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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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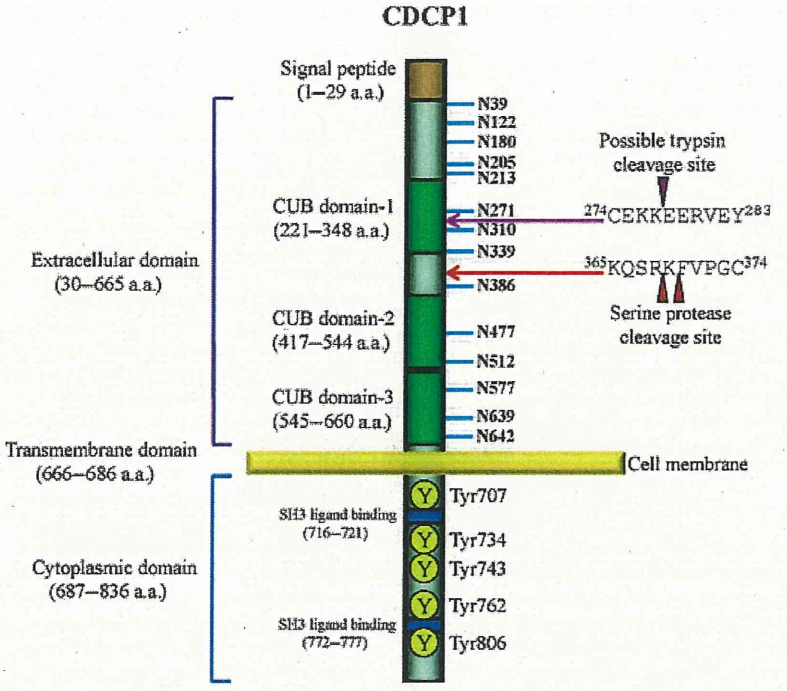

Fig. 1. Schematic illustration of human CUB domain-containing protein 1 (CDCP1) structures including the signal peptide. Derived CDCP1 encodes a protein of 836 amino acids (a.a.). Signal peptide (1–29 a.a.; red box) and an extracellular domain (30–665 a.a.) including CUB domains (CUB domain-1: 221–348 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.

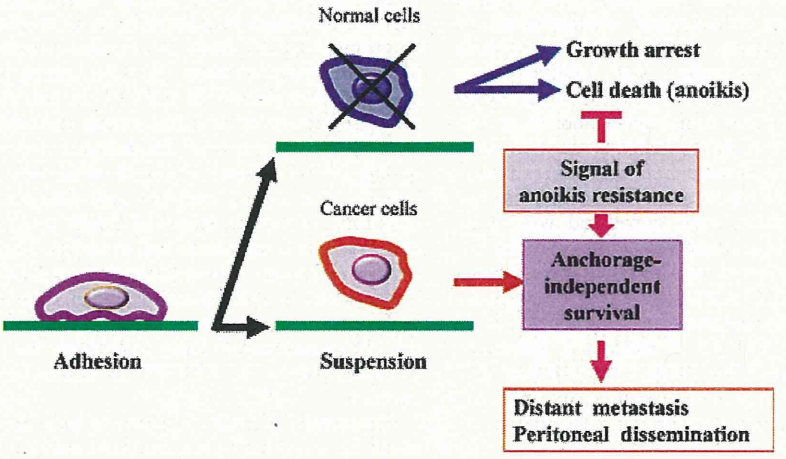

Fig. 2. Anoikis resistance: a key function of tumor metastasis. Anoikis is physiologically important in the maintenance of homeostasis and tissue architecture. In contrast, anoikis resistance is outstanding characteristics of cancer cells during tumor progression and metastasis. This property indicates 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.

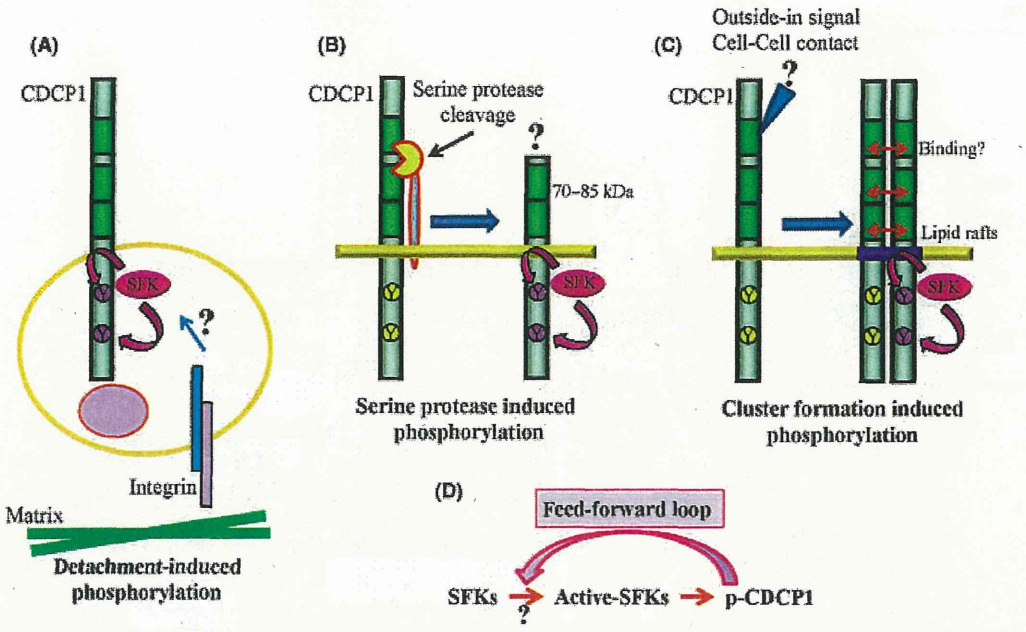

Fig. 3. Phosphorylation of CUB domain-containing protein 1 (CDCP1) is mediated by a number of cellular events. (A) Detachment cell-induced phosphorylation of CDCP1. Connection of cells to laminin 5 via integrins results in dephosphorylation of CDCP1,[8] suggesting that integrins affect detachment-induced 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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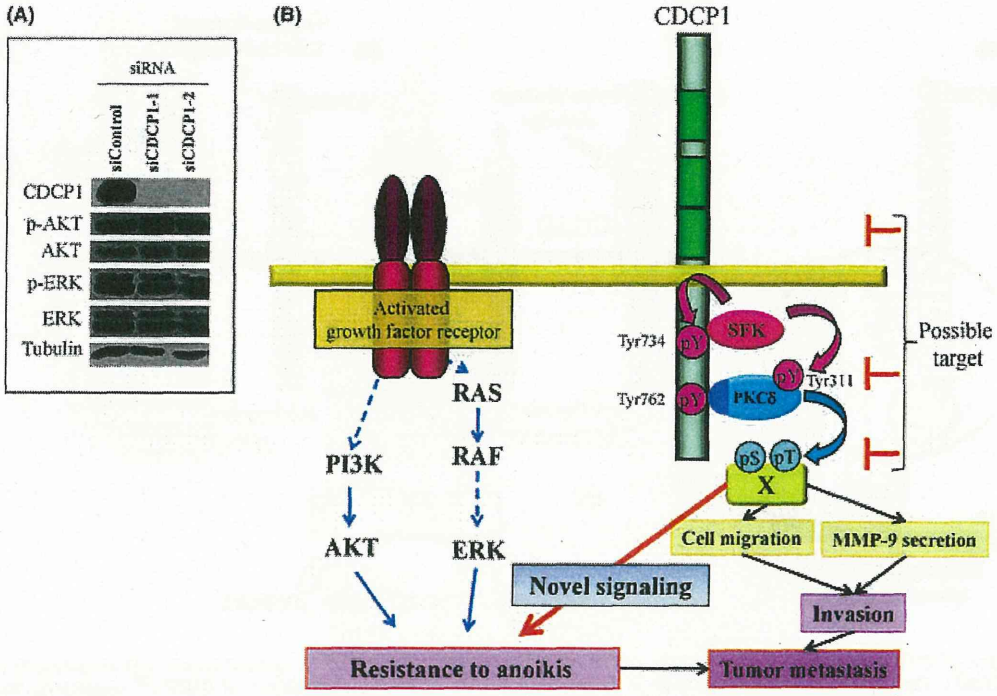

Fig. 4. Signal cascade of CUB domain-containing protein 1 (CDCP1) during tumor metastasis. (A) The pancreatic cancer cells (BxPC3) treated with CDCP1 siRNA were analyzed by immunoblotting with the indicated antibodies. Note that inhibition of CDCP1 expression does not significantly affect the phosphorylation 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

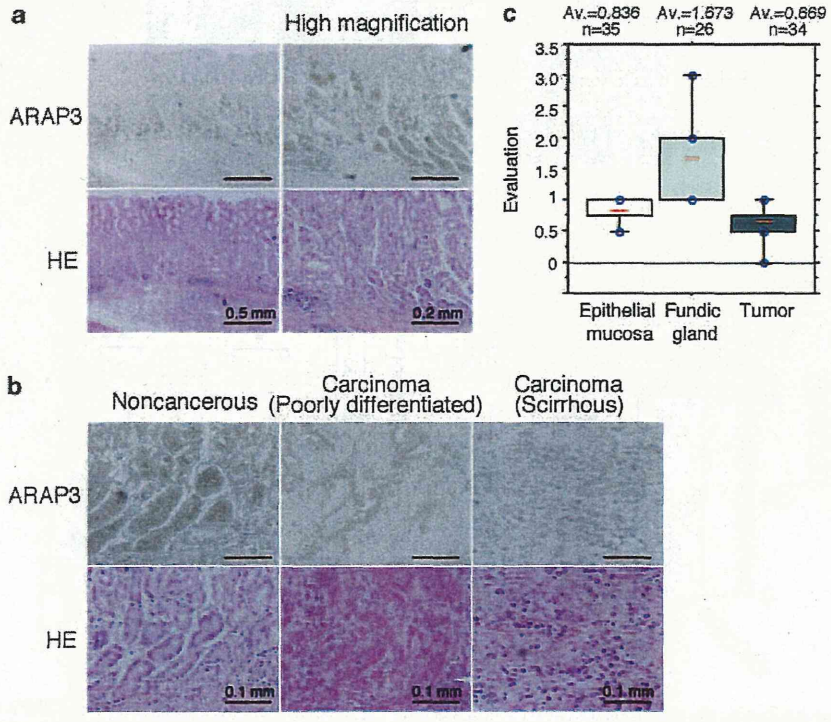

Figure 1 Immunohistochemical staining of ARAP3. Non-cancerous (**a**) and cancerous (**b**) human gastric tissues are shown. The intensity of staining was evaluated and arbitrarily scored from 0 to 3. The scores from samples of non-cancerous, cancerous and fundic gland tissues are shown in (**c**). Upper 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

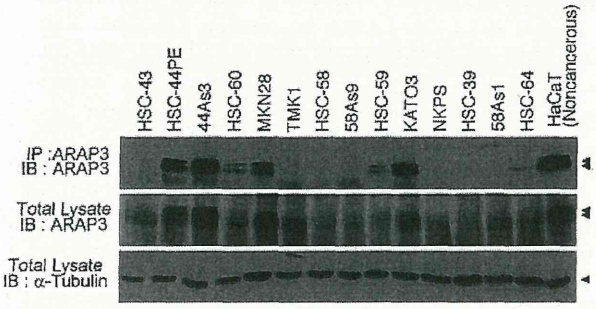

Figure 2 Expression of ARAP3 in human gastric cancer cell lines. Whole-cell lysates from non-scirrhous (MKN28, TMK1) and scirrhous human gastric cancer cell lines were immunoprecipitated with anti-ARAP3 antibody, and subsequently detected by immunoblotting. A human epithelial cell line, HaCaT, was used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

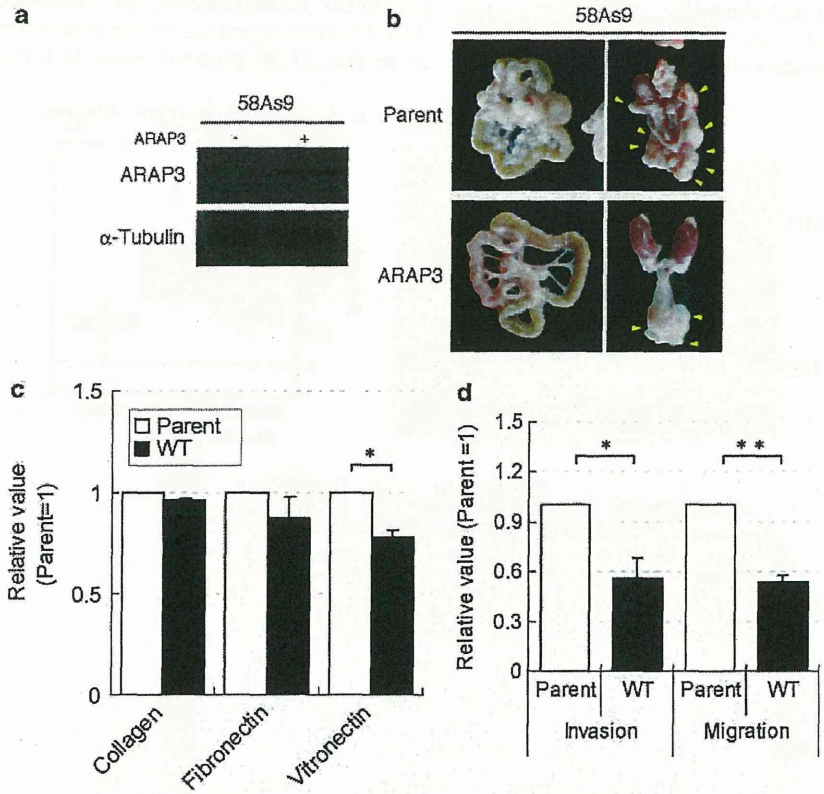

Figure 3 Functions of ARAP3 in the scirrhous gastric carcinoma cell line. The 58As9 cell line overexpressing ARAP3 was generated. Expression of ARAP3 was verified with immunoblotting (**a**). These cells were injected intraperitoneally into BALB/c nude mice ($n=4$). Mice were killed 21 days after injection 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* ($*P<0.05$ $**P<0.01$).