Cytoplasmic p120ctn Regulates the Invasive Phenotypes of E-Cadherin-Deficient Breast Cancer

Tatsuhiro Shibata, Akiko Kokubu, Shigeki Sekine, Yae Kanai, and Setsuo Hirohashi

From the Pathology Division, National Cancer Center Research Institute, Tokyo, Japan

In a search for signaling molecules that act downstream of E-cadherin inactivation in cancer, we examined the expression and localization of E-cadherin-associated proteins in lobular carcinoma, in which the E-cadherin gene is frequently inactivated, and found that E-cadherin down-regulation correlated with the cytoplasmic localization of p120ctn. Similar cytoplasmic localization of p120ctn and growth factor-induced accumulation of tyrosine-phosphorylated p120ctn in the protrusive domain were observed in E-cadherin-deficient breast cancer cells. Down-regulation of endogenous p120ctn by RNA interference promoted stress fiber formation and induced a flattened morphology with an increase of Rho-GTPase activity; it also reduced the development of membranous protrusions and migratory activity in E-cadherin-deficient breast cancer cells. Inactivation of E-cadherin in cancer cells is associated with the conversion from epithelial to mesenchymal phenotype, which also occurs in physiological conditions such as developmental processes. Cytoplasmic localization of p120ctn accompanied by E-cadherin down-regulation was observed in mesoderm cells that had undergone epithelial-mesenchymal transition during early mouse embryogenesis. Collectively, our results suggest that cytoplasmic p120ctn may contribute to the invasive phenotype of E-cadherin-deficient breast cancer cells. (*Am J Pathol* 2004, 164:2269–2278)

Cell-cell interactions, mediated by adhesion molecules, play vital roles in developmental morphogenesis, tissue remodeling, and carcinogenesis.^{1–3} One of the major epithelial cell adhesion molecules, E-cadherin, is frequently down-regulated in various human cancers, and reduced expression of this molecule correlates with the emergence of malignant characteristics, such as loss of epithelial morphology, invasiveness, and metastasis.^{3–5} Moreover the E-cadherin gene is somatically inactivated in diffuse-type gastric cancer and lobular carcinoma, which exhibit a highly invasive and metastatic phenotype, and carry a poor prognosis.^{6–9} On the other hand, during developmental processes, transcriptional repressors,

such as the snail and ZEB families cause down-regulation of E-cadherin gene expression and epithelial-to-mesenchymal morphological transition, and increase cell motility during the various processes of organogenesis.^{10–12} Thus, regulation of E-cadherin activity is one of the fundamental mechanisms underlining morphogenesis from both a pathological and a physiological point of view.

Although it is well known that E-cadherin is an invasion suppressor in many cancers,⁵ the way in which it negatively regulates the invasive potential of cancer cells is poorly understood. E-cadherin has been thought to act like glue, thus presenting a barrier against cell movement. Recently it has been called into question that only such a passive role of intercellular adhesion is contributed to cancer cell invasion. The E-cadherin complex contains many cytoplasmic signaling molecules, including α -, β -, and γ -catenin, p120ctn, and Shc,^{13–16} and E-cadherin has been shown to be implicated not only in cell-cell adhesion but also in epithelial polarity, cell migration, growth, and survival.^{17,18} The diverse signals emanating from the E-cadherin complex are therefore likely to be altered by the inactivation of E-cadherin resulting in the various phenotypes of cancer.

With this possibility in mind, in the present study, we focused on the expression and localization of p120ctn in E-cadherin-deficient cancers. Among E-cadherin-interacting molecules, p120ctn has been identified as a potential regulator of cell motility. Dynamic regulation of the actin cytoskeleton is essential to cell morphology and movement, and the Rho family of small GTPases plays various important roles in these processes.^{19–21} Overexpression of p120ctn drastically alters epithelial morphology and promotes cell motility by regulating members of the Rho family.^{22–24} Furthermore in the embryo of *Drosophila melanogaster*, p120ctn cooperates with α -catenin to regulate epithelial cell movement.²⁵ In addition, altered expression of p120ctn has been reported in various human cancers, including colon, gastric, and breast cancers.^{26–28}

Supported in part by a grant-in-aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor, and Welfare, Japan; and by a Grant-in-Aid for Scientific Research (Encouragement of Young Scientist) from the Japan Society for the Promotion of Science (to T.S.).

Accepted for publication February 27, 2004.

Address reprint requests to Setsuo Hirohashi, M.D., Pathology Division, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo, 104-0045, Japan. E-mail: shirohas@ncc.go.jp.

Our study showed that down-regulation of E-cadherin resulted in the cytoplasmic localization of p120ctn in breast cancers. Down-regulation of endogenous p120ctn in E-cadherin-deficient breast cancer cells prevented invasive phenotypes. Interestingly cytoplasmic localization of p120ctn accompanied with E-cadherin down-regulation was also observed in mesoderm cells that had undergone epithelial-mesenchymal transition during early mouse embryogenesis.

Materials and Methods

Immunohistochemistry

Sixty routinely processed, formalin-fixed, and paraffin-embedded blocks of lobular carcinoma were obtained from the National Cancer Center Hospital. Staged mouse embryos were obtained by natural mating of BALB/c mice, fixed in 4% formalin, and embedded in paraffin wax. Immunohistochemistry was performed using 4- μ m sections of formalin-fixed, paraffin-embedded specimens as described.²⁹ The sections were autoclaved for antigen retrieval and blocked in either 2% normal swine serum (DAKO Japan, Kyoto, Japan) for lobular carcinoma or M.O.M. mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) for mouse embryo specimens. They were then incubated with mouse monoclonal antibodies against E-cadherin ($\times 100$, DAKO Japan), β -catenin ($\times 100$, Transduction Laboratories, Lexington, KY), p120 ($\times 100$, Transduction Laboratories), and with a rabbit polyclonal antibody against E-cadherin ($\times 100$; Santa Cruz, Santa Cruz, CA). The antibodies were diluted in 2% normal swine serum (DAKO Japan) or in M.O.M. diluent (Vector Laboratories) for application to lobular carcinoma and mouse embryo specimens, respectively. For fluorescence immunohistochemistry, an Alexa 488 Fluor-conjugated anti-mouse antibody and an Alexa 594 Fluor-conjugated anti-rabbit antibody (Molecular Probes, Eugene, OR) were used as secondary antibodies.

Cell Lines

The human breast cancer cell lines MCF7, BT474, SKBR-3, ZR75-50, MDA-MB231, and MDA-MB435S were obtained from the American Type Culture Collection (Manassas, VA).

Fluorescence Immunocytochemistry

The cells were grown on type I collagen-coated glass (Iwaki, Tokyo, Japan), and fixed with 4% paraformaldehyde. Mouse monoclonal antibodies against p120ctn and tyrosine 228-phosphorylated p120ctn ($\times 100$, Transduction Laboratories), and a rabbit polyclonal antibody against E-cadherin were used as primary antibodies. An Alexa 488 Fluor-conjugated anti-mouse antibody and an Alexa 594 Fluor-conjugated anti-rabbit antibody were used as secondary antibodies. Alexa 594 Fluor-conjugated phalloidin (Molecular Probes) was used for the detection of actin fibers. The immunofluorescent images were captured

with a charge-coupled device camera (CoolSNAPfx; Roper Scientific Japan, Tokyo, Japan) and analyzed using Meta-morph software (Roper Scientific Japan).

Immunoblotting and Immunoprecipitation

For immunoblot analysis, the cells were lysed in lysis buffer [10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Triton X, and complete proteinase inhibitor cocktail tablet (Roche, Mannheim, Germany)]. Twenty μ g of protein of each cell was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5 to 15%), blotted onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA), and incubated with mouse monoclonal antibodies against E-cadherin ($\times 400$, Transduction Laboratories), α -catenin ($\times 400$, Zymed, South San Francisco, CA), β -catenin ($\times 500$, Transduction Laboratories), γ -catenin ($\times 400$, Transduction Laboratories), p120ctn ($\times 400$, Transduction Laboratories), and β -tubulin ($\times 400$, Santa Cruz). The immunocomplexes were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). For immunoprecipitation, the cells were lysed in lysis buffer (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 1% Triton X, and complete proteinase inhibitor cocktail tablet) and precleared with protein G-conjugated Sepharose beads (Amersham Pharmacia Biotech). Then rabbit polyclonal antibody against p120ctn (Santa Cruz) and protein-G-conjugated Sepharose beads were added. After washing with lysis buffer, immunoprecipitants were analyzed by immunoblotting with mouse monoclonal antibodies against phosphotyrosine ($\times 1000$, Upstate Biotechnology, Lake Placid, NY) and tyrosine 228-phosphorylated p120ctn ($\times 500$).

Plasmids and Transfection

Mouse E-cadherin cDNA (a gift from Dr. Takeichi, Kyoto University, Kyoto, Japan) was subcloned into pEF-1/Myc-His (Invitrogen, Carlsbad, CA). One μ g of each plasmid was transfected into SKBR-3 grown on type I collagen-coated glass using LipofectAmine 2000 (Invitrogen).

RNA Interference

Several double-strand (ds) RNAs were initially tested for their ability to down-regulate endogenous p120ctn expression. For the production of the dsRNA finally used in the experiments, sense and anti-sense RNA were synthesized using T7 RNA polymerase (Megashortscript; Ambion, Austin, TX) and the following oligonucleotides 5'-GTGGAC-CATGCACTGCATGCCTATAGTGAGTCGTATTAC-3' and 5'-AAGGCATGCAGTGCATGGTCCTATAGTGAGTCGTATTAC-3' for p120ctn, and 5'-GAGGTCTCTCTCACCAC-CTCCTATAGTGAGTCGTATTAC-3' and 5'-GTGGAGGTG-GTGAGAGAGACCTATAGTGAGTCGTATTAC-3' for GFP (used as a control). After annealing at 37°C, the dsRNA was purified by phenol/chloroform extraction and ethanol precipitation, then transfected using Oligofectamine (Invitrogen).

Figure 1. Cytoplasmic localization of p120ctn in lobular carcinoma. The expressions of E-cadherin (A), β -catenin (B), and p120ctn (C) in a specimen of lobular carcinoma are shown. The sections were incubated with mouse monoclonal antibodies against E-cadherin, β -catenin, and p120ctn. Membranous expression of E-cadherin, β -catenin, and p120ctn in normal mammary epithelium (arrowheads). E-cadherin expression was lost and β -catenin expression was reduced, while p120ctn was localized in the cytoplasm of cancer cells (arrows). **D:** Double-immunofluorescence analysis of E-cadherin (red) and p120ctn (green) shows that co-localization of p120ctn and E-cadherin in the lateral membranes of the normal mammary gland epithelial cells (yellow) and cytoplasmic localization of p120ctn in the E-cadherin-deficient cancer cells (arrow). Original magnifications, $\times 200$ (A–C).

Pull-Down Assay of the Rho Small GTPase

The Rho and Rac GTP pull-down assay was performed by using an EZ-detect Rho/Rac activation kit (Pierce, Rockford, IL) with minor modification. The cells were lysed in 25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1% Nonidet P-40, 1 mmol/L dithiothreitol, 5% glycerol, and protease inhibitors. One-twentieth of cell lysates were subjected to immunoblotting. Cell lysates were mixed with 40 μ g of GST-Rhotekin-RBD or 20 μ g of GST-Pac-PBD and incubated with glutathione-conjugated beads at 4°C for 30 minutes. Beads were washed four times with lysis buffer and bound proteins were analyzed by immunoblotting with rabbit polyclonal antibody against Rho ($\times 400$, Upstate Biotechnology) and mouse monoclonal antibody against Rac ($\times 400$, Pierce).

Migration Assay

SKBR-3 cells were trypsinized 16 hours after the transfection of 20 nmol/L dsRNA. The transfected SKBR-3 cells (5×10^5) were grown in serum-free medium on polycarbonate transwell filters with a pore size of 8 μ m (Kurabo, Tokyo, Japan). To ensure efficient adhesion, the transwell filters were precoated with 0.3 mg/ml of type I collagen. Because SKBR-3 cells are reported to migrate in response to heregulin stimulation,³⁰ heregulin- β epidermal growth factor domain (5 ng/ml, Upstate Biotech-

nology) was added to the bottom chamber. After incubation for 16 hours, the cells that had transmigrated through the pores were fixed, and the number of cells in five fields was counted by microscopy at $\times 200$ magnification. Each experiment was performed in triplicate transwells and was repeated at least three times.

Results

Cytoplasmic Localization of p120ctn in Lobular Carcinoma

Loss of E-cadherin expression in epithelial cells will disrupt the E-cadherin complex located in the lateral membrane and concentrated in the adherens junction. In a search for signaling molecules that act downstream of E-cadherin inactivation in cancer, we immunohistochemically screened the expression and localization of E-cadherin and E-cadherin-associated proteins in 60 cases of lobular carcinoma. E-cadherin expression was completely lost in most of the lobular carcinomas (55 specimens, 91.6%; Figure 1A and Table 1). In accordance with this, β -catenin expression was also significantly decreased in these specimens (Figure 1B). In contrast, we observed cytoplasmic localization of p120ctn in these E-cadherin-deficient lobular carcinomas (Figure 1C, Table 1). The expression of other E-cadherin-associated

Table 1. Expression of E-Cadherin and p120ctn in Lobular Carcinoma

E-cadherin	p120 ctn	
	Cytoplasmic	Membranous
Positive	0	5
Negative	55	0

proteins will be described elsewhere (α -catenin, γ -catenin, and Shc). Double-immunofluorescence analysis of E-cadherin and p120ctn expression revealed that p120ctn and E-cadherin were co-localized in the lateral membranes of the normal mammary epithelial cells, and that p120ctn was localized in the cytoplasm of E-cadherin-deficient cancer cells (Figure 1D). We also observed similar cytoplasmic p120ctn expression in diffuse-type gastric cancers (data not shown).

Cytoplasmic Localization of p120ctn in Breast Cancer Cell Lines

We next analyzed the expression of E-cadherin and catenins in various breast cancer cell lines. MCF-7 and BT474 cells showed E-cadherin expression and tight intercellular adhesion (Figure 2A, lanes 1 and 2; Figure 2B, 1 and 2). In contrast, SKBR-3, ZR75-50, MDA-MB231, and MDA-MB435S cells lost epithelial cell compaction and showed a rather round (SKBR-3 and ZR75-50) or fibroblastic (MDA-MB231 and MDA-MB435S) morphology (Figure 2B). SKBR-3 cells are reported to harbor a homozygous deletion of the E-cadherin gene,³¹ and completely lacked E-cadherin expression (Figure 2A, lane 3). Expression of E-cadherin was also undetectable in ZR75-50, MDA-MB231, and MDA-MB435S cells (Figure 2A; lanes 4, 5, and 6). Prolonged exposure of the blotted membrane revealed a small amount of E-cadherin protein in ZR75-50 and MDA-MB231 cells (data not shown). As in the lobular carcinomas, β -catenin expression was significantly reduced in SKBR-3, ZR75-50, and MDA-MB435S cells. The expression of γ -catenin was also reduced in MDA-MB435S cells compared with the other cell lines (Figure 2A, lanes 3, 4, and 6). In contrast to β - and γ -catenin, no significant reductions in α -catenin or p120ctn expression were observed in any of the breast cancer cells (Figure 2A). Immunofluorescence analysis of p120ctn expression in the breast cancer cell lines revealed cytoplasmic localization of p120ctn in SKBR-3, ZR75-50, MDA-MB231,³¹ and MDA-MB435S cells, and membranous expression in MCF-7 and BT474 cells (Figure 2B).

p120ctn Localization Is Regulated by E-Cadherin in Breast Cancer Cells

To determine whether p120ctn localization is regulated by E-cadherin, we used SKBR-3 cells that completely lack E-cadherin. Exogenous E-cadherin cDNA was introduced into these cells, and the expression and localization of p120ctn was examined. Cells that express the

Figure 2. A: Immunoblot analysis of E-cadherin and catenins in breast cancer cell lines. Twenty μ g of proteins extracted from each cell line (lane 1, MCF7; lane 2, BT474; lane 3, SKBR-3; lane 4, ZR75-50; lane 5, MDA-MB231; lane 6, MDA-MB435S) were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with the antibodies indicated on the left. Anti- β -tubulin antibody was used as a loading control. **B:** Cytoplasmic localization of p120ctn in breast cancer cell lines. The expression and localization of p120ctn in six human breast cancer cell lines (1, MCF7; 2, BT474; 3, SKBR-3; 4, ZR75-50; 5, MDA-MB231; 6, MDA-MB435S) are shown. The cells were grown on type I collagen-coated glass and fixed with 4% paraformaldehyde. A monoclonal antibody against p120ctn was used as the primary antibody, and an Alexa 488 Fluor-conjugated anti-mouse antibody was used as the secondary antibody. p120ctn was localized predominantly in the membranes of MCF7 and BT474 cells, whereas it was found mainly in the cytoplasm of SKBR-3, ZR-75-50, MDA-MB231, and MDA-MB435S cells. Weak nuclear p120ctn immunoreactivity was also observed (arrow). Scale bar, 10 μ m.

E-cadherin cDNA showed an increase in β -catenin protein, but no significant increase in p120ctn expression was observed compared with the mock transfectant (Figure 3A). In SKBR-3 cells, p120ctn was localized in the cytoplasm and was particularly concentrated in membranous protrusions, in which thick actin fibers also accumulated (Figure 3B). Overexpression of exogenous E-cadherin caused p120ctn to move to the cell-cell boundary, and therefore reduced its cytoplasmic expression (Figure 3C).

Figure 3. The localization of p120ctn is regulated by E-cadherin expression. **A:** Immunoblot analysis of SKBR-3 cells after transfection with mock (lane 1) or E-cadherin cDNA transfection (lane 2). The expression of E-cadherin, β-catenin, and p120ctn was examined. **B:** Immunofluorescence analysis of p120ctn expression and actin fibers in SKBR-3 cells. The cells were grown on type I collagen-coated glass and fixed. After incubation with a mouse monoclonal antibody against p120ctn, an Alexa 488 Fluor-conjugated anti-mouse antibody was used as secondary antibody (left). Alexa 594 Fluor-conjugated phalloidin was used for the detection of actin fibers (right). p120ctn was concentrated in membranous protrusions where thick actin fibers also accumulated (arrows). **C:** Immunofluorescence analysis of p120ctn (left) and E-cadherin (right) expression in E-cadherin-transfected SKBR-3 cells. Mouse monoclonal antibodies against p120ctn and a rabbit polyclonal antibody against E-cadherin were used as the primary antibodies. An Alexa 488 Fluor-conjugated anti-mouse antibody and an Alexa 594 Fluor-conjugated anti-rabbit antibody were used as the secondary antibodies. Note that p120ctn has moved to the cell-cell boundary of E-cadherin-expressing cells (arrows). Scale bars, 10 μm.

Down-Regulation of Endogenous p120ctn Expression Reduces Membranous Protrusion Formation and Promotes Stress Fiber Production

To explore the roles of endogenous p120ctn in E-cadherin-deficient breast cancer cells more directly, we tried to down-regulate endogenous p120ctn expression using an RNA interference (RNAi) method. We tested several dsRNAs and found one (corresponding to nucleotides 2006 to 2026) that reduced all endogenous isoforms of p120ctn expression to a level that was hardly detectable by immunofluorescence. Immunoblot analysis of the p120ctn-dsRNA-transfected SKBR-3 cells revealed more than 80% reduction in endogenous p120ctn expression compared with control dsRNA-transfected cells (Figure 4A, lanes 2 to 4). There was no difference in cell viability between the p120ctn and control dsRNA-transfected cells. We then examined the morphologies of the p120ctn-dsRNA and control-dsRNA-transfected cells. The p120ctn-dsRNA-transfected cells showed a more flattened morphology and had fewer protrusions but more stress fibers than the surrounding nontransfected cells (Figure 4B).

Down-Regulation of p120ctn Increased Rho GTPase Activity in SKBR-3 Cells

The Rho family of small GTPase regulates intracellular cytoskeleton and cell morphology,^{19,20} and p120ctn has been shown to regulate Rho GTPase activity.^{22,23} Therefore we next examined whether p120ctn is implicated in the regulation of Rho GTPase in E-cadherin-deficient breast cancer cells. The activities of Rho GTPase and Rac GTPase were measured in p120ctn-dsRNA and control dsRNA-treated SKBR-3 cells by pull-down assay. We detected no significant change of Rac GTP between p120ctn dsRNA and control dsRNA-treated cells (Figure 5, lanes 3 and 4). In contrast, p120ctn-dsRNA-treated SKBR-3 cells showed significantly increased Rho-GTP compared to the control cells (Figure 5, lanes 1 and 2).

Down-Regulation of p120ctn Reduces Cell Migration

E-cadherin-deficient cancer cells, such as breast and gastric cancer cells, have been reported to show a highly motile phenotype.³³ To determine whether cytoplasmic p120ctn plays any role in cell migratory activity, we measured the heregulin-stimulated migratory activity of SKBR-3 cells transfected with either p120ctn or control dsRNA. As shown in Figure 6, p120ctn-dsRNA treatment significantly reduced migratory activity compared with the control-dsRNA-transfected cells. We observed a similar reduction in migratory activity in p120ctn-dsRNA-transfected MDA-MB231 cells (data not shown).

Figure 4. Down-regulation of endogenous p120ctn by RNA interference. **A:** Immunoblot analysis of p120ctn expression in SKBR-3 cells transfected with either p120ctn-dsRNA and control dsRNA. Proteins were extracted from SKBR-3 cells transfected with various concentrations of p120ctn-dsRNA (**lane 2**, 10 nmol/L; **lane 3**, 20 nmol/L; **lane 4**, 40 nmol/L), control dsRNA (**lane 5**, 10 nmol/L; **lane 6**, 20 nmol/L; **lane 7**, 40 nmol/L) and untransfected SKBR-3 cells (**lanes 1** and **8**). Twenty μ g of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with antibodies against p120ctn and β -tubulin. **B:** Double-immunofluorescence analysis of p120ctn (**top**) and actin fibers (**bottom**) in p120ctn-dsRNA-transfected SKBR-3 cells. The **arrow** indicates cells exhibiting p120ctn down-regulation. p120ctn-dsRNA-transfected cells showed a flattened morphology and fewer membranous protrusions but more stress fibers and peripheral actin cytoskeleton than the surrounding nontransfected cells. Scale bar, 10 μ m.

Heregulin Stimulated Tyrosine-Phosphorylated p120ctn Accumulation in the Protrusive Membrane of SKBR-3 Cells

To elucidate how p120ctn is implicated in the downstream pathway of heregulin-mediated cell migration, we examined whether heregulin induces p120ctn phosphorylation in SKBR-3 cells because several growth factors stimulate tyrosine phosphorylation of p120ctn.^{14,34} Using antibodies against phosphotyrosine and tyrosine-228-

Figure 5. Rho GTPase pull-down assay of dsRNA-treated SKBR-3 cells. SKBR-3 cells transfected with either p120ctn-dsRNA (**lanes 1** and **3**) or control dsRNA (**lanes 2** and **4**) (20 nmol/L) were lysed and incubated with GST-Rhotekin-RBD or GST-Pac-PBD. Bound proteins were analyzed by immunoblotting with a rabbit polyclonal antibody against Rho (**top**, **lanes 1** and **2**) and a mouse monoclonal antibody against Rac (**top**, **lanes 3** and **4**). Part of cell lysates were also subjected to immunoblotting for total Rho and Rac expression (**bottom**).

phosphorylated p120ctn, we detected tyrosine-phosphorylated p120ctn in heregulin-stimulated SKBR-3 cells (Figure 7A, lanes 2 and 4) as well as other breast cancer cell lines (data not shown). We then analyzed the subcellular localization of tyrosine-228-phosphorylated p120ctn in heregulin-stimulated SKBR-3 cells. As shown in Figure 7B (right, arrows), tyrosine-228-phosphorylated p120ctn predominantly accumulated in the protrusive domain.

Cytoplasmic Localization of p120ctn in Early Mouse Embryo

Because down-regulation of E-cadherin expression also occurs in various developmental processes,^{35,36} we investigated whether cytoplasmic p120ctn expression is detectable during embryogenesis in the mouse. In the early mouse embryo (day 7.5), mesoderm cells, which lack E-cadherin expression, segregate from the embryonic ectoderm and move into the amniotic cavity (Figure 8, A and B). At this stage, β -catenin expression is reduced in the mesoderm cells (Figure 8C). We observed cytoplasmic localization of p120ctn in ingressing mesoderm cells (Figure 8D).

Figure 6. Down-regulation of endogenous p120ctn reduces SKBR-3 cell migration. The number of cells that transmigrated through the pores of a transwell filter was counted after 16 hours. The means of five separate fields in three transwells are shown. The data represent the results of three independent experiments. p120ctn-dsRNA significantly reduced SKBR-3 cell migration by *t*-test ($P < 0.0001$).

Discussion

In this study, we address the question of how E-cadherin functions as an invasion suppressor in breast cancer. Inactivation of E-cadherin correlates significantly with the emergence of malignant characteristics, such as invasiveness and metastasis, in cancer cells. Although one of the major functions of E-cadherin in epithelial cells is to maintain tight cell-cell adhesion, recent studies revealed cell adhesion molecules to play more dynamic roles in cell motility, growth, and survival.^{17,18} To elucidate the signal pathways affected by E-cadherin inactivation in cancer, we searched for changes in the expression and localization of E-cadherin-associated molecules in E-cadherin-deficient breast cancers.

In the present study, we found that one of these E-cadherin-associated molecules, p120ctn, was invariably localized in the cytoplasm of E-cadherin-deficient breast cancer cells. Dillion and colleagues²⁸ previously reported altered p120ctn expression in breast cancers. In agreement with their findings, we noted the frequent disappearance of membranous p120ctn in lobular carcinoma. In addition, we detected cytoplasmic p120ctn expression in these breast cancer cells. An immunoblot analysis of p120ctn expression in breast cancer cell lines showed that the expression level of p120ctn did not differ significantly between E-cadherin-positive and E-cadherin-negative cancer cells. Moreover we confirmed the cytoplasmic localization of p120ctn in E-cadherin-deficient breast cancer cell lines by immunofluorescent cytochemistry.

Because p120ctn is directly associated with E-cadherin, it is likely that down-regulation of E-cadherin will result in the relocalization of p120ctn from the lateral membrane to the cytoplasm. Indeed p120ctn has been reported to be localized predominantly in the lateral membranes of highly confluent MDCK cells, but to move to the cytoplasm when these cells are growing at a low density and membranous E-cadherin levels are decreased.²⁴ We also observed recruitment of p120ctn to the membrane in E-cadherin-transfected SKBR-3 cells and this result supports the idea that the membranous localization of p120ctn may depend primarily on E-cadherin expression. Recently it has also been reported that nuclear localization of p120ctn is regulated by a nuclear export signal sequence located in the carboxyl-terminus of p120ctn splice variants and is counteracted by E-cadherin expression.³⁷ We occasionally observed weak nuclear p120ctn expression in lobular carcinomas and E-cadherin-deficient breast cancer cell lines (Figure 2B). Nuclear p120ctn may therefore also be an important outcome of E-cadherin inactivation in breast cancers.

Despite its direct association with cadherins, it is unclear whether p120ctn positively or negatively regulates the adhesive activity of E-cadherin. Overexpression of p120ctn or of a mutant E-cadherin with defective p120ctn binding produced different results in various types of cells.³⁸⁻⁴⁰ Analysis of a p120ctn-deleted colon cancer cell line showed that p120ctn stabilizes E-cadherin protein and positively regulates cell adhesion.⁴¹ In addition to its possible role in regulating E-cadherin, overexpres-

Figure 7. Heregulin stimulated accumulation of tyrosine phosphorylated p120ctn in the protrusive domain of SKBR-3 cells. **A:** SKBR-3 cells were serum-starved overnight (**lanes 1 and 3**) and stimulated with heregulin (5 ng/ml) for 30 minutes (**lanes 2 and 4**). The cells were lysed and immunoprecipitated with a rabbit polyclonal antibody against p120ctn. Immunoprecipitants were analyzed by immunoblotting with mouse monoclonal antibodies against phosphotyrosine (**top, lanes 1 and 2**) and tyrosine 228-phosphorylated p120ctn (**top, lanes 3 and 4**). Total p120ctn were detected by a mouse monoclonal anti-p120ctn antibody (**bottom**). **B:** Immunofluorescence analysis of tyrosine 228-phosphorylated p120ctn expression in heregulin-stimulated SKBR-3 cells. The cells were grown on type I collagen-coated glass and unstimulated (**left**) or stimulated with heregulin as above (**right**). After incubation with a mouse monoclonal antibody against tyrosine 228-phosphorylated p120ctn, an Alexa 488 Fluor-conjugated anti-mouse antibody was used as secondary antibody. Tyrosine 228-phosphorylated p120ctn was concentrated in the protrusive domain (**right, arrows**). Scale bar, 10 μ m.

Figure 8. Cytoplasmic expression of p120ctn in early mouse gastrulation. H&E staining (A) and immunohistochemical analyses of E-cadherin (B), β -catenin (C), and p120ctn (D) expression in early mouse embryo (day 7.5) are shown. In embryonic ectoderm cells (arrowhead) that express E-cadherin, both β -catenin and p120ctn were localized in the membrane. Expression of E-cadherin and β -catenin disappeared and reduced in mesoderm cells, respectively (arrow), which are characterized by a round morphology. In these cells, p120ctn was predominantly localized in the cytoplasm. Original magnifications, $\times 200$.

sion of p120ctn alters the epithelial morphology of MDCK and COS7 cells, producing membranous extensions and decreased focal adhesion.^{22–24} We observed similar morphological changes in p120ctn-overexpressing mouse mammary gland epithelial cells and E-cadherin-positive breast cancer cells. In SKBR-3 cells, p120ctn was concentrated in membranous protrusions in which thick actin bundles were also accumulated. Such localization also supports the possibility that p120ctn is involved in actin reorganization.

Previous studies showing the involvement of p120ctn in epithelial morphology have depended on the overexpression of exogenous p120ctn in E-cadherin-positive epithelial cells.^{22–24} It is therefore difficult to assess the physiological roles of p120ctn in E-cadherin-deficient cancer cells from these studies. We used an RNA interference technique to down-regulate endogenous p120ctn expression to see whether endogenous p120ctn plays any role in the malignant phenotypes of cancer cells. Recently a similar approach has shown the physiological roles of p120ctn in regulating E-cadherin protein stability.^{42,43} Down-regulation of p120ctn expression in SKBR-3 cells led to an increase in stress fiber formation and produced a flattened morphology with few protrusions. Epithelial morphology depends on cell-cell or cell-matrix interactions and the cytoskeletal network, and members of the small GTPase families, Rho, Rac, and Cdc42, play major roles in these processes.²⁰ Because Rho stabilizes stress fiber formation and Rac promotes the development of membranous protrusions such as

lamellipodia and filopodia,^{19,20} we measured whether p120ctn down-regulation affects the activity of Rho small GTPase families in SKBR-3 cells. Down-regulation of p120ctn increased Rho-GTP, suggesting that p120ctn negatively regulates Rho in SKBR-3 cells. We could not detect any significant change of Rac-GTP in p120ctn dsRNA-treated SKBR-3 cells. Previous studies have shown that overexpression of exogenous p120ctn down-regulates^{22,23} or up-regulates⁴⁴ Rho, and unaffected⁴⁴ or up-regulates Rac.^{23,24} It has also been reported that p120ctn regulates Rho activity in a cell-context-dependent manner.⁴⁴ Our results show that cytoplasmic endogenous p120ctn may play an important role in regulating members of the Rho family in some E-cadherin-deficient cancer cells.

Cytoskeletal reorganization and rapid turnover of the focal adhesion mediated by members of the Rho family are essential in allowing cells to migrate.⁴⁵ Overexpression of p120ctn has been reported to promote the motility of E-cadherin-negative fibroblast cells.^{23,24} In addition to causing alterations in their epithelial morphology, the migration of SKBR-3 cells was significantly impaired by p120ctn down-regulation. Interestingly heregulin, a ligand for c-erbB tyrosine kinase family, is required for the efficient migration of SKBR-3 cells.³⁰ Thus when E-cadherin is inactivated, other signals, that can be by-passed by p120ctn overexpression, may also be required for the migration of some cancer cells. p120ctn is phosphorylated by Src oncoprotein and after stimulation by various growth factors (epithelial growth factor, platelet-derived

growth factor, and colony-stimulating factor),^{14,34} and Cozzolino and colleagues⁴⁴ have shown that the amino-terminus deleted p120ctn, which lacks several tyrosine residues including tyrosine 228, abrogates motility of keratinocyte induced by epithelial growth factor. Therefore we reasoned that phosphorylation may modulate p120ctn's function on cell migration and examined the phosphorylation status of p120ctn in heregulin-stimulated SKBR-3 cells. Heregulin stimulation robustly induced tyrosine phosphorylation of p120ctn. We also applied a monoclonal antibody against phosphorylated tyrosine 228 residue of p120ctn, which is phosphorylated by Src.⁴⁶ This specific antibody recognized similar phosphorylated p120ctn proteins as those detected by an antibody against pan-phosphotyrosine, suggesting that tyrosine 228 is dominantly phosphorylated in heregulin-stimulated SKBR-3 cells. Using this phospho-specific antibody, we further analyzed subcellular localization of tyrosine-phosphorylated p120ctn. We found that tyrosine-phosphorylated p120ctn was specifically concentrated in the protrusive domain of SKBR-3 cells. This suggests that membranous tyrosine-phosphorylated-p120ctn may have positive roles in cell migration. Some direct regulators of the Rho family have been reported to work cooperatively with p120ctn,^{22,23} however, how these proteins orderly recruit or localize to a specific membranous region is still open to question. Further detailed analysis will uncover how tyrosine-phosphorylation of p120ctn is implicated to regulate dynamic process of cancer cell migration.

Dynamic regulation of E-cadherin expression is also required in many developmental processes including epithelial-mesenchymal transition.^{35,36,47} Such morphological alterations have been observed in cancers with E-cadherin inactivation. We attempted to determine whether cytoplasmic p120ctn exists under these physiological conditions. For that purpose, we examined the expression of p120ctn during gastrulation of the mouse embryo when drastic morphological changes occur. During this process, the embryonic mesoderm established in the primitive streak dissociates from the embryonic ectoderm. Down-regulation of E-cadherin expression by the snail transcriptional repressor is essential for the formation of this mesoderm⁴⁸ and overexpression of p120ctn disrupts the normal gastrulation process.⁴⁹ It is quite similar to the ones we observed in lobular carcinoma that expression of E-cadherin and β -catenin was undetectable and reduced in ingressing mesoderm cells. In accordance with this similarity, p120ctn is localized in the cytoplasm of these cells. During organogenesis, mesoderm cells are characterized by their mesenchymal morphology and increased motility. Therefore p120ctn may also play important roles in the formation and features of the mesoderm.

References

1. Takeichi M: Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* 1995, 7:619–627

2. Gumbiner BM: Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 1996, 84:345–357
3. Hirohashi S: Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* 1998, 153:333–339
4. Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D, Birchmeier W: E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* 1991, 113:173–185
5. Vleminckx K, Vakaet L, Mareel M, Fiers W, van Roy F: Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 1991, 66:107–119
6. Kanai Y, Oda T, Tsuda H, Ochiai A, Hirohashi S: Point mutation of the E-cadherin gene in invasive lobular carcinoma of the breast. *Jpn J Cancer Res* 1994, 85:1035–1039
7. Bex G, Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C, van Roy F: E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J* 1995, 14: 6107–6115
8. Oda T, Kanai Y, Oyama T, Yoshiura K, Shimoyama Y, Birchmeier W, Sugimura T, Hirohashi S: E-cadherin gene mutations in human gastric carcinoma cell lines. *Proc Natl Acad Sci USA* 1994, 91:1858–1862
9. Becker KF, Atkinson MJ, Reich U, Nekarda H, Siewert JR, Hoffer H: E-cadherin mutations provide clues to diffuse type gastric carcinomas. *Cancer Res* 1994, 54:3845–3852
10. Battle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A: The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2000, 2:84–89
11. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA: The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000, 2:76–83
12. Comijn J, Bex G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F: The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 2001, 7:1267–1278
13. Ozawa M, Baribault H, Kemler R: The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J* 1989, 8:1711–1717
14. Reynolds AB, Daniel J, McCrean PD, Wheelock MJ, Wu J, Zhang Z: Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol Cell Biol* 1994, 14:8333–8342
15. Shibamoto S, Hayakawa M, Takeuchi K, Hori T, Miyazawa K, Kitamura N, Johnson KR, Wheelock MJ, Matsuyoshi N, Takeichi M, et al: Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes. *J Cell Biol* 1995, 128:949–957
16. Xu Y, Guo DF, Davidson M, Inagami T, Carpenter G: Interaction of the adaptor protein Shc and the adhesion molecule cadherin. *J Biol Chem* 1997, 272:13463–13466
17. Watabe M, Nagafuchi A, Tsukita S, Takeichi M: Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J Cell Biol* 1994, 127:247–256
18. Hermiston ML, Gordon JL: In vivo analysis of cadherin function in the mouse intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. *J Cell Biol* 1995, 129:489–506
19. Etienne-Manneville S, Hall A: Rho GTPases in cell biology. *Nature* 2002, 420:629–635
20. Nobes CD, Hall A: Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol* 1999, 144:1235–1244
21. Machesky LM, Hall A: Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. *J Cell Biol* 1997, 138:913–926
22. Anastasiadis PZ, Moon SY, Thoreson MA, Mariner DJ, Crawford HC, Zheng Y, Reynolds AB: Inhibition of RhoA by p120 catenin. *Nat Cell Biol* 2000, 2:637–644
23. Noren NK, Liu BP, Burridge K, Kreft B: p120 catenin regulates the actin cytoskeleton via Rho family GTPases. *J Cell Biol* 2000, 150:567–580
24. Grosheva I, Shtutman M, Elbaum M, Bershadsky AD: p120 catenin affects cell motility via modulation of activity of Rho-family GTPases:

- a link between cell-cell contact formation and regulation of cell locomotion. *J Cell Sci* 2001, 114:695–707
25. Magie CR, Pinto-Santini D, Parkhurst SM: Rho1 interacts with p120ctn and alpha-catenin, and regulates cadherin-based adherens junction components in *Drosophila*. *Development* 2002, 129:3771–3782
26. Thoreson MA, Reynolds AB: Altered expression of the catenin p120 in human cancer: implications for tumor progression. *Differentiation* 2002, 70:583–589
27. Jawhari AU, Noda M, Pignatelli M, Farthing M: Up-regulated cytoplasmic expression, with reduced membranous distribution, of the src substrate p120(ctn) in gastric carcinoma. *J Pathol* 1999, 189:180–185
28. Dillon DA, D'Aquila T, Reynolds AB, Fearon ER, Rimm DL: The expression of p120ctn protein in breast cancer is independent of alpha- and beta-catenin and E-cadherin. *Am J Pathol* 1998, 152:75–82
29. Sekine S, Shibata T, Kokubu A, Morishita Y, Noguchi M, Nakanishi Y, Sakamoto M, Hirohashi S: Craniopharyngiomas of adamantinomatous type harbor beta-catenin gene mutations. *Am J Pathol* 2002, 161:1997–2001
30. Schelfhout VR, Coene ED, Delaey B, Thys S, Page DL, De Potter CR: Pathogenesis of Paget's disease: epidermal heregulin-alpha, motility factor, and the HER receptor family. *J Natl Cancer Inst* 2000, 92:622–628
31. van de Wetering M, Barker N, Harkes IC, van der Heyden M, Dijk NJ, Hollestelle A, Klijn JG, Clevers H, Schutte M: Mutant E-cadherin breast cancer cells do not display constitutive Wnt signaling. *Cancer Res* 2001, 61:278–284
32. Feltes CN, Kudo A, Blaschuk O, Byers SW: An alternatively spliced cadherin-11 enhances human breast cancer cell invasion. *Cancer Res* 2002, 62:6688–6697
33. Handschuh G, Candidus S, Luber B, Reich U, Schott C, Oswald S, Becke H, Hutzler P, Birchmeier W, Hofler H, Becker KF: Tumour-associated E-cadherin mutations alter cellular morphology, decrease cellular adhesion and increase cellular motility. *Oncogene* 1999, 18:4301–4312
34. Reynolds AB, Herbert L, Cleveland JL, Berg ST, Gaut JR: p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo. *Oncogene* 1992, 7:2439–2445
35. Hirai Y, Nose A, Kobayashi S, Takeichi M: Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. II. Skin morphogenesis. *Development* 1989, 105:271–277
36. Oda H, Tsukita S, Takeichi M: Dynamic behavior of the cadherin-based cell-cell adhesion system during *Drosophila* gastrulation. *Dev Biol* 1998, 203:435–450
37. van Hengel J, Vanhoenacker P, Staes K, van Roy F: Nuclear localization of the p120(ctn) Armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression. *Proc Natl Acad Sci USA* 1999, 96:7980–7985
38. Yap AS, Niessen CM, Gumbiner BM: The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J Cell Biol* 1998, 141:779–789
39. Aono S, Nakagawa S, Reynolds AB, Takeichi M: p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells. *J Cell Biol* 1999, 145:551–562
40. Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB: Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. *J Cell Biol* 2000, 148:189–202
41. Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy F, Reynolds AB: A novel role for p120 catenin in E-cadherin function. *J Cell Biol* 2002, 159:465–476
42. Davis MA, Ireton RC, Reynolds AB: A core function for p120-catenin in cadherin turnover. *J Cell Biol* 2003, 163:525–534
43. Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V, Kowalczyk AP: Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. *J Cell Biol* 2003, 163:535–545
44. Cazzolino M, Stagni V, Spinardi L, Campioni N, Fiorentini C, Salvati E, Alema S, Salvatore AM: p120 catenin is required for growth factor-dependent cell motility and scattering in epithelial cells. *Mol Biol Cell* 2003, 14:1964–1977
45. Worthylake RA, Lemoine S, Watson JM, Burridge K: RhoA is required for monocyte tail retraction during transendothelial migration. *J Cell Biol* 2001, 154:147–160
46. Mariner DJ, Anastasiadis P, Keilhack H, Bohmer FD, Wang J, Reynolds AB: Identification of Src phosphorylation sites in the catenin p120ctn. *J Biol Chem* 2001, 276:28006–28013
47. Tanaka-Matakatsu M, Uemura T, Oda H, Takeichi M, Hayashi S: Cadherin-mediated cell adhesion and cell motility in *Drosophila* trachea regulated by the transcription factor Escargot. *Development* 1996, 122:3697–3705
48. Carver EA, Jiang R, Lan Y, Oram KF, Gridley T: The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol Cell Biol* 2001, 21:8184–8188
49. Paulson AF, Fang X, Ji H, Reynolds AB, McCrea PD: Misexpression of the catenin p120(ctn)1A perturbs *Xenopus* gastrulation but does not elicit Wnt-directed axis specification. *Dev Biol* 1999, 207:350–363