Oncogenic Ras/ERK Signaling Activates CDCP1 to Promote Tumor Invasion and Metastasis

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Abstract

Involvement of Ras in cancer initiation is known, but recent evidence indicates a role in cancer progression, including metastasis and invasion; however, the mechanism is still unknown. In this study, it was determined that human lung cancer cells with Ras mutations, among other popular mutations, showed significantly higher expression of CUB domain–containing protein 1 (CDCP1) than those without. Furthermore, activated Ras clearly induced CDCP1, whereas CDCP1 knockdown or inhibition of CDCP1 phosphorylation by Src-directed therapy abrogated anoikis resistance, migration, and invasion induced by activated-Ras. Activation of MMP2 and secretion of MMP9, in a model of Ras-induced invasion, was found to be regulated through induction of phosphorylated CDCP1. Thus, CDCP1 is required for the functional link between Ras and Src signaling during the multistage development of human malignant tumors, highlighting CDCP1 as a potent target for treatment in the broad spectrum of human cancers associated with these oncogenes.

Implications: CDCP1 protein induced by oncogenic Ras/Erk signaling is essential for Ras-mediated metastatic potential of cancer cells. Mol Cancer Res; 12(10); 1449–59. ©2014 AACR.

Introduction

Cancers originate from genetic alterations such as the loss of tumor suppression genes and the acquisition of tumor promoting or oncogenic genes, which results in uncontrolled cell growth or behavior (1, 2). The ras family of genes is the most frequently activated oncogene in human cancers. They encode 3 closely related Ras proteins, H-Ras, K-Ras, and N-Ras, which are localized at the inner plasma membrane, bind GDP and GTP, and possess intrinsic GTPase activity. It was originally proposed that Ras activation is essential for rather initiation step during multistep carcinogenesis models by causing aberrant cell growth, although it has later been implicated in virtually all aspects of malignant cancer phenotypes, including proliferation, adhesion, invasion, and survival of cells in suspension, so-called anoikis resistance (3–5). Activation of Ras is known to trigger several downstream signaling pathways, including the Raf–MEK–ERK and phosphoinositide 3-kinase (PI3K)-AKT pathways, whereas the precise mechanism in which Ras regulate later process of cancer progression, such as invasion and metastasis, is not well understood.

CUB domain–containing protein 1 (CDCP1), also known as SIMA135, gp140, and Trask (6–8), is a transmembrane glycoprotein and a major substrate of Src family kinases (SFK) in cancer cells (9). According to the activation of SFKs during the progression of cancers, CDCP1 is tyrosine phosphorylated and directly binds to protein kinase Cδ (PKCδ) in a phosphorylation-dependent manner (10). Phosphorylated CDCP1 recruits PKCδ to the plasma membrane causing activation of this molecule, which results in the acquirement of anoikis resistance (9), enhanced cell migration and invasion, and metalloproteinase secretion in vitro (11). The CDCP1–PKCδ pathway is required in vivo for distant metastasis of lung cancer cells in a mouse model and for the peritoneal dissemination of gastric scirrhou carcinoma (9, 12). Recent reports also suggest that cleavage of CDCP1 at the extracellular domain to produce 70-kDa form might have a role in the activation of CDCP1 signaling (13, 14).

Immunohistochemical analysis recently revealed that CDCP1 expression levels in various human cancer tissues showed significant correlation with poor prognosis of patients (11, 15, 16), suggesting the amount of CDCP1 protein might have impact on the characteristics of cancers. However, no clear information was available on the factors that affect the expression levels of CDCP1. Therefore, we first analyzed the factors that affect the expression of CDCP1.
in human lung cancer cell-lines and tissues in this study and discovered that high CDCP1 expression shows correlation with the presence of Ras mutations. We demonstrated that activated Ras actually induces the expression of CDCP1. It was further demonstrated that induction of CDCP1 expression by Ras along with tyrosine phosphorylation of CDCP1 by SFKs is required for Ras-mediated oncogenic phenotypes, such as anoikis resistance, migration, and invasion.

Materials and Methods

Plasmids, antibodies, reagents, and siRNA

Human CDCP1 with a FLAG tag at the C terminus (CDCP1-F) and the cytoplasmic mutant of CDCP1 (CDCP1, Y734F (Yr734 to Phe) with a FLAG tag at the C terminus (Y734F-F)) were cloned into pcDNA 3.1 (Invitrogen). Active Ras (H-Ras-12V) and wild-type H-Ras plasmids were purchased from Clontech (Ras Dominant-Negative Vector Set). AKT, p-AKT (Ser473), ERK (p44/p42 MAP kinase Antibody), p-ERK [phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibodies], and p-Src (pY416) antibodies were purchased from Cell Signaling. Anti-phosphotyrosine (4G10) and anti-matrix metalloproteinases (MMP) 9 (clone EP1254) antibodies were purchased from Millipore. HA and pan-Src (Src2) antibodies were purchased from Santa Cruz Biotechnology. FLAG M2 and tubulin antibodies were from Sigma. CDCP1 and p-CDCP1 (Y734) antibodies were prepared as described previously (9). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were purchased from Amersham Pharmacia. The SFK inhibitor PP2 and its structural analog PP3 were purchased from Calbiochem-Novabiochem Ltd. The AKT inhibitor LY294002, the ERK1/2 inhibitor PD98059, MMP2/MMP9 inhibitor II, and MMP inhibitor II (MMP1, MMP3, MMP7, and MMP9 inhibitor) were purchased from Calbiochem. Active Ras plasmids were purchased from Clontech. The siRNA of K-Ras was purchased from Santa Cruz Biotechnology. The siRNA of CDCP1 was described previously (17).

Cell culture and inhibitor treatment

Seventy-nine human lung cancer cell lines, validation of the property in these cells was described previously (18, 19), were maintained in RPMI 1640 or DMEM medium with 10% fetal bovine serum (FBS) at 37°C in 5% CO2. In the carcinogenesis model of human cervical cancer (HCK), HCK1T-E cells were established by transduction of bTERT and HPV E6E7 (20) and their subline HCK cells were grown in DMEM medium with 10% FBS at 37°C in 5% CO2. To investigate the effect of PP2 treatment, cells were treated with 10 μM PP2, an inhibitor of SFK, or 10 μM PP2. In PD98059, LY294002, MMP2/MMP9 inhibitor II, and MMP inhibitor II, cells were treated with 20 μM of each inhibitor or control DMSO.

Quantitative RT-PCR

CDCP1 gene expression was measured by a TaqMan Gene Expression assay (Applied Biosystems). The Assay Kit contains a fluorescein amide dye-based assay for the CDCP1 gene and human HPRT1 was used as an endogenous control. PCR was carried out in duplicate using 5 ng DNA as a template. Primers (assay ID: Hs01080405_m1) recognized the boundary of exons 3 to 4 and probe concentrations were optimized for each target according to the manufacturer’s instructions. The PCR program consisted of 95°C for 15 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves for the expression of the target and control were generated using serially diluted normal human lung tissue DNA. Data analysis was carried out using ABI prism 7900HT Sequence Detection Software v2.3.

Western blotting

Cell lysates were prepared with protease inhibitors in a buffer [10 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EGTA, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 10 μg/mL aprotinin, 1 mmol/L sodium orthovanadate (Na3VO4), and 100 μg/mL leupeptin]. The protein concentration was measured by BCA Protein Assay (Pierce). Samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). After blocking (Blocking One, Nakarai Tesque), the membrane was probed with antibodies for detection. The membrane was probed with HRP-conjugated anti-mouse or anti-rabbit (1:4,000) to visualize the reacted antibody. Images were captured by a molecular imager GS-800 (Bio-Rad).

Tissue samples and immunohistochemical analysis

Forty patients with lung adenocarcinoma underwent curative pulmonary resections from 2000 to 2007 at the National Cancer Center Hospital (NCCH; Tokyo, Japan). Twenty cases of lung adenocarcinoma contained mutations in Ras and the remaining cases contained wild-type Ras. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded slides using the avidin–biotin complex methods, as described previously (21). A specific antibody against CDCP1 (rabbit polyclonal antibody, 1:500) was used as the primary antibody. Staining in the absence of primary antibody provided the negative control. Staining on each slide was evaluated by 1 researcher in 3 independent observations. Immunoreactivity was scored semiquantitatively according to the estimated area of positive tumor cells (0% of area = 0% to 100% of area = 10) and the level of staining (standard-0, <5% reacting cells: standard-1, <20% reacting cells: standard-2, <50% reacting cells: standard-3, >80%). The level of intensity between standard-0 and standard-1 is level-0, scored as 0; level-1 is between standard-1 and standard-2, scored as 1; level-2 is between standard-2 and standard-3, scored as 2; level-3 is over standard-3 and is scored as 3. The total score is the sum of each score in an area × the score of the level.

Viral vector construction and transduction

Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen). In brief, C-terminally FLAG-tagged wild type and mutant CDCP1 (CDCP1-FLAG, CDCP1-Y734F-FLAG) and C-terminally HA-tagged Fyn (Fyn-2HA) were recombined into entry vectors by BP reaction (Invitrogen). These segments were
recombined with a lentiviral vector, CSII-CMV-ΔRA (a gift from Dr Miyoshi, RIKEN), by LR reaction (Invitrogen) to generate CSII-CMV-CDCP1-FLAG, ΔCDCP1-Y734F-FLAG, and Δ-Fyn-2HA. The production of recombinant lentivirus with the vesicular stomatitis virus G glycoprotein (VSV-G) was as previously described (22).

Soft-agar colony assay

Six-well tissue culture plates were coated with a layer of RPMI 1640, 10% FBS containing 0.5% agarose. Subconfluent cells were treated with EDTA, washed twice in PBS, and resuspended in RPMI 1640 or DMEM 10% FBS at 6 × 10^3 cells/mL. Then, a 500 μL cell sample was added to 1 mL of RPMI 1640 or DMEM 10% FBS containing 0.5% agarose (final 0.3%). The cells were plated onto the coated tissue culture plates, allowed to solidify, and then placed in a 37°C incubator. After 30 days, colonies were scanned with a GS-800 Calibrated Densitometer (Bio-Rad) and the numbers of colonies/wells were counted. Soft-agar assays were performed 3 times.

Measurement of cell death

Trypan blue staining (0.4%; GIBCO) was used to visualize cell death. The cells were transfected with or without siRNA targeting CDCP1. After 24 hours, the cells were resuspended into 2-methacryloyloxyethyl phosphorylcholine (MPC)–coated plate (Nunc). After 48 hours, cells were treated with trypan blue stain reagent (Gibco). The number of cells positive or negative for trypan blue staining was determined by counting cells on 4 slides for 2 plates (5 fields per slide) at a magnification of ×20, and the percent of trypan blue–positive cells was calculated as the number of trypan blue–positive cells/total number of cells. The results are expressed as the mean of three independent experiments.

Gelatin zymography

Gelatin zymography was conducted with a polyacrylamide gel containing gelatin (0.8 mg/mL) as described previously (24). Twenty microliters of supernatant of culture medium without serum was used for zymogram. The SDS-polyacrylamide gel was incubated for 72 hours in the incubation buffer at 37°C. Enzyme activity was visualized as negative staining with Coomassie brilliant blue.

Results

Activating Ras mutation induces CDCP1 expression in non–small cell lung cancer

CDCP1 is strongly expressed in colon, gastric, kidney, pancreatic, and lung cancers (11, 12, 15, 16, 25). In lung, kidney, and pancreatic cancers, immunostaining of CDCP1 showed that high expression correlates with poor prognosis of the patients (11, 15, 16). We examined CDCP1 gene expression in 58 non–small cell lung cancer (NSCLC) cell lines by quantitative RT-PCR (qRT-PCR) analysis. Mutations in K-Ras, N-Ras, B-Raf, EGFR, LKB1 or p53, and amplification of Myc are found in these cell lines (Supplementary Table S1), which are associated with lung cancer malignancies (26–28). Ras mutations were found in 13 cell lines at K-Ras-G12, 2 cell lines at K-Ras-Q61, and 4 cell lines at N-Ras-G12. Double mutations at N-Ras and B-Raf were exceptionally found in a cell line H2087 (Supplementary Table S1). It was demonstrated that the levels of CDCP1 gene expression were significantly higher in NSCLC cell lines with Ras mutations (mean = 1.566 ± 0.258, P = 0.0089) than those with wild-type Ras (mean = 0.992 ± 0.148), and even higher in cells with Ras/B-Raf mutations (mean = 1.613 ± 0.212, P = 0.007) than in cells without mutations in these oncogenes (mean = 0.968 ± 0.124; Fig. 1A and Supplementary Fig. S1). On the other hand, no significant difference was observed with other common mutations such as EGFR, LKB1, and p53 (Table 1). In addition, 21 small cell lung cancer cell lines without RAS mutations had significantly lower expression of CDCP1 than NSCLC cell lines (Supplementary Fig. S2).
We also examined the expression of CDCP1, ERK1/2 proteins, and phosphorylation of ERK1/2 in NSCLC cells with Ras/B-Raf mutations (Fig. 1B). CDCP1 protein expression was elevated in those cells and the ratio of phosphorylated ERK1/2, an index of Ras activity, was increased (Fig. 1C). In addition, significantly higher levels of CDCP1 were observed in human lung cancer tissues with Ras mutations (mean = 19.5 ± 1.393; P = 0.009) than in tissues without mutations (mean = 13.0 ± 1.355; Fig. 1D and E). The method by which we scored the intensity of CDCP1 staining is illustrated in Supplementary Fig. S3.

Because existence of Ras mutations showed significant correlation with CDCP1 expression in NSCLC, we tested whether activated Ras directly regulate CDCP1 expression. CDCP1 gene expression was induced by overexpression of activated Ras (H-Ras-12V) in H322 cells that have wild-type K-Ras (Fig. 2A). At the same time, the expression of CDCP1 protein was also elevated (Fig. 2B). On the other hand, knockdown of K-Ras in A549 cells attenuated CDCP1
protein (Fig. 2C). PD98059, an inhibitor of MEK that is downstream of the Ras signal, reduced expression of CDCP1 protein and mRNA in A549 cells (Fig. 2D and E). The phosphorylation of ERK was significantly reduced at 6 hours after treatment of PD98059 whereas the expression of CDCP1 was also significantly decreased at that time (Supplementary Fig. S4A and S4B).

These observations brought us to the conclusion that active Ras induces CDCP1 expression in NSCLCs, probably through the authentic MEK-ERK signaling pathway.

CDCP1 links Ras and SFK signaling and regulates anoikis resistance, cell migration, and invasion in NSCLC cells

We postulated that CDCP1 might link oncogenic signaling of Ras and SFKs, based on the fact that activation of Ras signaling induces CDCP1 expression (Figs. 1 and 2) and SFKs lead to the phosphorylation of CDCP1 for signal transduction from CDCP1 to PKCδ (9, 10). Although the cooperative effect of Ras and SFKs has been reported by several groups (29, 30), the molecular mechanism linking Ras and SFK signaling has not yet been clearly described.

In H322 cells, expression of H-Ras-12V resulted in marked induction of CDCP1 protein, although CDCP1 is not tyrosine phosphorylated because of low activity of SFKs in these cells (Fig. 3A), compared with A549 cells in which Fyn, a member of the SFKs, is dominantly activated (9). When H-Ras-12V was expressed in H322Fyn, a stable Fyn-expressing line of H322 cells, a similar amount of CDCP1 with marked tyrosine phosphorylation was induced (Fig. 3A). H-Ras-12V expression induced soft-agar colony

Table 1. Association of CDCP1 expression with genetic alterations in NSCLC cell lines

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*pBy the Fisher exact test.

Figure 2. Induction of CDCP1 is regulated by Ras in NSCLC cells. A, analysis of CDCP1 mRNA expression by qRT-PCR in H322 cells transfected with wild-type (H-Ras-WT) or active Ras (H-Ras-12V) plasmid. B, H322 cells transfected with each plasmid were analyzed by immunoblotting. Each cells was cultured by serum-depleted medium. C, A549 cells were treated with K-Ras siRNA for 72 hours and detected expression of CDCP1. D, A549 cells treated with PD98059 or DMSO for 24 hours were analyzed by immunoblotting. E, analysis of CDCP1 mRNA expression by qRT-PCR in A549 cells treated with PD98059 or DMSO for 24 hours. Each data are the means ± SD from three experiments. * P values in a Student t test < 0.005. Black arrowheads denote band corresponding to CDCP1. White arrowhead indicates nonspecific bands.
formation and anoikis resistance in H322Fyn cells but not in H322 cells (Fig. 3B and C). CDCP1 siRNA in H322Fyn cells inhibited those oncogenic properties induced by H-Ras-12V expression (Fig. 3A–C: siCDCP1). The same result was obtained by using an independent set of siRNA for CDCP1. Cell proliferation was not altered in these cells (Fig. 3D), consistent with previous reports (9).

To confirm the role of CDCP1 in the oncogenic function of Ras, we analyzed whether overexpression of CDCP1 could rescue the biologic effect of Ras–ERK pathway inhibition by a MEK inhibitor PD98059 in A549 cells. Overexpression of wild-type CDCP1 but not Y734F mutant CDCP1, which lacks the SFK binding site, rescued the reduction in soft-agar colony formation (Fig. 3F), cell migration (Fig. 3G), invasion (Fig. 3H), and anoikis resistance (Supplementary Fig. S5A) caused by Ras–ERK signaling inhibition (Fig. 3E). The level of cell growth was also inhibited by PD98059, which was not significantly rescued by expression of CDCP1 (Supplementary Fig. S5B), suggesting that CDCP1 is not involved in the regulation of cell proliferation among the oncogenic phenotypes induced by the Ras–ERK pathway.

These results strongly suggest that Ras along with SFKs utilize CDCP1 to regulate the metastatic properties of tumor cells, including anoikis resistance, migration, and invasion.

**Oncogenic Ras signaling activates SFKs and promotes CDCP1-dependent invasion through MMPs regulation in HCK**

To further investigate the role of CDCP1 in Ras-mediated cancer invasion, we utilized the model of Ras-induced invasion of human cervical keratinocytes (HCK) in which the effect of activated H-Ras is visible by raft assay. Expression of oncogenic H-Ras-12V in HCK (HCK1T-E-Hras) causes anchorage-independent growth and induced cell invasion activity in vitro (20). Outstanding phosphotyrosine-containing proteins with molecular mass of 130 and 70 kDa were detected by anti-phosphotyrosine antibody in HCK1T-E-Hras cells but not in HCK1T-E cells (Fig. 4A). Knockdown of CDCP1 or depletion of CDCP1 by

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Figure 3. CDCP1 links Ras and SFK signaling and regulates anoikis resistance, cell migration, and invasion in NSCLC cells. A, A549, H322, and H322Fyn cells treated with H-Ras-12V and/or CDCP1 siRNA were analyzed by immunoblotting. B, the effect of H-Ras-12V on anchorage independence was determined by soft-agar assay. C, the effect of H-Ras-12V on cell death in suspension culture was determined by Trypan blue staining as a percentage. D, cell proliferation in suspension culture of each cells were determined with a cell proliferation ELISA BrdUrd Kit. E, A549 cells that stably expressed CDCP1 tagged with FLAG (CDCP1-F), a CDCP1 mutant tagged with FLAG (Y734F-F), and control pcDNA3.1 plasmid (pcDNA) were treated with PD98059 for 24 hours and analyzed by immunoblotting. F, the effect of CDCP1-F or Y734F-F on anchorage independence in A549 cells were determined by soft-agar assay. G, rescue of cell migration by phosphorylated CDCP1. H, rescue of cell invasion by phosphorylated CDCP1. Black arrowheads indicate phosphorylated CDCP1. White arrowheads indicate cross-reactive bands. Each data are the means ± SD from three experiments. * P values in a Student t test < 0.005. Black arrowheads denote band corresponding to CDCP1. White arrowhead indicates nonspecific bands.
immunoprecipitation using anti-CDCP1 antibody resulted in marked decrease of these proteins, suggesting that these 130 and 70-kDa phosphoproteins induced by Ras was mostly CDCP1 (Fig. 4A and Supplementary Fig. S6) and these results were also confirmed by anti-CDCP1 antibody. The enhancement of the phosphorylated ERK1/2 was observed in HCK1T-E-Hras cells compared with HCK1T-E cells (Fig. 4B), which was well corresponded to the level of CDCP1 expression. SFK activity was attenuated by treatment with CDCP1 siRNA or PD98059 in HCK1T-E-Hras cells (Fig. 4B), supporting the possible role of CDCP1 as an activator of SFKs recently shown in melanoma cells (31).

We used 3-dimensional collagen raft assays, which mimic in vivo tissue invasion of cancers, and detected marked expression of CDCP1 in the area of invasion into the collagen raft. This is reminiscent of cancer invasion of the submucosal layer in HCK1T-E-Hras cells (Fig. 4C). Treatment with CDCP1 siRNA, a SFK inhibitor PP2 or PD98059, inhibited cell invasion into the collagen raft in HCK1T-E-Hras cells (Fig. 4D).

Then, the involvement of matrix metalloproteinases (MMP) was examined because they are critical regulators of tumor invasion (32). The activation of MMP9 secretion was induced in HCK1T-E-Hras cells according to the acquisition of invasive phenotype, whereas treatment with PP2 or PD98059 diminished the secretion of MMP9 (Fig. 5A). Same result was observed in treatment with CDCP1 siRNA as previously reported in pancreatic cancer.

**Figure 4.** Oncogenic Ras signaling activates SFKs and promotes CDCP1-dependent invasion in 3-dimensional (3D) collagen raft culture of HCK. A, HCK1T-E cells, HCK1T-E cells stably expressing H-Ras12V (HCK1T-E-Hras) and HCK1T-E-Hras cells treated with CDCP1 siRNA were analyzed by phosphotyrosine antibody. Black arrowhead indicated CDCP1. B, HCK cells treated with each CDCP1 siRNA, PP2, or PD98059 were analyzed by immunoblotting. C, the invasive ability of the cells in 3D collagen raft culture was determined by 2 weeks culture. Top panel is stained with CDCP1 antibody, middle panel is stained with H&E. Lower panel showed quantification of CDCP1 expression in tissue area of 3D collagen raft compared with invasion area by ImageJ. D, HCK1T-E-Hras cells treated with CDCP1 siRNA, PP2, or PD98059 were cultured for 4 weeks in 3D collagen rafts and cell invasion was detected by H&E. Blue arrow show the invaded each HCK cells. Black arrowheads denote band corresponding to CDCP1.
cells (11). The molecular size of MMP9 in HCK cells was also confirmed by Western blotting using anti-MMP9. Moreover, the activation of MMP2 protein was detected in HCK1T-E-Hras cells, whereas treatment with CDCP1 siRNA, PP2, or PD98059 reduced active-MMP2 and increased pro-MMP2 (Fig. 5A and B) antibody. The position of MMP2 bands was confirmed by zymogram and Western blotting in HCK cells along with HT1080 cells (Fig. 5A and Supplementary Fig. S7). Treatment of MMP2/MMP9 inhibitor II or MMP inhibitor II, which inhibits a series of MMPs including MMP9 but not MMP2, suppressed invasion in HCK1T-E-Hras cells (Fig. 5C and D). The suppressive effect of the MMP2/MMP9 inhibitor II in cell invasion was also observed in A549 cells, H-Ras-12V-expressing H322Fyn cells and HT1080 cells, of which invasion was reported to be regulated by MMP2 and MMP9 (33, 34) (Supplementary Fig. S8). On the other hand, no significant change of cell growth and cell migration was observed in these cells by treatment with MMP2/MMP9 inhibitor II (Supplementary Fig. S9). The suppressive effect of the MMP2/MMP9 inhibitor II in cell invasion was also observed in A549 cells, H-Ras-12V-expressing H322Fyn cells and HT1080 cells, of which invasion was reported to be regulated by MMP2 and MMP9 (33, 34) (Supplementary Fig. S8). On the other hand, no significant change of cell growth and cell migration was observed in these cells by treatment with MMP2/MMP9 inhibitor II (Supplementary Fig. S9). Therefore, CDCP1 regulates cell invasion induced by H-Ras-12V, possibly through the control of MMP2 activation and MMP9 secretion in this model of cancer invasion by activated Ras.

These results indicate that CDCP1, induced by Ras and tyrosine-phosphorylated by SFKs, is essential for progression in the tissue invasion model of cancer. It is indicated that CDCP1 is a common oncogenic effector of Ras, and SFK proteins in cancers.

**Discussion**

Activating Ras mutations are detected in approximately 90% of pancreatic, 50% of colon, 30% of lung, and 10% of gastric cancers and correlated with poor prognosis and malignancy in various human cancers (2, 35). High CDCP1 expression is also linked with poor prognosis in several cancers (11, 15, 16). We found that CDCP1 expression in human lung cancer tissue is significantly higher in the presence of activated Ras (Fig. 1E). In 58 NSCLC cell lines, higher CDCP1 gene expression was found in a subset with Ras/B-Raf mutations, but not with common mutations such as EGFR, LKB1, or p53 (Table 1). Activated Ras stimulates a number of effectors, including the Raf–MEK–ERK and PI3K–AKT pathways (36). Our data show that the activity of ERK downstream of K-Ras regulates the expression of CDCP1 (Fig. 2D and E). It is currently under investigation how the CDCP1 expression is regulated by activation of ERK, whereas a recent study indicates the expression of CDCP1 is regulated by Hypoxia inducible factor-1 (37), which might be a candidate for induction of CDCP1 as one of the downstream effect of ERK.
Role of CDCP1 in Ras-Mediated Tumor Metastasis

(38). On the other hand, there are three activator protein-1 (AP-1) binding sites in the promoter region of CDCP1 (from -2836 to -1 nucleotide sequences). Because ERK activation is known to enhance AP-1 activity (39), it is possible that the Ras–ERK pathway might regulate CDCP1 expression through AP-1. The Ras–ERK pathway is activated in many types of human cancer and is recognized as a driving force for tumor initiation and progression (4). Our data demonstrate that part of the malignant characteristics induced by activated Ras, such as anoikis resistance, migration, and invasion are dependent upon the upregulation of CDCP1 protein. It is notable that oncogenic Ras promotes both proliferative and metastatic potential of cancers, whereas induction of CDCP1 is only responsible for the metastatic potential induced by Ras.

Knockdown of CDCP1 protein blocks cell motility and invasion in vitro and in vivo (11, 12). A recent study suggested that CDCP1 signaling is accompanied by a decrease in phosphorylation of focal adhesion kinase (FAK), indicating a role for CDCP1 in tumor invasion and metastasis through reduced adhesion at least in part (40) and overexpression of CDCP1 actually induces round morphology of MDA-468 breast cancer cells and reduces cell adhesion (8). Activated Ras also induces FAK dephosphorylation and promotes disassembly and turnover of focal adhesion (41). It seems that CDCP1 regulates intensity of focal adhesion as a downstream effector of Ras. On the other hand, we recently showed that CDCP1 regulates secretion of MMP9 (11). In this study, we observed that MMP2 activation and MMP9 secretion were induced by activated Ras and these functions were reduced by CDCP1 siRNA or PD98059, an inhibitor of MEK downstream of Ras (Fig. 5A). On the contrary, using farnesyl transferase inhibitors, which affect H-Ras function, reduced MMP9 (42). It is well known that MMPs have major roles during the tumor invasion through extracellular matrix degradation (32). Actually, specific MMP2 and MMP9 inhibitor reduced invasion of HCK cells (Fig. 5C and D). Recently, we reported that CDCP1 may be involved in a lipid raft-dependent secretary pathway (43). It is reasonable to speculate that CDCP1 also plays a role in Ras-induced cell motility and invasion by regulating focal adhesion and MMP2 activation and MMP9 secretion. Moreover, CDCP1 is associated with MT1–MMP, which is an activator of both MMP2 and MMP9 (43). It is speculated that CDCP1 can regulate activation of MMP2 and MMP9 through membrane trafficking of MT1–MMP to the same intracellular compartment.

Mutations in Ras and EGFR have been well characterized in human lung cancers. Recently, the activating mutation of K-Ras was shown to be responsible for resistance to EGFR inhibitors in chemorefractory cancer cells (44, 45). The significant difference in pathologic appearance between clinical specimens of NSCLC with EGFR mutations and Ras mutations (46) revealed a difference in the nature of these signaling pathways and their control of malignant phenotypes. Because predominant activation of the Ras–ERK pathway might contribute to the specific phenotype, CDCP1 expression may be a useful and universal marker of Ras–ERK pathway activation and resistance to EGFR inhibitors. Recently, it was reported that expression of CDCP1 is regulated by the ERK pathway through stimulation of EGFR in ovarian cancer cells (47). Our data also support CDCP1 is under regulation of the ERK pathway in NSCLC samples, although we could not see positive relationship between EGFR activation and CDCP1 expression. Further systematic histologic analysis of various cancer specimens with CDCP1 antibody will be required to validate the clinicopathologic features associated with CDCP1 overexpression.

We showed that CDCP1 was the main tyrosine-phosphorylated protein induced by expression of activated Ras in the HCK cells, which brings an invasion promoting activity (Fig. 4A and C). It was recently suggested that cleaved form of 70-kDa CDCP1 might be critical for activation of CDCP1 signaling (13, 14). It seems that activated Ras highly induces 70-kDa CDCP1 in HCK cells in addition to intact 135-kDa CDCP1 (Fig. 4A and B). The induction was almost proportional for both 2 forms of CDCP1 in NSCLC cells (Figs. 2B and 3A), whereas some cell lines with activated Ras such as HCC44, H2087 express large amount of 70-kDa CDCP1 (Fig. 1B), suggesting possible regulation of CDCP1 cleavage by activated Ras. Our results do not directly indicate selective induction of 70-kDa CDCP1 by activated Ras whereas it is indicated that 70-kDa CDCP1 might also have essential roles in the progression of NSCLC cells. As knockdown of CDCP1 from the HCK1T-E-Hras cells suppressed the activation of Src family kinases, induction of CDCP1 by activated Ras was responsible for SFK activation in this model (Fig. 4B). This effect was not apparent in some cell lines such as H322 or A549, indicating the requirement for other unknown factor(s) for activation of SFK by CDCP1. It is already known some of the substrates of Src such as P130Cas and Ossa can activate SFKs through association with their regulatory domain (48, 49), as CDCP1 can also activate Src family kinases possibly through similar mechanism (31). The possibility that activated Ras could activate SFKs through induction of CDCP1 in a context-dependent manner, will give novel insight to the invasion mechanism of cancer through activation of these oncogenes. The cooperative effect of Ras and Src influences the development of cancers such as pancreatic neoplasia (30); however, there is no direct evidence to indicate whether these signals in malignant stage of tumor engage in crosstalk to control cellular function or separately modulate common phenotypes. We demonstrated in this study that expression and tyrosine phosphorylation of CDCP1 by Ras and SFKs, respectively, are essential for acquisition of the metastatic and invasive properties of cancer cells, suggesting that CDCP1 links the Ras and SFK signals during the cancer progression. As we described in a previous paper, the CDCP1 pathway did not significantly support cell growth or tumorigenicity, whereas it clearly regulates tumor metastasis (9). We also noticed in this study that CDCP1 is essential in Ras-mediated metastatic properties, but not in Ras-mediated proliferation effect (Fig. 3D). There is one recent report showing that CDCP1 possesses tumor-suppressive function...
(50). At this moment, we have no evidence that CDCP1 has the opposite effect on tumorigenicity and on metastasis just like TGFβ in context-dependent manners. Further study will be required to resolve this apparent discordance.

The significance of CDCP1 as a regulator of metastatic aspects of progressed cancers under the control of the Ras and SFK signal pathways naturally indicates that CDCP1 might be an ideal therapeutic target of metastatic cancers. The utilization of CDCP1 as a target molecule will be especially advantageous when combined with conventional antiproliferative drugs for the treatment of progressed tumors, considering that CDCP1 controls their metastatic and invasive potential without significantly affecting proliferation. Moreover, it might be universal and powerful approach because CDCP1 is a major phosphotyrosine-containing membrane protein in various types of solid tumors located at the node of 2 major oncogenic pathways.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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Oncogenic Ras/ERK Signaling Activates CDCP1 to Promote Tumor Invasion and Metastasis

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