6D and E). Moreover, inhibition of the association between CDCP1 and PKC $\delta$ , by expressing the C2 domain of PKC $\delta$ , suppressed the tyrosine phosphorylation of PKC $\delta$  and increased the level of apoptosis in A549 cells in a suspension condition at the same time (Fig. 6F and G). It is speculated that CDCP1-mediated tyrosine phosphorylation and the activation of PKC $\delta$  lead to the suppression of apoptosis in A549 cells.

Tyrosine phosphorylation of PKC $\delta$  is a critical regulatory factor for PKC $\delta$  activity and results in the elevation of both tyrosine phosphorylation and the activity of PKC $\delta$  in various cells stimulated with substances such as phorbol esters, growth factors, and hormones (21, 22, 23, 27, 29). It was also reported that tyrosine phosphorylation of PKC $\delta$  by Src actually increased PKC $\delta$  activity (1, 11). On the other hand, several reports have shown that active PKC $\delta$  possesses an antiapoptotic function. For example, the activation of PKC $\delta$  by fibroblast growth factor has an antiapoptotic effect in PC12 cells (34) and a reduction of PKC $\delta$  activity by using a kinase-dead mutant of PKC $\delta$  induced apoptosis in lung cancer cells (7). Further evidence that supports PKC $\delta$  as a suppressor of apoptosis includes the requirement for active PKC $\delta$  during cell transformation mediated by insulin-like growth factor I receptor (23) and the induction of anchorage-independent growth and increased metastatic potential of breast cancer cells overexpressing PKC $\delta$  (17, 18). Our observation that tyrosine-phosphorylated PKC $\delta$  serves an antiapoptotic function in lung cancer cells supports these reports, although it appears that PKC $\delta$  has both proapoptotic and antiapoptotic functions, which are dependent on the specific circumstances and modes of action (4).

Taken together, it is strongly suggested that CDCP1 is a docking protein between SFKs and PKC $\delta$  and that CDCP1-SFK complex-dependent PKC $\delta$  phosphorylation plays a significant role in the control of anoikis resistance in lung adenocarcinoma cells. Further study is required to identify the signal downstream of tyrosine-phosphorylated PKC $\delta$ .

Finally, this study suggests that CDCP1 is a novel regulator of anoikis resistance under the control of SFKs in lung adenocarcinoma cells and that PKC $\delta$ , which is associated with and conditionally phosphorylated by the CDCP1-SFK complex, is a good candidate as a signal mediator of anoikis resistance. It was found that CDCP1 is essential *in vivo* for lung cancer metastasis in the mouse model (Fig. 7), indicating that CDCP1 is actually a modulator of the later processes of cancer metastasis through the regulation of anoikis. Further investigation of the specific functions of CDCP1 in normal cells and its disorders in cancer may yield important information that will help determine a clinical target for lung cancer metastasis.

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#### REFERENCES

1. Benes, C., and S. P. Soltoff. 2001. Modulation of PKC $\delta$  tyrosine phosphorylation and activity in salivary and PC-12 cells by Src kinases. *Am. J. Physiol. Cell Physiol.* **280**:C1498–C1510.
2. Benes, C. H., N. Wu, A. H. Elia, T. Dharia, L. C. Cantley, and S. P. Soltoff. 2005. The C2 domain of PKC $\delta$  is a phosphotyrosine binding domain. *Cell* **121**:271–280.
3. Bhatt, A. S., H. Erdjument-Bromage, P. Tempst, C. S. Craik, and M. M. Moasser. 2005. Adhesion signaling by a novel mitotic substrate of Src kinases. *Oncogene* **24**:5333–5343.
4. Brodie, C., and P. M. Blumberg. 2003. Regulation of cell apoptosis by protein kinase C  $\delta$ . *Apoptosis* **8**:19–27.
5. Brown, T. A., T. M. Yang, T. Zaitsevskaja, Y. Xia, C. A. Dunn, R. O. Sigle, B. Kundsen, and W. G. Carter. 2004. Adhesion or plasmin regulates tyrosine phosphorylation of a novel membrane glycoprotein p80/gp140/CUB domain-containing protein 1 in epithelia. *J. Biol. Chem.* **279**:14772–14783.
6. Buhring, H. J., S. Kuci, T. Conze, G. Rathke, K. Bartolovic, F. Grunebach, M. Scherl-Mostageer, T. H. Brummendorf, N. Schweifer, and R. Lammers. 2004. CDCP1 identifies a broad spectrum of normal and malignant stem/progenitor cell subsets of hematopoietic and nonhematopoietic origin. *Stem Cells* **22**:334–343.
7. Clark, A. S., K. A. West, P. M. Blumberg, and P. A. Dennis. 2003. Altered protein kinase C (PKC) isoforms in non-small cell lung cancer cells: PKC $\delta$  promotes cellular survival and chemotherapeutic resistance. *Cancer Res.* **63**:780–786.
8. Fidler, I. J., E. Gruys, M. A. Cifone, Z. Banes, and C. Bucana. 1981. Demonstration of multiple phenotypic diversity in a murine melanoma of recent origin. *J. Natl. Cancer Inst.* **67**:947–956.
9. Frisch, S. M., and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124**:619–626.
10. Frisch, S. M., and R. A. Screaton. 2001. Anoikis mechanisms. *Curr. Opin. Cell Biol.* **13**:555–562.
11. Gschwendt, M., K. Kielbassa, W. Kittstein, and F. Marks. 1994. Tyrosine phosphorylation and stimulation of protein kinase C  $\delta$  from porcine spleen by src *in vitro*. Dependence on the activated state of protein kinase C  $\delta$ . *FEBS Lett.* **347**:85–89.
12. Hanahan, D., and R. A. Weiberg. 2000. The hallmarks of cancer. *Cell* **100**:57–70.
13. Hisano, C., R. Tanaka, H. Fujishima, H. Ariyama, T. Tsuchiya, T. Tatsumoto, K. Mitsugi, M. Nakamura, and S. Nakano. 2003. Suppression of anoikis by v-Src but not by activated c-H-ras in human gallbladder epithelial cells. *Cell Biol. Int.* **27**:415–421.
14. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-direct mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51–59.
15. Hooper, J. D., A. Zijlstra, R. T. Aimes, H. Liang, G. F. Claassen, D. Tarin, J. E. Testa, and J. P. Quigley. 2003. Subtractive immunization using highly metastatic human tumor cells identifies SIMA135/CDCP1, a 135 kDa cell surface phosphorylated glycoprotein antigen. *Oncogene* **22**:1783–1794.
16. Khwaja, A., P. Rodriguez-Viciana, S. Wennstrom, P. H. Warne, and J. Downward. 1997. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* **16**:2783–2793.
17. Kiley, S. C., K. J. Clark, S. K. Duddy, R. D. Welch, and S. Jaken. 1999. Increased protein kinase C  $\delta$  in mammary tumor cell: relationship to transformation and metastatic progression. *Oncogene* **18**:6748–6757.
18. Kiley, S. C., K. J. Clark, M. Goodnough, D. R. Welch, and S. Jaken. 1999. Protein kinase C  $\delta$  involvement in mammary tumor cell metastasis. *Cancer Res.* **59**:3230–3238.
19. Kronfeld, I., G. Kazimirsky, P. S. Lorenzo, S. H. Garfield, P. M. Blumberg, and C. Brodie. 2000. Phosphorylation of PKC $\delta$  on distinct tyrosine residues regulates specific cellular functions. *J. Biol. Chem.* **275**:35491–35498.
20. Kyo, S., M. Nakamura, T. Kiyono, Y. Maida, T. Kanaya, M. Tanaka, N. Yatabe, and M. Inoue. 2003. Successful immortalization of endometrial glandular cells with normal structural and functional characteristics. *Am. J. Pathol.* **163**:2259–2269.
21. Li, W., H. Mischak, J. C. Yu, L. M. Wang, J. F. Mushinski, M. A. Heidarani, and J. H. Pierce. 1994. Tyrosine phosphorylation of protein kinase C-delta in response to its activation. *J. Biol. Chem.* **269**:2349–2352.
22. Li, W., J. C. Yu, P. Michieli, J. F. Beeler, N. Ellmore, M. A. Heidarani, and J. H. Pierce. 1994. Stimulation of the platelet-derived growth factor beta receptor signaling pathway activates protein kinase C-delta. *Mol. Cell. Biol.* **14**:6727–6735.
23. Li, W., Y. X. Jiang, J. Zhang, L. Soon, L. Flechner, V. Kapoor, J. H. Pierce,

- and L. H. Wang. 1998. Protein kinase C- $\delta$  is an important signaling molecule in insulin-like growth factor I receptor-mediated cell transformation. *Mol. Cell. Biol.* **18**:5888–5898.
24. Michel, J. B. 2003. Anoikis in the cardiovascular system: known and unknown extracellular mediators. *Arterioscler. Thromb. Vasc. Biol.* **23**:2146–2154.
25. Naviaux, R. K., E. Costanzi, M. Haas, and I. M. Verma. 1996. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J. Virol.* **70**:5701–5705.
26. Payford, M. P., and M. D. Schaller. 2004. The interplay between Src and integrins in normal and tumor biology. *Oncogene* **23**:7928–7946.
27. Popoff, I. J., and J. P. Dean. 1999. Activation and tyrosine phosphorylation of protein kinase C delta in response to B cell antigen receptor stimulation. *Mol. Immunol.* **36**:1005–1016.
28. Scherl-Mostageer, M., W. Sommergruber, R. Abseher, R. Hauptmann, P. Ambros, and N. Schweifer. 2001. Identification of a novel gene, CDCP1, overexpressed in human colorectal cancer. *Oncogene* **20**:4402–4408.
29. Soltoff, S. P., and A. Toker. 1995. Carbachol, substance P, and phorbol ester promote the tyrosine phosphorylation of protein kinase C  $\delta$  in salivary gland epithelial cells. *J. Biol. Chem.* **270**:13490–13495.
30. Song, J. S., P. G. Swann, Z. Szallasi, U. Blank, P. M. Blumberg, and J. Rivera. 1998. Tyrosine phosphorylation-dependent and -independent associations of protein kinase C- $\delta$  with Src family kinase in the RBL-2H3 mast cell line: regulation of Src family kinase activity by protein kinase C- $\delta$ . *Oncogene* **16**:3357–3368.
31. Summy, J. M., and G. E. Gallick. 2003. Src family kinases in tumor progression and metastasis. *Cancer Metastasis Rev.* **22**:337–358.
32. Wei, L., Y. Yang, and Q. Yu. 2001. Tyrosine kinase-dependent, phosphatidylinositol 3'-kinase, and mitogen-activated protein kinase-independent signaling pathways prevent lung adenocarcinoma cells from anoikis. *Cancer Res.* **61**:2439–2444.
33. Wei, L., Y. Yang, X. Zhang, and Q. Yu. 2004. Altered regulation of Src upon cell detachment protects human lung adenocarcinoma cells from anoikis. *Oncogene* **23**:9052–9061.
34. Wert, M. M., and H. C. Palfrey. 2000. Divergence in the anti-apoptotic signaling pathways used by nerve growth factor and basic fibroblast growth factor (bFGF) in PC12 cells: rescue by bFGF involves protein kinase C $\delta$ . *Biochem. J.* **352**:175–182.
35. Windham, T. C., N. U. Parikh, D. R. Siwak, J. M. Summy, D. J. McConkey, A. J. Kraker, and G. E. Gallick. 2002. Src activation regulates anoikis in human colon tumor cell lines. *Oncogene* **21**:7797–7807.
36. Yeatman, T. J. 2004. A renaissance for Src. *Nat. Rev. Cancer* **4**:470–480.
37. Zhong, M., Z. Lu, and D. A. Foster. 2002. Downregulation PKC $\delta$  provides a PI3K/Akt-independent survival signal that overcomes apoptotic signals generated by c-Src overexpression. *Oncogene* **21**:1071–1078.