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Tumorigenesis and Neoplastic Progression

CUB-Domain-Containing Protein 1 Regulates Peritoneal Dissemination of Gastric Scirrhous Carcinoma

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CUB-domain-containing protein 1 (CDCP1) is a type-I transmembrane protein that is highly expressed in colon, breast, and lung cancers. We recently revealed that CDCP1 is associated with and phosphorylated by Src family kinases and is involved in the regulation of anchorage independence of certain lung cancer cell lines. In this study, we examined whether CDCP1 is involved in the regulation of tumor progression of scirrhous gastric cancer, which is a diffusely infiltrative carcinoma with high invasion potential. Expression and phosphorylation levels of CDCP1 correlated with the invasive potential of scirrhous gastric cancers. Reduction of CDCP1 expression by siRNA suppressed migration, invasion, and anchorage independence without affecting the proliferation of highly invasive scirrhous gastric cancer cells. However, CDCP1 overexpression promoted gastric cancer cell migration with low potential of invasion. Loss of CDCP1 suppressed invasion and dissemination of cancer cells that were orthotopically implanted in the gastric wall of nude mice. Expression and phosphorylation of CDCP1 were also detected in cancer cells of surgically resected tissues of human scirrhous gastric cancer by immunohistochemical analysis. Our results suggest that CDCP1 promotes invasion and peritoneal dissemination of cancer cells through the regulation of cell migration and anchorage independence. Therefore, it is both a potential prognostic and therapeutic target in certain types of gastrointestinal cancers, and suppression of its phosphorylation might be a useful strategy for modulating cancer metastasis. *(Am J Pathol 2008, 172:1729–1739; DOI: 10.2353/ajpath.2008.070981)*

CUB-domain-containing protein 1 (CDCP1) was first identified as the product of a gene preferentially expressed in colon cancer cells compared to normal tissue.[1] The CDCP1 gene contains nine exons, and the first mRNA transcript to be described is ~6.4 kb in length. Further studies report that CDCP1 mRNA is expressed on hematopoietic stem cells, mesenchymal stem cells, neuronal progenitor cells,[2,3] human epidermoid carcinoma cell lines,[4,5] lung, breast, and prostate carcinoma cell lines.[1,6]

The CDCP1 protein, also described as SIMA135 and Trask,[4,6] is a type I transmembrane protein containing three putative CUB (complement protein subcomponents C1r/C1s, urchin embryonic growth factor, and bone morphogenic protein 1) domains that are characterized by immunoglobulin-like folds and are involved in protein-protein interaction.[7,8] CDCP1 is phosphorylated by Src family kinases (SFKs) and previous studies have suggested possible roles of CDCP1 in cellular adhesion and cell-cycle regulation.[5,6,9] We recently purified a major phosphoprotein detected in the suspension culture of anchorage-independent lung cancer cells and identified it as the CDCP1 protein by mass spectrometry. We revealed that tyrosine phosphorylated CDCP1 has a novel role in regulating the anchorage independence of lung cancer cells by linking cell signaling from SFKs to protein kinase Cδ.[10]

Scirrhous gastric carcinoma diffusely infiltrates a broad region of the stomach and frequently associates with metastasis to lymph nodes and peritoneal dissemination. The process of peritoneal dissemination involves several steps, including invasion, migration, anchorage-independent growth, and proliferation in the peritoneum. Signaling mediated by tyrosine phosphorylation of some transmembrane proteins, including ephrin-B1 and fibro-

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blast growth factor receptor II is reported to be involved in the progression of scirrhous gastric cancer cells.[11,12]

CDCP1 is frequently expressed in gastric cancer cells, and expression and tyrosine phosphorylation levels of CDCP1 are associated with high invasiveness of tumors. These observations led us to examine in this study whether disruption of CDCP1-mediated signaling suppresses invasion and peritoneal dissemination of scirrhous gastric carcinoma. We show in this study that reduction of CDCP1 expression suppresses the migration and anchorage independence of highly invasive gastric cancer cell lines, and inhibits the peritoneal dissemination of these cells. Our results suggest that CDCP1 represents a rational therapeutic target and suppression of its phosphorylation is a strategy for modulating the metastasis of some types of cancers.

Materials and Methods

Plasmids, Antibodies, and Reagents

Plasmids encoding full-length cDNAs of human CDCP1, and the cytoplasmic domain mutant of CDCP1 Y734F (Tyr734 to Phe) have already been described.[10] The pan-Src antibody (Src2) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-Src family (pY416) antibody was purchased from Cell Signaling (Beverly, MA). The FLAG M2 antibody and the α-tubulin antibody were purchased from Sigma (St. Louis, MO). The monoclonal antibody for phosphotyrosine (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody against CDCP1 and tyrosine-phosphorylated CDCP1 [p-CDCP1 (Tyr734)] were obtained from MBL Co., Ltd. (Woburn, MA) as described previously.[10] Fibronectin (bovine), collagen type I, and Matrigel basement membrane matrix were purchased from Sigma, Nitta Gelatin Inc. (Osaka, Japan), and BD Biosciences (San Jose, CA), respectively.

Cell Culture and Transfection

Gastric carcinoma cell lines (HSC-59, HSC-60, 44PE, 44As3, 58, 58As1, 58As9) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. In suspension culture, cells were seeded on a 2-methacryloyloxyethyl phosphorylcholine-coated plate (Nunc, Roskilde, Denmark). For transfection, cells were seeded on a cell culture plate at 1.5×10^5 cells per six-well plate and transfection was performed after 14 hours. Expression plasmids were transfected by Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Short Interfering RNA (siRNA) Treatment

Two sets of siRNAs of CDCP1 were synthesized as follows (Invitrogen). CDCP1 sense no. 1: 5′-UAAUGUUGCUUUCUCGUGGCAGAGC-3′; CDCP1 antisense no. 1: 5′-GCUCUGCCACGAGAAAGCAACAUUA-3′; CDCP1 sense no. 2: 5′-AUAGAUGAGCGGUUUGCAAUGCUGA-3′; CDCP1 antisense no. 2: 5′-UCAGCAUUGCAAACCGCUCAUCUAU-3′. The control siRNA (scramble II duplex: 5′-GCGCGCUUUGUAGGAUUCGdTdT-3′) was purchased from Dharmacon (Lafayette, CO). siRNAs were incorporated into cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Assays were performed 72 hours after treatment.

To generate a stably expressing siRNA system, the BLOCK-iT Pol II miR RNAi expression vector kit (Invitrogen) was used. The target sequence of CDCP1 has been described previously.[10] Cells stably expressing the CDCP1 siRNA (miCDCP1) and the control LacZ siRNA (miLacZ) were established through transfection of miR RNAi vectors that holds these sequence and selection in medium containing blasticidin S (Invivogen) at a concentration of 10 μg/ml.

Western Blotting

Cell lysates were prepared with protease inhibitors in PLC 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 (Na_3VO_4), and 100 μg/ml leupeptin]. Protein concentration was measured by BCA protein assay (Pierce, Rockford, IL). Samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). After the blocking of the membrane with blocking buffer (Blocking One; Nakarai Tesque, Kyoto, Japan), the membrane was probed with antibodies for detection. The membrane was further probed with horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:4000) to visualize the antibody. The images were captured by molecular imager GS-800 (Bio-Rad, Hercules, CA).

Cell Attachment Assay

Cancer cells were detached by phosphate-buffered saline (−) [PBS(-)] containing EDTA (2 mmol/L) and replated onto the chamber slides coated with either collagen type I (100 μg/ml; Nitta Gelatin, Inc.), fibronectin (50 μg/ml, Sigma), or Matrigel (85 μg/ml, BD Biosciences). After incubation for 30 minutes, unattached cells were removed by washing the slides in PBS(−) several times, and the remaining cells were stained with Giemsa's solution. The number of attached cells on each substrate was counted.

Cell Migration Assay

Migration assay was performed using modified transwell chambers with a polycarbonate nucleopore membrane (BD Falcon, Franklin Lakes, NJ). Precoated filters (6.5 mm in diameter, 8-μm pore size, fibronectin 10 μg/ml) were rehydrated with 100 μl of medium. Then, 4×10^4 cells (HSC-59, 44As3, and 58As9) and 8×10^4 cells (HSC-60) in 100 μl of serum-free RPMI 1640 were seeded onto the upper part of each chamber, whereas the lower compartments were filled with 600 μl of the same medium

with 10% fetal bovine serum (FBS). After incubation for 14 hours at 37°C, nonmigrated cells on the upper surface of the filter were wiped out with a cotton swab, and the migrated cells on the lower surface of the filter were fixed and stained with Giemsa's stain solution (azur-eosin-methylene blue solution; Muto Pure Chemical, Co., Tokyo, Japan). The totals of migrated cells were determined by counting cells in five microscopic fields per well at a magnification of]times]100, and the extent of migration was expressed as the average number of cells per microscopic field. Cell migration assay were performed three times.

Matrigel Invasion Assay

Invasion of tumor cells into the Matrigel was monitored as described previously.[13] Gastric cancer cells treated with CDCP1 siRNA or control siRNA for high invasion potential were detached with Hanks' balanced salt solutions ($HBSS^-$) containing 2 mmol/L of EDTA and seeded on the Matrigel (100 $\mu g/cm^2$). After being cultured in the RPIM 1640 medium with 10% FBS for 17 hours, the cells were fixed with 4% paraformaldehyde in PBS and stained with Giemsa's stain solution. The number of invaded cells were counted in five microscopic fields per well at a magnification of ×100, and the extent of invasion was expressed as the average number of cells per microscopic field. Matrigel invasion assays were performed three times.

Soft Agar Colony Assay

Six-well culture plates were coated with a layer of RPMI 1640 and 10% FBS containing 0.5% UltraPure agarose (Invitrogen). Subconfluent 44As3 cells transfected the miR RNAi vector expressing clones were treated with EDTA, washed in PBS twice, and resuspended in RPMI 1640, 10% FBS at 6×10^3 cells/ml. Then, a 500-μl cell sample was added to 1 ml of RPMI 1640, 10% FBS containing 0.5% UltraPure agarose (final 0.33%). 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 using GS-800 calibrated densitometer (Bio-Rad) and the numbers of colonies/well were counted. Soft agar assays were performed three times.

In Vivo *Tumor Cell Invasion Assay*

The animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the guidelines for animal experiments in the National Cancer Center. Peritoneal dissemination of tumors was examined by intraperitoneal injection of 5×10^6 gastric cancer cells suspended in 0.3 ml of RPMI 1640 medium into 6-week-old BALB/c nude mice (Clea Japan, Inc., Tokyo, Japan). The mice ($n = 6$) were sacrificed 2 weeks after injection, and peritoneal dissemination was evaluated. Orthotopic implantation of gastric cancer cells into BALB/c nude mice has been described previously.[11] Briefly, 1×10^6 cells were inoculated into the middle wall of the greater curvature of the glandular stomach using a 30-gauge needle. The mice ($n = 12$) were sacrificed at 15 to 16 days after the orthotopic transplantation of the cancer cells, and the tumors were examined macroscopically. To determine the effect on the tumor growth in nude mice, 44As3 clones (3×10^6 cells/0.3 ml of serum-free medium) were subcutaneously injected into the right flank of mice. Mice were euthanized at 30 days. The results are expressed as the mean weight of tumors (g) from three mice ± SE.

Tissue Samples and Immunohistochemical Analysis

We obtained 10 paraffin-embedded tumor tissues of gastric scirrhous carcinoma in 2006 at the National Cancer Center Hospital. The study population consisted of five men (50%) and five women (50%). Paraffin blocks were sectioned in slices and subjected for immunohistochemical stains using the indirect polymer method with Envision reagent (DAKO, Carpinteria, CA). Antigen retrieval was performed by placing sections in the citrate buffer and heating in a microwave pressure cooker according to the manufacturer's instructions. All sections were incubated with specific antibodies against CDCP1 (rabbit polyclonal antibody; dilution, 1:100) and tyrosine-phosphorylated CDCP1 [p-CDCP1 (Tyr734): rabbit polyclonal antibody; dilution, 1:400].

Results

CDCP1 Affects Migration and Anchorage Independence of Gastric Cancer Cells

To examine the involvement of CDCP1 for progression of tumors, we analyzed cell lines of scirrhous gastric carcinoma. HSC-59, HSC-60, HSC-44PE, and HSC-58 were originally established from the patients of scirrhous gastric carcinoma, and highly invasive sublines were further selected from these parent cells (44As3 from HSC-44PE; 58As1 and 58As9 from HSC-58).[14,15] Expression of CDCP1 was remarkably elevated in highly invasive HSC-44PE, HSC-58 cells, whereas higher expression and phosphorylation levels of CDCP1 was observed in invasive sublines compared with less invasive HSC-59 and HSC-60 cells (Figure 1A).[14]

Cell proliferation, migration, invasion, loss of cell adhesion, and anchorage-independent growth are major functions of the cancer cells required during dissemination. We next examined whether the reduction of CDCP1 affects these functions of gastric cancer cells. The treatment of cells with two independent siRNA of CDCP1 effectively reduced the CDCP1 expression level in 44As3 cells and 58As9 cells (Figure 1B). Proliferation of both cell lines was not significantly affected (Figure 1D). To check whether the expression of CDCP1 affects the cell-extracellular matrix adhesion, the degree of cell attachment to type I collagen, fibronectin, and Matrigel was analyzed.

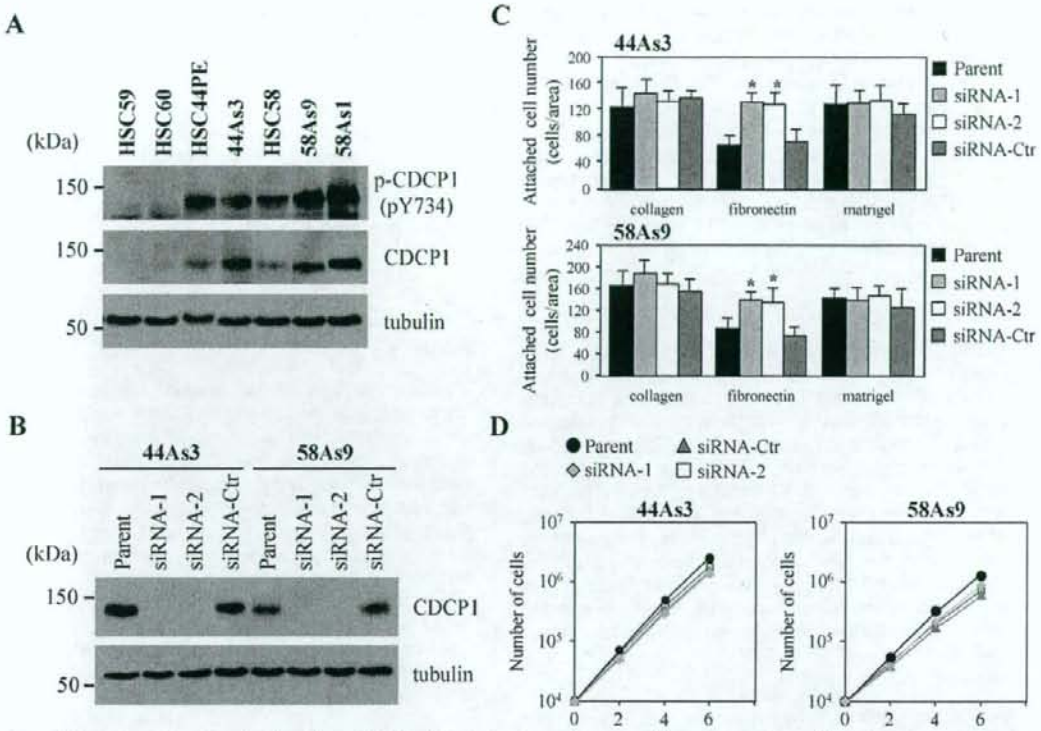

Figure 1. Expression and tyrosine phosphorylation of CDCP1 is higher in invasive gastric cancer cell lines. **A:** Lysates from cells as indicated were subjected to immunoblotting with anti-CDCP1 and anti-phospho-CDCP1 (Tyr734) in each cell lysate. HSC-59 and HSC-60 of noninvasive cell lines and HSC-44PE, 44As3, HSC-58, 58As9, and 58As1 of invasive cell lines were seeded onto each cell culture plate. **B:** Cellular levels of CDCP1 were analyzed 72 hours after treatment with siRNAs by Western blotting using α-tubulin as a loading control. Expression of CDCP1 was reduced in cells treated with CDCP1 siRNA (siRNA-1, -2) compared with siRNA control (siRNA-Ctr) and parental cell (parent). **C:** 44As3 and 58As9 cells treated with CDCP1 siRNA (siRNA-1, -2) or control siRNA (siRNA-Ctr) were detached by EDTA, and replated on the chamber slides coated with either collagen type I (100 μg/ml), fibronectin (50 μg/ml), or Matrigel (85 μg/ml). After incubation for 30 minutes, unattached cells were removed by washing the slides in PBS(−) several times, and the remaining cells were stained with Giemsa's solution. The number of attached cells on each substrate was counted, and the results from three independent experiments, each in duplicate, are shown as the mean ± SD. The **asterisks** indicate differences from the control cells. $^*P < 0.01$. **D:** Cell growth in 44As3 and 58As9 cells were shown by the number of cells. Approximately 1×10^4 cells were seeded onto cell culture plates with medium. The growth medium was changed every 2 days and cell numbers were counted by Coulter particle counter z1 (Beckman).

The number of attached cells was increased in 44As3 and 58As9 cells treated by siRNA of CDCP1 on fibronectin, but reduction of CDCP1 expression did not cause significant change in either cell line on type I collagen and Matrigel (Figure 1C).

Interestingly, reducing the amount of CDCP1 protein by siRNA inhibited migration and invasion of 44As3 and 58As9 cells by *in vitro* transwell assay (Figure 2A). On the other hand, overexpression of CDCP1 in less invasive HSC-59 and HSC-60 cells increased cell migration (Figure 2C). In this case, Y734F mutant of CDCP1, which lacks the phosphorylation site by SFKs[5,10] did not affect cell migration (Figure 2, B and C). These results suggest that signaling mediated by tyrosine phosphorylation of CDCP1 regulates the invasion of gastric cancer cells via cell migration.

We recently found that CDCP1 protein regulates anchorage-independent growth of lung adenocarcinoma in a tyrosine phosphorylation-dependent manner.[10] Tyrosine phosphorylation of CDCP1 was also maintained in the suspension culture of gastric cancer cell lines (Figure 3A), therefore we checked whether CDCP1 can regulate the anchorage-independent growth of gastric cancer cells. We established stable clones of 44As3 cells, miCDCP1-1 and miCDCP1-2, in which the expression of CDCP1 protein was suppressed by siRNA (Figure 3B). Both of these two clones formed significantly fewer colonies in soft agar compared with control miLacZ clones (Figure 3C), suggesting that CDCP1 is also required for the anchorage independence of highly invasive gastric cancer cells.

Reduction of CDCP1 Expression Attenuates Dissemination of Gastric Cancer Cells

Gastric scirrhous carcinoma frequently associates with peritoneal dissemination through a process by which cancer cells perforate the gastric serosa and become exfoliated and free, then attach on the surface of the

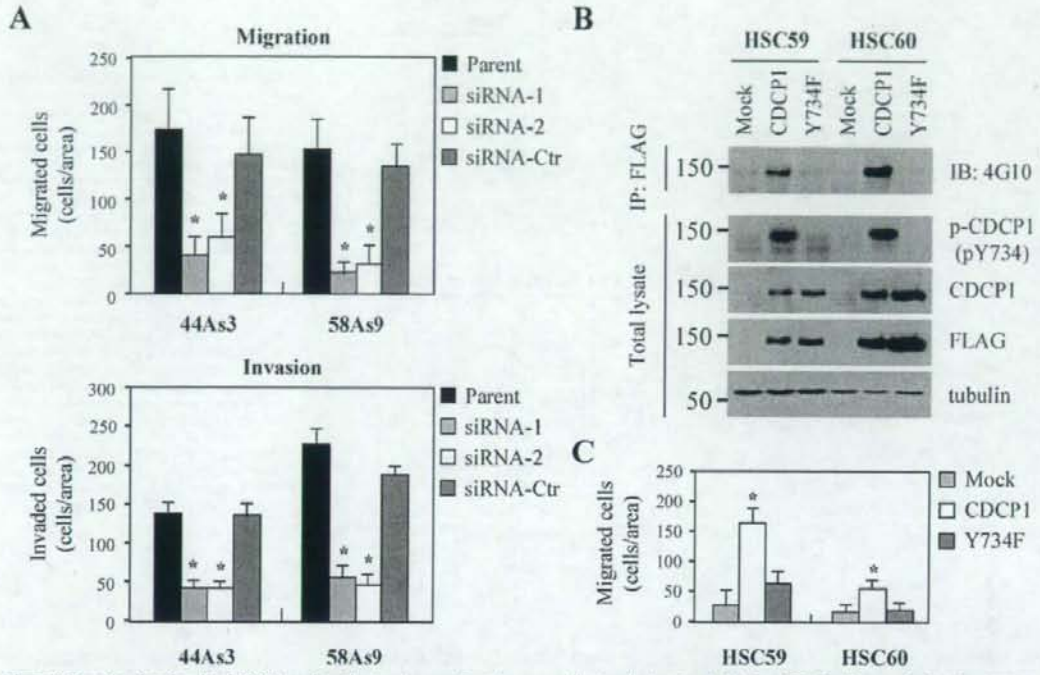

Figure 2. Tyrosine-phosphorylated CDCP1 regulates the invasiveness of gastric cancer cells via cell migration. **A:** 44As3 and 58As9 cells treated with either CDCP1 siRNA (siRNA-1, -2), control siRNA (siRNA-Ctr), or untreated parent cells (parent) were plated onto a Transwell membrane in serum-free medium. In the lower chamber, medium containing 10% FBS was added as a chemoattractant. After 14 hours of incubation for migration and 17 hours for invasion, the wells were harvested and cells that migrated to the lower surface of the membrane were counted. The results from three independent experiments, each in duplicate, are shown as the mean values ± SD. The **asterisks** indicate differences from the cells treated with control siRNA. *$P < 0.01$. **B:** CDCP1 or CDCP1 mutant (Y734F) tagged with FLAG were transiently transfected in HSC-59 and HSC-60 cells of noninvasive potential. After 24 hours, cells were treated EDTA and collected. The lysates were subjected to immunoblotting with the indicated antibody or immunoprecipitation of total CDCP1 and Y734F using FLAG M2 antibody and immunoblotting with anti-phosphotyrosine antibody (4G10). **C:** The transiently transfected cells as indicated were seeded onto a Transwell membrane in serum-free medium. In the lower chamber, medium containing 10% FBS was added. After 12 hours of incubation, the wells were harvested, and cells that migrated to the lower surface of the membrane were counted. The results from three independent experiments, each in duplicate, are shown as the mean values ± SD. The **asterisks** indicate differences from the cells transfected with mock. *$P < 0.01$.

peritoneum and start to invade. We further examined the effect of CDCP1 on tumor progression *in vivo* using a model system of peritoneal dissemination.[14,15] When control clones (miLacZ-1, -2) were injected intraperitoneally into nude mice, severe carcinomatous peritonitis was observed in miLacZ clones as previously described (Figure 4A; a–c).[15] Innumerable whitish nodules were observed in the mesentery of almost all mice injected with miLacZ clones (Figure 4Ac). In addition, many tumor nodules of miLacZ clone were observed in the peritoneal cavity, including the omentum (Figure 4Ac). On the other hand, when the miCDCP1 clones (miCDCP1-1, -2) in which the expression of CDCP1 is stably suppressed by siRNA, was injected, dissemination of cancer cells was apparently modest (Figure 4A; d–f). Large numbers of tumor nodules of control clones were observed in the mesentery, whereas, miCDCP1-1 and miCDCP1-2 clones formed smaller numbers of colonies in the mesentery (Figure 4B). On the contrary, no significant change in the tumor growth was observed in any 44As3 clone subcutaneously implanted in nude mice (Figure 4C).

To further evaluate the effect of CDCP1 on the process of tumor invasion and dissemination, we orthotopically implanted gastric cancer cells in the gastric submucosa of nude mice. At 15 to 16 days after implantation, 67% of mice injected with miLacZ clones formed a tumor mass involving the omentum. On the other hand, such local invasion into the omentum was less frequently observed (17%) in mice implanted with miCDCP1 clones (Table 1). The dissemination and invasion of miLacZ clones was observed in several tissues including mesenteric sheets and liver surface (67% and 58%). However, we rarely observed cancer dissemination in mesenteric sheets and invasion in liver surface in mice implanted with miCDCP1 clones (17% and 17%) (Figure 5A, Table 1). Additionally, gastric cancer cells in mice hepatic stroma were checked by histological analysis. None of the cancer cells was detected in hepatic stroma when there were no nodules in the liver surface (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). These results suggest that CDCP1 is required for peritoneal dissemination and invasion of scirrhous gastric carcinomas. Stromal fibrosis is an important

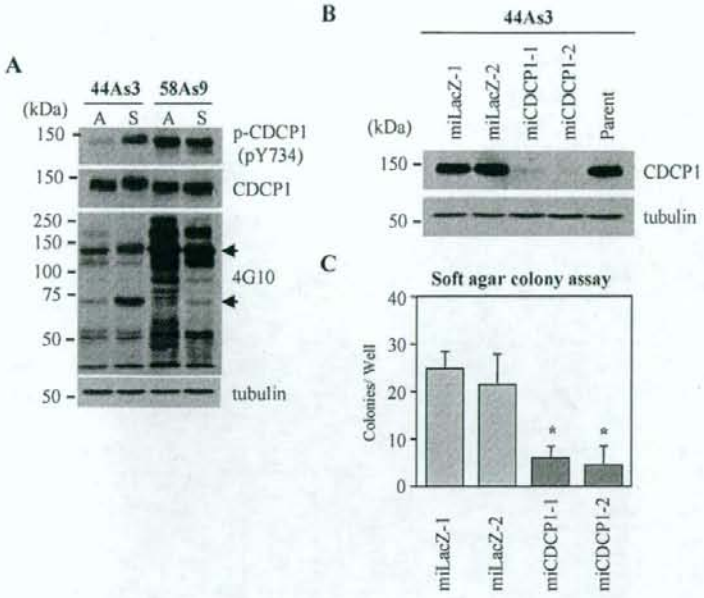

Figure 3. CDCP1 confers anchorage independence in highly invasive gastric cancer cells. **A:** 44As3 and 58As9 cells of highly invasive potential cultured for 24 hours in both adhesion and suspension condition were collected and subjected to immunoblotting with anti-CDCP1, anti-phospho-CDCP1 (Tyr734), or anti-phosphotyrosine (4G10) antibody. A, adhesion; S, suspension. The **black arrow** indicates CDCP1. **B:** CDCP1-defective 44As3 clones (miCDCP-1 and miCDCP1-2) were generated by miR RNAi expression vector kit (Invitrogen). miLacZ-1 and miLacZ-2 were control clones. The expression of CDCP1 in each clone cultured for 24 hours in 2-methacryloyloxyethyl phosphorylcholine-coated plates was examined by Western blotting using CDCP1 antibody. The concentration of total protein in each clone was confirmed by the same membrane rehybridized with anti-α-tubulin antibody. **C:** Each CDCP1-defective clone and control clone was seeded onto soft agar plates (3×10^3 cells). Colonies equal to and larger than 0.5 mm in diameter were counted after 30 days. The results from three independent experiments, each in duplicate, are shown as the mean values ± SD. The **asterisks** indicate differences from the cells with control miLacZ-1. $^*P < 0.01$.

character of scirrhous gastric carcinoma. We examined whether CDCP1 affects stromal fibrosis of scirrhous carcinoma by azan staining, however no significant change of stromal fibrosis was observed in orthotopically implanted gastric cancer cells with or without CDCP1 (Figure 5B).

Tyrosine Phosphorylation of CDCP1 Associates with Invasion of Human Gastric Cancer Cells

When 44As3 cells were intraperitoneally injected, many tumor nodules were observed not only in the mesentery sheets, but also in the parietal and visceral peritoneum, which leads to carcinomatous peritonitis (Figure 6A, dissemination).[11] To further evaluate the involvement of CDCP1 in the formation of peritoneal dissemination of cancer cells, the expression and phosphorylation level of CDCP1 in the disseminated tumor nodules was monitored through immunohistochemical analysis. Antibodies against CDCP1 and against phospho-Tyr734 of CDCP1 revealed that CDCP1 was exclusively expressed and phosphorylated at Tyr734 in the invaded cancer cells, but not in stromal cells derived from mice including fibroblasts (Figure 6A). We and other researchers previously observed that CDCP1 is tyrosine phosphorylated at Tyr734 by SFKs.[5,10] Compared to 44As3 cells cultured *in vitro* under normal tissue plate culture, elevation in the activity of SFK was detected in tumor nodules formed by the same 44As3 cells in nude mice, as judged by the antibody recognizing the phosphorylation of Tyr416 of SFK [Figure 6B; p-Src (pY416)]. Moreover, an outstanding elevation of the phosphorylation level of CDCP1 was observed in 44As3 tumor nodules in nude mice (Figure 6B; 44As3-i.p.−1, −2), suggesting a possible role of CDCP1 phosphorylation in scirrhous carcinoma cells with the peritoneal dissemination.

We further examined the expression and phosphorylation of CDCP1 in human scirrhous gastric cancer tissues by immunohistostaining. Although the expression of CDCP1 was very faintly observed in normal gastric mucosa, it was clearly detected in gastric cancer cells invading the gastric wall (Figure 7). Intense staining of tyrosine-phosphorylated CDCP1 was also observed in cancer cells (black arrowhead), but not in stromal tissues including endothelial cells of veins and proprial muscle (white arrowhead). No detectable signal was observed in the immunohistostaining of cancer cells using the second antibody alone (data not shown). Elevated expression and tyrosine phosphorylation of CDCP1 was observed in ~30% of the scirrhous-type gastric cancer tissues (n = 10) as compared with normal tissue. In addition, the expression of CDCP1 was also observed in some of human nonscirrhous gastric cancer tissue (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Further analysis of the expression and phosphorylation of CDCP1 is required to elucidate the role of CDCP1 in the nonscirrhous gastric cancer.

Discussion

Highly invasive potential is one of the major characteristics of scirrhous carcinoma, which is a major cause of the poor prognosis of this type of cancer. Using two scirrhous gastric cancer cell lines with highly invasive character, we show for the first time that CDCP1 modifies cancer

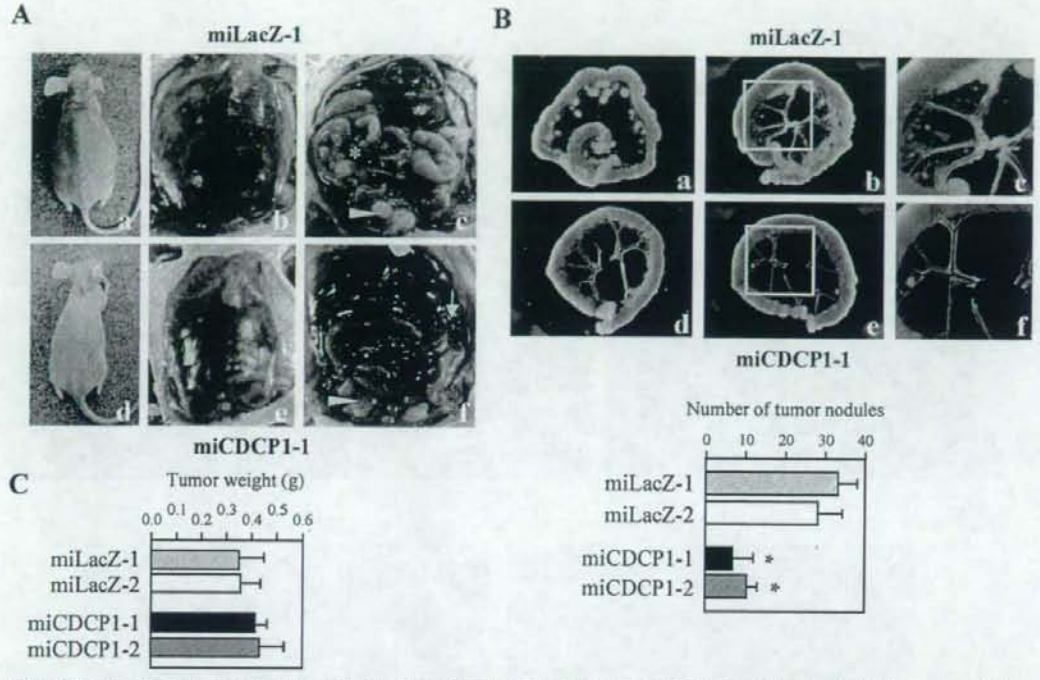

Figure 4. Disruption of CDCP1 expression suppressed the peritoneal dissemination of 44As3 cells. Each CDCP1-defective clone (miCDCP1) and control clone (miLacZ) was injected intraperitoneally into nude mice (5×10^6 cells/mice). **A:** Representative appearance of peritoneal dissemination at 21 days after the injection of miLacZ-1 clone (**a–c**) or miCDCP1-1 clone (**d–f**) is shown. Carcinomatus peritonitis and abdominal distension because of bloody ascites was observed in miLacZ clones (**a** and **b**). **c** and **f:** The **asterisk** indicates dissemination of cancer nodules in the mesentery and **arrows** indicate the tumor mass, including omentum. **Arrowheads** indicate the tumor nodules of cancer cells disseminated around the rectouterine region. **B:** Representative dissected intestinal loops from two mice injected with miCDCP1-1 and miLacZ-1 clones, respectively. **Top:** Representative appearance of peritoneal dissemination 2 weeks after the injection of miLacZ-1 clone (**a–c**) or miCDCP1-1 clone (**d–f**) is shown. **c** and **f** show high magnification of the mesentery in the **middle panels** (**yellow box**). The **red arrow** indicates local invasion of tumor nodules in the mesentery. **Bottom:** Tumor nodules equal to and larger than 1.0 mm diameter were counted after 16 days (n = 6). Error bars indicate the SD. The **asterisks** indicate differences from the cells with control miLacZ-1. $^*P < 0.01$. **C:** The effect of CDCP1 on the tumor growth in nude mice was determined as described under Materials and Methods. The data represent the weight (g) of tumors from miCDCP1 clones and miLacZ clones (n = 3). Error bars indicate the SD.

invasion and dissemination *in vivo*. Disruption of CDCP1 suppressed the migration and anchorage-independent growth of these scirrhous cancer cells. In addition, knockdown of CDCP1 suppressed peritoneal dissemination of both ectopically and orthotopically injected scirrhous cancer cells and invasion of orthotopically implanted scirrhous cancer cells toward the gastric wall and into the liver and the mesentery.

From the original identification, CDCP1 was estimated to be important in the progression of human tumors. Using cDNA chip hybridization techniques to search for genes preferentially expressed in solid tumors relative to normal tissues, Scherl-Mostageer and colleagues[1] identified Est sequences corresponding to CDCP1 cDNA. In another approach to identify tumor-associated proteins, Hooper and colleagues[4] used subtractive immunization techniques to generate antibodies toward cell surface epitopes preferentially expressed by highly metastatic relative to nonmetastatic carcinomas, and identified a cell surface glycoprotein named SIMA135, identical to CDCP1. We recently discovered a novel function of CDCP1 in cancer progression. It was revealed that CDCP1 is a SFK-binding phosphoprotein that regulates the anoikis resistance of human lung adenocarcinoma. In suspension culture, CDCP1-SFK complex is required for the phosphorylation of PKCδ, which was shown to be a key molecule for anoikis resistance downstream of CDCP1 signaling. Moreover, CDCP1 affects the late phase of metastasis of lung adenocarcinoma *in vivo*, possibly through regulation of anoikis resistance.[10]

Scirrhous gastric carcinoma diffusely infiltrates a broad region of the stomach and is frequently associated with metastasis to lymph nodes and peritoneal dissemination. In this study, we found that the expression levels

Table 1. Dissemination at 15 to 16 Days after Orthotopic Implantation of 44As3 Gastric Cancer Cells

Cell	Omentum	Mesenterium	Liver
miLacZ (15 days)	8/12 (67)	8/12 (67)	7/12 (58)
miCDCP1 (16 days)	2/12 (17)	2/12 (17)	2/12 (17)

Number of mice bearing tumor at the site per total number of mice bearing tumor (%).

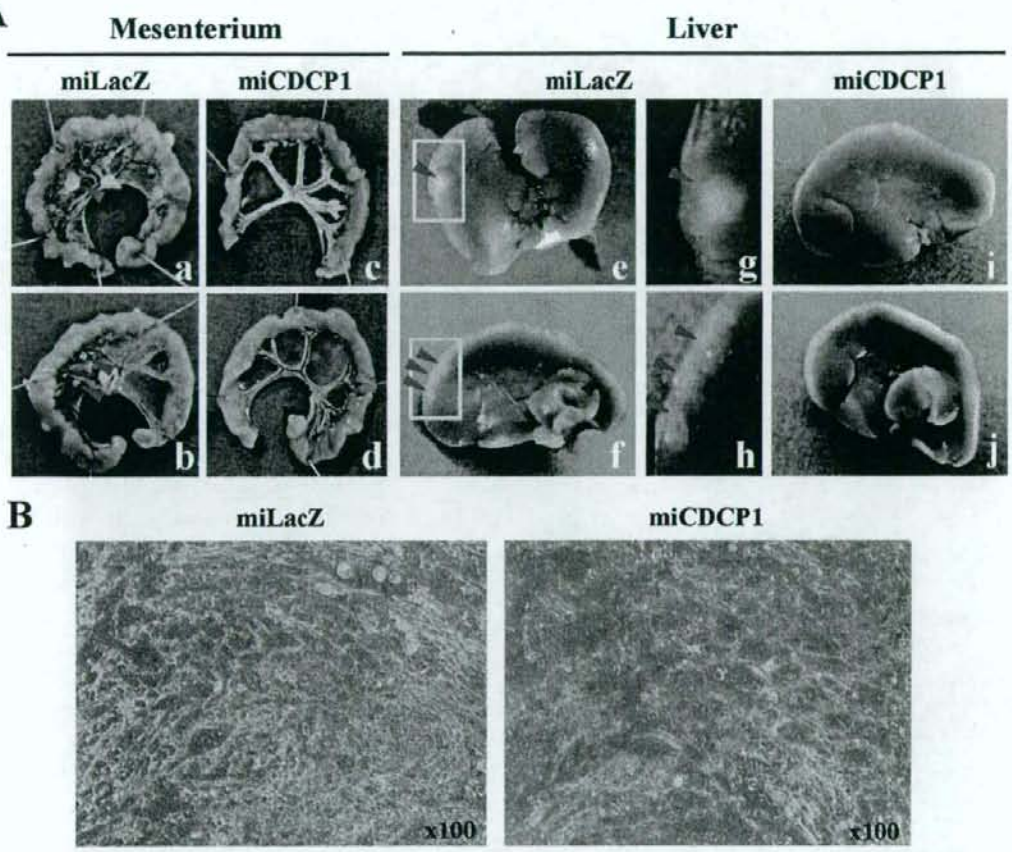

Figure 5. Disruption of CDCP1 suppressed the peritoneal dissemination of orthotopically implanted 44As3 cells. **A:** Each CDCP1-defective clone (miCDCP1) and control clone (miLacZ) was orthotopically implanted in submucosa of the gastric wall ($n = 12$) as described in the Materials and Methods. The representative macroscopic views of dissected organs 15 days (miLacZ) and 16 days (miCDCP1) after implantation including mesenterium (**a–d**) and liver (**e–j**) are shown. **g** and **h** show high magnification of the liver in the **top panels** (**yellow box**). **Red arrow** indicates tumor nodules. **B:** Stromal fibrosis of each miCDCP1 and miLacZ clone orthotopically implanted in mice submucosa was detected by azan staining. The region with stromal fibrosis was observed as blue stain.

of CDCP1 were higher in the cells of invasive scirrhous gastric carcinoma than in the cells of less invasive type (Figure 1A). This expression pattern of CDCP1 prompted us to examine the involvement of CDCP1 in the invasion and dissemination of scirrhous gastric cancer cells.

It was also shown for the first time that CDCP1 regulates cell migration using gastric cancer cells (Figure 2, A and C). Cell migration mediates some of the functions in the process of cancer cell invasion and is generally presumed to be regulated by cell adhesion molecules. For example, integrins bind extracellular matrix and link to the signaling pathway inside the cells[16] and the turnover of integrin contacts may promote cell migration.[17] CDCP1 contains three CUB domains, which are thought to play biological roles by mediating protein-protein interaction, in various molecules such as galectins, DMBT1,[18] sperm adhesine,[19] neuropilin,[20] and proteases including Bmp1/C-proteinase, Tolloid, and MASP.[7] Recently, it is reported that some type of CUB domain binds to fibronectin and modulates interaction of the other matrix components.[21] Moreover, CDCP1 interacts with a number of adhesion and matrix proteins including cadherins and syndecans.[6] It is also proposed that special subsets of integrin affect phosphorylation of CDCP1.[22] Thus, CDCP1 may modulate cell adhesion. Actually, overexpressions of CDCP1 in MDA-468 cells develop a rounded shape and grow in a loose cell adhesion[6] and we show that the loss of CDCP1 increases the attachment of 44As3 and 58As9 cells on fibronectin (Figure 1C). Therefore, CDCP1 might regulate cell migration by affecting the cell adhesion to extracellular matrix in the cell invasion assay *in vitro* and in the process of stromal invasion of tumors *in vivo*.

In this study, the requirement of tyrosine phosphorylation site, Y734, in cell migration, is strongly suggested because wild-type CDCP1, but not Y734 mutant, promoted cell migration when expressed in less invasive gastric cells (Figure 2C). This result may be consistent with the observation that the phosphorylation levels of

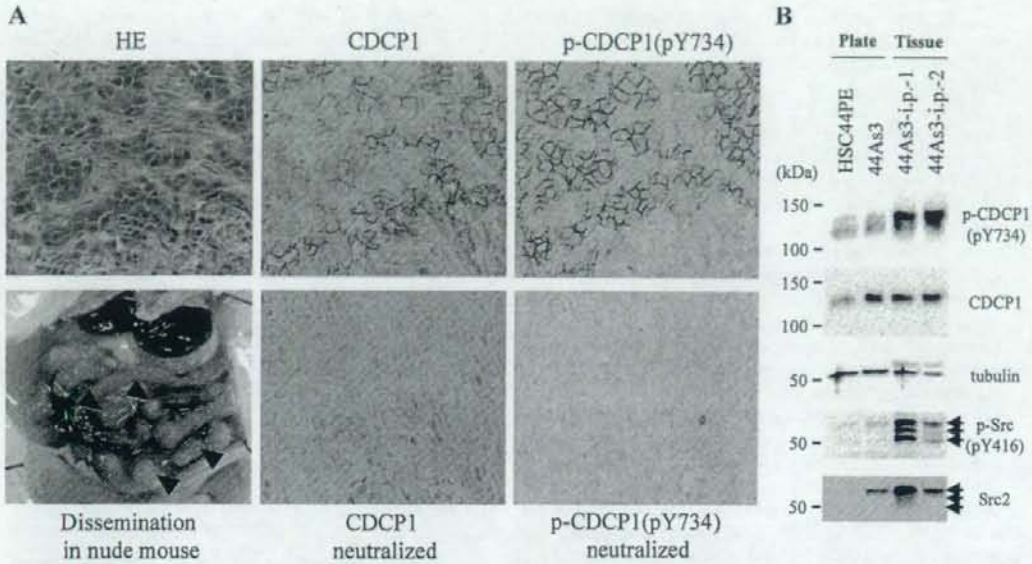

Figure 6. Tyrosine phosphorylation of CDCP1 is up-regulated in the tumor nodules of peritoneal dissemination. **A:** Histology of the tumor nodules 14 days after peritoneal dissemination of 44As3 cells. **Left:** Representative macroscopic views of organs 14 days after implantation are shown (dissemination). **Black arrowheads** indicate the tumor of dissemination in nude mouse. The **left panels** show H&E staining. **Middle:** Staining of tumor nodules using anti-CDCP1 antibody and neutralized with CDCP1 peptide. **Right:** Staining of tumor nodules using anti-phospho-CDCP1 (pY734) antibody and neutralized with phospho-CDCP1 peptide. **B:** 44As3 cells were cultured onto cell culture plate (plate) or injected intraperitoneally into nude mice (tissue). Each lysate was collected and subjected to immunoblotting with anti-CDCP1, anti-phospho-CDCP1 (Tyr734), anti-pan-Src (Src2), anti-phospho-Src family (pY416), or anti-phosphotyrosine (4G10) antibody. α-Tubulin as a loading control. **Black arrows** indicate Src family kinases.

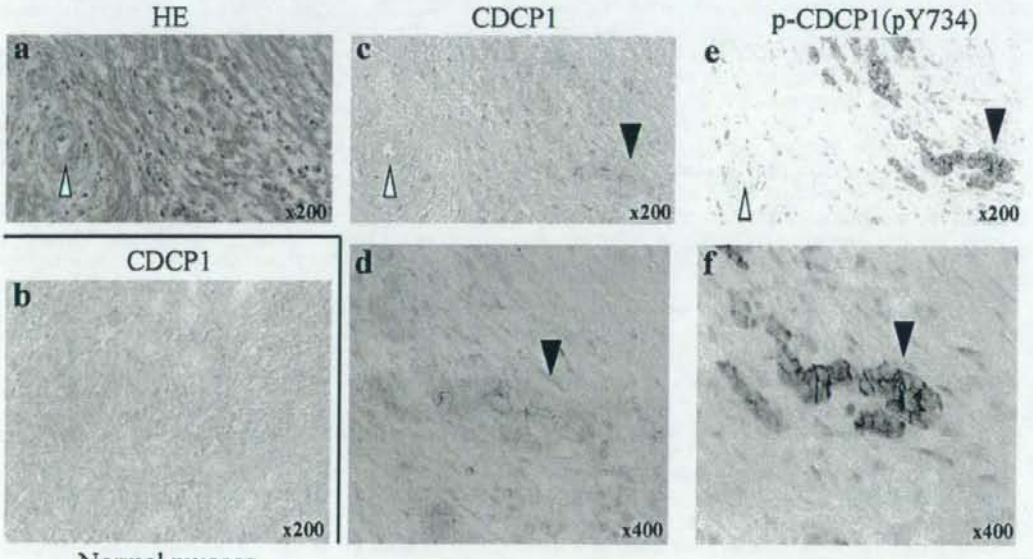

Figure 7. Expression and tyrosine phosphorylation of CDCP1 is detected in the invasive site of human scirrhous tissue. Human scirrhous tissue was stained with H&E (**a**) and either CDCP1 (**c** and **d**) or phospho-CDCP1 [p-CDCP1(pY734)] (**e** and **f**). Normal mucosa was stained with CDCP1 (**b**). **Black arrowheads** indicate the area of invasive tumor and **white arrowheads** indicate blood vessels (**c** and **e**). High magnification of the invasive site of the tumor is shown in **d** and **f**, respectively. Note that expression and phosphorylation of CDCP1 was detected in invasive sites of human scirrhous tissue but not detected in normal tissue including blood vessel (**c–f**). Original magnifications: ×200 (**c**, **e**); ×400 (**d**, **f**).

CDCP1 correlated with the levels of cell motility in gastric cancer cells between 44As3 and 58As9 of invasive type and HSC-59 and HSC-60 of less invasive type (Figure 1A; and Figure 2, A and C). Moreover, phosphorylated CDCP1 was identified in a screen for adhesion-dependent phosphorylation in an epithelial wound model.[22] Based on these findings, tyrosine phosphorylation of CDCP1 might have a critical role in cell migration, although the precise molecular mechanism of regulation needs to be clarified.

We recently reported that tumor cells expressing CDCP1 regulate anchorage-independent growth in lung cancer cells and the phosphorylation of CDCP1 is important for the signal of resistance to detachment-induced apoptosis.[10] CDCP1 is one of the major phosphotyrosine-containing proteins in suspension condition as well in the adherent condition in both gastric cancer cells and in lung cancer cells. Anchorage-independent growth was also regulated by CDCP1 in the invasive gastric cancer cells 44As3 and 58As9 by siRNA experiments (Figure 3, B and C). Several theories have been proposed to explain the mechanism of peritoneal dissemination in human gastric cancer.[23–25] It has been suggested that cancer cells are detached from primary lesions and freed into the peritoneal cavity, to colonize the peritoneum and induce cancerous peritoneum. Anchorage-independent growth is a form of resistance to apoptosis induced by the loss of cell signals generated from interaction with extracellular matrix. Therefore, CDCP1 might be required for cell detachment from primary lesions and survival in the peritoneal cavity of scirrhous gastric cancer cells.

Loss of CDCP1 attenuates invasion and dissemination of highly invasive gastric cancer cells *in vivo* (Figure 4, A and B; and Figure 5A). Moreover, we found that the phosphorylation level of CDCP1 in tumor nodules disseminated to mesentery is much higher than in cells growing in cell culture plate (Figure 6B). These results indicate that cancer cells expressing phosphorylated CDCP1 might be involved in the process of peritoneal dissemination of cancer cells via cell migration and anchorage-independent growth. It might be important to examine the phosphorylation state of CDCP1 *in vivo* in a wide range of cancers other than gastric cancers and lung cancers to determine the organ-specific roles of CDCP1 in cancer progression.

Because early clinical diagnosis of scirrhous gastric carcinoma is difficult, peritoneal dissemination or invasion to lymph nodes has frequently occurred before diagnosis. Histological analysis revealed that expression and phosphorylation of CDCP1 was detected in the invasive site of human scirrhous tissues (Figure 7). It might be useful to examine the expression and phosphorylation level of CDCP1 in a surgical specimen of scirrhous gastric carcinomas to predict the prognosis of the tumors. Dissemination is a frequent form of the recurrence of scirrhous gastric carcinoma, which serves as a major factor determining the prognosis. In addition, our study shows that disruption of CDCP1 suppresses the infiltration of cancer cells into liver and the peritoneal dissemination in animal models. CDCP1 is considered to be a prognosis factor of gastric scirrhous carcinoma, and the inhibition of a specific cellular signal originating from CDCP1 phosphorylation might be a good candidate for regulating its invasion and dissemination.

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ARTICLE
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Expression and Tyrosine Phosphorylation of Crk-Associated Substrate Lymphocyte Type (Cas-L) Protein in Human Neutrophils

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ABSTRACT

Crk-associated substrate lymphocyte type (Cas-L) protein, also known as human enhancer of filamentation 1 (Hef1) or neural precursor cell-expressed, developmentally down-regulated gene 9 (Nedd9), belongs to the Cas family of adapter proteins, which are involved in integrin signaling. Previous reports showed that Cas-L is expressed preferentially in lymphocytes and epithelial cells. Cas-L mediates signals from integrins, T-cell receptors, B-cells receptors, and transforming growth factor beta, leading to cell movement and cell division. Here, we report the expression of Cas-L in neutrophils. Cas-L was tyrosine-phosphorylated when human neutrophils were stimulated by fMLP, tumor necrosis factor-alpha (TNF), or lipopolysaccharide. The tyrosine phosphorylation of Cas-L in fMLP- or TNF-stimulated neutrophils was further enhanced by adhesion of the cells to their substrates. Cas-L was found to be localized at focal adhesions in stimulated neutrophils based on immunofluorescence microscopy. These findings suggest that Cas-L is one of the targets of inflammatory cytokines and is also modulated by cell adhesion process in neutrophils. J. Cell. Biochem. 105: 121–128, 2008.

KEY WORDS: Cas-L; fMLP; Hef1; LIPOPOLYSACCHARIDE; Nedd9; NEUTROPHIL; TUMOR NECROSIS FACTOR ALPHA

The Cas (Crk-associated substrate) family of proteins are docking proteins that mediate integrin-initiated signal transduction pathways [O'Neill et al., 2000; Chodniewicz and Klemke, 2004; Defilippi et al., 2006]. The family consists of $p130^{Cas}$, Cas-L/Hef1/Nedd9, and Efs/sin (embryonal Fyn substrate/Src-interacting protein). They have characteristic primary structure: an N-terminal SH3 domain, a substrate domain that contains multiple YXXP motifs, an Src binding sequence, and a C-terminal helix-turn-helix domain. The founding member, $p130^{Cas}$, was originally cloned as a major tyrosine-phosphorylated protein in v-*crk*-transformed cells [Sakai et al., 1994] and was subsequently shown to mediate integrin signals, actin reorganization, cell migration, anoikis, bacterial infection, and cancer progression [Defilippi et al., 2006].

Cas-L/Hef1/Nedd9 was cloned because it is one of the proteins that are down-regulated during neural development (Nedd9 = neural precursor cell-expressed, developmentally down-regulated gene 9) [Kumar et al., 1992], it is a human protein that elicits filamentous budding in yeast (Hef1 = human enhancer of

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filamentation) [Law et al., 1996], and is a 105-kDa tyrosine-phosphorylated protein in lymphocytes (Cas-L = Crk-associated substrate in lymphocytes) [Minegishi et al., 1996].

In contrast to the ubiquitous expression of $p130^{Cas}$ among adherent cells, Cas-L is preferentially expressed in epithelial cells and lymphocytes. In adherent cells, Cas-L is involved in integrin signaling, transforming growth factor beta (TGF-β) signaling, proteasomal regulation, apoptosis, cell migration, and cell division [Law et al., 1996, 1998; Zheng and McKeown-Longo, 2002; Feng et al., 2004; Pugacheva and Golemis, 2005]. In hematological cells, most studies of Cas-L have focused on the role of Cas-L in lymphocytes. Cas-L is tyrosine-phosphorylated by β1 integrin stimulation and CD3 stimulation in T cells, leading to interleukin-2 production [Minegishi et al., 1996; Kanda et al., 1997; Iwata et al., 2002]. In B cells, Cas-L is tyrosine phosphorylated by β1 integrin ligation, BCR signaling, and CXCL12 signaling [Astier et al., 1997a,b; Seo et al., 2005]. Furthermore, Cas-L-deficient mice showed a defect in marginal B-cell development and a cell migration defect in both B and T cells [Seo et al., 2005, 2006].

Neutrophils are components of natural immune system and form the first line defence against invading bacteria and fungi. Various stimuli including chemokines, inflammatory cytokines, and toll like receptor ligands activate neutrophils. Especially, fMLP, TNF-a, and lipopolysaccharide elicit the activation of many signaling pathways leading to the effector functions of neutrophils. Focal adhesion proteins such as paxillin, focal adhesion kinase (FAK), and PYK2, are already reported to be phosphorylated and to play important roles in stimulated neutrophils [Graham et al., 1994; Fuortes et al., 1999; Ryu et al., 2000]. However, the expression of Cas-L in neutrophils has only been suggested in a few microarray analyses [Subrahmanyam et al., 2001; Theilgaard-Monch et al., 2005] and Cas family proteins in neutrophils have not been analyzed further. Therefore, we examined the expression of Cas-L protein in neutrophils and the tyrosine phosphorylation of Cas-L in response to fMLP, TNF-α, or lipopolysaccharide.

MATERIALS AND METHODS

REAGENTS

Ficoll and the enhanced chemiluminescence (ECL) Western blotting system were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Conray was purchased from Mallinckrodt (St. Louis, MO). Rabbit polyclonal antibodies against p38 MAP kinase, Tyr^{401}-phosphorylated $p130^{Cas}$, and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody against $p130^{Cas}$ was purchased from BD-Transduction Laboratories (San Jose, CA). Mouse monoclonal antibody against Hef1/Cas-L was purchased from Immuquest (Cleveland, UK). Goat anti-mouse IgG antibody conjugated with alkaline phosphatase was purchased from Invitrogen (Carlsbad, CA). Phospho-specific antibodies to Cas (αCas-pYDVP, αCas-pYDpYV) were generated as described previously [Huang et al., 2006]. Anti-CD18 (L130) antibody and normal mouse IgG1κ were purchased from BD-Pharmingen (San Jose, CA).

AlexaFluor 546-conjugated phalloidin was purchased from Invitrogen.

PREPARATION OF NEUTROPHILS

Human neutrophils and mononuclear cells (PBMC) were prepared from cells of healthy adult donors using dextran sedimentation, centrifugation with Conray-Ficoll, and hypotonic lysis of contaminated erythrocytes as described previously [Suzuki et al., 1999]. Neutrophil fractions contained >95% neutrophils. PBMC fractions contained 75–85% lymphocytes, 15–25% monocytes, and <1% neutrophils. Lymphocytes were further purified from mononuclear cells by centrifugal elutriation in a Hitachi SRR6Y elutriation rotor (Hitachi, Tokyo, Japan) [Yuo et al., 1992]. Cells were suspended in Hanks balanced salt solution (HBSS) containing 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid (HEPES) at pH 7.4.

WESTERN BLOTTING

Cells were suspended in HBSS containing 10 mM HEPES (pH 7.4) and treated with fMLP for various times at 37°C. The reactions were terminated by adding 10% by volume of trichloroacetic acid (Wako, Tokyo). After 1 h of incubation on ice, the cells were centrifuged and the pellet was washed twice with 10 mM dithiothreitol/acetone. The pellet was resuspended in 1.3× sample buffer (4% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol, and a trace amount of bromophenol blue dye in 125 mM Tris aminomethane hydrochloride, pH 6.8), sonicated, heated at 100°C for 5 min, and frozen at −20°C until use. Samples were subjected to 10% SDS gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane in a buffer containing 100 mM Tris, 192 mM glycine, and 20% methanol at 2 mA/cm^2 for 1.5 h at 25°C. Residual binding sites on the membrane were blocked by incubating the membrane in Tris-buffered saline (pH 7.6) containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) and 5% nonfat dried milk for 2 h at 25°C. The blots were washed in Tris-buffered saline containing 0.1% Tween 20 (TBST) and then incubated with the appropriate antibody overnight at 4°C. After three washings with TBST, the membrane was incubated with anti-rabbit immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase or alkaline phosphatase, and the antibody complexes were visualized using the ECL detection system (Amersham) or NBT/BCIP (Promega) as directed by the manufacturer. All the experiments were repeated to confirm the reproducibility.

IMMUNOFLUORESCENCE MICROSCOPY

Actin organization was analyzed using confocal laser scanning microscopy. Neutrophils (5×10^6 ml^{-1}) suspended in HBSS were treated with fMLP on FCS-coated glass cover slips at 37°C for 5 min. After incubation, cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). Cells were blocked with 5% bovine serum albumin in PBS at 4°C. Cells were then incubated with αCas-pYDVP or αCas-pYDpYV at room temperature for 3 h. Cells were washed three times with PBS and incubated with AlexaFluor 546-conjugated phalloidin (0.2 $U ml^{-1}$) and AlexaFluor 488-conjugated goat anti-rabbit

antibody (Molecular Probes) in the dark at room temperature for 30 min. Fluorescence images were photographed using a confocal laser scanning microscope (Zeiss LSM 510, Welwyn, Garden City, UK).

RESULTS

Cas-L IS EXPRESSED IN HUMAN NEUTROPHILS

We examined the expression of Cas-L in human neutrophils using the monoclonal antibody from BD-Transduction Laboratories that reacts with both $p130^{Cas}$ and Cas-L (Fig. 1A). After the preparation of cells, the cells were fixed with trichloroacetic acid [Kobayashi et al., 2002] to prevent the artificial degradation of proteins by highly active proteases in neutrophils during the lysis procedure [Kato et al., 2004]. This was done because we could not detect bands of Cas family proteins after the neutrophils were lysed in the RIPA buffer with protease inhibitors (PMSF and leupeptin) nor directly in the 1.3× sample buffer (data not shown).

K562 cells expressed $p130^{Cas}$, but not Cas-L as previously noted [Minegishi et al., 1996] (Fig. 1A, lane 1). Lymphocytes and mononuclear cells expressed Cas-L as a doublet of p105Cas-L and p115Cas-L and expressed a trace amount of $p130^{Cas}$ (Fig. 1A, lanes 2,3). In contrast, neutrophils only expressed only p105Cas-L, and the expression of $p130^{Cas}$ was not detected (Fig. 1A, lane 4). We also used Cas-L/Hef1-specific monoclonal antibody from Immunoquest, which does not cross-react with $p130^{Cas}$. This antibody did not detect $p130^{Cas}$ in K562 cells (Fig. 1B, lane 1), but did detect bands corresponding p105Cas-L and p115Cas-L (Fig. 1B, lanes 2–4). Thus, human neutrophils express p105Cas-L, but do not express p115Cas-L nor $p130^{Cas}$.

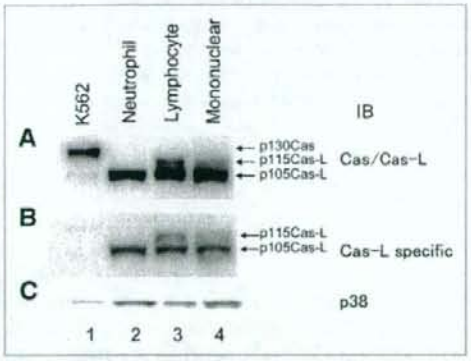

Fig. 1. Expression of Cas-L in human neutrophils. Lysates from K562 cells, human neutrophils, human lymphocytes, and human mononuclear cells were elctrophoresed and transferred to nitrocellulose membrane. A: Immunoblotting with anti-Cas/Cas-L antibody (BD Transduction) that reacts with both p130Cas and Cas-L. B: Immunoblotting with Cas-L-specific antibody (Immunoquest). C: Immunoblotting with anti-p38 antibody.

TYROSINE PHOSPHORYLATION OF Cas-L IN fMLP-STIMULATED NEUTROPHILS

We examined the tyrosine phosphorylation of Cas-L in human neutrophils stimulated by fMLP (fMLF), a bacteria-derived chemokine for neutrophils. Because Cas family proteins including Cas-L, are implicated in the adhesion process [Nojima et al., 1995; Minegishi et al., 1996], we examined the tyrosine phosphorylation of Cas-L in the adhesion or in suspensions of neutrophils. We used phospho-specific anti-Cas antibodies because immunoprecipitation was not possible after the fixation of cells using trichloroacetic acid (TCA). Three types of anti-phospho-Cas antibody were used. The anti-pY410 phospho-Cas antibody (Cell Signaling) detects the phosphorylated YAXP sequence found in both $p130^{Cas}$ and Cas-L. The αCas-pYDXP antibody [Huang et al., 2006] was raised against the pYDXP sequences, which are the binding consensus sequences for the Crk SH2 domain [Songyang et al., 1993; Sakai et al., 1994] and exist multiply in the substrate domain of Cas family proteins. The αCas-pYDpYV antibody was raised against the pYDpYV sequence, which is conserved in $p130^{Cas}$ and Cas-L proteins and is the binding site for the Src SH2 domain [Nakamoto et al., 1996; Tachibana et al., 1997].

When neutrophils were allowed to adhere to an FBS-coated dish for 30 min and were then stimulated by fMLP, the tyrosine phosphorylation of Cas-L detected by anti-pYAXP was prominent 1 min after fMLP stimulation, peaked from 5 to 20 min and declined after 40 min (Fig. 2A), although the time course varied somewhat among donor individuals (data not shown). The αCas-pYDpYV antibody similarly detected phosphorylation of Cas-L. In contrast, phosphorylation detected by the αCas-pYDpYV antibody peaked 1 min after fMLP stimulation and began to decline after 5 min (Fig. 2A).

When we stimulated neutrophils in suspension, the tyrosine phosphorylation of Cas-L was detected 1 min after stimulation, declining rapidly thereafter (Fig. 2A), and the phosphorylation was not as prominent as found for adhesion, indicating that the signal from adhesion enhanced the persistence and extent of Cas-L phosphorylation.

The tyrosine phosphorylation of Cas-L was dependent on the concentrations of fMLP in both the suspension and adhesion conditions (Fig. 2B). The tyrosine phosphorylation of Cas-L in suspension increased with the dosage of fMLP up to 10^{-5} M (Fig. 2B). In contrast, the tyrosine phosphorylation of Cas-L in adhesion increased up to the concentration of 10^{-6} M and further increase was not detected at 10^{-5} M (Fig. 2B).

We also examined the effect of neutrophil adhesion to various extracellular matrix proteins on the fMLP-induced tyrosine phosphorylation of Cas-L (Fig. 2C). Neutrophils express LFA-1, Mac-1, α4β1 integrin, and α2β1 integrins. FBS contains ligands for LFA-1, Mac-1, and α4β1 integrins, but fibrinogen can only serve as a ligand for Mac-1, and fibronectin serves as a ligand for Mac-1 and α4β1 integrins [Heit et al., 2005]. In contrast, type I collagen serves as a ligand for LFA-1 and α2β1 integrins [Garnotel et al., 1995]. Fibrinogen and fibronectin supported the fMLP-induced tyrosine phosphorylation of Cas-L as well as did FBS, but the tyrosine phosphorylation on collagen was weaker (Fig. 2C).

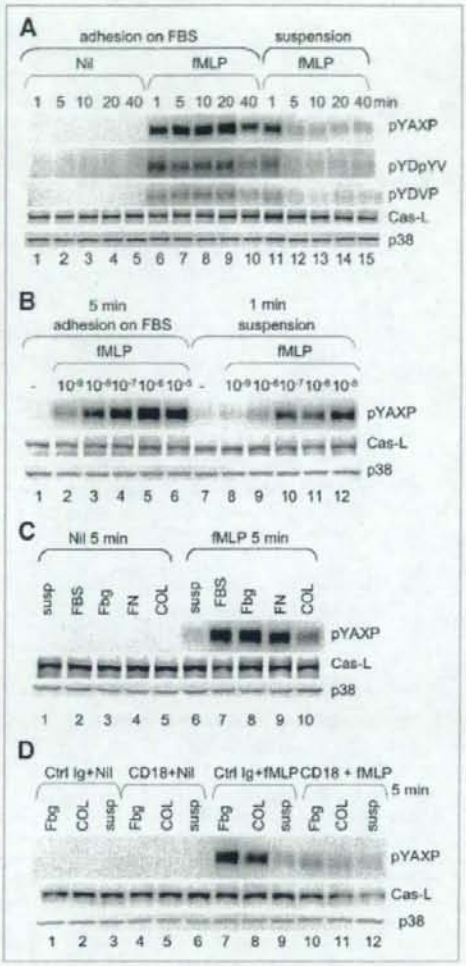

Fig. 2. Tyrosine phosphorylation of Cas-L in fMLP-stimulated neutrophils. A: Human neutrophils either adherent to an FBS-coated dish or in suspension were stimulated with 10^{-7} M fMLP at 37°C for the indicated times. B: Neutrophils adherent to the FBS-coated dish were stimulated for 5 min and neutrophils in suspension were stimulated for 1 min with the indicated concentrations of fMLP at 37°C. C: Neutrophils in suspension or adherent to the dishes coated with the indicated substrates were stimulated with 10^{-7} M fMLP at 37°C for 5 min. D: Neutrophils in suspension or adherent to the dishes coated with fibrinogen or collagen were stimulated with 10^{-7} M fMLP at 37°C for 5 min in the presence of either anti-CD18 or control murine IgG1κ. The tyrosine phosphorylation and expression of Cas-L was analyzed by immunoblotting using antibodies against the phosphorylated form of Cas and against total Cas. The expression of p38 was examined as a loading control. Cell lysates equivalent to 2.5×10^5 cells were loaded onto each lane.

To further clarify the mechanisms of the tyrosine phosphorylation of Cas-L, we inhibited β2 integrin using anti-CD18 blocking antibody. Blocking β2 integrin reduced the tyrosine phosphorylation of Cas-L in fMLP-stimulated neutrophils on fibrinogen and on collagen to the level comparable to that in suspension (Fig. 2D).

TYROSINE PHOSPHORYLATION OF Cas-L IN TNF-α-STIMULATED NEUTROPHILS

We investigated the tyrosine phosphorylation of Cas-L in human neutrophils stimulated with tumor necrosis factor-alpha (TNF-α). Isolated neutrophils were allowed to adhere to an FBS-coated dish and were stimulated with 10 U ml^{-1} TNF-α. Neutrophils were fixed with TCA at each sampling time and were then analyzed. Tyrosine phosphorylation of Cas-L detected by the anti-pYAXP antibody (anti-p-Cas-L antibody) was observed 10 min after stimulation, peaked after 20 min, and persisted for more than 90 min (Fig. 3A). We then examined the dose-response of tyrosine phosphorylation of Cas-L induced by TNF-α-stimulation. The phosphorylation was most prominent at a concentration of 10 U ml^{-1} TNF-α and declined when neutrophils were stimulated with 100 U ml^{-1} or at 1,000 U ml^{-1} of TNF-α (Fig. 3B). The tyrosine phosphorylation of

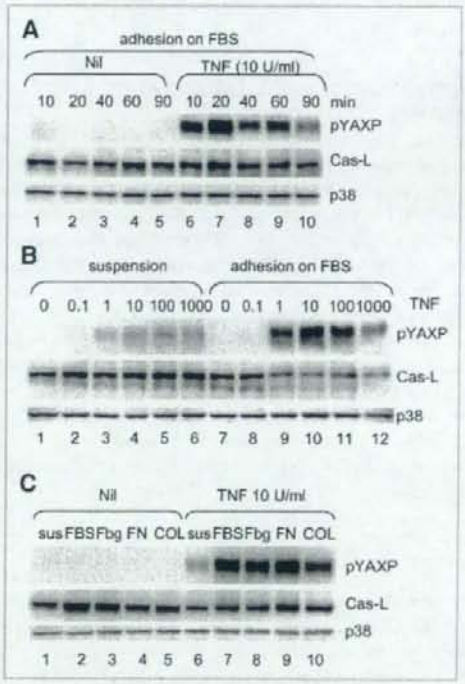

Fig. 3. Tyrosine phosphorylation of Cas-L in TNF-α-stimulated neutrophils. A: Human neutrophils adherent to an FBS-coated dish were stimulated with 10 U ml^{-1} TNF-α at 37°C for the indicated times. B: Neutrophils in suspension or adherent to an FBS-coated dish were stimulated with the indicated concentrations of TNF-α at 37°C for 20 min. C: Neutrophils in suspension or adherent to the dishes coated with the indicated substrates were stimulated with 10 U ml^{-1} TNF-α at 37°C for 20 min.

suspension (Fig. 3B). We also examined TNF-α-induced tyrosine phosphorylation of Cas-L when neutrophils were placed on various extracellular matrix proteins. Fibrinogen and fibronectin supported the TNF-induced tyrosine phosphorylation of Cas-L as well as did FBS, but tyrosine phosphorylation on collagen was weaker (Fig. 3C).

TYROSINE PHOSPHORYLATION OF Cas-L IN LPS-STIMULATED NEUTROPHILS

We examined whether Cas-L is also tyrosine phosphorylated by stimulation with lipopolysaccharide (LPS), a Toll-like receptor ligand. The tyrosine phosphorylation of Cas-L was detected after 40 min of stimulation with 1 $\mu g\,ml^{-1}$ LPS and declined thereafter (Fig. 4A). The tyrosine phosphorylation of Cas-L was weaker when neutrophils were stimulated with 100 $ng\,ml^{-1}$ LPS (Fig. 3E), but the phosphorylation of Cas-L did not increase even when neutrophils were stimulated with >1 $\mu g\,ml^{-1}$ LPS (Fig. 4B). In contrast to the phosphorylation in response to fMLP and TNF-α, LPS-induced phosphorylation of Cas-L was prominent in suspension and was not enhanced when neutrophils were allowed to adhere on an FBS-coated dish (Fig. 4B). However, neutrophils that adhered to the extracellular matrix proteins, fibronectin and collagen showed slightly reduced tyrosine phosphorylation in response to LPS stimulation (Fig. 4C).

PHOSPHORYLATED Cas-L IS LOCALIZED TO FOCAL ADHESIONS IN NEUTROPHILS

We investigated the localization of phosphorylated Cas-L in LPS- or fMLP-stimulated adherent neutrophils (Fig. 5D–L). Unstimulated neutrophils kept in suspension were used as controls (Fig. 5A–C). αCas-pYDXP or αCas-pYVpYD was used to visualize the localization of phosphorylated Cas-L in neutrophils. The phosphorylated Cas-L in the LPS- or fMLP-stimulated neutrophils stained in a dot-like pattern at the edge of adhesion sites (Fig. 5E,H,K) and co-localized with vinculin, a focal adhesion marker (Fig. 5D), or with F-actin (Fig. 5G). However, when the leading edge was formed from a gross actin bundle, Cas-L was localized in dot-like structures both under the leading edge and in the tail region, and did not colocalize with actin bundle. This suggests that the localization of Cas-L was not affected by the formation of a leading edge (Fig. 5J–L). Phosphorylated Cas-L was not observed in unstimulated neutrophils (Fig. 5A–C).

DISCUSSION

We demonstrated that: neutrophils expressed p105CasL, but not p115Cas-L nor $p130^{Cas}$; Cas-L was tyrosine-phosphorylated in response to fMLP, TNF-α, and LPS in neutrophils; tyrosine phosphorylation of Cas-L in neutrophils was enhanced by adhesion; and tyrosine-phosphorylated Cas-L was localized to focal adhesions in neutrophils.

The roles of Cas-L in lymphocytes have been investigated extensively [Minegishi et al., 1996; Kanda et al., 1997, 1999; Tachibana et al., 1997; Astier et al., 1997b; Ohashi et al., 1998, 1999; Kamiguchi et al., 1999; Hogg et al., 2003; Iwata et al., 2005; Seo et al., 2005], but the expression of Cas-L in phagocytes has only been

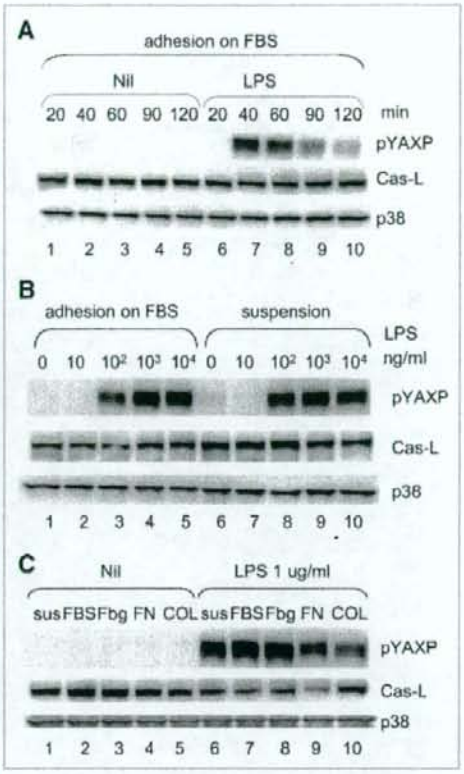

Fig. 4. Tyrosine phosphorylation of Cas-L in LPS-stimulated neutrophils. A: Human neutrophils adherent to an FBS-coated dish were stimulated with 1 $\mu g\,ml^{-1}$ LPS at 37°C for the indicated times. B: Neutrophils in suspension or adherent to the FBS-coated dish were stimulated with the indicated concentrations of LPS at 37°C for 40 min. C: Neutrophils in suspension or adherent to the dishes coated with the indicated substrates were stimulated with 1 $\mu g\,ml^{-1}$ LPS at 37°C for 40 min.

reported in monocyte-lineage cells (osteoclasts) [Zhang et al., 2002]. In one microarray analysis [Subrahmanyam et al., 2001], mRNA for Cas-L was up-regulated when neutrophils were exposed to bacteria. In another microarray analysis [Theilgaard-Monch et al., 2005], mRNA for Cas-L increased with the differentiation of granulocytes. However, the expression of Cas-L at the protein level has not previously been reported. Cas-L is vulnerable to proteases as already reported for other cell types [Law et al., 2000; O'Neill and Golemis, 2001; Nourry et al., 2004; Zheng and McKeown-Longo, 2006] and neutrophils are rich in proteases, which often cause the artificial degradation of proteins during the preparation of cell lysates [Kato et al., 2004]. We could not detect the expression of Cas-L when neutrophils were lysed with RIPA buffer nor with 1.3× sample buffer. Instead, we detected Cas-L only after neutrophils were fixed with trichloroacetic acid (TCA) to inactivate proteases (Fig. 1). This may be one reason why Cas-L has not previously been reported in neutrophils at the protein level. Fixation with trichloroacetic acid is

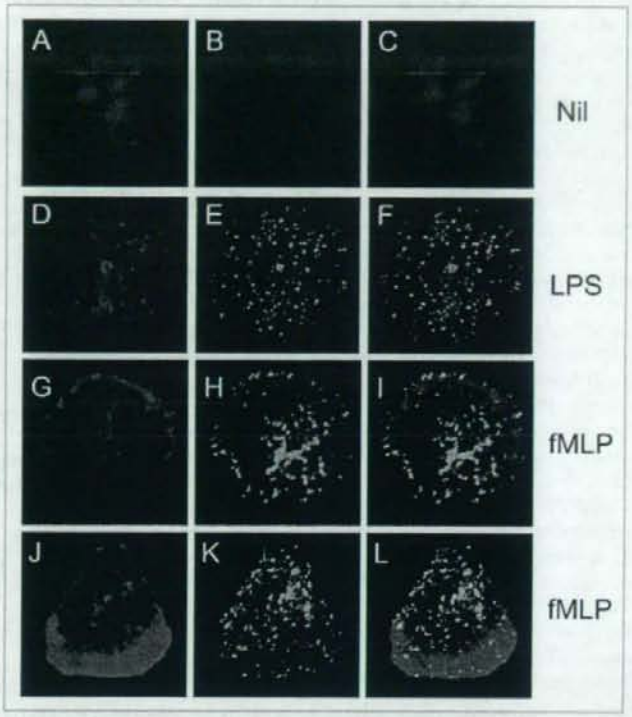

Fig. 5. Subcellular localization of phosphorylated Cas-L in neutrophils. Unstimulated human neutrophils were kept in suspension (A–C). Human neutrophils adherent to a fibrinogen-coated dish were stimulated with 1 $\mu g\ ml^{-1}$ LPS for 40 min (D–F). Human neutrophils adherent to an FBS-coated dish were stimulated with 10^{-7} M fMLP for 5 min (G–L). Cells were fixed with 4% paraformaldehyde and were stained with anti-pYDpYV (B,E,H) or anti-pYDXP(K), together with anti-vinculin (D) or phalloidin (A,G,J).

a powerful method for the prevention of protein degradation. In contrast to the lysis buffers that release proteases by the destruction of the protease-rich granules in neutrophils, TCA fixes the protease-rich granules without destroying their structure. However, TCA fixation prevents the examination of protein binding by co-immunoprecipitation. Cas-L has been reported to bind Crk or Crk-L to transduce signals to downstream effectors [Minegishi et al., 1996; Manie et al., 1997; Sattler et al., 1997; Astier et al., 1997b; Ohashi et al., 1999]. Therefore, it is possible that Crk or Crk-L could act as downstream adaptor molecules from Cas-L in neutrophils.

Cas-L is reportedly tyrosine-phosphorylated in response to β1 integrin, T-cell receptors, B-cell receptors, calcitonin, and TGF β1 [Minegishi et al., 1996; Kanda et al., 1997, 1999; Tachibana et al., 1997; Astier et al., 1997a,b; Kamiguchi et al., 1999; Zhang et al., 1999; Zheng and McKeown-Longo, 2002]. Here, we described the tyrosine phosphorylation of Cas-L in response to fMLP, TNF-α, and LPS in neutrophils. The receptors for fMLP are seven transmembrane G-protein-coupled receptors. The tyrosine phosphorylation of Cas-L in response to G-protein-coupled receptor stimulation has been reported with calcitonin [Zhang et al., 1999]. The response to CXCL12, which is mediated by CXCR4, another G-protein-coupled receptor, was impaired in Cas-L-deficient lymphocytes [Seo et al., 2005], and the tyrosine phosphorylation of Cas-L in response to CXCL12 is reported in T cells [Regelmann et al., 2006]. Although TNF-α was reported to induce the degradation of Cas-L in epithelial cells [Law et al., 2000], our results provide the first evidence of the tyrosine phosphorylation of Cas-L by cytokines. Focal adhesion proteins such as paxillin, PYK2, and FAK are involved in toll-like receptor signaling [Williams and Ridley, 2000; Zeisel et al., 2005]. However, the involvement of Cas family members in the innate immune system has not previously been reported. LPS-induced tyrosine phosphorylation of Cas-L in neutrophils, together with the chemokine- or cytokine-induced tyrosine phosphorylation of Cas-L, suggests that Cas-L would be one of the targets of inflammatory stimuli in neutrophils.

The tyrosine phosphorylation of Cas-L in neutrophils was greatly enhanced or prolonged by adhesion, especially when neutrophils were stimulated with fMLP or TNF-α (Figs. 2 and 3). Although the weak fMLP-induced phosphorylation in suspension was not dependent on β2 integrins, the adhesion-induced enhancement of tyrosine phosphorylation in fMLP stimulation was mediated by β2 integrins (Fig. 2D). Furthermore, the tyrosine-phosphorylated Cas-L was localized to focal adhesions in neutrophils (Fig. 5). Therefore, the tyrosine phosphorylation of Cas-L appears to be involved in cell

adhesion and cytoskeletal change, as already reported for other cell types such as lymphocytes and epithelial cells [Law et al., 1996; Minegishi et al., 1996]. The tyrosine phosphorylations of Cas-L were variable depending on extracellular matrix proteins (Figs. 2C, 3C, and 4C), suggesting the involvement of specific integrins in the phosphorylation process. However, the Cas-L phosphorylation in fMLP and TNF stimulation is also observed in suspension, suggesting that the phosphorylation occurs before integrin stimulation and that the integrin signaling enhance this phosphorylation. In contrast, LPS-mediated tyrosine phosphorylation of Cas-L is independent on adhesion and is delayed. Toll like receptors may produce signals that are delayed and are independent on integrins in neutrophils. Another possible explanation may be the existence of small numbers of contaminating monocytes in neutrophil preparation [Sabroe et al., 2004]. Those monocytes may produce multiple cytokines that cause neutrophil activation without adhesion. This might also explain the delay of phosphorylation of Cas-L by LPS. In any case, LPS-induced phosphorylation of Cas-L in neutrophils reflects the physiological phenomenon, because neutrophils in vivo are not pure and are influenced by neighboring cells.

In this study, we used human neutrophils to investigate the expression and tyrosine phosphorylation of Cas-L. However, there are no specific inhibitors of Cas-L; thus, it is difficult to investigate the role of Cas-L in human neutrophils. Neutrophils express p105Cas-L and not p115Cas-L (Fig. 1). p115Cas-L is a Ser/Thr phosphorylated form of Cas-L [Law et al., 1998]. The depletion of p115Cas-L impairs cell migration in T cells and p105Cas-L could not substitute the loss of p115Cas-L [Regelmann et al., 2006]. Therefore, it is estimated that p105Cas-L present in neutrophils and p115/p105Cas-L present in lymphocytes might play different roles in cell migration and in other biological functions. In fact, the investigation of neutrophils from Cas-L deficient mice showed that Cas-L deficient neutrophils migrated to fMLP faster than wildtype neutrophils in Boyden chamber assay (Seo et al., in preparation). This result is in contrast to the fact that Cas-L deficient lymphocytes are less responsive to CXCL-12, CXCL-13, and CCL21 [Seo et al., 2005], suggesting the differential roles of p115Cas-L and p105Cas-L.

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A Novel RNA-Binding Protein, Ossa/C9orf10, Regulates Activity of Src Kinases To Protect Cells from Oxidative Stress-Induced Apoptosis▽†

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During the process of tumor progression and clinical treatments, tumor cells are exposed to oxidative stress. Tumor cells are frequently resistant to such stress by producing antiapoptotic signaling, including activation of Src family kinases (SFKs), although the molecular mechanism is not clear. In an attempt to identify the SFK-binding proteins selectively phosphorylated in gastric scirrhous carcinoma, we identified an uncharacterized protein, C9orf10. Here we report that C9orf10 (designated Ossa for *o*xidative *s*tress-associated *S*rc *a*ctivator) is a novel RNA-binding protein that guards cancer cells from oxidative stress-induced apoptosis by activation of SFKs. Exposure to oxidative stress such as UV irradiation induces the association of Ossa/C9orf10 with regulatory domains of SFKs, which activates these kinases and causes marked tyrosine phosphorylation of C9orf10 in turn. Tyrosine-phosphorylated Ossa recruits p85 subunits of phosphatidylinositol 3-kinase (PI3-kinase) and behaves as a scaffolding protein for PI3-kinase and SFKs, which activates the Akt-mediated antiapoptotic pathway. On the other hand, the carboxyl terminus of Ossa has a distinct function that directly binds RNAs such as insulin-like growth factor II (IGF-II) mRNA and promotes the extracellular secretion of IGF-II. Our findings indicate that Ossa is a dual-functional protein and might be a novel therapeutic target which modulates the sensitivity of tumors to oxidative stress.

Tumor cells are exposed to oxidative stress in various situations in vivo. Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2) are generated by exposure of cancer cells to hypoxia, followed by reperfusion; radiotherapy; photodynamic therapy; and some chemotherapeutic agents such as cisplatin (6, 25). This production of ROS generally induces apoptosis, whereas some tumor cells become resistant to this kind of apoptosis by some mechanism such as elevated expression of antioxidant thiols in the cells (17, 27).

Src family kinases (SFKs) play important roles in various cell functions such as cell proliferation, cell adhesion, and cell migration (26), and the activities of SFKs often correlate with the malignant potential of cancer and a poor prognosis (37). Activation of c-Src is observed after the cells are exposed to oxidative stress (1, 9, 11, 34), and the activation of SFKs contributes to the resistance to apoptosis induced upon cellular stress. For instance, treatment of cells with oxidative stress such as UV irradiation or H_2O_2 causes apoptotic cell death, which is rescued by expression of v-Src (28). In our attempt to identify the key molecules that promote the expansion of gastric scirrhous carcinoma in vivo by mediating signals from activated SFKs, we identified an uncharacterized protein, C9orf10.

C9orf10 (*Homo sapiens* chromosome 9 open reading frame 10) was originally found by the human genome sequence project as an annotated protein, and the gene was mapped to chromosome 9q22.31 (12). C9orf10 was recently detected within the Purα-containing mRNA-protein complex in the brain, although no functional information about this protein is available (14). We show that C9orf10 protects cells from apoptosis through activation of SFKs in response to oxidative stress. The kinase activity of SFKs is regulated by two intramolecular interactions. The inactive form is achieved by interaction of the SH2 domain with the phosphorylated C-terminal tail and association of the SH3 domain with a polyproline type II helix formed by the linker region between the SH2 domain and the catalytic domain (30). C9orf10 functions as a novel activator of SFKs that unfolds the inactive form of SFKs by association with both the SH2 and SH3 domains of SFKs. Tyrosine phosphorylation of C9orf10 is induced by the activated SFKs in turn, producing scaffolds to recruit phosphatidylinositol 3-kinase (PI3-kinase) and activate PI3-kinase–Akt signaling, which plays a key role in protecting cancer cells from oxidative stress-induced apoptosis. Therefore, we named C9orf10 Ossa (*o*xidative *s*tress-associated *S*rc *a*ctivator).

We also showed that the carboxyl terminus of Ossa directly binds to RNA, suggesting a distinct role for Ossa as an RNA-binding protein. As one of the target RNAs, Ossa directly binds to insulin-like growth factor II (IGF-II) mRNA, which subsequently enhances the extracellular secretion of IGF-II. Because an increase in IGF-II promotes cell proliferation, the RNA-binding function of Ossa may also contribute to the survival of cancer cells in vivo.

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