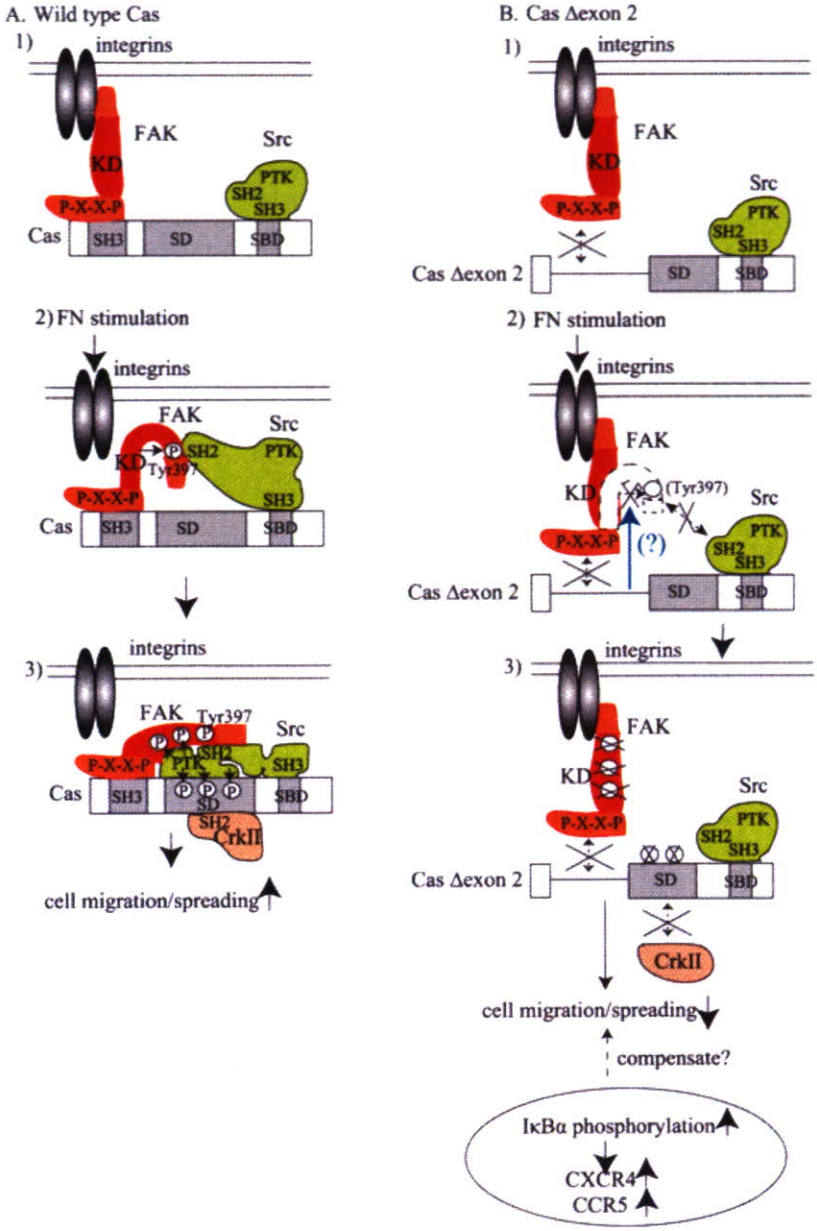

Figure 8 (A) Models for a signaling network involving wild-type Cas. (1) Cas binds to the proline-rich region of FAK through its SH3 domain and binds to the SH3 domain of Src through its Src binding domain (SBD) in unstimulated cells. (2) Upon FN stimulation, integrin clustering promotes FAK autophosphorylation at Tyr397, which creates a binding site for the SH2 domain of Src. (3) FAK/Src binding leads to the conformational activation of Src and results in an activated FAK/Src signaling complex. FAK/Cas binding and activated Src are linked to enhanced tyrosine-phosphorylation of Cas. Tyrosine-phosphorylated Cas binds to CrkII SH2 domain through the SD domain with preference for YDXP motifs and the Cas/CrkII complex plays a key role in cell migration/spreading. (B) Models for a signaling network involving Cas Δexon 2. (1) Cas Δexon 2 binds to Src but cannot bind to FAK in unstimulated cells because Cas binds to proline-rich region of FAK through its SH3 domain, which is missing from Cas Δexon 2. (2) Upon FN stimulation, FAK cannot be auto-phosphorylated by an unknown mechanism (possibly involving Cas SH3) and fails to bind to the SH2 domain of Src. Impaired FAK/Src complex leads to reduced activation of the FAK/Src signaling complex. (3) Because FAK is not tyrosine-phosphorylated and Src is not activated, Cas Δexon 2 cannot be tyrosine-phosphorylated and binding of CrkII to Cas Δexon 2 is impaired. Owing to impaired FAK/Cas/Src/CrkII complex, Cas exon 2-deficiency results in delay in cell migration/spreading. Cas exon 2-deficiency also enhances the expression of CXCR4 and CCR5, which may be dependent on IκBα phosphorylation. These factors may be up-regulated to compensate for the cellular functions affected by Cas exon 2-deficiency.

medium (DMEM) with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C with 5% CO_2. Experiments were performed between three and five passages.

Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation were performed essentially as previously described (Huang *et al.* 2002). Proteins were extracted by lysing cells in ice-cold 1% Triton lysis buffer [50 mM Tris–HCl PH 8.0, 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 1 mM Na_3VO_4]. For Western blotting, samples were separated by SDS-PAGE and probed with indicated antibodies. Positive signals were visualized with an enhanced chemiluminescence system (Amersham, Uppsala, Sweden). For immunoprecipitation, 500 μg protein aliquots were incubated with the indicated antibodies for 2 h at 4 °C and subsequently with Protein A-Sepharose (Invitrogen) for 1 h at 4 °C. Beads were washed 4 times with 1% Triton lysis buffer and boiled in sample buffer prior to SDS-PAGE analysis.

Cell stimulation with FN

Serum-starved cells were removed from the culture dishes by 0.05% trypsin treatment and were resuspended in DMEM. Culture dishes were coated overnight with 10 μg/mL FN (Chemicon, Temecula, CA) at 4 °C. The suspended cells were then plated on FN-coated dishes and incubated at 37 °C for various periods of time as described previously (Iwahara *et al.* 2004).

Wound healing cell migration assay

The wound healing cell migration assay was performed according to a method used previously (Honda *et al.* 1999). In brief, cells were first grown to confluence in plastic culture dishes, and a wound was made in the cell monolayer using a sterile micropipette tip. Then cells were washed 3 times with PBS and cultured at 37 °C in DMEM containing 10% FBS. Cell movement was assessed 3, 6, 9 and 12 h after wounding. The percentage of reduced distance between the nuclei of cells at each time period relative to the distance between two rims in the cleared field at the beginning was taken as the index.

Cell spreading assay

The cell spreading assay was performed as previously described (Honda *et al.* 1999). In brief, serum-starved cells were removed from the culture dishes by exposure to 0.05% trypsin–EDTA, and 2×10^5 cells in a volume of 1 mL DMEM were added to 35 mm tissue culture dishes coated with 10 μg/mL FN. The dishes were incubated at 37 °C for the indicated periods of time. Single cells that were phase-bright with rounded morphology were scored as non-spread, whereas those that possessed a flattened shape and looked phase-dark were scored as spread. The number of spread cells was calculated as percentage of the total cells in eight independent fields.

Immunofluorescence

Immunofluorescence was performed as previously described (Nakamoto *et al.* 1997). Cells were grown on FN-coated coverslips (Matsunami, Osaka, Japan) for 90 min. They were washed 3 times with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS. The fixed cells were washed twice with PBS and permealized with 0.2% Triton-X in PBS. The cells were rinsed and then blocked in PBS plus 3% bovine serum albumin (Sigma). Primary antibodies were used at the following dilutions for 3 h at room temperature in a humidified chamber: 1 : 200 for anti-Cas2, and 1 : 200 for hVIN-1. The coverslips were washed 3 times with PBS and treated with secondary antibodies at the recommended dilutions. After three washes with PBS, the coverslips were mounted in a 1 : 2 mixture of glycerol and PBS. The cells were examined with a LSM5 PASCAL confocal microscopic system (Carl Zeiss, Germany).

Microarray analysis

Total RNA was extracted from primary fibroblasts using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA from each sample were labeled using One-cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA) and hybridized with a GeneChip slide (Mouse Genome 430 2.0 Array, Affymetrix). Hybridization was performed at 45 °C for 16 h. After hybridization, slides were washed, dried and scanned using the GeneChip Scanner 3000 (Affymetrix). The array results were analyzed using GeneSpring (Agilent Technologies, Santa Clara, CA).

Quantitative real-time RT-PCR analysis

To confirm the differences in expression levels of the genes identified, we used fluorescent-based quantitative real-time RT-PCR with a TaqMan probe. RT-PCR was performed in 20 μL reaction mixtures containing 4 μL of 5 × LightCycler Taqman Master (Roche), 200 nM each primer and 100 nM Universal Probe-Library probe (Roche, Basel, Switzerland). Amplification reaction was carried out in a 384-well reaction plate in a spectrofluorimetric thermal cycler (ABI PRISM 7900 Sequence Detector, Applied Biosystems, Foster City, CA). A threshold cycle (Ct) for each sample was calculated by the point in which the fluorescence exceeded the threshold limit. To normalize the samples for loading total RNA equivalent, the second real-time PCR assay was performed targeting the 18S ribosomal RNA gene.

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Hyperphosphorylated cortactin in cancer cells plays an inhibitory role in cell motility by regulating tyrosine phosphorylation of p130Cas

Running title: hyperphosphorylated cortactin inhibits cell motility

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Abstract

Cortactin is frequently overexpressed in cancer cells, and changes of the levels of its tyrosine phosphorylation have been observed in several cancer cells. However, how the expression level and phosphorylation state of cortactin would influence the ultimate cellular function of cancer cells is unknown. In this study, we analyzed the role cortactin in gastric and breast cancer cell lines using RNA interference technique, and found that knockdown of cortactin inhibited cell migration in a subset of gastric cancer cells with a lower level of its tyrosine phosphorylation, whereas it greatly enhanced cell migration and increased tyrosine phosphorylation of p130Cas in other subsets of cells with hyperphosphorylated cortactin. Consistent results were obtained when hyperphosphorylation of cortactin was induced in MCF7 breast cancer cells by expressing Fyn tyrosine kinase. Additionally, immunostaining analysis demonstrated that knockdown of hyperphosphorylated cortactin resulted in recruitment of p130Cas to focal adhesions. These results suggest that cortactin hyperphosphorylation suppresses cell migration possibly through the inhibition of membrane localization and tyrosine phosphorylation of p130Cas.

Keywords: tyrosine phosphorylation, cortactin, p130Cas, small interfering RNA (siRNA), Fyn kinase and cell motility.

Introduction

Protein phosphorylation by tyrosine kinases functions as a major switch in cellular biological signaling events through modulating protein-protein interaction and protein conformation. Substrates of Src family kinases (SFKs) play essential roles in various cellular events by mediating tyrosine phosphorylation-dependent signals. Since cortactin was originally identified as a v-Src substrate, it has been shown to play a critical role in the organization of the cytoskeleton (1). The cortactin gene *EMS1* is located on chromosome 11q13, a region amplified in several cancers such as head and neck squamous carcinoma and breast cancer, (2-6). Cortactin is a modular protein that contains several motifs and domains involved in protein-protein interactions. An N-terminal acidic domain mediates its binding to Arp2/3, which regulates actin assembly, followed by an adjacency of six-and-a-half tandem repeats of 37 amino acids called cortactin repeats domain. There is a proline-rich domain immediately upstream of the SH3 domain, which also contains tyrosine residues phosphorylated by Src family kinases (7, 8). At the carboxyl terminus, there is a Src homology 3 (SH3) domain binding to several proteins including cortactin binding protein1 (CortBP1/Shank2) (9) and N-WASP (10). Cortactin is a substrate of tyrosine kinases including SFKs, Fer and Syk (11-13), and of serine/threonine kinases including Erk and PAK (14, 15). Among the Src family, Fyn kinase seems to play a specific role in the cortactin function in some tumors, since highly

phosphorylated cortactin was shown to associate with Fyn kinase in metastatic murine melanoma in our previous study (16). However, how cortactin phosphorylation affects intercellular signaling pathways for cell dynamics control and other functions is not understood.

It has been demonstrated that cell motility involves coordination of multiple signaling pathways regulating cell-substrate adhesion or actin polymerization (17, 18). A docking protein, p130Cas (Crk-associated substrate) is one of the key components of integrin-mediated signaling pathways, which conducts cell migration and actin filament reorganization in a tyrosine phosphorylation-dependent manner (19, 20). The C-terminal domain of p130Cas has both consensus SH3 and SH2 domains binding sites for SFKs, which are mainly responsible for the phosphorylation of p130Cas (21).

In this study, we investigated the role of cortactin in human gastric cancer cell lines using RNA interference (RNAi) technique, and discovered that knockdown of cortactin led to suppression of cell migration of the cells in which phosphorylation of cortactin is at basal level, while it increased cell migration of the cells in which cortactin is highly phosphorylated. It was also observed that knockdown of cortactin resulted in enhancement of cell motility of breast cancer cell line MCF7 in which phosphorylation level of cortactin was elevated by exogenously introduced Fyn kinase. In both cases, marked elevation in tyrosine phosphorylation of p130Cas was specifically and consistently observed by knockdown of hyperphosphorylated cortactin. We propose that tyrosine phosphorylation of cortactin may function as a molecular switch buffering the change in cell motility.

Results:

Effect on cortactin knockdown on cell migration of gastric cancer cells

Levels of tyrosine phosphorylation of cortactin were examined in human gastric cancer cell lines HSC57, HSC44As3, HSC44PE and HSC58As9 established in NCCRI, Japan (22), along with a breast carcinoma cell line MCF7 which possesses amplification of cortactin gene, *EMS1*(2). Expression of total cortactin was at similar levels (Figure 1A, lower panel), while there was a significant difference in tyrosine phosphorylation among these cell lines (Figure 1A, upper panel). HSC57, HSC44As3 and MCF7 cells exhibited a low level of tyrosine phosphorylation of cortactin, whereas HSC44PE and HSC58As9 showed hyperphosphorylation of cortactin as also indicated by the ratio of tyrosine phosphorylated cortactin to total cortactin (Figure 1A).

We knocked down cortactin expression by small interfering RNA (siRNA) to investigate the function of cortactin in the regulation of cell motility in various cancer cells using Transwell assay. It was confirmed by immunoblotting that more than 80% of cortactin expression was down-regulated at 72 hours after initiation of siRNA treatment (Figure 1B).

Interestingly, knockdown of cortactin increased cell migration in HSC44PE and HSC58As9 cells with hyperphosphorylated cortactin, while it impaired cell motility in HSC57, Hsc44As3 and MCF7 cells with a low level of phosphorylated cortactin (Figure 1C). Essentially similar effect on cell migration was seen by another siRNA, cort-siRNA2 (data not shown). To further confirm the migration promoting effect of cortactin siRNA in HSC44PE cells, we expressed mouse cortactin (mcort-WT) which is well conserved to human cortactin by the retrovirus vector (4), along with the mutant mouse cortactin (mcort-Mut) which lacks all 3 putative tyrosine phosphorylation sites by exchanging tyrosine residues 421, 466 and 482 to phenylalanine (F421F466F482). Wild type cortactin but not the mutant cortactin could block the elevation of cell migration induced by siRNA of cortactin in HSC44PE cells suggesting that cortactin is actually suppressing migration of HSC44PE cells in a tyrosine phosphorylation-dependent manner. These results indicate that cortactin might differentially exert negatively- and positively- regulating functions in cell migration depending on its tyrosine phosphorylation.

Cortactin was originally identified as a substrate of SFKs. Among SFKs expressing in solid tumor cells, Fyn kinase was shown to play central roles of in the tyrosine phosphorylation of cortactin in murine melanoma cells in our previous study (16). Treatment by a Src family specific inhibitor PP2 significantly reduced tyrosine phosphorylation of cortactin in HSC44PE cells (Supplementary Figure A), suggesting that Src family kinases are actually responsible for tyrosine phosphorylation of cortactin in HSC44PE cells. Relatively high expression of Fyn kinase along with stable association between Fyn and cortactin was observed in HSC44PE cells (Supplementary Figure B & C), suggesting the possibility that Fyn kinase is involved in hyperphosphorylation of cortactin in HSC44PE cells.

Knockdown of cortactin enhanced tyrosine phosphorylation of p130Cas in the cells with hyperphosphorylation of cortactin

By knockdown of cortactin, tyrosine phosphorylation of a 125~130KDa protein was remarkably enhanced in HSC44PE cells but not in HSC57 cells (Figure 2A). In HSC44PE cells treated with cortactin siRNA, dramatically increased tyrosine phosphorylation of p130Cas was observed using phospho-specific antibody of p130Cas (P-Cas460Y) at the exactly same position where the 125~130KDa protein detected by 4G10 (Figure 2B), while anti-phospho-FAK (Tyr397) and phospho-paxillin (Tyr118) antibodies failed to detect a significant change of phosphorylation state (Figure 2B). We also generated another phospho-specific antibody against p130Cas (P-Cas766Y: See Materials and Methods), and found consistent elevation in tyrosine phosphorylation of p130Cas by P-Cas766Y antibody in HSC44PE cells treated with 2 independent siRNAs for cortactin (Figure 2B). On the other hand, the level of phosphorylation of p130Cas was not significantly elevated in HSC57 cells (Figure

2B). There was no clear changes in the phosphorylation level of FAK or paxillin by the cortactin siRNAs in either HSC44PE cells or HSC57 cells (Figure 2B).

To confirm the role of hyperphosphorylated cortactin in these cells, we also generated MCF7 cell lines which have elevated tyrosine phosphorylation of cortactin. Original MCF7 cells showed high expression of cortactin with the minimum level of tyrosine phosphorylation (Figure 1A) and low expression of Fyn kinase (Figure 2C). A vector expressing the Flag-tagged Fyn kinase was introduced into MCF7 cells, and several clones with stable expression of Fyn kinase were isolated, and two of these clones were named as MCF-Fyn1 and MCF-Fyn2. The amount of Fyn protein in both cells was about 10-fold greater than that of endogenous Fyn kinase in original MCF7 cells (Figure 2C). Marked hyperphosphorylation of cortactin was observed in these two clones where Fyn kinase was introduced while original MCF7 cells or mock transfected cells (MCF-Vec) were not (Figure 2C). Knockout of cortactin greatly enhanced tyrosine phosphorylation of p130Cas both in MCF-Fyn1 cells and MCF-Fyn2 cells, but not in MCF7-Vec cells (Figure 2D and data not shown). These consistent results suggest that hyperphosphorylated cortactin may inhibit tyrosine phosphorylation of p130Cas in both HSC44PE and MCF-Fyn cells. Knockdown of cortactin also increased cell motility in both MCF-Fyn1 and MCF-Fyn2 cells while it blocked cell migration in parental MCF7 cells and MCF-Vec cells (Figure 1C, Figure 2E and data not shown). Similar results were obtained in the study using gastric cancer cells and it was demonstrated that hyperphosphorylation of cortactin switches the response of cell motility.

Since many reports have shown the significant role of phosphorylation of p130Cas in cell migration, we examined whether knockdown of p130Cas by siRNA impairs the cell motility of HSC44PE cells treated or non-treated with cortactin siRNA. Double treatment of Cas-siRNA and cort-siRNA completely impaired cell motility as well as treatment by Cas-siRNA alone, showing that p130Cas plays a dominant role in the regulation of cell motility of HSC44PE cells (Figure 3B). These results indicate the possibility that hyperphosphorylated cortactin may suppress cell migration through inhibiting tyrosine phosphorylation of p130Cas.

Recruitment of p130Cas to focal adhesion by treatment of cortactin siRNA

To further investigate the cause of tyrosine phosphorylation of p130Cas induced by knockdown of cortactin, we analyzed the subcellular localization of p130Cas, cortactin and focal adhesion proteins in HSC44PE cells. Total cortactin was widely expressed near the cell membrane in HSC44PE cells (Figures 4A and 4B) while phosphorylated cortactin detected by a phospho-specific antibody against cortactin Y421 is appeared at specific domain which seems to be as focal adhesion within the cell membrane (Figure 4B). It was further demonstrated that phosphotyrosine-containing cortactin was clearly colocalized with vinculin which is expressed at focal adhesion (Figure 4C). By the treatment with cortactin siRNA, both the signals detected

by total cortactin and cortactin Y421 were significantly weakened, suggesting these signals are cortactin-specific (Figure 4A).

In the control cells, p130Cas is mainly distributed in cytoplasm and only slightly expressed at focal adhesion (Figure 5B, upper panels). When cortactin is knocked down, a substantial amount of p130Cas comes to locate at focal adhesion (Figure 5B, lower panels), suggesting the loss of cortactin induced membrane localization of p130Cas. On the other hand, tyrosine phosphorylated p130Cas detected by phospho-specific antibody was specifically localized at the focal adhesion although the amount of tyrosine phosphorylation was greatly increased by the suppression of cortactin expression (Figure 5A). This observation may support the model that tyrosine phosphorylated cortactin expressed predominantly in focal adhesions interferes with the localization of p130Cas at the focal adhesion, which causes tyrosine phosphorylated p130Cas. Loss of hyperphosphorylated cortactin could therefore recruit p130Cas to the focal adhesion, which results in tyrosine phosphorylation of p130Cas, followed by enhancement of cell motility.

Discussion:

We explored the role of cortactin in cell motility by using RNAi technique in several gastric cancer cell lines that show various phosphorylation states of cortactin. It was revealed that knockdown of cortactin results in enhanced cell motility along with increased tyrosine phosphorylation of p130Cas in the cells that have hyperphosphorylated cortactin, whereas it impairs cell migration in the cells with a low level of tyrosine phosphorylated cortactin. In addition, knockdown of hyperphosphorylated cortactin caused recruitment of p130Cas to focal adhesion, which might result in enhanced cell migration. In this study, for the first time, we demonstrated that cortactin has a dual function in the regulation of cell motility, which depends upon its tyrosine phosphorylation state.

Amplification and overexpression of cortactin gene, *EMS1* have been identified in various cancers (2, 3, 6, 23). In our previous study, overexpression and elevated tyrosine phosphorylation of cortactin was selectively observed in the metastatic subgroup of murine melanoma cells with high migratory potential (16). Based on these results, we first hypothesized that the hyperphosphorylated cortactin may promote cell migration in cancer cells. In this study, gastric cancer cells that have hyperphosphorylated cortactin showed at least similar or higher migration potential compared with those cells with basal levels of tyrosine phosphorylation of cortactin (Figures 1A and C). However, unexpectedly, knockdown of cortactin in these cells further enhanced cell migration. The effect of cortactin knockdown was opposite in the cells with a low level of cortactin phosphorylation. This paradoxical outcome by cortactin knockdown was also confirmed in breast cancer cells MCF7, which expressed a significant

amount of cortactin although the level of tyrosine phosphorylation was quite low. Introduction of Fyn kinase to MCF7 cells significantly enhanced the tyrosine phosphorylation of cortactin. In this condition, loss of cortactin by siRNA enhanced cell migration while it had a negative effect on cell migration in the parental cells. Based on this finding, cortactin hyperphosphorylation may be induced as a negative feedback mechanism when cells acquire highly migratory potential.

Some of studies have shown that knockdown of cortactin results in impaired cell motility in hepatocellular carcinoma and head and neck squamous cell carcinoma cells (24, 25) while other studies have shown no significant effect on cell motility (8, 26), although all these studies lack information on the phosphorylation states of cortactin. Since it is suggested that cortactin differentially functions in cell migration depending on its phosphorylation state, the previous controversial results from the knockdown of cortactin should be revaluated from the viewpoint of its tyrosine phosphorylation state. A previous study shows positive effect of the cortactin phosphorylation on the cell migration by overexpression of normal cortactin and phosphorylation-defective mutants in the endothelial cells (27). Although this study does not mention about level of tyrosine phosphorylation of endogenous cortactin either, it might be weak in normal endothelial cells. Therefore positive effect of cortactin expression shown in the study might be consistent with our result using HSC57 or MCF7 cells, while dominant-negative effect of phosphorylation-defective mutant might indicate some different function of cortactin in the endothelial cells.

In our observation, knockdown of cortactin induced outstanding enhancement in tyrosine phosphorylation of p130Cas. Since there is no significant change in the phosphorylation of other substrates of SFKs such as FAK and paxillin, it is indicated that there exists some specific mechanism underlying the regulation of tyrosine phosphorylation state of p130Cas by cortactin. Unphosphorylated p130Cas is known to be mainly expressed in cytoplasm as shown in the Fig 5B, while localization of p130Cas in the focal adhesion is thought to be required for tyrosine phosphorylation of p130Cas (28, 29). The results of immunocytostaining indicate that tyrosine phosphorylated cortactin predominantly exists in the focal adhesion, and thus purging p130Cas from focal adhesion to lose the chance of being phosphorylated. Therefore, knockdown of cortactin might give chance for p130Cas to localize at the focal adhesion to be phosphorylated (Figure 5). p130Cas was originally found as a prominent substrate of SFKs including c-Src and Fyn during the transformation of cells (21, 30). It has been demonstrated that phosphorylated p130Cas in focal adhesion plays a regulatory role in cell spreading and cell migration (19,31-33), and knockdown of p130Cas actually abrogated cell migration in our gastric cancer cells HSC44PE even with the absence of cortactin (Figure 3A and B). These results indicate that activation of p130Cas-mediated signal might be

responsible for the enhancement of cell motility in the cells where hyperphosphorylated cortactin is knocked down.

Our study sheds new light on the crosstalk between cortactin and p130Cas, both of which are Src substrates. Such cross-regulation between SFKs substrates might give more optimized outcome out of overlapping or conflicting effects of SFKs signals. However, there is still a possibility that phosphorylated cortactin may also transduce specific signals regulating cell migration, which is independent of a function of p130Cas. As far as we examined, there was no significant effect on the morphology, cell-matrix adhesion or numbers of focal adhesions in HSC44PE cells by suppression of cortactin expression (Figure 5A and data not shown). On the other hand, it has been suggested that cortactin originally support the cell migration through several pathways and therefore its tyrosine phosphorylation may have an inhibitory effect on these pathways. Binding of cortactin with N-WASP via its SH3 domain may synergize in causing actin polymerization preceding cell migration, which is promoted by a serine kinase Erk and conversely inhibited by Src kinase (34). We illustrated all these signal pathways of phosphorylated cortactin in a schema (Figure 6). In summary, tyrosine phosphorylation of cortactin might act as a unique and naïve switch in the regulation of cell motility. Our study offers new insight into cortactin for understanding its biological function in cancer progression.

Materials and Methods

Cells culture

Human breast cancer cell line MCF7 was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS; GIBCOBRL, LIFE TECHNOLOGIES) and 50 ug/mL penicillin-streptomycin antibiotics. Human gastric cancer cell lines HSC57, HSC44As3, HSC44PE and HSC58As9 was supplied by Central Animal Laboratory, National Cancer Center Institute, Tokyo, Japan (Yanagihara *et al.*, 2004). They were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics.

Antibodies and Reagents

Anti-phosphotyrosine (4G10) and anti-cortactin (clone 4F11) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies Fyn3 and anti-FAK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-paxillin and anti-Green fluorescent protein (GFP) antibodies were purchased from Zymed (San Francisco, CA) and MBL (Nagoya, Japan) respectively. Anti-phospho-paxillin (Tyr118), anti-phospho-FAK (Tyr397) and anti-phospho-cortactin (Tyr421) antibodies were purchased from Cell Signaling Technology (Danvers, MA), Upstate Biotechnology (Lake Placid, NY) and Chemicon (Boronia Victoria, Australia) respectively. Anti-Flag-M2 antibody was obtained from SIGMA (Sigma, USA). Polyclonal antibodies against p130Cas (Cas2 & Cas3) and phospho-specific polyclonal

antibody P-Cas460Y were used as described previously (30, 35). Another phospho-specific antibody P-Cas766Y was generated by immunizing rabbits with a synthetic phospho-peptide, CMEDpYDpYVHL, which includes the phosphotyrosine-containing motifs in the Src-binding domain of p130Cas, after being conjugated with thymoglobulin. As secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (Amersham) were used. Polylysine, fibronectin (FN) and cycloheximide were purchased from SIGMA. Inhibitor of SFKs, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP2) and the inactive structural analog 4-amino-7-phenylpyrazol [3,4-d] pyrimidine (PP3) were obtained from Calbiochem-Novabiochem Ltd.

RNAi experiments for cortactin and p130Cas

Two independent small interfering RNA (siRNA) of human cortactin, cort-siRNA1 and cort-siRNA2 for RNAi experiment were generated by Invitrogen Life Technologies. Cort-siRNA1 targets cortactin mRNA at 5'-CCCAGAAAGACUAUGUGAAAGGGUU-3' and Cort-siRNA2 targets at 5'-GGAGAAGCACGAGUCACAGAGAGAU-3'. The siRNA of human p130Cas was also generated by Invitrogen Life Technologies, using the target sequence 5'-CCAAGAUCUUUGUGGCGCACAGCAA-3'. For transient transfection of siRNA, the cells were plated at 1.5 X 10^5 per well on a six-well plate. Transfection of siRNAs was performed with Lipofectamine™ 2000 (Invitrogen Co.). After transfection for 72 hours, the cells were harvested for the biochemical analyses.

Infection of retroviral constructs

Retrovirus vector were used to express mouse cortactin fused with GFP (pjl6) and F421F466F482 triple mutant of mouse cortactin fused with GFP (pJL12)(4). Briefly, the retroviral vector and the packaging construct pCL-10A1 were cotransfected into 293T cells and culture fluid was harvested 72 hours post-transfection. HSC44PE cells were infected with the viral fluid in the presence of 4 mg/ml polybrene, and the infected cells were selected in the presence of 800 μg/ml Geneticin G418 (SIGMA) for a period of 2~3 weeks.

Establishment of stable MCF7 clones expressing Fyn kinase

Breast cancer cells MCF7 were grown in DMEM containing 10% FBS and antibiotics. On the night before transfection, the cells were seeded onto a 10-cm dish at a density of 9.0 X 10^5. Transfection of a vector expressing Fyn kinase with a tag of Flag (Fyn-Flag) and an original vector (pcDNA3.1) was performed according to the manufacture's instructions. Twenty-four hours after transfection, the cells were subjected to selection by Geneticin G418 (SIGMA) at a concentration of 800 μg/ml for a period of 2~3 weeks.

Immunoblotting and immunoprecipitation

For immunochemical analysis, cells were cultured in the incubator at 37℃ with 5% CO_2 for 48~72 hours, before the cells were lysed in 1% Triton X-100 buffer (50mM HEPES,

150mM NaCl, 10% glycerol, 1% Triton-X 100, 1.5mM $MgCl_2$, 1mM EGTA, 100mM NaF, 1mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1mM phenylmethylsulfony fluoride), and insoluble material was removed by centrifugation for 10 min. Protein concentration was analyzed by BCA Protein Assay (PIERCE) and the protein aliquots were separated by SDS-PAGE. Gels were transferred to the polyvinylidene difluoride membrane (Millipore) and subjected to immunoblotting. After blocking with 5% skim milk in TBST (100mM Tris-HCl pH 8.0, 150mM NaCl, 0.05% Tween 20) for 1h, blots were incubated with primary antibodies. In the case of 4G10, blocking was performed with Blocking One Solution (Nakarai Co.). Membranes were then washed three times with TBST, incubated with HRP-conjugated secondary antibodies for 30 min at RT, washed three times by TBST again and once by TBS (100mM Tris-HCl pH 8.0, 150mM NaCl), and visualized by autoradiography using a chemiluminescence reagent (Western Lighting, Perkin Elmer).

For immunoprecipitation, 500 μg of protein was mixed with 2 μg of antibodies and incubated for 1 hour on ice, and then samples were rotated with protein G-Sepharose beads (Amersham Pharmacia) for 2 hours at 4℃. The beads were washed three times with 1% Triton X-100 buffer and boiled in sample buffer (2% SDS, 0.1M Tris-HCl, pH6.8, 10% glycerol, 0.01% bromphenol blue, 0.1M dithiothreitol) for SDS-PAGE analysis.

Immunocytostaining

Around 5 $\times 10^4$ cells were plated on 12-mm circle cover glasses on a 24-well plate, which were grown in DMEM with 10% FBS at 37℃ with 5% CO_2 for 24 hours. The 12-mm circle cover glasses were coated by fibronectin (FN) 10 μg/ml in PBS overnight before seeding the cells. Then cells were fixed in 4% paraformaldehyde in 0.1M sodium phosphate (pH 7.0) for 5 min, washed three times with PBS, and permeabilized with 0.1% Triton X-PBS for 10 min before blocking with 5% bovine serum albumin with TBST (0.15M NaCl, 1% Tris, pH 7.0, 0.05% Tween 20) for 10 min. Then the cells were incubated with the first antibody for 1 hour at room temperature. Cells were washed with PBS three times, and incubated with appropriate second Alexa antibodies (Molecular Probe) (1:500) in 5% goat serum with 3% BSA/TBST. All cover glasses were mounted in 1.25% DABCO, 50% PBS and 50% glycerol. The staining was visualized using a Radiance 2100 confocal microscopic system (Bio-Rad).

Cell migration assay

Migration assay was performed using modified Transwell chambers with polycarbonate Nucleopore membrane (FALCON™, BD, US). Precoated filters (6.5 mm in diameter, 8-μm pore size, FN 10 μg/ml) were rehydrated with 100 μl medium. Then, 4 x 10^4 cells in 100 μl serum-free DMEM supplemented were seeded onto the upper part of each chamber, whereas the lower compartments were filled with 600 μl of the same medium with 10% FBS. Following incubation for 8 hours at 37°C, non-migrated cells on the upper surface of

the filter were wiped out with a cotton swab, and the migrated cells on the lower surface of the filter were fixed and stained with Giemsa's Stain Solution (Azur-Eosin-Methylene Blue Solution, MUTO PURE CHEMICALS, Co. Japan). The total number of migrated cells was determined by counting cells in five microscopic fields per well at a magnification of 100X, and the extent of migration was expressed as the average number of the cells per microscopic field.

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Titles and legends to figures

Figure 1. Changes in cell motility by knockdown of cortactin in gastric and breast cancer cells. (A) To evaluate expression and tyrosine phosphorylation of cortactin, cell lysates of gastric and breast cancer cell lines were immunoprecipitated with anti-cortactin antibody (2μg/mL), and immunoblotted by anti-phosphotyrosine antibody (4G10) and anti-cortactin antibody. The positions of cortactin isoforms (p85/80) are indicated by arrows. MCF7, HSC57 and HSC44As3 cells exhibited a low level of tyrosine phosphorylation of cortactin, whereas HSC44PE and HSC58As9 showed hyperphosphorylation of cortactin. Quantification of tyrosine phosphorylated cortactin was noted under the panel. The expression of total cortactin is shown in the bottom panel. **(B)** Amounts of cortactin in these cell lines at 72 hours after siRNA treatment are shown. **(C)** Cell motility in various cancer cell lines was evaluated by numbers of migrated cells on the membrane. Knockdown of cortactin by siRNA led inhibition of cell migration in HSC57, HSC44As3 and MCF7 cells with hypophosphorylated cortactin, whereas it resulted in increase of cell migration in HSC44PE and HSC58As9 cells with hyperphosphorylated cortactin. **(D)** HSC44PE cells (Parent) and HSC44PE cells stably expressed mouse cortactin (mcort-WT) or F421F466F482 triple mutant of mouse cortactin (mcort-Mut) fused with GFP were treated with or without cortactin siRNA. Cells were lysed 72 hours after treatment and immunoblotted for cortactin and GFP. The concentration of total proteins was confirmed by the same membrane rehybridized with anti-α-tubulin antibody. Cortactin siRNA downregulates endogenous human cortactin (endo) but not exogenous mouse cortactins (exo). Tyrosine phosphorylation of mouse cortactins was analyzed by immunoprecipitation of total cortactin and immunoblotting with anti-phosphotyrosine antibody (4G10). The quantification of immunoprecipitated cortactin is shown in the bottom (IB: cortactin). **(E)** Effect of the rescue of mouse cortactin expression on cell migration in cortactin knockdown HSC44PE cells were analyzed as described. Rescue of tyrosine phosphorylated cortactin (mcort-WT) affected the inhibition of cells migration but non-tyrosine phosphorylated cortactin (mcort-Mut) did not.

Figure 2. Analysis of cell motility in MCF clones with hyperphosphorylated cortactin. (A) HSC57 and HSC44PE cells were treated with cortactin siRNA for 72 hours, the whole cell lysates were subjected to immunoblotting assay by antibody 4G10 to see phosphotyorsine-containing proteins. The phosphotyrosine-containing protein around 120~130KDa was significantly enhanced as indicated by a square bracket. **(B)** Marked elevation of phosphorylation of p130Cas at tyrosine 460 and tyrosine 766 in HSC44PE cells was confirmed by specific anti-phospho-Cas antibodies (P-Cas460Y and P-Cas766Y) using two independent cortactin siRNAs. Immunoblotting by anti-cortactin, anti-Cas2, anti-phospho-FAK (Tyr397), anti-phospho-paxillin (Tyr118) and anti-tubulin antibodies are also shown. **(C)** Stable

clones of MCF7 cells expressing Flag-tagged Fyn kinase (MCF-Fyn1 and MCF-Fyn2) or vector alone (MCF-Vec) were established (see Materials and Methods). Left panels show immunoblotting by anti-Flag, anti-Fyn3 and anti-tubulin antibodies to demonstrate high expression of Flag-Fyn in these clones. Right panels indicate that the tyrosine phosphorylation of cortactin was markedly induced in MCF-Fyn1 and MCF-Fyn2 clones with no significant change in total cortactin expression. **(D)** Enhancement of phosphorylation of p130Cas at tyrosine 460 and tyrosine 766 in MCF-Fyn1 cells treated with two independent cortactin siRNAs was confirmed by specific anti-phospho-Cas antibodies (P-Cas460Y and P-Cas766Y). **(E)** Effect of cortactin siRNA on cell migration of MCF7-Fyn clones was analyzed as described.

Figure 3. Knockdown of p130Cas blocks cell migration of HSC44PE cells regardless to the expression of cortactin. (A & B) HSC44PE cells were treated with either cortactin siRNA or p130Cas siRNA alone, or both and cell migration was analyzed as described. (A) Expression of cortactin, p130Cas (Cas2) and phosphorylated p130Cas (pCas460Y) was analyzed to check the effect of each siRNA.(B) By treatment with siRNA of Cas, cell migration of HSC44PE cells was totally inhibited regardless of the amount of cortactin expression.

Figure 4. Localization of cortactin and tyrosine phosphorylated cortactin in HSC44PE cells. (A) HSC44PE cells were transfected with cortactin siRNA or control siRNA for 72 hours followed by immunocytostaining with anti-cortactin (green) and anti-phospho-cortactin (Tyr421: red) antibody. **(B)** HSC44PE cells were immunostaied with anti-phospho-cortactin (Tyr421: red) and anti-cortactin (green) antibodies. **(C)** HSC44PE cells were immunostained with anti-phospho-cortactin (Tyr421: red) and anti-vinculin (green) antibodies. Merged image (Merge) indicates that of tyrosine phosphorylated cortactin localizes at focal adhesions which are stained by anti-vinculin.

Figure 5. Changes in localization of p130Cas by treatment with cortactin siRNA. (A) HSC44PE cells were transfected with cortactin siRNA or control siRNA for 72 hours, before localization of phosphorylated p130Cas was analyzed by immunocytostaining with P-Cas766Y antibody. Anti-vinculin antibody was used to visualize focal adhesions. Merged images indicate that tyrosine phosphorylated p130Cas which was greatly increased by cortactin siRNA specifically localizes at focal adhesion. **(B)** In HSC44PE cells treated with control or cortactin siRNA, localization of p130Cas was analyzed by anti-Cas3 antibody. Potion of p130Cas shows distinct colocalization with vinculin at focal adhesion in cells treated with cortactin siRNA, while staining of vinculin and p130Cas did not significantly overlapped in cells treated with control siRNA.

Figure 6. Schematic view of multiple roles of cortactin in the regulation of cancer cell motility.

Figure. 1

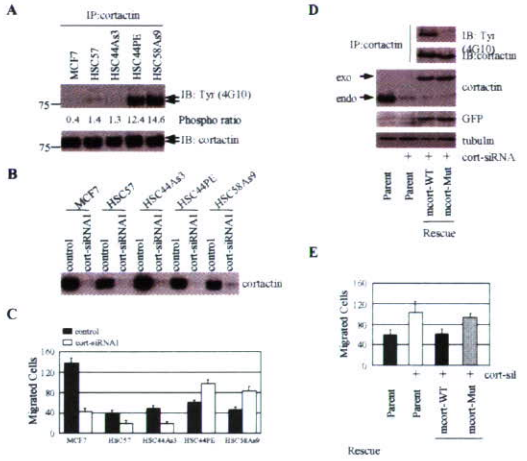

Figure. 2

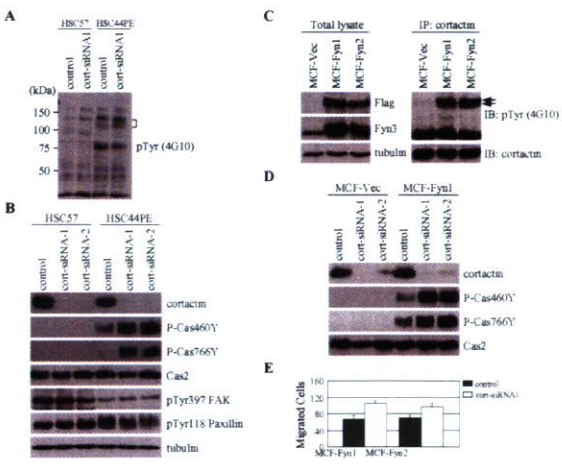

Figure. 3

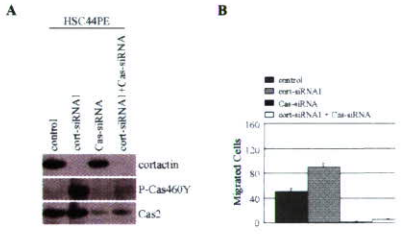

Figure. 4

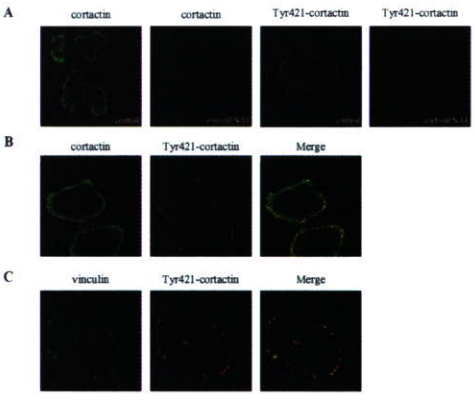

Figure. 5

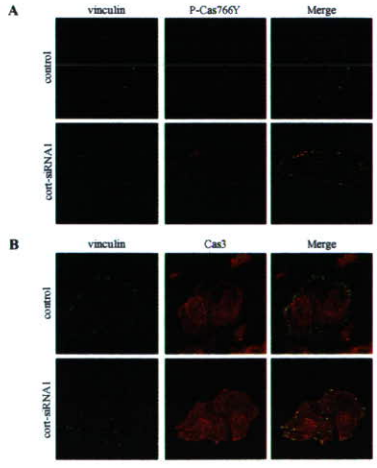

Figure. 6