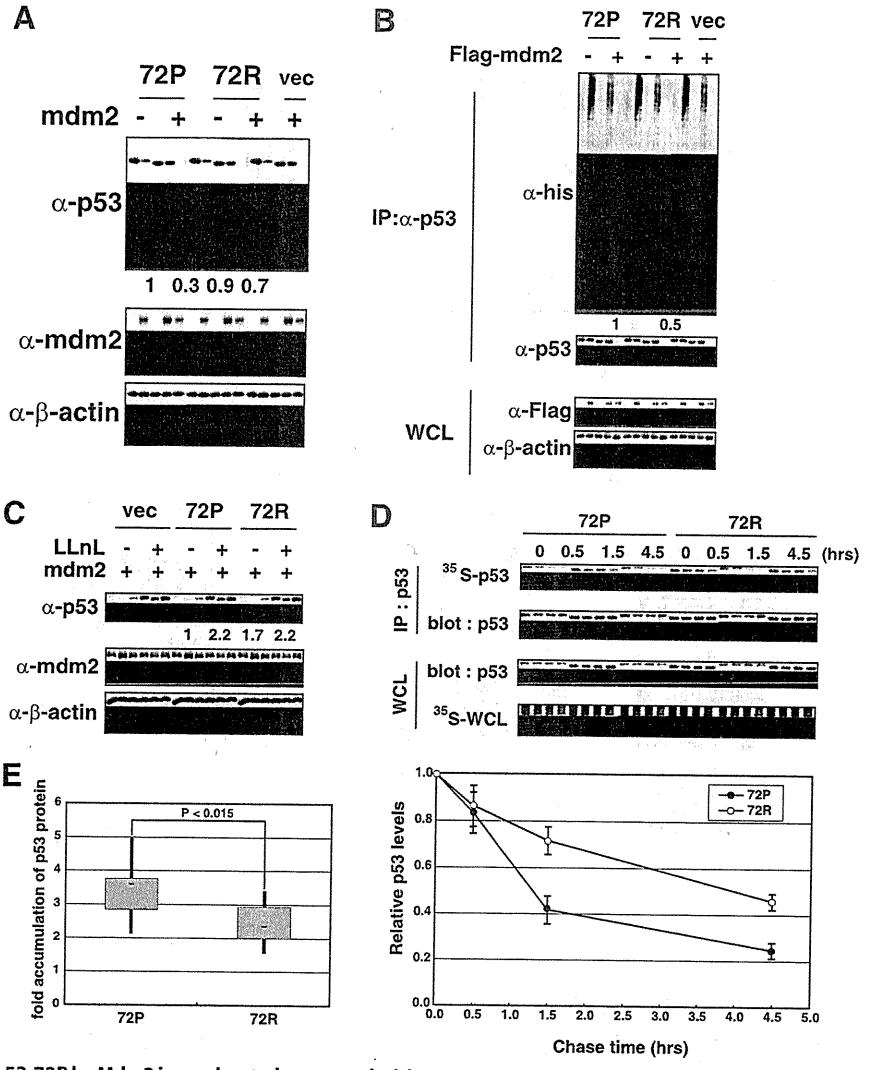

FIGURE 4. **Degradation of p53-72P by Mdm2 is accelerated compared with p53-72R.** *A*, pcDNA3-p53-72P or -72R together with N-terminally c-Myc-tagged Mdm2 (pCMV-Myc-Mdm2; Mdm2 expressed from a CMV promoter) or control empty vector were introduced into H1299 cells (4.4×10^5 cells/10-cm dish) and analyzed by Western blotting. 0.44 μg of p53 and 4 μg of Mdm2 were transfected. Cells were harvested 21 h post-transfection. Levels of p53 (normalized by β-actin) were quantified and are shown *below* the panels. *B*, pcDNA3-p53-72P or -72R (0.35 μg) together with His_6-tagged ubiquitin (2.2 μg) and N-terminally FLAG-tagged Mdm2 (pSG-FLAG-Hdm2, 1.42 μg) or control empty vector (*vec*) (1.42 μg) were introduced into H1299 cells (6×10^5 cells/10-cm dish), and cells were harvested 27 h post-transfection. To detect ubiquitinated p53 efficiently, Mdm2 was expressed at a low level using expression plasmid pSG-F-Hdm2 (Hdm2 is under the control of SV40 promoter, which is much weaker than CMV promoter). p53 was immunoprecipitated (*IP*) with anti-p53 polyclonal antibody (FL393), and immunoprecipitated samples and whole cell lysates (*WCL*) were analyzed by Western blotting. Western blot analyses of immunoprecipitates were performed with the anti-His antibody to detect ubiquitinated p53 (*upper panel*) or with FL393 antibody to detect nonubiquitinated p53 (*lower panel*). Levels of ubiquitinated p53 (normalized by nonubiquitinated p53) were quantified and are shown *below* the panels. *C*, pMX-p53-72P or -72R (0.5 μg) together with pCMV-Myc-Mdm2 (4.5 μg) or control empty vector (4.5 μg) were introduced into H1299 cells (4.4×10^5 cells/10-cm dish). Where indicated, cells were treated with LLnL (50 μM) 16 h post-transfection. Cells were harvested 21 h post-transfection and analyzed by Western blotting. Experiments were performed in triplicate, and representative images are shown. Levels of p53 were quantified (normalized by β-actin) and the relative p53-72P and -72R levels are shown *below* the panel. *D*, pcDNA3-p53-72P or -72R (0.4 μg) together with pCMV-Myc-Mdm2 (3.6 μg) were introduced into H1299 cells (4×10^5 cells/10-cm dish). Cells were pulse-labeled 20 h post-transfection for 30 min and then cultured in chase medium for 1.5 h. Following incubation, cells were harvested at the indicated time points. p53 was immunoprecipitated, and the levels of labeled p53 were detected by autoradiography. Total p53 protein levels were analyzed by Western blotting. Experiments were performed in triplicate, and representative images are shown. Levels of p53 were quantified (normalized by total p53) and the relative p53-72P and -72R levels are shown *below* the panel. *E*, immortalized peripheral lymphocytes from healthy donors were subjected to LLnL treatment. Cells derived from 10 homozygotes each for p53-72P and -72R were subjected to analysis. Each sample was run in triplicate and analyzed by Western blotting (supplemental Fig. S3). Quantification was performed using Image J software. Fold accumulation of p53 protein after LLnL treatment was calculated for each sample and shown as a *box plot*.

antibody to detect the amount of His-tagged ubiquitinated p53s. Again, ubiqitination was more prominent in p53-72P than p53-72R (supplemental Fig. S2).

To further test whether the variants differ in degradation levels mediated by the proteasome pathway, we treated cells co-expressing Mdm2 and p53-72R or -72P with LLnL, a proteasome inhibitor. As shown in Fig. 4*C*, the p53-72P level was significantly increased by LLnL treatment compared with p53-72R, showing that p53-72P is more susceptible to degradation by the proteasome pathway. In addition, both variants were expressed at similar levels after LLnL treatment, supporting the idea that differences in the expression levels of both variant

TABLE 1
Over-representation of p53–72P homozygotes in lung cancer cases with gains of the *mdm2* gene in their tumors

p53 genotype	No. of cases: MDM2 normal	No. of cases: MDM2 gain[a]	Odd ratio (95% confidence interval)	*p* value by Fisher's exact test
	%	%		
R/R + R/P	48 (94)	19 (79)	Reference	
P/P	3 (6)	5 (21)	4.21 (0.94–22.2)	0.101

[a] Copy number ratio >1.25 in tumors by array comparative genomic hybridization analysis using MCG Cancer array-800.

proteins were the result of proteasomal degradation. We also performed [^{35}S]methionine pulse-chase experiments to determine the half-lives of p53 variant proteins. Cells co-expressing Mdm2 and p53-72R or -72P were pulse-labeled, and p53 protein levels were monitored for 4.5 h. As shown in Fig. 4*D*, the half-life of p53-72R was significantly longer than p53-72P, demonstrating that p53-72R is more resistant to Mdm2-mediated degradation.

We next utilized peripheral lymphocytes immortalized using Epstein-Barr virus to analyze the degradation of endogenously expressed p53 proteins. We selected 10 cells each that were homozygous for p53-72P or -72R. To minimize the difference between cell lines, they were also selected based on the criteria that they were derived from healthy donors who were Japanese, male, nonsmoking, and aged 30–50 years old. As shown in supplemental Fig. S3 and Fig. 4*E*, when cells were treated with LLnL, accumulation of p53 protein was more pronounced in cells with p53-72P, confirming the result obtained for exogenously expressed p53 proteins. Collectively, it was shown that ubiquitination by Mdm2 and subsequent degradation is more enhanced in p53-72P than -72R.

Cancer Patients Carrying p53-72P Are Over-represented in Patients with Gain in the mdm2 Gene—The nature of p53-72P being more sensitive to degradation by Mdm2 than p53-72R raises the possibility that homozygotes for the p53-72P allele are more susceptible to developing cancer by up-regulation of Mdm2 expression. We therefore analyzed the copy number of the *mdm2* gene in tumors in combination with genotypes for the p53 polymorphism at codon 72 in the germ line. Seventy five lung cancer cases, consisting of 39 adenocarcinomas, 2 bronchioalveolar carcinomas, 25 squamous cell carcinomas, 2 small cell lung carcinomas, 7 large cell neuroendocrine carcinoma cases, that were available for information both on p53 genotypes in their peripheral blood cells and the *mdm2* gene copy numbers in their tumor cells were subjected to analysis (Table 1). These cases were selected from a Japanese population of lung cancer cases that showed a frequency of the p53-72P allele higher than control individuals in a previous case-control study based on the criterion that information on the *mdm2* gene copy numbers in their tumor was available (19). In fact, the frequency of the p53-72P allele in these 75 cases (*i.e.* 0.41) was higher than that of controls (*i.e.* 0.33), although the difference did not reach statistical significance ($p = 0.070$ by Fisher's exact test). Among the 75 cases, 24 (32%) showed gain of the *mdm2* gene (ratio of test signal/reference signal >1.25) in their tumors. The fraction of p53-72P homozygotes was notably higher in patients with *mdm2* gains in their tumors than without (21 *versus* 6%, $p = 0.101$ by Fisher's exact test). Although further study is required with more test cases, these data suggest that p53-72P individuals develop lung cancer at a higher frequency upon increase of the *mdm2* gene copy number and support our results showing that p53-72P is more susceptible to Mdm2-mediated degradation.

Phosphorylation of Ser-6 Is More Enhanced in p53-72R than -72P under Basal and Damaged Conditions—Ser-6, Ser-15. and Thr-18 are the phosphorylation sites within the N-terminal transactivation domain that are conserved among vertebrates. Phosphorylation of Ser-15 and Thr-18 plays important roles in the regulation of p53 activity; however, although it has been reported that Ser-6 is phosphorylated under damaged or basal conditions, the biological significance of Ser-6 phosphorylation remains elusive (6). Because we found that Ser-6 is strongly phosphorylated in p53-72R compared with p53-72P in Saos2 cells (Fig. 2), we further analyzed under which conditions Ser-6 is phosphorylated. As shown in Fig. 5*A*, upon γ-ray irradiation, the Ser-6 phosphorylation level is increased, and p53-72R is phosphorylated at a higher level than p53-72P in Saos2 cells. In this experiment, the difference in Ser-6 phosphorylation without DNA damage was also confirmed. Under the same conditions, phosphorylation of Ser-15 was induced upon γ-ray irradiation, but no difference was detected between variants with or without γ-ray irradiation. To further analyze p53 phosphorylation under damaged conditions, we obtained cell lines stably expressing both p53s in HCT116 p53(−/−) cells. As shown in supplemental Fig. S4*A*, both cell lines expressed p53-72R or -72P at similar levels and induced p21 upon DNA damage, showing a normal p53 response in these cell lines. Using these cell lines, phosphorylation of Ser-6 under basal conditions and upon DNA damage was analyzed. As shown in Fig. 5*B* and supplemental Fig. S4, *B* and *C* upon γ-ray or UV irradiation, adriamycin or 5-fluorouracil treatment resulted in increased Ser-6 phosphorylation, and under all conditions, p53-72R showed elevated phosphorylation levels compared with p53-72P. Again, phosphorylation of Ser-15 was induced upon γ-ray irradiation, but no difference was detected between variants (Fig. 5*B*).

Phosphorylation of Ser-6 Is Required for p53 Transactivation under Basal Conditions and upon Activation of TGF-β Signaling—To analyze the biological function of Ser-6 phosphorylation, we constructed p53 mutants carrying Ser to Ala conversions at codon 6. Wild-type as well as mutant p53s were expressed in H1299 cells, and the induction of representative p53 target gene products (p21, Bax, PIG-3, and Mdm2) were analyzed by Western blotting. As shown in Fig. 5*C*, all four p53 target gene products were induced by wild-type p53 as expected. Interestingly, wild-type p53 induced p21 more effectively than S6A mutant, demonstrating the involvement of Ser-6 phosphorylation in p21 induction. In addition, as shown in Fig. 5*D*, p53-72R induced p21 more strongly than p53-72P, likely reflecting the difference in Ser-6 phosphorylation levels between proteins. The elevated expression of p21 in p53-72R-expressing cells was also demonstrated in HCT116 p53(−/−) cells stably expressing p53-72P or -72R and in immortalized human peripheral lymphocytes (supplemental Figs. S4*A* and S5*A*).

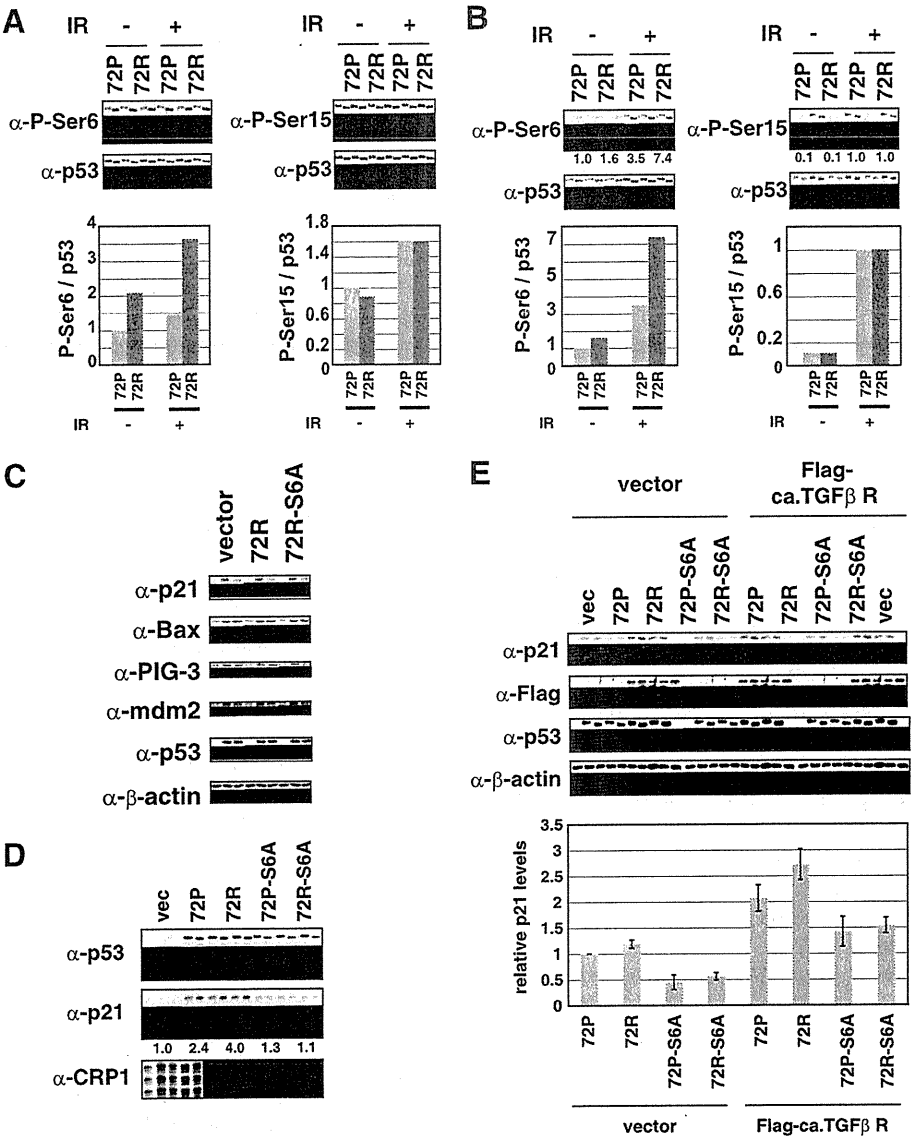

FIGURE 5. **Phosphorylation of p53 at Ser-6 was enhanced in p53-72R compared with -72P, and p53-dependent p21 expression was enhanced in cells expressing p53-72R.** *A*, phosphorylation of p53 at Ser-6 and Ser-15 in Saos2 cells expressing p53-72P or -72R was analyzed by Western blotting. Cells (2.2×10^6 cells/10-cm dish) were transfected with pMX-p53-72P or -72R, treated with γ-ray (20 gray) 24 h post-transfection, and harvested 50 h post-transfection. As in Fig. 2, p53 proteins were immunoprecipitated and analyzed using anti-phospho-Ser-6 p53 antibody (*upper panel*) and anti-p53 antibody (*lower panel*). Levels of phospho-Ser-15 are shown for comparison. To detect phospho-Ser-15 and total p53 in *right panels*, whole cell lysates were analyzed by Western blotting. Relative phosphorylation levels (normalized by total p53 levels) are shown *below* the panels. *B*, HCT116 p53(−/−) cells stably expressing p53-72P or -72R (6.7×10^6 cells/10-cm dish) were treated with γ-ray (20 gray), and cells were harvested 2 h post-irradiation. Levels of phospho-Ser-6 and -15 were analyzed as in *A*. *C*, H1299 cells (4×10^5 cells/10-cm dish) were transfected with pMX-p53-72R or 72R-S6A mutant (0.3 μg) together with pcDNA3 (2.7 μg). Cells were harvested 26 h post-transfection, and analyzed by Western blotting. *D*, wild-type or mutant (S6A) pMX-p53-72P or -72R (1.63 μg) was introduced into H1299 cells (2.4×10^6 cells/10-cm dish), and cells were harvested 29 h post-transfection. Expression of p53, p21, and cytoskeletal CRP1 (as a loading control) was analyzed by Western blotting. The experiment was performed with p53-72P and -72R expressed at similar levels. The levels of p21 and 23-kDa CRP1 were quantified using Image J software, and p21 levels were normalized by CRP1 levels. *E*, H1299 cells (2.4×10^6 cells/10-cm dish) were transfected with pMX-p53-72P or 72R (1.63 μg) and ca. TGF-βR (6.54 μg). Cells were harvested 29 h post-transfection. Expression of ca. TGF-βR was detected by anti-FLAG antibody. Experiments were performed in triplicate, and representative images are shown. The levels of p21 (normalized by β-actin) were quantified using Image J software. The mean ± S.D. of p21 levels was calculated and is shown in the *bottom column*.

It has been reported that the activation of MAPK promotes the phosphorylation of p53 at Ser-6 and Ser-9 (25). Phosphorylation at these sites facilitates the interaction of p53 with activated Smad2 or Smad3 and the subsequent recruitment of p53-Smad2/3 complexes to TGF-β-responsive target promoters (25). As shown in supplemental Fig. S4*D*, we also have confirmed MAPK-dependent phosphorylation of p53 at Ser-6. It has also been shown using H1299 cells that the expression of p53 with amino acid conversions from Ser to Ala at codon 6 or 9 impaired the ability of p53 to enhance TGF-β-mediated expression of the p21 gene (25). Because we detected a significant difference in Ser-6 phosphorylation between the variants, we analyzed whether TGF-β-mediated expression of the p21 gene differs between them. We analyzed TGF-β-dependent up-regulation of p21 by introducing constitutively active TGF-β receptor I (ca. TGF-βR) with p53 variants. It was confirmed

that introduction of ca. TGF-βR results in activation of the TGF-β pathway in H1299 cells, as judged from Smad2 phosphorylation (supplemental Fig. S5*B*). As shown in supplemental Fig. S5*B* and Fig. 5*E*, without TGF-β signaling, p53-72R induced p21 more efficiently than p53-72P, confirming the results shown in Fig. 5*D*. When ca. TGF-βR was co-transfected with p53s, TGF-β-dependent up-regulation of p21 was observed, and this induction was significantly stronger in cells expressing p53-72R. Enhanced induction efficiency of p21 in p53-72R-expressing cells with or without ca. TGF-βR was also confirmed by quantitative real time PCR (supplemental Fig. S5*C*). The difference between variants was abolished when Ser to Ala conversions were introduced at Ser-6, showing that the difference in p21 induction was brought about from differences in Ser-6 phosphorylation levels (Fig. 5*E*). Collectively, these results indicate that Ser-6 phosphorylation is important for p53 transactivation activity under basal conditions and upon activation of TGF-β signaling, and enhanced Ser-6 phosphorylation in p53-72R results in stronger induction of p21.

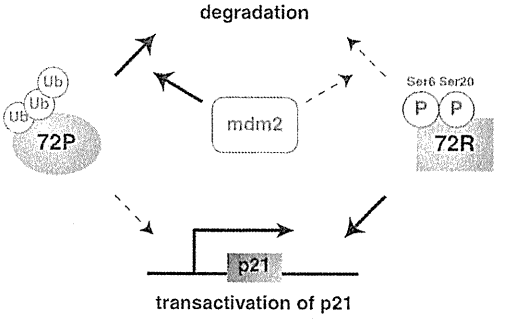

FIGURE 6. **Common polymorphism of p53 affects phosphorylation and degradation of p53 protein.** Phosphorylation of Ser-6 and -20 is enhanced in p53-72R compared with p53-72P. Difference in protein structure and phosphorylation of Ser-20 affects Mdm2-mediated degradation of p53 protein, whereas phosphorylation of Ser-6 affects transactivation ability of p53 protein.

DISCUSSION

The polymorphism of p53 at codon 72 is unique to humans and is very common. For example, 44% of Japanese are homozygous for p53-72R and 11% are homozygous for p53-72P (19). Cancer susceptibility and clinical outcome differ among individuals having the two variants; therefore, the impact of understanding the molecular basis for the difference between p53-72P and -72R is huge. Recently, using chimeric p53 protein containing N-terminal mouse p53 (amino acids 1–34) and human p53 (amino acids 32–393), it was shown that codon 72 polymorphism-specific effects of human p53 require N-terminal 31 amino acids of human p53 (26). In addition, we found that p53-72R and -72P proteins differ in structure, especially in the N-terminal region, by partial proteolytic digestion of the proteins. We speculated that differences in the protein structure may change the affinity of p53 variants with kinases that modify p53, especially in the N-terminal region, and we found that the variants differ in phosphorylation levels at Ser-6 and -20. We actually found that strength of association with Chk2 kinase differs between p53-72P and p53-72R (supplemental Fig. S6). We do not know the precise mechanism of the differential association of the variants with Chk2, and it is an interesting issue to clarify in future research.

It has been reported that p53 phosphorylated at Ser-20 escapes from degradation by Mdm2, leading to stabilization of the protein (6), whereas phosphorylation of Ser-6 is required for TGF-β-dependent induction of p21 and p15INK4b (25). We found that phosphorylation of these sites is enhanced in p53-72R, and consequently, the two p53 polymorphic variants differ in the stabilization of proteins and TGF-β-dependent and -independent induction of p21, both of which are important for the tumor-suppressive function of p53 (Fig. 6).

In this study, we have shown for the first time that the *mdm2* gene gain in tumors is more frequent in lung cancer cases homozygous for the p53-72P allele than with other genotypes. Although this association should be further validated in other sets of lung cancer cases, the present result demonstrates the possibility that p53-72P homozygotes develop lung cancer at a higher frequency upon gain of the *mdm2* gene and supports our data showing that p53-72P is more susceptible to Mdm2-mediated degradation. It will be interesting to determine whether such an association is also observed in patients with other types of cancer.

Previously, it was shown that the expression of p21 mRNA was altered by p53 codon 72 polymorphism, and the Pro allele variant was associated with decreased p21 mRNA levels compared with Arg allele (27). In this study, we have also shown that p21 expression was decreased in p53-72P compared with -72R and was dependent on p53 Ser-6 phosphorylation. The physiological relevance of Ser-6 phosphorylation remains unknown; however, it was shown recently to be required for TGF-β signaling. TGF-β is a potent growth inhibitor with tumor suppressing activity, and TGF-β-mediated growth suppression is mediated by p53 (28). TGF-β cooperates with p53 to induce p21, and this induction requires p53 to be phosphorylated at N-terminal Ser residues, including Ser-6 (25). We have shown that p53-72P was less phosphorylated at Ser-6 compared with p53-72R under all conditions studied, and TGF-β-dependent and -independent induction of p21 was attenuated in p53-72P-expressing cells. Previously, we have shown that phosphorylation of Ser-6 does not affect binding of p53 to p21 promoter (8). Therefore, Ser-6 may affect other aspects of p21 promoter activation, such as cofactor recruitment to the promoters.

The results shown in this study collectively reveal a novel difference in p53 polymorphic variants at codon 72. Although several molecular mechanisms to explain the difference in tumor suppression function of the variants have been reported, our results also reveal a novel difference in the variants through differences in protein structure and phosphorylation levels at Ser-6 and -20. Understanding the molecular mechanism leading to differences in the tumor suppression potential of the two variants is very important for cancer prevention and therapy. Our results may provide basic knowledge to develop novel cancer therapy or prevention strategies on the basis of the genotype of p53.

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Review Article

Roles of CUB domain-containing protein 1 signaling in cancer invasion and metastasis

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Tumor metastasis is a complex multistep process by which cells from the primary tumor invade tissues, move through the vasculature, settle at distant sites and eventually grow to form secondary tumors. Altered tyrosine phosphorylation signals in cancer cells contribute to a number of aberrant characteristics involved in tumor invasion and metastasis. CUB domain-containing protein 1 (CDCP1) is a substrate of Src family kinases and has been shown to regulate anoikis resistance, migration and matrix degradation during tumor invasion and metastasis in a tyrosine phosphorylation-dependent manner. Knockdown of CDCP1 blocks tumor metastasis or peritoneal dissemination *in vivo*, without significantly affecting cell proliferation. Moreover, expression levels of CDCP1 are of prognostic value in several cancers. Here, we summarize the studies on CDCP1, focusing on structure and signal transduction, to gain insight into its role in cancer progression. Understanding the signaling pathways regulated by CDCP1 could help establish novel therapeutic strategies against the progression of cancer. (*Cancer Sci* 2011; 102: 1943–1948)

Metastatic cancers acquire various biological properties during the process of cancer progression. Their ability to migrate, invade and survive in adverse conditions, including hypoxia, malnutrition, immunological attack, oxidative stress or absence of cell adhesion might be instrumental in allowing cancer cells to invade distant organs through tissues and vessels.[1] In order to find effective therapeutic approaches targeting metastatic cancers, it is essential to understand the mechanism by which they achieve these characteristic abilities associated with metastatic potential.

It is widely accepted that both receptor- and non-receptor-type tyrosine kinases are closely associated with cancer cell behavior as direct cellular mediators of extracellular stimuli. Src kinase, a non-receptor tyrosine kinase, was originally identified as a form of viral oncogene able to transform fibroblasts, and was later shown to be the regulator of various cellular signaling events. To date, several members of Src family kinases (SFK) have been identified, which play crucial roles in the regulation of cell attachment, movement and proliferation, as well as cell–cell contact.[2]

In 2007, we noticed prominent tyrosine phosphorylation of unique 75- and 135-kDa proteins in the anchorage-independent subset of non-small-cell lung cancer (NSCLC) cell lines in suspension culture conditions.[3] Using large-scale purification and mass spectrometry analysis, we discovered that these 75- and 135-kDa phosphoproteins were different forms of CUB domain-containing protein 1 (CDCP1). Further study revealed that CDCP1 is a key regulator of cell survival in suspension conditions, also known as anoikis resistance.[3] In this review, we summarize the biological roles of CDCP1 in cancers in order to assess its potential as a therapeutic target in metastatic cancers.

Molecular cloning and structure of CDCP1

The gene encoding CDCP1 was first cloned in 2001 from colon cancer cells, because of its preferential expression in these cell lines compared with normal tissue.[4] The CDCP1 protein is a type-I transmembrane glycoprotein, also known as SIMA135,[5] gp140[6] and Trask.[7] Previously, a part of the CDCP1 protein was described as an unknown 80-kDa protein tyrosine phosphorylated in response to loss of integrin α6β4-mediated human keratinocyte adhesion on laminin5.[8]

CDCP1 is a protein comprising 836 amino acids, consisting of a 29-residue amino terminal signal peptide, an extracellular domain, a transmembrane domain and a cytoplasmic domain containing 150 amino acids (Fig. 1). The extracellular domain contains three CUB (complement protein subcomponents C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein 1) domains that are characterized by immunoglobin-like folds and might be involved in protein–protein interactions. It has been suggested that CUB domains play essential roles in developmental processes such as embryogenesis and organogenesis.[9] The cytoplasmic domain contains five conserved tyrosine residues that can be phosphorylated. In addition, two proline-rich stretches that could potentially bind Src homology 3 (SH3)-containing proteins are present in the cytoplasmic domain.[6] CDCP1 is heavily glycosylated and contains 14 putative *N*-glycosylation sites.

Full-length CDCP1 (135–140 kDa) is known to undergo protease cleavage in various cancer cells[3,7,10,11] and keratinocytes.[8] This cleavage of the extracellular domain of CDCP1 at R368, K369 (Fig. 1) results in generation of a smaller C-terminal membrane protein, described as the 70–85-kDa fragment of CDCP1. The native protease that cleaves this site has not been determined, although treatment with a trypsin inhibitor reduced the level of cleaved CDCP1 in MDA-MB-468 cells.[7] Plasmin and MT-SP1 proteases can also cleave full-length CDCP1 *in vitro*.[6,7]

Role of CDCP1 in cancer metastasis as a regulator of anoikis resistance

Anoikis is a form of apoptosis triggered by the loss of cell survival signals generated through interaction with the extracellular matrix. Resistance to anoikis has been described as a prerequisite for cancer cells during tumor metastasis or peritoneal dissemination (Fig. 2).[1] Because the property of anoikis resistance is unique to metastatic cancer cells, it might be a good

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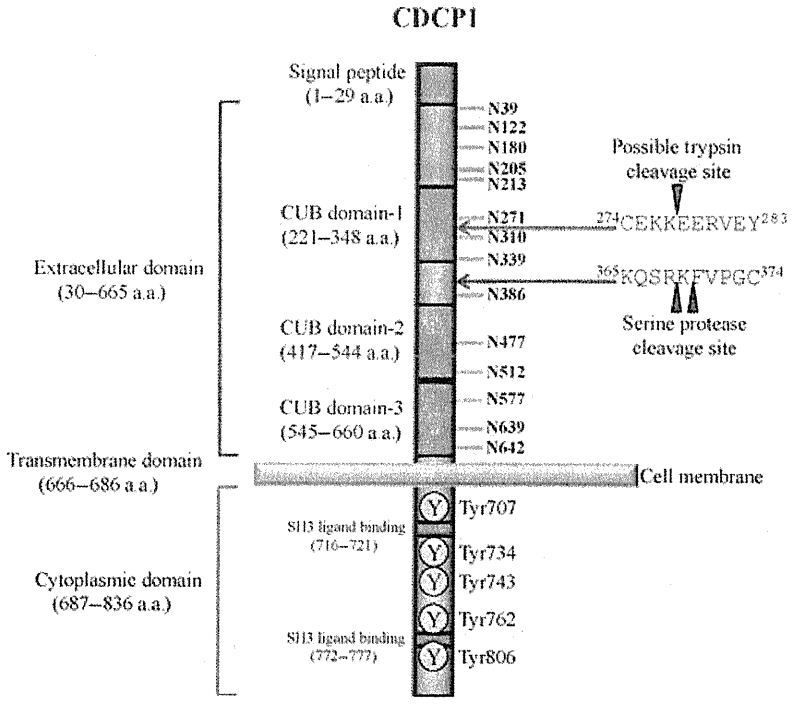

Fig. 1. Schematic illustration of human CUB domain-containing protein 1 (CDCP1) structures including the signal peptide. Derived CDCP1 encodes a protein of 836 amino acids (a.a.). Signal peptide (1–29 a.a.; red box) and an extracellular domain (30–665 a.a.) including CUB domains (CUB domain-1: 221–348 a.a., CUB domain-2: 417–544 a.a. and CUB domain-3: 545–660 a.a., respectively; green boxes), a transmembrane domain (666–86 a.a.) and a cytoplasmic domain (687–836 a.a.). In the extracellular domain, consensus 14 *N*-glycosylation sites are indicated by light blue lines. Serine protease (red arrow) and possible trypsin (pink arrow) cleavage sites are shown. In the cytoplasmic domain, intracellular tyrosine residues are indicated by yellow circles, and two possible SH3 ligand-binding domains are indicated by blue boxes.

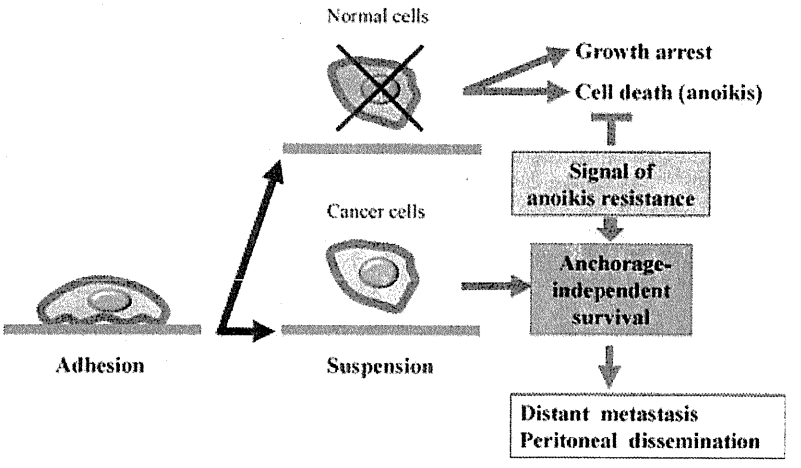

Fig. 2. Anoikis resistance: a key function of tumor metastasis. Anoikis is physiologically important in the maintenance of homeostasis and tissue architecture. In contrast, anoikis resistance is outstanding characteristics of cancer cells during tumor progression and metastasis. This property indicates the existence of survival signals in suspending tumor cells, which is normally supported by cell–matrix interactions.

target for anti-metastasis therapy, which has minimal side-effects for normal tissue cells.

We first noticed that knockdown of Fyn or Yes, members of SFK in A549 lung cancer cells, abrogates the soft agar colony formation without significantly affecting the phosphoinositide 3-kinase (PI3K)–AKT or MEK–ERK pathways, suggesting the existence of a novel SFK-dependent signaling pathway supporting anchorage-independent survival of A549 cells.[3] After investigation of various anchorage-dependent and anchorage-independent lung cancer cells, CDCP1 was identified as a major phosphotyrosine-containing protein in anchorage-independent lung cancer cells that physically associate with Fyn. It was observed that anchorage-dependent lung cancer cells achieved resistance to cell death in suspension culture when CDCP1 was overexpressed along with Fyn kinase, while the effect was not observed with Y734F mutant of CDCP1, which lacks the phosphorylation site of SFK, or Fyn alone.[3] Finally, it was shown that CDCP1, as a substrate of SFK, actually supports survival of

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lung cancer cells in suspension culture conditions by inducing anoikis resistance.

Further experiments using RNAi knockdown of CDCP1 demonstrated that CDCP1 promotes the formation of metastatic nodules of lung adenocarcinoma cells in a mouse model[3] and enhances peritoneal dissemination of gastric scirrhous carcinoma in a mouse orthotopically implanted tumor model.[11] Outstanding elevation of the phosphorylation level of CDCP1 was observed in disseminated tumor nodules of gastric cancer cells in nude mice compared with standard culture conditions of these cells *in vitro*.[11] It was indicated that CDCP1 actually regulates the metastatic potential of solid tumors through regulation of resistance to anoikis.

Interestingly, CDCP1 does not obviously affect cell growth or survival in the attached cells or in a mouse xenograft model using lung adenocarcinoma and scirrhous gastric carcinoma cells.[3,11] Thus, inhibition of CDCP1 function might be quite a unique and specific therapeutic approach for metastatic cancers distinct from conventional drugs.

CDCP1 expression and cancer

Previous studies revealed that the CDCP1 protein is expressed on hematopoietic stem cells, mesenchymal stem cells and neuronal progenitor cells,[12,13] and we and other researchers have shown that CDCP1 is highly expressed in various human cancer cells including melanoma,[14] lung,[3] pancreatic,[10] renal cell,[15] colon, liver, gastric, kidney, breast and prostate carcinoma cell lines.[5] Studies using 25 breast cancer patient samples demonstrated that expression of *CDCP1* mRNA is regulated by CpG methylation in the promoter region.[16] Moreover, *CDCP1* mRNA expression in K562 and Jurkat hematopoietic cells is also inversely correlated with CpG methylation.[17]

CDCP1 staining of colon cancer and adjacent normal tissue suggested a correlation between tumor malignancy and staining intensity of CDCP1.[5] During histological examinations of CDCP1 in human cancer specimens, we and other researchers revealed that there are subsets of tumors with relatively high CDCP1 expression. These subsets make up as many as 77 of 230 renal cell carcinoma, 60 of 200 lung cancer and 53 of 145 pancreatic cancer cases, and are significantly associated with poor prognosis in relation to disease-free and overall survival.[10,15,18] However, a recent report showed that low but not high levels of CDCP1 expression were correlated with poor prognosis in 23 of 110 cases of endometrial adenocarcinoma.[19] Recently, it has also been suggested that kidney cancer tissues expressing membrane-localized CDCP1 have a worse prognosis than those with cytoplasmic expression of CDCP1.[20] It is likely that total expression of CDCP1 in cancers is generally associated with poor prognosis, but further information and more precise analysis will be required to determine the implications of subcellular localization and tyrosine phosphorylation of this protein.

Recently, it was reported that the *CDCP1* gene is induced by hypoxia-inducible factor (HIF)-1 and HIF-2, linked to the loss of the von Hippel-Lindau (VHL) tumor suppressor gene in clear cell renal cell carcinoma (CC-RCC) cells.[20] This is the first report of transcriptional control of *CDCP1* gene induction in cancer cells. Hypoxia-inducible factor regulates the expression of target genes, even in the case of tumor progression, and HIF can be degraded by the proteasome in the presence of the VHL protein.[21] In CC-RCC, the VHL gene is inactive in 80% of cases.[21] Hypoxic tumor cells are especially aggressive, metastatic and resistant to cancer therapy.[22] Thus, CDCP1 expression might regulate the malignancy of hypoxic tumor cells, and the CDCP1 protein might be a good therapeutic target for hypoxic tumors.

Induction of phosphorylated CDCP1 in cancer cells

CDCP1 is a major substrate of SFK, including Src, Fyn and Yes.[3,5–7] A key feature of the CDCP1 signaling pathway was elucidated by the structural analysis of Benes *et al.*,[23] who demonstrated that tyrosine phosphorylation of CDCP1 by SFK is required for binding to the unique C2 domain of Protein kinase Cδ (PKCδ). Specifically, it was shown that SFK initially induce phosphorylation at Tyr734 of CDCP1, resulting in binding of SFK to this site, promotion of phosphorylation at a further tyrosine residue, Tyr762, and recruitment of PKCδ to CDCP1 at phospho-Tyr762, mediated by a specific C2 domain of PKCδ, demonstrated to be a novel type of phosphotyrosine-binding motif.[23] We showed that the association of phosphorylated CDCP1 with PKCδ causes enzymatic activation of PKCδ, as analyzed by determination of the phosphorylation status of Thr505 of PKCδ.[10] Activation of PKCδ by CDCP1 has been shown to result in multiple malignant phenotypes of cancer cells, including anoikis resistance, cell migration and matrix degradation. At present there is no clear information on the oncogenic substrates or the downstream signaling pathway of PKCδ.

CDCP1 was the most prominent phosphotyrosine-containing protein in several NSCLC cell lines in suspension culture as first reported by our group,[3] and this phosphorylation might be triggered by interruption of integrin binding to the extracellular matrix (Fig. 3A).[3] It was demonstrated that inhibition of cell binding to laminin 5, using anti-laminin 5 antibody, increased phosphorylation of the C-terminal portion of CDCP1 in keratinocytes.[8] Subsequently, treatment of rounded keratinocytes with trypsin was shown to cause loss of the full-length 140-kDa phosphorylated CDCP1 and appearance of a cleaved 80-kDa phosphorylated CDCP1.[6] A recent report showed that proteolysis of CDCP1 induced its tyrosine phosphorylation (Fig. 3B).[24] However, this type of detachment-induced cleavage of CDCP1 is not obvious in most cancer cells we have examined. It is therefore possible that there is some mechanism in cancer cells to protect CDCP1 from detachment-induced cleavage, although the relationship between cell detachment, proteolysis of CDCP1 and phosphorylation of CDCP1 is not clear. In cancer cells, phosphorylation of both full-length and cleaved CDCP1 is mediated by cell detachment. Dephosphorylation of CDCP1 occured at around 48 h after adhesion when we examined the A549 cells.[3] The switch between cell detachment and adhesion is important in various stages of tumor progression during anoikis resistance, cell migration and invasion. The mechanism of interchange between phosphorylation and dephosphorylation of CDCP1 and its relationship to matrix adhesion might be crucial to the processes of cancer invasion and metastasis. Further studies will be required to fully understand the regulation of tyrosine phosphorylation of CDCP1 in physiological conditions and cancers.

Structurally, CDCP1 appears to be a membrane receptor that could be activated by unidentified specific ligands. It was demonstrated that clustering of CDCP1, using beads coated with anti-CDCP1 antibody, leads to its phosphorylation.[25] It has been suggested that extracellular signaling through the CUB domain of CDCP1 assembles CDCP1 into clusters and recruits them to detergent-resistant microdomains known as lipid rafts (Fig. 3C).[25] The CUB domain is clearly involved in protein–protein interaction.[26] Although their functions have not been clarified, the tetraspanin CD9,[27] *N*- and P-cadherin, and syndecan-1 and syndecan-4[7] were found to interact with CDCP1 through the CUB domain. The CUB domain might also have the potential to form homo-dimers. Identification of proteins that activate CDCP1 through association with its extracellular domain might prove crucial for further understanding the biological roles of this protein.

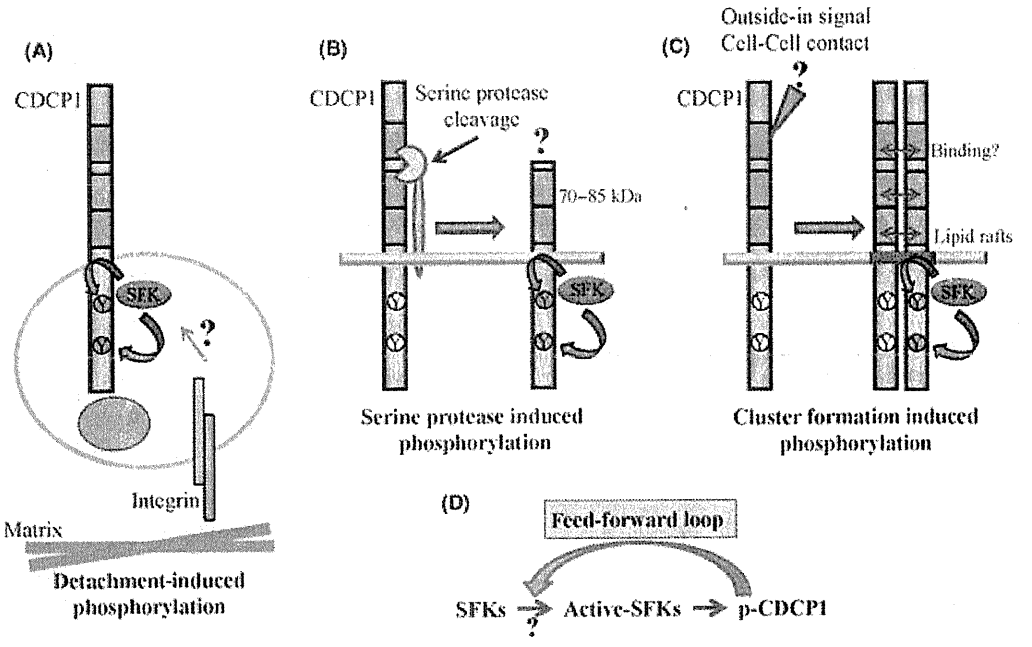

Fig. 3. Phosphorylation of CUB domain-containing protein 1 (CDCP1) is mediated by a number of cellular events. (A) Detachment cell-induced phosphorylation of CDCP1. Connection of cells to laminin 5 via integrins results in dephosphorylation of CDCP1,[8] suggesting that integrins affect detachment-induced phosphorylation of CDCP1. (B) Serine protease (yellow packman) cleaves full-length CDCP1 (135–140 kDa) to generate a C-terminal fragment (70–85 kDa), and this might induce the phosphorylation of CDCP1. (C) Outside-in signal, including cell–cell contact through the CUB domain, assembles CDCP1 clusters that are recruited to lipid rafts (purple line) and might activate Src family kinases (SFK).[25] (D) The means of initial activation of SFK is unknown; however, phosphorylation of CDCP1 by SFK activates SFK. This 'feed-forward loop' (pink ribbon) might be important for tumor progression.

Interestingly, overexpression of CDCP1, or even Y743F and Y806F mutants of CDCP1, in A375 cells enhanced SFK activity and promoted melanoma metastasis (Fig. 3D).[14] It is usually postulated that association between SFK and CDCP1 is dependent on tyrosine phosphorylation of CDCP1, because we first purified CDCP1 using a Fyn SH2 domain,[3] known to be a phosphotyrosine-binding motif.[2] Because Y734 is not necessarily required for the activation of SFK by CDCP1, it is possible that CDCP1 could also bind to the SH3 domain of Src through its proline-rich regions. Molecules that bind to the SH3 region of SFK can induce activation of SFK, as in the case of p130Cas.[28,29] Although the mechanism of initial activation of SFK is not clear, their activation by CDCP1 might be important for maintenance of constitutively elevated activity of SFK in tumor progression. Understanding the activation mechanism of SFK is necessary for the study of CDCP-induced tumor progression.

Role of CDCP1 signaling in solid tumors

CDCP1 is a major phosphotyrosine-containing protein in a wide range of solid tumors. Accumulating *in vitro* and *in vivo* evidence suggests that CDCP1 is a master regulator of tumor metastasis, through the control of multiple biological processes including anoikis resistance, cell migration, cell invasion, matrix metalloproteinase (MMP) secretion and invadopodia formation (Fig. 4B). It was also confirmed that knockdown of CDCP1 does not affect the PI3K–AKT or MEK–ERK pathways, as shown in Figure 4A, indicating that CDCP1–PKCδ signaling is a novel regulator of anoikis resistance, distinct from the major common pathways (Fig. 4B).[3,10]

We have demonstrated that phosphorylation of CDCP1 promotes cell migration *in vitro* and peritoneal dissemination *in vivo* in mice, using 44As3 human gastric cancer cell lines.[11] It was also reported that CDCP1 is required for tumor dissemination of HeLa cells in a chick embryo metastasis model.[30] In pancreatic cancer cell lines, it was observed that phosphorylation of CDCP1 activates PKCδ, regulating the secretion of MMP-9 and stimulating cell migration and invasion.[10] Additionally, the expression of a CDCP1 mutant, Y734F, inhibits cell survival, cell migration and invasion.[3,10,11] The evidence from our studies thus far indicates that activated PKCδ, induced by phosphorylated CDCP1, is the main mediator of properties associated with cancer metastasis and invasion. It was also demonstrated that reduction of CDCP1 expression in CC-RCC cells suppressed cell migration and that this could be rescued by overexpression of a constitutively active mutant of PKCδ.[20] A recent study suggests that the activation of CDCP1–PKCδ is accompanied by a decrease in focal adhesion kinase phosphorylation, which might be responsible for regulation of cell adhesion and motility.[31] Although it is reported that active PKCδ possesses both anti-apoptotic and apoptotic functions,[32] activation of PKCδ by fibroblast growth factor has an anti-apoptotic effect in PC12 cells,[33] and reduction of PKCδ activity, by an inactivated-kinase PKCδ mutant, induced apoptosis in lung cancer cells.[34] It is currently suggested that activated PKCδ in a complex with phosphorylated CDCP1 at the cytoplasmic membrane, or at membrane rafts, might have pro-metastatic and pro-invasive potential.

Taken together, the evidence suggests that the membrane protein CDCP1 has potential to be an ideal therapeutic target in tumor metastasis. The development of a monoclonal antibody against CDCP1 to control CDCP1-mediated signaling is currently in progress. Recent biological screening of a phage display combinatorial antibody library for anti-cancer activity identified an antibody recognizing CDCP1.[35] This antibody

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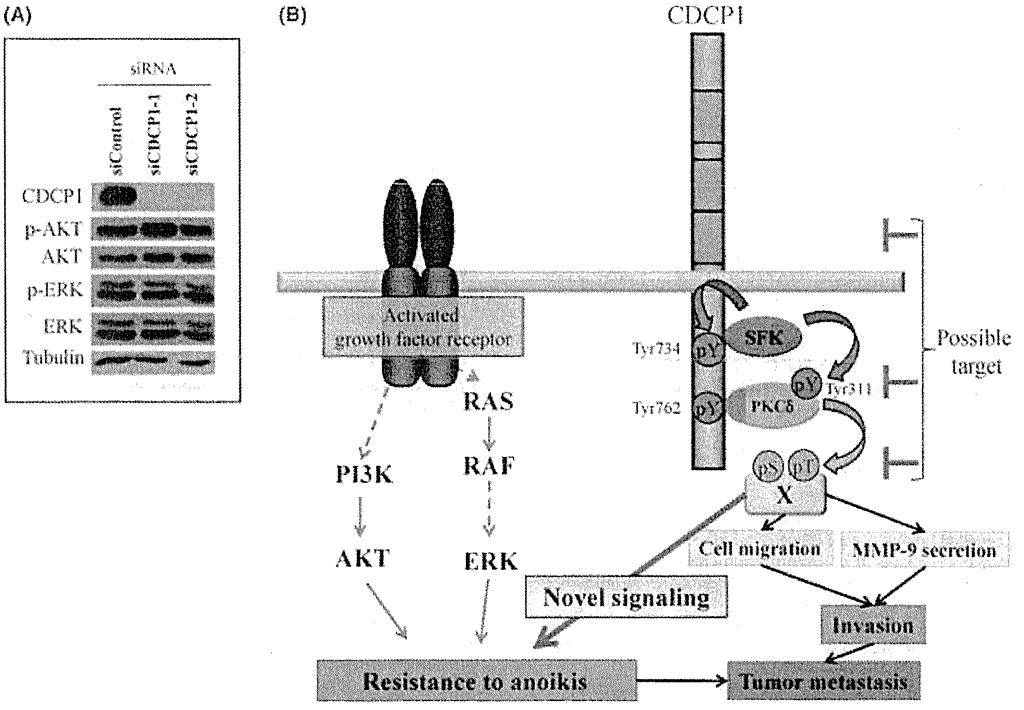

Fig. 4. Signal cascade of CUB domain-containing protein 1 (CDCP1) during tumor metastasis. (A) The pancreatic cancer cells (BxPC3) treated with CDCP1 siRNA were analyzed by immunoblotting with the indicated antibodies. Note that inhibition of CDCP1 expression does not significantly affect the phosphorylation of AKT (p-AKT) and ERK (p-ERK), while it blocks the metastatic properties of these cells.[10] (B) CDCP1 signaling mediated by Src family kinases (SFK) is a novel pathway of anoikis resistance independent of the RAS–RAF–MEK–ERK and PI3K–AKT pathways. Activated PKCδ phosphorylates downstream factors (X) at serine or threonine residues and the CDCP1–PKCδ complex eventually converts the tyrosine phosphorylation signal to a serine/threonine signal. Events downstream of PKCδ regulate invasion, including cell migration, protease secretion and anoikis resistance, and causes tumor metastasis. Inhibition of the CUB domain, CDCP1–PKCδ binding, downstream factors are all possible targets for cancer therapy.

inhibited cell migration and invasion in PC-3 prostate cancer cells. When anti-CDCP1 antibody was coupled to the cytotoxin saporin, either directly or indirectly through a secondary antibody, it induced death of PC-3 cells. This anti-CDCP1 antibody conjugated with saporin also significantly inhibited primary tumor growth and metastasis of PC-3 cells to lymph nodes in mice.[36] More recently, a human monoclonal antibody specifically targeting CDCP1 was shown to inhibit anchorage-independent colony formation in soft agar, experimental metastasis in chick embryo and enhanced cell death in indirect immunotoxin experiments using HeLa cells.[37] While CDCP1 has multiple roles in cancer progression, it has a minimal contribution to general cell proliferation. This suggests that it might be advantageous to target CDCP1 signaling to eliminate tumor metastasis, and that a combination of such an approach with conventional anti-cancer drugs might be powerful. It should also be taken into consideration that the CDCP1 protein is detectable in normal epithelial cells of the colon,[5] liver hepatocytes,[36] cells of the epidermis,[25] primary cultures of foreskin keratinocytes,[6] cells of hematopoietic lineages, and mesenchymal and neural progenitor cells.[12,13] To date, little is known about the phosphorylation of CDCP1 in normal human tissues. Wong *et al.* reported that CDCP1 is expressed in normal epithelial tissues but not in mesenchymal or central nervous system tissues, and that phosphorylation of CDCP1 is detected at the apices of intestinal villi, which are frequently shed into the lumen[38] and detached lung epithelial cells.[39] The phosphorylation of CDCP1 is tightly regulated in normal tissues but dysregulated in human cancers. Consistent with this, the phosphorylation of CDCP1 is detected in human gastric cancer cells invading the gastric wall[11] and peripheral areas of lung tumor cell nests,[18] while phosphorylated CDCP1 is not detected in adjacent normal tissues. Thus, phosphorylation of CDCP1 is a promising target of tumor progression including tumor metastasis.

Conclusion

CDCP1 is a phosphotyrosine-containing membrane protein and a critical regulator of the metastatic and invasive potential of solid cancers. The CDCP1–PKCδ pathway appears to be an ideal therapeutic target in metastasized cancers. Further studies will be required to understand what kind of stimulation triggers overexpression and tyrosine phosphorylation of CDCP1 in cancers. It is also important to obtain accurate knowledge of the cancer subtypes in which therapy targeting CDCP1 signaling is advantageous. In addition, understanding the physiological role of CDCP1 by, for example, gene targeting in mice, might be beneficial for predicting the side-effects of CDCP1-targeted therapy. Finally, it is expected that molecules participating in the CDCP1 signal will emerge as targets of new strategies for the prevention of and therapy for human cancer invasion and metastasis.

Disclosure Statement

The authors have no conflict of interest.

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ORIGINAL ARTICLE

ARAP3 inhibits peritoneal dissemination of scirrhous gastric carcinoma cells by regulating cell adhesion and invasion

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During the analysis of phosphotyrosine-containing proteins in scirrhous gastric carcinoma cell lines, we observed an unusual expression of Arf-GAP with Rho-GAP domain, ankyrin repeat and PH domain 3 (ARAP3), a multimodular signaling protein that is a substrate of Src family kinases. Unlike other phosphotyrosine proteins, such as CUB domain-containing protein 1 (CDCP1) and Homo sapiens chromosome 9 open reading frame 10/oxidative stress-associated Src activator (C9orf10/Ossa), which are overexpressed and hyperphosphorylated in scirrhous gastric carcinoma cell lines, ARAP3 was underexpressed in cancerous human gastric tissues. In this study, we found that overexpression of ARAP3 in the scirrhous gastric carcinoma cell lines significantly reduced peritoneal dissemination. *In vitro* studies also showed that ARAP3 regulated cell attachment to the extracellular matrix, as well as invasive activities. These effects were suppressed by mutations in the Rho-GTPase-activating protein (GAP) domain or in the C-terminal two tyrosine residues that are phosphorylated by Src. Thus, the expression and phosphorylation state of ARAP3 may affect the invasiveness of cancer by modulating cell adhesion and motility. Our results suggest that ARAP3 is a unique Src substrate that suppresses peritoneal dissemination of scirrhous gastric carcinoma cells.
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Keywords: ARAP3; scirrhous gastric carcinoma; Rho-GAP; cell-ECM adhesion; invasion

Introduction

The prognosis of scirrhous gastric carcinoma is poor because peritoneal dissemination and rapid submucosal invasion make it refractory to cancer treatments. Therefore, the discovery of novel therapeutic targets that control the progression of scirrhous gastric carcinoma is urgently needed. However, progress has been limited by the lack of knowledge about the factors and signaling pathways that mediate peritoneal dissemination and invasion.

Previous studies have shown that Src family tyrosine kinases (SFKs) are likely to be involved in these processes. For example, increased expression and kinase activity of SFKs frequently occurs during the transformation of precancerous cells (Frame, 2002). Activation of SFKs is also associated with tumor progression and metastasis, as well as with characteristic activities of cancer cells including proliferation, differentiation, motility, cell-extracellular matrix (ECM) adhesion, cell–cell adhesion and invasion (Brown and Cooper, 1996; Frame, 2002).

Recently, we reported that Src substrates such as CUB domain-containing protein 1 (CDCP1) (Uekita *et al.*, 2008) and Homo sapiens chromosome 9 open reading frame 10/oxidative stress-associated Src activator (C9orf10/Ossa) (Tanaka *et al.*, 2009) are hyperphosphorylated in the peritoneal nodules formed after inoculating the scirrhous gastric carcinoma cell line 44As3 into BALB/c nude mice. In our studies, we also detected another substrate of Src, Arf-GAP with Rho-GTPase-activated protein (GAP) domain, ankyrin repeat and PH domain 3 (ARAP3), in these nodules. However, immunohistochemical analysis showed that the expression level of ARAP3 was surprisingly higher in normal gastric glands than in cancerous tissue, unlike CDCP1 and C9orf10/Ossa.

ARAP3 functions as an effector of phosphoinositide 3-kinase (PI3K) by binding to phospatidyl-inositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) through pleckstrin homology (PH) domains (Krugmann *et al.*, 2002). On activation of PI3K signaling, ARAP3 is thought to be recruited to the plasma membrane, where it regulates lamellipodia formation and growth factor signaling (Kowanetz *et al.*, 2004; Krugmann *et al.*, 2004, 2006). ARAP3 is tyrosine-phosphorylated by Src mainly at the two tyrosine residues at the C-terminus (I *et al.*, 2004), whereas the biological role of tyrosine phosphorylation of ARAP3 is still unclear. In addition, the Rho-GAP domain of ARAP3 modulates the activity of Rho-family small GTPases, which regulate cytoskeletal dynamics as well as cancer invasion and metastasis. However, the

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relationship between these functions of ARAP3 and cancer phenotypes is not understood well.

In this study, we show that overexpression of ARAP3 in 58As9 cells, a highly metastatic scirrhous gastric carcinoma cell line, inhibited peritoneal dissemination in a mouse model. Furthermore, mutations to either the Rho-GAP domain or tyrosine phosphorylation sites of ARAP3 failed to inhibit peritoneal dissemination. ARAP3 also inhibited rapid cell-ECM adhesion and cell invasion *in vitro*. Our results support the hypothesis that ARAP3 suppresses the peritoneal dissemination of scirrhous gastric carcinoma cells.

Results

Expression of ARAP3 in human gastric tissues and tumor cell lines

Expression of ARAP3 in human gastric tissues was examined by immunohistochemical staining using an anti-ARAP3 antibody. ARAP3 is expressed primarily on the luminal side of the fundic gland (Figure 1a). In human gastric cancer tissues, ARAP3 expression was lower than in non-cancerous tissue (Figures 1b and c). Furthermore, the expression level of ARAP3 was lower in the undifferentiated type of gastric cancer than in the differentiated type (data not shown).

We also examined the expression of ARAP3 in 14 human gastric cancer cell lines (Figure 2). The expression of ARAP3 was very low or undetectable in 10 of these cell lines.

Roles of ARAP3 in peritoneal dissemination

To study the functional importance of ARAP3 in the peritoneal dissemination of scirrhous gastric carcinoma, we introduced ARAP3 expression vectors into 58As9 and NKPS scirrhous gastric carcinoma cell lines that expressed relatively little endogenous ARAP3 (Figure 2). Both the 58As9 and NKPS cell lines are highly metastatic (Yanagihara *et al*., 2005; Tanaka *et al*., 2009). After establishing 58As9 and NKPS cells that stably expressed wild-type ARAP3, we injected the cells into the peritoneal cavities of BALB/c nude mice (Figure 3 and Supplemental Figure 1). Compared with the parental 58As9 cell, the ARAP3-overexpressing cells formed fewer nodules on the mesentery and fatty tissues adjacent to the uterus (Figure 3b, lower panels). Essentially the same results were obtained from the analysis of ARAP3-expressing NKPS cells (Supplemental Figure 1B).

Effects of ARAP3 expression on the characteristics of gastric cancer cells in vitro

As the adhesiveness and invasiveness of scirrhous gastric carcinoma cell lines have a significant effect on peritoneal dissemination, we examined the effect of ARAP3 overexpression on the attachment of 58As9 cells to several ECM proteins, such as fibronectin, collagen

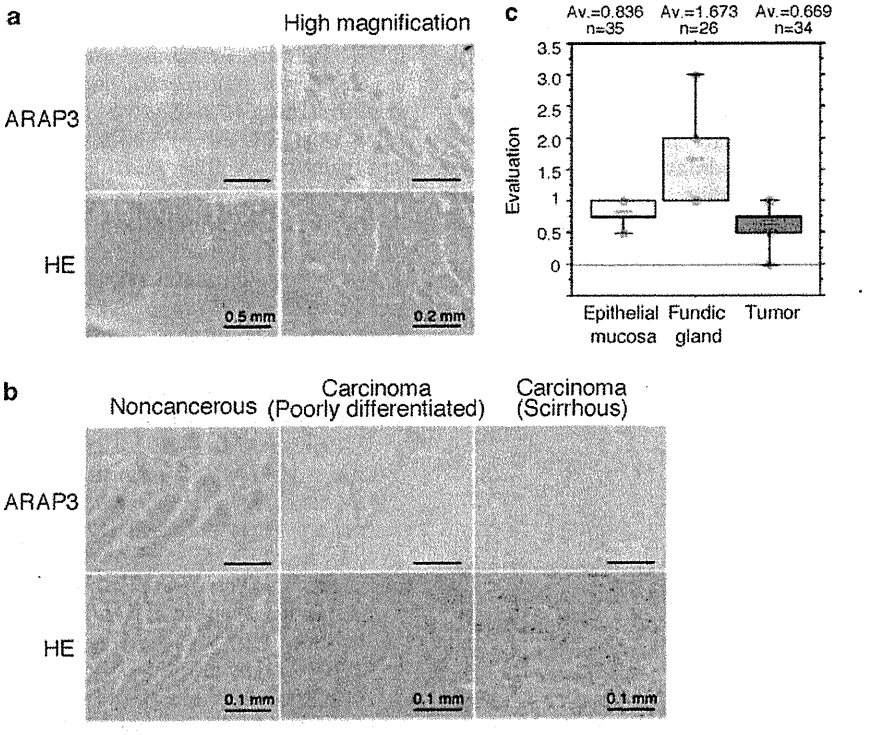

Figure 1 Immunohistochemical staining of ARAP3. Non-cancerous (**a**) and cancerous (**b**) human gastric tissues are shown. The intensity of staining was evaluated and arbitrarily scored from 0 to 3. The scores from samples of non-cancerous, cancerous and fundic gland tissues are shown in (**c**). Upper and lower quartiles are indicated with boxes, and minimum and maximum values are indicated with bars. Blue dots indicate the scores of all samples. Average scores (Av., red line) and sample numbers (n) are also shown. ARAP3 staining was detected in the fundic gland of the non-cancerous gastric tissues and was decreased in many cancerous tissues.

and vitronectin. Overexpression of ARAP3 partially suppressed the attachment of 58As9 cells to fibronectin and vitronectin (Figure 3c), but did not affect their adhesion to collagen. In addition, we performed an *in vitro* invasion assay using the 58As9 cell line with or without the overexpression of ARAP3. As shown in Figure 3d, ARAP3 clearly suppressed cell migration and invasion in 58As9 cells. ARAP3 also suppresses the cell migration activities and invasiveness of NKPS cells (Supplemental Figure 1C). However, overexpression of ARAP3 did not affect the cell growth (Supplemental Figure 2).

We also examined the effect of knocking down ARAP3 expression by using RNA interference in the 44As3 scirrhous gastric carcinoma cell line that expressed relatively high endogenous levels of ARAP3 (Figure 2a). ARAP3 expression was reduced in clones R3 and R5, as shown by immunoblotting (Figure 4A). Underexpression of ARAP3 promoted the attachment of 44As3 cells to fibronectin and vitronectin *in vitro* (Figure 4A). Moreover, an *ex vivo* adhesion assay, which measures the adhesiveness of gastric cancer cells to the mesothelium, showed the same results (Figure 4C). These results demonstrate that expression of ARAP3 may reduce the peritoneal dissemination of scirrhous gastric carcinoma cells by inhibiting cell-ECM adhesion and cell invasion. As activities of cell adhesion and invasion are strongly affected by morphological

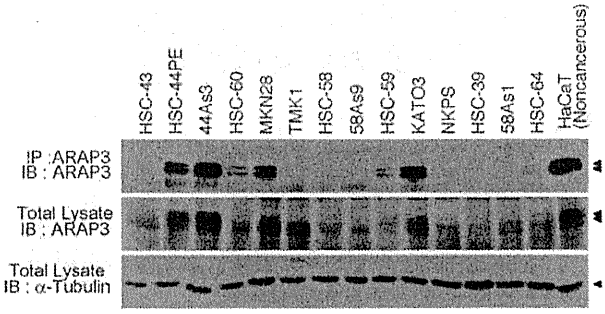

Figure 2 Expression of ARAP3 in human gastric cancer cell lines. Whole-cell lysates from non-scirrhous (MKN28, TMK1) and scirrhous human gastric cancer cell lines were immunoprecipitated with anti-ARAP3 antibody, and subsequently detected by immunoblotting. A human epithelial cell line, HaCaT, was used as a non-cancerous control. The expression of ARAP3 was diminished in 10 of the 14 gastric cancer cell lines tested.

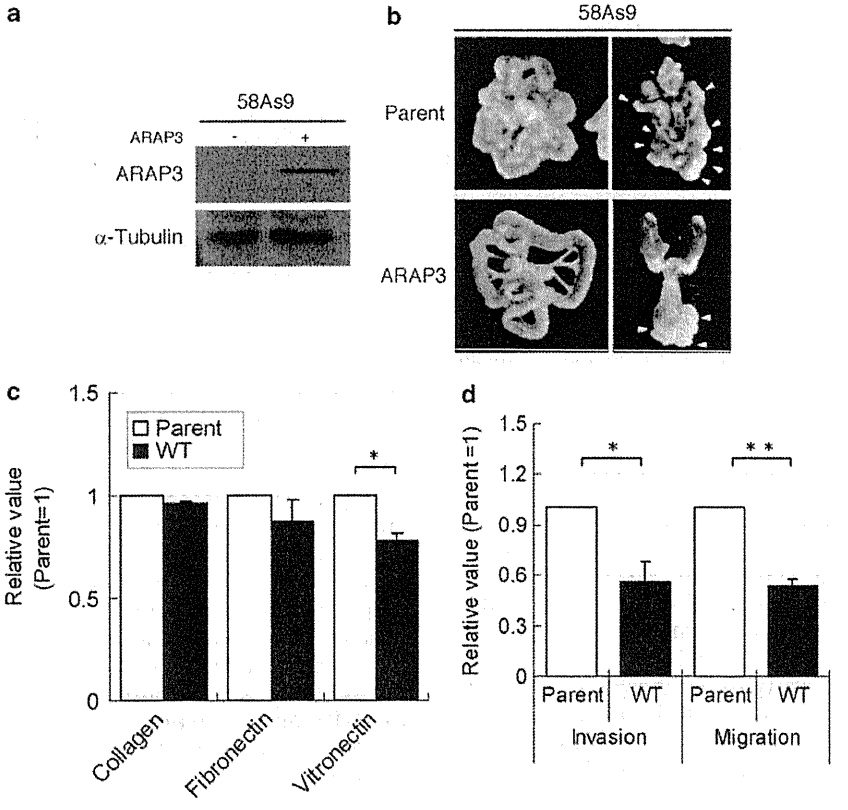

Figure 3 Functions of ARAP3 in the scirrhous gastric carcinoma cell line. The 58As9 cell line overexpressing ARAP3 was generated. Expression of ARAP3 was verified with immunoblotting (**a**). These cells were injected intraperitoneally into BALB/c nude mice ($n=4$). Mice were killed 21 days after injection and the peritoneal tissues were resected. (**b**) Tumors are indicated by yellow arrow heads. Cell adhesion to ECM proteins was also examined (**c**). Invasiveness was assessed *in vitro* by cell migration through a matrigel-coated or non-coated cell culture insert (**d**). ARAP3 expression suppressed cancerous activities of the scirrhous gastric carcinoma cell line *in vitro* and *in vivo* ($^*P<0.05$ $^{**}P<0.01$).

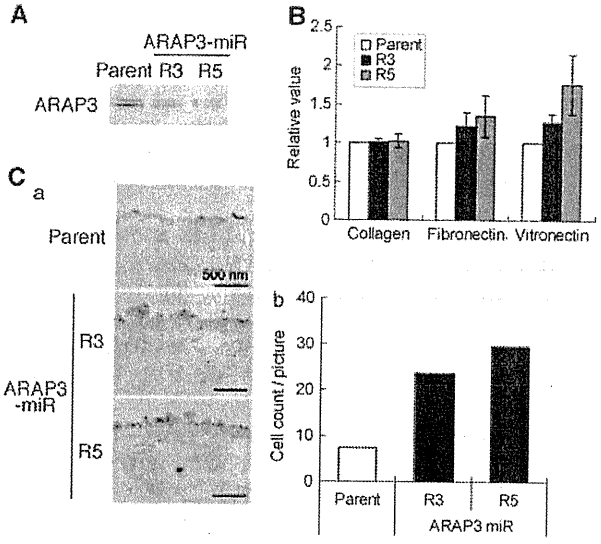

Figure 4 Expression of ARAP3 regulated cell-ECM attachment. ARAP3 was knocked down in 44As3 scirrhous gastric carcinoma cells by a microRNA knockdown system (R3 and R5 cells (**A**)). Cell attachment assays were performed with these cells *in vitro* (**B**) and *ex vivo* (**C**). In the *ex vivo* assay, the attachment of cancer cells to the mesentery was visualized by hematoxylin and eosin staining (**C**(a)) and quantified by cell counts (**C**(b)). Knockdown of ARAP3 increased the attachment of gastric carcinoma cells to both the ECM and mesentery.

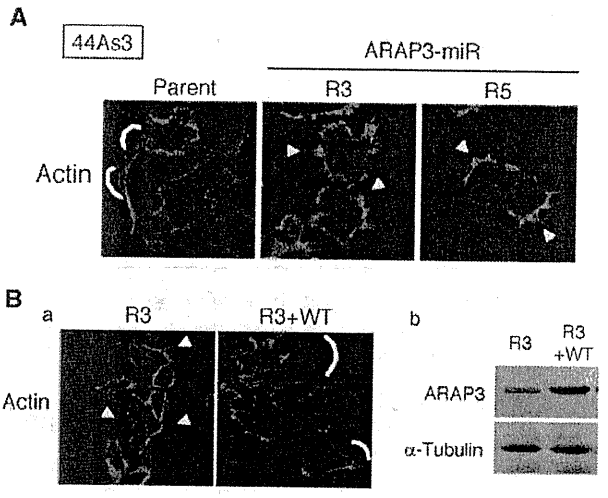

Figure 5 Expression of ARAP3 affected the morphology of the 44As3 cell line. Phalloidin staining for ARAP3 knockdown 44As3 cells (R3 and R5 cells) was performed (**A**). Wild-type ARAP3, which contained silent mutations in the microRNA target sequence, was stably introduced into R3 cells. Morphological changes were shown by phalloidin staining (**B**a) and expression of ARAP3 was detected by western blotting (**B**b). Formation of lamellipodia is indicated with yellow lines and filopodia is marked with yellow arrow heads.

changes (Carragher and Frame, 2004), we examined whether the expression of ARAP3 also affects the cell morphology of the gastric carcinoma cell line. Phalloidin staining showed that R3 and R5 cells formed filopodia instead of the lamellipodia observed in parental 44As3 cells (Figure 5A). Furthermore, introduction of wild-type ARAP3 that contained silent mutations in the target sequence of microRNA into R3 cells suppressed filopodia formation and recovered lamellipodia formation (Figure 5Ba). Expression of mutant ARAP3 was detected with western blotting (Figure 5Bb).

Differential roles of functional domains of ARAP3 in cell adhesion and cell invasion in vitro

As ARAP3 is a multimodular protein (Figure 6A), we investigated the structure–function relationships of ARAP3 domains to peritoneal dissemination. We established three 58As9 cells that expressed mutant ARAP3: (1) an Arf-GAP domain mutant (R532K), (2) a Rho-GAP domain mutant (R942L) and (3) a mutant that lacks both putative tyrosine phosphorylation sites (2YF, Y1403/1408F) (I *et al*., 2004) (Figure 6A). The level of tyrosine phosphorylation of the ARAP3 2YF mutant was significantly reduced compared with R532K or R942L in 58As9 cells (Figure 6B). As expression levels of ARAP3 were associated with cell morphology in gastric cancer cells (Figure 5), we performed phalloidin staining for 58As9 cells overexpressing ARAP3 mutants. R532K mutant ARAP3 suppressed filopodia formation more strongly than R942L or 2YF mutants (Figure 6C). None of the ARAP3 mutants affected the growth of 58As9 cells (Supplemental Figure 2).

We also studied the effect of overexpression of each mutant ARAP3 on cell-ECM adhesion and invasion. Overexpression of the R532K mutant ARAP3 reduced the attachment of 58As9 cell lines to ECM proteins, but overexpression of the R942L and 2YF mutants did not have any effect (Figure 7A).

As overexpression of wild-type ARAP3 could inhibit the metastasis of 58As9 cells, we performed an *in vitro* migration and invasion assay with the ARAP3 mutant cells. The results of this assay were similar to the attachment assay. Specifically, overexpression of the R532K mutant inhibited cell migration and invasion, but the R942L and 2YF mutants did not have any effect (Figure 7B).

These results suggest that both Rho-GAP function and tyrosine phosphorylation of ARAP3 are necessary to suppress cell-ECM adhesion and invasion of gastric cancer cells. As ARAP3 can inhibit RhoA, which is required for cell invasion (Itoh *et al*., 1999), we tested whether inhibition of RhoA signal by Y27632, one of the general inhibitors of Rho-associated coiled-coil kinase (ROCK), can also suppress the invasiveness of ARAP3 mutant expressing 58As9 cells. As expected, Y27632 could modestly suppress the invasiveness of the cells (Figure 7Bc).

Functional domains of ARAP3 critical for suppressing peritoneal dissemination in vivo

To investigate whether expression of mutant ARAP3 affects peritoneal dissemination *in vivo*, we injected the

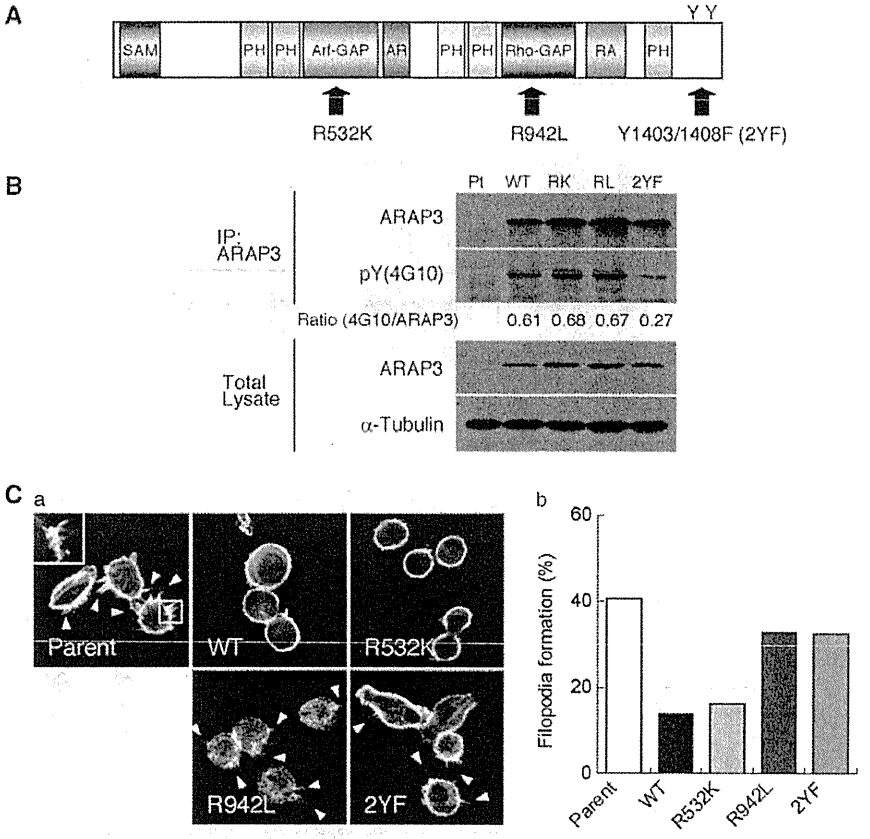

Figure 6 Expression of ARAP3 mutants. Mutations were introduced into the Arf-GAP (R532K) or Rho-GAP (R942L) domain, or into the two putative tyrosine phosphorylation sites (2YF) of ARAP3 (**A**). Expression and tyrosine phosphorylation profiles of 58As9 cells expressing mutant ARAP3 were verified by immunoprecipitation and immunoblotting. The relative tyrosine phosphorylation levels of ARAP3 (phosphorylated ARAP3/total ARAP3) were also calculated (**B**). Morphologies of cells expressing mutant ARAP3 are shown in (**C**). Phalloidin staining was performed on these cells. R532K mutant suppressed the filopodia formation that was observed in the parental cell line, but the R942L and 2YF mutants were not. Filopodia are indicated by white arrows. A highly magnified image of the area that is indicated by the white line is also shown in the image. Quantification of filopodia formation was performed by calculating the percentage of filopodia-positive cells (**C**b). Over 100 cells were counted. SAM, sterile alpha motif; PH, pleckstrin homology domain; AR, ankyrin repeats; RA, Ras association domain (nearly conserved).

cells intraperitoneally into BALB/c nude mice (Figure 8). Parental 58As9 cells showed severe peritoneal dissemination with ascitic fluid as previously described (Yanagihara *et al*., 2005). The R532K mutant suppressed production of ascitic fluid equally effectively, whereas the R942L and 2YF mutants were less effective (Figure 8A). Consistent with this result, expression of R532K, but not R942L and 2YF, suppressed the peritoneal dissemination of 58As9 cells, which was quantified by counting the number of mesentery nodules formed (Figure 8B). These results show that ARAP3 inhibits peritoneal dissemination *in vivo*, and that both the Rho-GAP domain and phosphotyrosine residues at the C-terminus are important for this function.

Discussion

This study showed that ARAP3 is expressed in normal fundic gland mucosa, but its expression is reduced in poorly differentiated carcinomas. ARAP3 inhibited not only cell-ECM attachment and cell invasion *in vitro* but also peritoneal dissemination of 58As9 cells *in vivo*. As adhesion to and invasion through the ECM are essential steps for peritoneal dissemination of scirrhous gastric carcinoma cells, it is hypothesized that ARAP3 suppresses peritoneal dissemination by regulating these processes enhanced in cancer cells.

We first identified ARAP3 while screening for phosphotyrosine proteins in mesentery nodules formed after inoculating nude mice with the 44As3 gastric carcinoma cell line. Unlike other phosphotyrosine proteins identified in this screening, such as FAK, CDCP1 and C9orf10/Ossa, ARAP3 was underexpressed in undifferentiated gastric cancers compared with normal fundic glands. Src pathways are implicated in cancer progression because SFK activity and expression and tyrosine phosphorylation of Src substrates are often elevated in advanced stages of cancer (Yeatman, 2004). Although ARAP3 is a Src substrate, our results showed that tyrosine phosphorylation of ARAP3 conferred

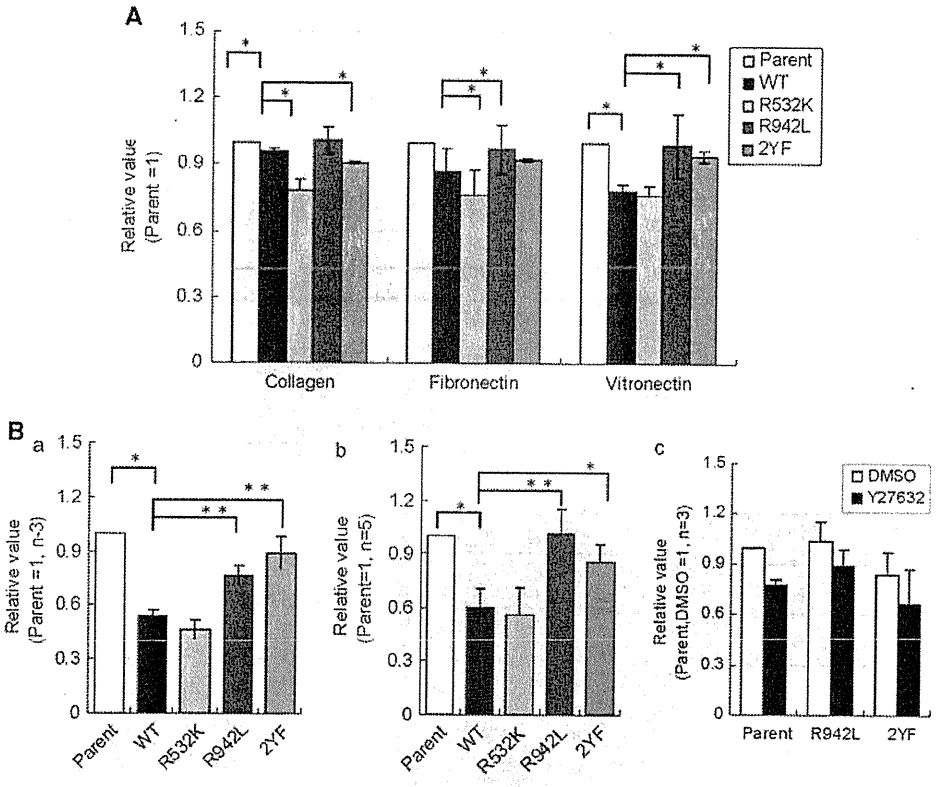

Figure 7 Effects of overexpression of ARAP3 mutants in 58As9 cell lines. Adhesiveness and invasiveness were examined by a cell attachment assay (**A**) and a cell migration and invasion assay (**Ba** and **Bb**, respectively). Overexpressed R942L and 2YF mutants did not rapidly adhere to ECM proteins or suppress cell migration and invasion. The Rho-associated coiled-coil kinase (ROCK) inhibitor Y27632 suppressed the invasiveness of 58As9 cells expressing R942L or 2YF ARAP3 (**Bc**). ARAP3 may suppress the invasiveness of 58As9 cells *in vitro* by inhibiting RhoA activity (*$P<0.05$ **$P< 0.01$).

a tumor suppressive activity. As a result, SFKs may phosphorylate not only oncogenic proteins but also tumor suppressor proteins such as ARAP3.

The ability of ARAP3 to suppress peritoneal dissemination was blocked by a mutation in the Rho-GAP domain, suggesting that this function is mediated by regulation of the Rho family small GTPases. In addition, loss of the C-terminal tyrosine phosphorylation sites of ARAP3 also reduced this suppressive function of ARAP3. However, it is not clear whether Rho-GAP activity and tyrosine phosphorylation of ARAP3 regulate the peritoneal dissemination using an overlapping mechanism or a signal pathway, as no information is available on the phosphotyrosine-mediated signaling of ARAP3. Although we could not detect any change in the activities of Rho family small GTPases in total cell lysate (data not shown), we cannot rule out the possibility that the activation of Rho occurs at the cell surface, because ARAP3 can localize and function there (Krugmann *et al.*, 2002), and its expression actually affected their cytoskeletal reorganization.

Rho family small GTPases are involved in a wide range of cellular processes, such as cell morphology, adhesion and motility. For example, activation of RhoA promotes the stabilization of focal contacts, which are important in the integrin-dependent cell-ECM attachment and cell invasion (Huveneers and Danen, 2009). Filopodia formation is necessary for cell migration and contact sites formation. In neuronal cells, RhoA activity is necessary for the formation of filopodial protrusion induced by RhoA/ROCK signaling (Chen *et al.*, 2006; Kim *et al.*, 2010). Furthermore, activation of mammalian diaphanous-related formin (mDia), which is known as an effector of RhoA, localizes at the filopodia, promotes actin filament assembly and contributes to filopodia formation (Faix and Grosse, 2006; Carramusa *et al.*, 2007; Sarmiento *et al.*, 2008). Therefore, ARAP3 may suppress the cell adhesion, migration and invasion of cancer cells by inhibiting RhoA signaling through its Rho-GAP domain.

ARAP3 is a multimodular protein that can bind to several molecules such as PI(3,4,5)P3 and Src homology 2 domain containing inositol 5-phosphatase 2 (SHIP2) *in vitro*, possibly through its sterile alpha motif domain (Krugmann *et al.*, 2002; Raaijmakers *et al.*, 2007). Our data revealed the essential role of the phosphotyrosine-containing region of ARAP3 for the first time. It might be required to identify molecules that bind to the C-terminal region of ARAP3 in a tyrosine phosphorylation-dependent manner. Further study is needed to determine

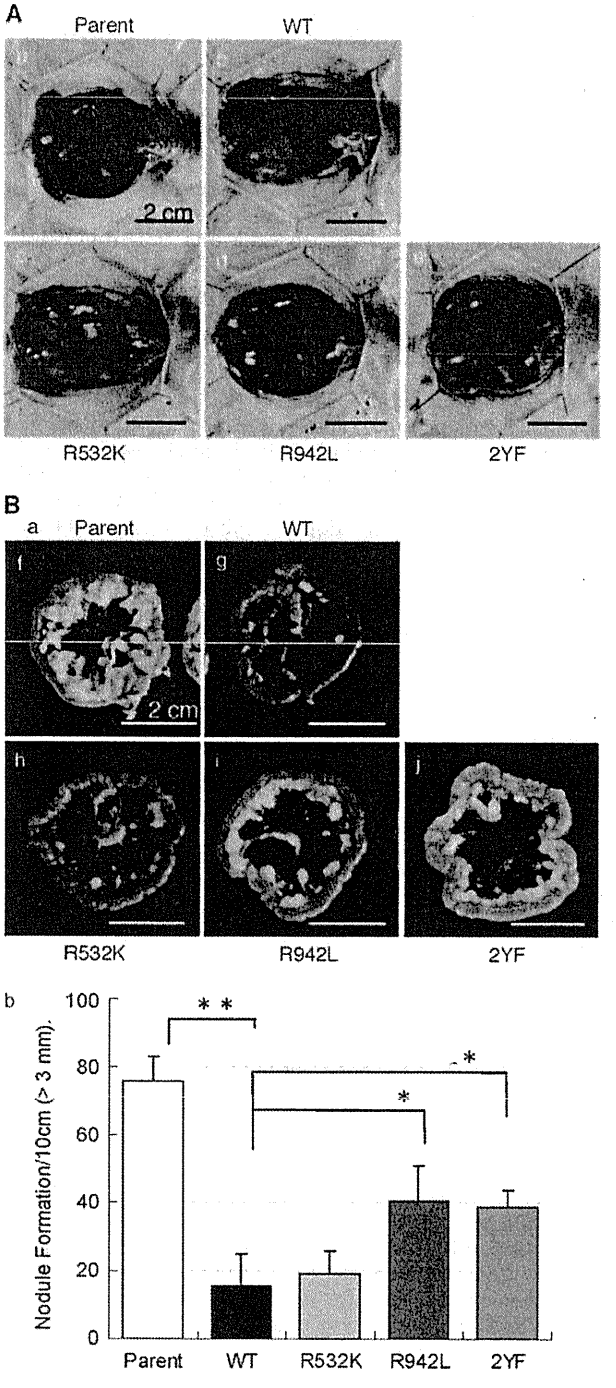

Figure 8 Expression of ARAP3 mutants affects peritoneal dissemination of gastric carcinoma cells. 58As9 cells expressing ARAP3 mutants were injected intraperitoneally into BALB/c nude mice (4×10^6 cells/mouse, $n = 5$). Nineteen days after injection, the R532K mutant ARAP3 suppressed ascites formation and peritoneal dissemination, but the R942L and 2YF mutants did not (**A** and **Ba**). Similarly, R532K also reduced the number of mesentery nodules formed after tumor cell inoculation, whereas R942L and 2YF did not (**Bb**) ($^*P<0.05$ $^{**}P<0.01$).

how the phosphotyrosine-dependent signal of ARAP3 suppresses cell-ECM attachment, migration and invasion of cancer cells.

We also observed that the Arf-GAP domain of ARAP3 regulated the internalization of epidermal growth factor receptor following epidermal growth factor stimulation (data not shown), similar to that reported in ARAP1 (Daniele *et al.*, 2008; Yoon *et al.*, 2008). However, judging from the results in this study, the Arf-GAP activity of ARAP3 does not significantly contribute to the suppression of peritoneal dissemination.

Several suppressors of integrin signaling or cell-ECM adhesion have been identified as possible therapeutic targets for preventing peritoneal dissemination of scirrhous gastric carcinoma (Nishimura *et al.*, 1996; Matsuoka *et al.*, 1998). Likewise, ARAP3, which regulates both cell-ECM adhesion and invasiveness, may be a novel therapeutic target. Small molecules that mimic the suppressive function of ARAP3, induce its expression or promote tyrosine phosphorylation of ARAP3 are anticipated to be effective drugs to prevent peritoneal dissemination of scirrhous gastric carcinoma cells.

Materials and methods

Materials

Anti-ARAP3 antibodies used for western blotting were purchased from Abcam (Cambridge, MA, USA), whereas those used for immunohistochemistry were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine (4G10) antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Sheep antimouse antibodies and sheep antirabbit antibodies were bought from GE Healthcare (Buckinghamshire, UK). Rabbit antigoat antibodies were purchased from Zymed (Carlsbad, CA, USA). Alexa 594-conjugated phalloidin was obtained from Molecular Probes (Carlsbad, CA, USA).

Immunohistochemical staining

Paraffin-embedded tissue samples of human scirrhous or non-scirrhous gastric carcinoma were obtained from the National Cancer Center Hospital (Tokyo, Japan). Sections on glass slides were rehydrated and autoclaved at 120 °C for 10 min to reactivate the antigen. Thereafter, the specimens were immunostained using the indirect polymer method with Envision reagent (Dako, Carlsbad, CA, USA) and ARAP3 antibodies (1:500 dilution). Finally, the stained sections were examined with an Olympus BX51 microscope (Tokyo, Japan).

Cell culture

Gastric cancer cell lines (HSC-39, HSC-43, HSC-44PE, 44As3, HSC-58, 58As1, 58As9, HSC-59, HSC-60, HSC-64, KATO3, NKPS, TMK1 and MKN28) were maintained in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. G418 (600 ng/ml; Sigma) or Blasticidin (10 ng/ml; Sigma) were also included in the growth medium to select for ARAP3-over-expressing or knockdown cells, respectively.

Construction of ARAP3 Stealth RNAi, miR RNAi and pDONAI-ARAP3 retroviral vectors

The ARAP3 gene was subcloned from a pEGFP-ARAP3 plasmid into a pDON-AI retrovirus vector, and then mutant

ARAP3 plasmids were constructed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA).

The miR RNAi design was based on the sequence of Stealth RNAi, which was designed using the BLOCK-iT RNAi designer tool (https://rnaidesigner.invitrogen.com/rnaiexpress/, Invitrogen, Carlsbad, CA, USA).

Phalloidin staining

Gastric cancer cells cultured on coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature. The samples were incubated with 3% bovine serum albumin for 1 h in Tween-Tris buffered saline (TTBS), followed by incubation with Alexa-594-conjugated phalloidin in TTBS for 45 min at room temperature, and coverslips were mounted on glass slides. The samples were examined using an Olympus IX-70 confocal laser-scanning microscope or an Olympus BX51 microscope for fluorescence microscopy. Phalloidin was used at 1:500 dilutions.

Immunoprecipitation and western blotting

Whole-cell lysates of gastric cancer cells were harvested by PLC lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid, 10% glycerol, 100 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 M phenylmethylsulfonyl fluoride), and used for immunoprecipitation and immunoblotting. Equal amounts of total protein were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies overnight at 4 °C, and then incubated with Horseradish peroxide (HRP)-conjugated secondary antibodies for 45 min at room temperature. Bands were detected on an X-ray film using an enhanced chemiluminescence system (Perkin elmer, Waltham, MA, USA)). Primary antibodies were used at dilutions of 1:1000 for ARAP3, 1:2000 for α-Tubulin and 1:5000 for phosphotyrosine.

Adhesion test

Cultured gastric cancer cell lines were dissociated with Hank's Balanced Salt Solution containing 0.25 mM EDTA. Cell suspensions (5×10^4 cells/well) were seeded into 24-well plates coated with collagen (Nitta gelatin, Osaka, Japan), fibronectin (Sigma) or vitronectin (TaKaRa, Shiga, Japan) according to the manufacturer's procedure. After incubating the plates for 30 min at 37 °C in a humidified atmosphere containing 5% CO_2, unattached cells were removed by washing with PBS, and then attached cells were trypsinized and counted with a Z1Coulter counter (Beckman Coulter, Brea, CA, USA).

Cell migration and invasion assays

Gastric cancer cells were dissociated with Hank's Balanced Salt Solution containing 0.25 mM EDTA. For the cell invasion assay, cell culture inserts (8.0 μM pore) were coated on the top and bottom surfaces with matrigel (10 μg/well) and fibronectin, respectively. In the cell migration assay, only the bottom surface was coated with fibronectin. Cells (2×10^4) suspended in serum-free RPMI1640 medium were seeded into each cell culture insert. These inserts were then transferred to a culture plate containing RPMI1640 medium with 5% FBS. After incubating at 37 °C in a humidified atmosphere containing 5% CO_2 for 8 h (migration assay) or 12 h (invasion assay), the cells were fixed with 4% paraformaldehyde and stained with Giemsa staining solution. Assays were performed in triplicate.

Ex vivo cell adhesion assay

Ten-millimeter square pieces of the peritoneal wall, including the peritoneum and abdominal skeletal muscle, were excised from 5-week-old BALB/c nude mice. The pieces were placed on top of a layer of 3% agarose gel in culture dishes, with the peritoneum side facing up. Equal numbers of parent 44As3 cells or 44As3 ARAP3 miR cells (4×10^6 cells/dish) were seeded onto the peritoneal tissue, and then incubated in RPMI 1640 medium containing 10% FBS, 50 μg/ml gentamicin, penicillin and streptomycin for 4 weeks. Finally, the cultured peritoneal walls were removed, fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis.

Inoculation of gastric tumor cells into nude mice

Gastric cancer cell lines were trypsinized, and aliquots of 4×10^6 cells were injected intraperitoneally into BALB/c nude mice purchased from CLEA Japan (Tokyo, Japan). After 3 weeks, the mice were killed and dissected. These experiments were approved by the Committee for Ethics of Animal Experimentation and conducted in accordance with the Guidelines for Animal Experiments in the National Cancer Center.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)