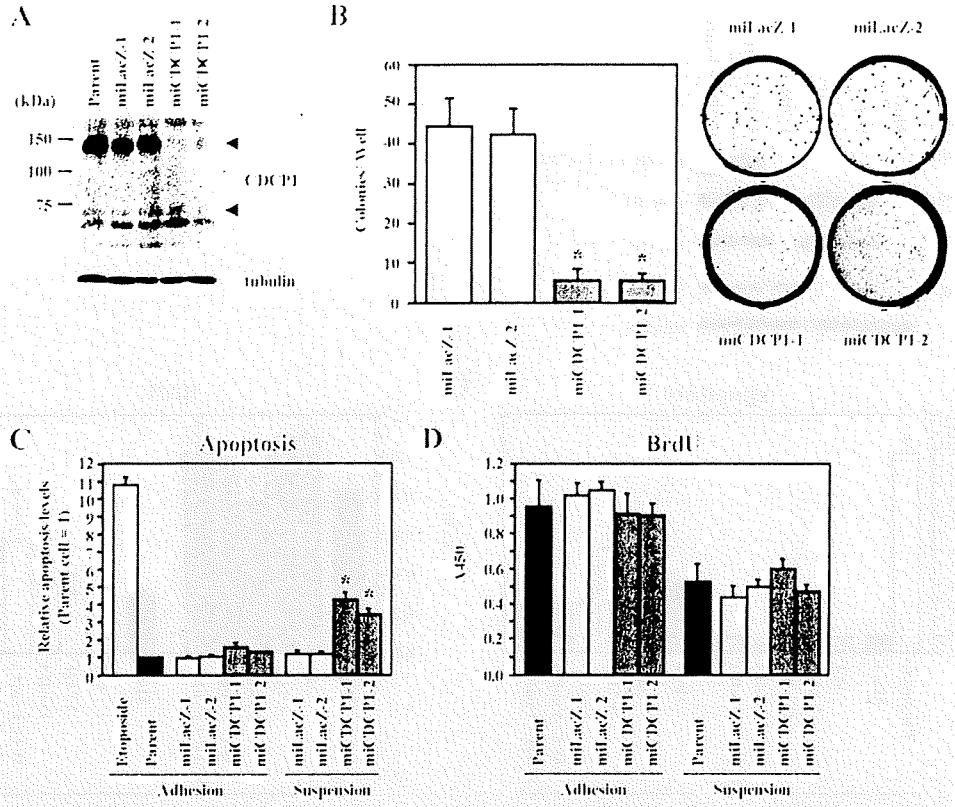

FIG. 4. CDCP1 confers anchorage independence by inhibiting apoptosis in suspended lung adenocarcinoma. (A) CDCP1-defective A549 cell clones (miCDCP1-1 and miCDCP1-2) were generated by an miR RNAi expression vector kit (Invitrogen). miLacZ-1 and miLacZ-2 were control clones. The expression of CDCP1 in each clone (1.5×10^5 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 (3×10^3 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 (1.0×10^4 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 μM). 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 (1.0×10^4 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 PKCδ by linking PKCδ to SFKs in a phosphorylation-dependent manner.

To check whether PKCδ can regulate anoikis in lung adenocarcinoma cells, cell apoptosis caused by the suspension of miCDCP1 and miLacZ clones was examined with or without PKCδ RNAi. As shown in Fig. 6D, PKCδ 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 PKCδ. Similar results were obtained from two other independent sets of siRNAs for PKCδ (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-PKCδ signal pathway affects anoikis resistance in A549 cells by overexpressing the C2 domain of PKCδ, which has been shown to be responsible for the association with tyrosine-phosphorylated CDCP1 (2). The HA-tagged C2 domain of PKCδ (C2HA) expressed in A549 cells was actually associated with phosphorylated CDCP1 (Fig. 6F, upper panel) and suppressed the tyrosine phosphorylation levels of PKCδ (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 PKCδ under suspension conditions and that PKCδ 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

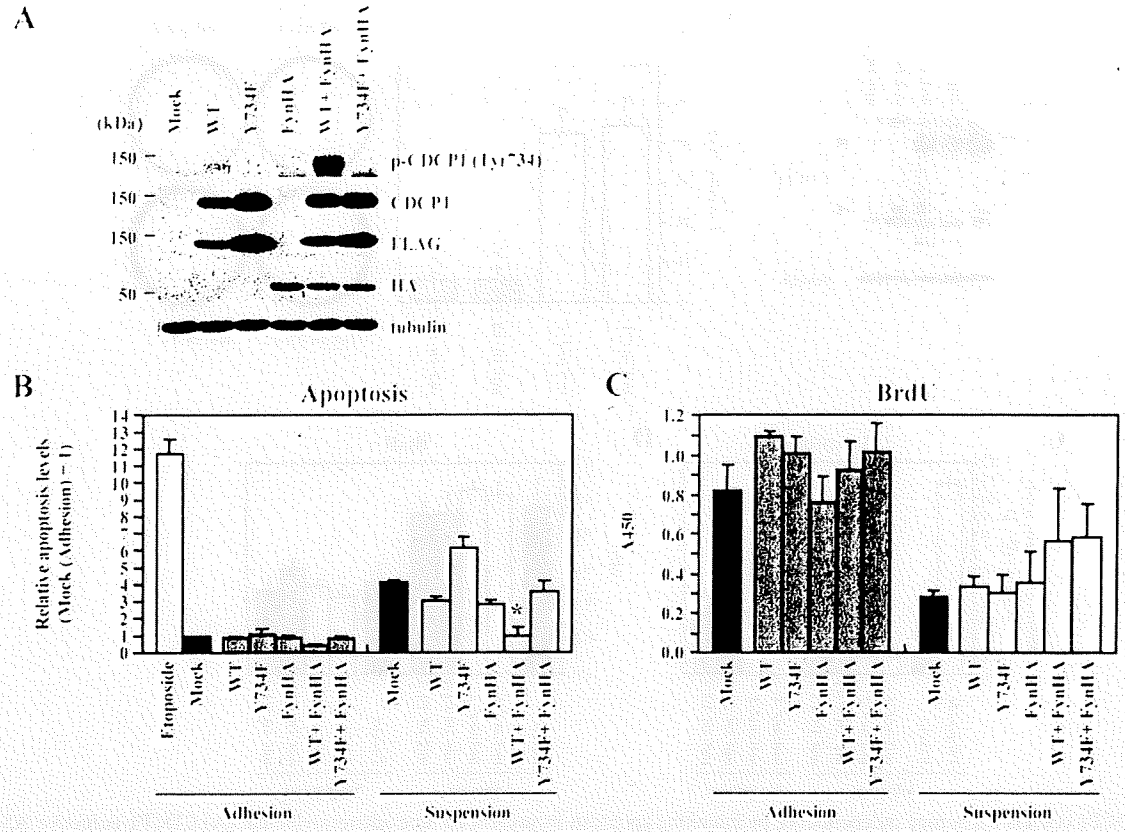

FIG. 5. Anoikis resistance was recovered by phosphorylated CDCP1 in H322 cells with low anchorage independence. (A) H322 cells that overexpressed CDCP1 (WT), a CDCP1 mutant (Y734F), and/or Fyn kinase tagged with HA (FynHA) was incubated for 24 h in MPC-coated plates. The cells were lysed and subjected to immunoblotting with the indicated antibodies. (B) Cells, as indicated (1.0×10^4 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 μM). 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 (1.0×10^4 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

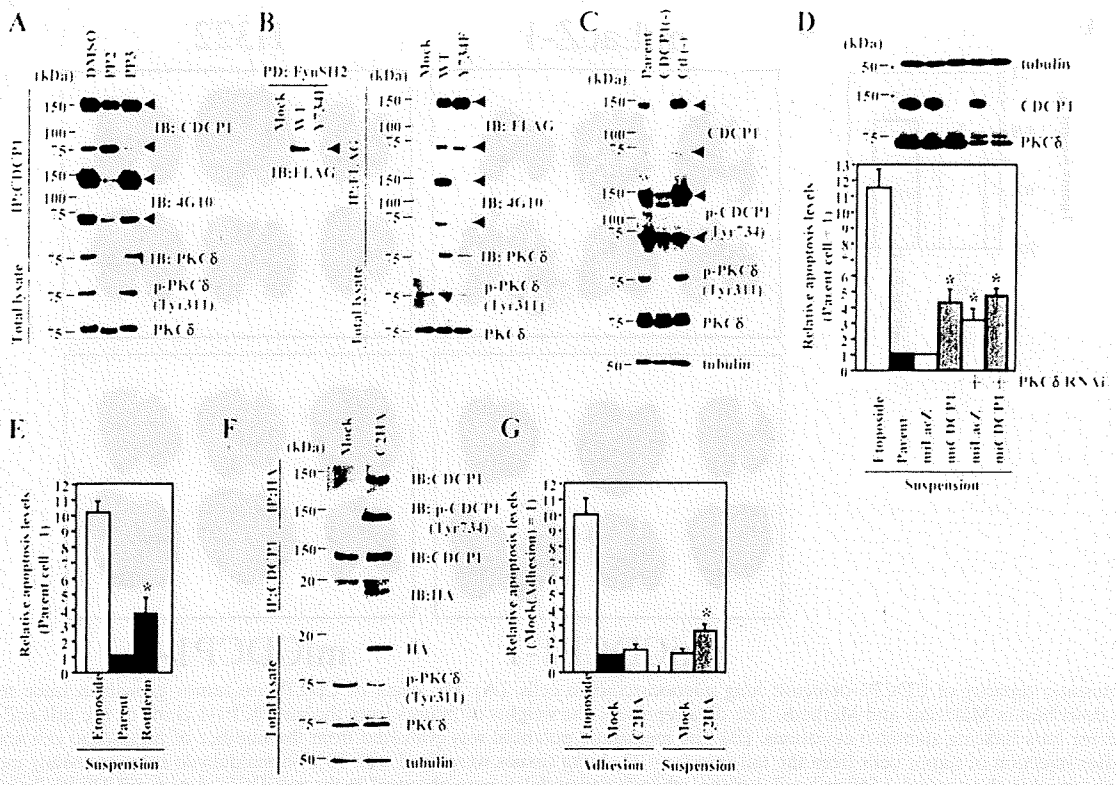

FIG. 6. PKCδ is a signaling molecule downstream of CDCP1 during anoikis resistance. (A) Treatment with the SFK inhibitor PP2 blocked the physical association between PKCδ and CDCP1 and at the same time suppressed phosphorylation of PKCδ at Tyr311. A549 cells treated with 10 μM of PP2 and 10 μM of PP3 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 PKCδ (p-PKCδ [Tyr311]) total cell lysate was used to detect the phosphorylation of PKCδ, and the expression of PKCδ 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 PKCδ. (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 PKCδ on apoptosis was determined by apoptosis assay. PKCδ stealth siRNA was transiently transfected into CDCP1-defective A549 cell clones and control miLacZ clones. After 48 h, each cell clone (1.0×10^4 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 μM). 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 PKCδ was determined by Western blotting with the indicated antibodies (top). (E) The effect of PKCδ activation on apoptosis was determined by apoptosis assay. A549 cells (1.0×10^4 cells) were seeded onto MPC-coated 96-well plates and treated or not with Rottlerin (5 μM). 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 PKCδ 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 PKCδ in A549 cells. (G) The cells transiently transfected with C2HA or mock vector, as indicated (1.0×10^4 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.

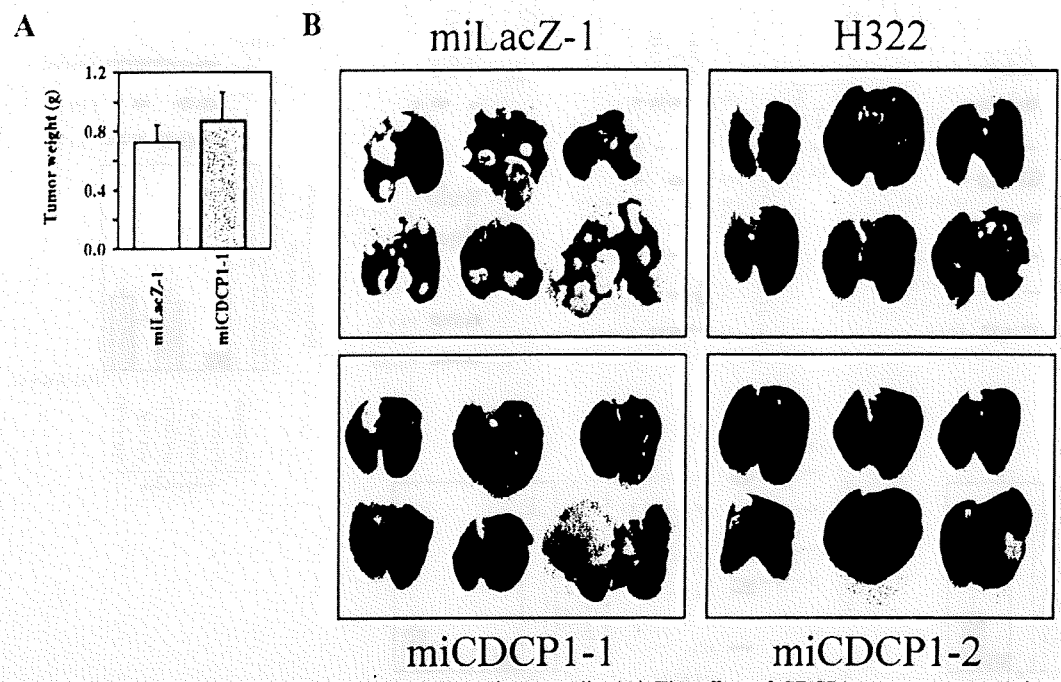

FIG. 7. Metastatic capacity of CDCP1-defective lung adenocarcinoma cells. (A) The effect of CDCP1 on tumor growth in nude mice was determined as described in Materials and Methods. The data represent the weights of tumors from the miCDCP1-1 clone or the miLacZ-1 clone (n = 3). The error bars indicate 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 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.

TABLE 1. Effects of CDCP1 downregulation on lung cancer metastasis in vivo[a]

Cells	Metastasis[b]	No. of nodules in lung[c]
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

[a] Mice were sacrificed 100 days after inoculation.
[b] Data are shown as the number of mice bearing tumors in the lung/total number of mice.
[c] Average number of metastatic tumor nodules larger than 2 mm in the lung per mouse.

Benes et al. (2) recently reported that the C2 domain of PKCδ associates with phosphorylated CDCP1. Several studies have also reported on the phosphorylation of PKCδ by SFKs (19, 30), but the regulatory mechanism of PKCδ phosphorylation remains unclear. Our study found that PKCδ 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 PKCδ, which suggests that CDCP1 mediates the phosphorylation of PKCδ by SFKs. We found that an increased level of apoptosis was observed with the treatment of siRNA for PKCδ 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 PKCδ, by expressing the C2 domain of PKCδ, suppressed the tyrosine phosphorylation of PKCδ 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 PKCδ lead to the suppression of apoptosis in A549 cells.

Tyrosine phosphorylation of PKCδ is a critical regulatory factor for PKCδ activity and results in the elevation of both tyrosine phosphorylation and the activity of PKCδ 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 PKCδ by Src actually increased PKCδ activity (1, 11). On the other hand, several reports have shown that active PKCδ possesses an antiapoptotic function. For example, the activation of PKCδ by fibroblast growth factor has an antiapoptotic effect in PC12 cells (34) and a reduction of PKCδ activity by using a kinase-dead mutant of PKCδ induced apoptosis in lung cancer cells (7). Further evidence that supports PKCδ as a suppressor of apoptosis includes the requirement for active PKCδ 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 PKCδ (17, 18). Our observation that tyrosine-phosphorylated PKCδ serves an antiapoptotic function in lung cancer cells supports these reports, although it appears that PKCδ 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 PKCδ and that CDCP1-SFK complex-dependent PKCδ 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 PKCδ.

Finally, this study suggests that CDCP1 is a novel regulator of anoikis resistance under the control of SFKs in lung adenocarcinoma cells and that PKCδ, 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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