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Alteration of Integrin-Dependent Adhesion and Signaling in EMT-Like MDCK Cells Established Through Overexpression of Calreticulin

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ABSTRACT

Calreticulin (CRT) is a multi-functional Ca^{2+}-binding molecular chaperone in the endoplasmic reticulum. We previously reported that kidney epithelial cell-derived Madin-Darby Canine Kidney cells were transformed into mesenchymal-like cells by gene transfection of CRT. In this study, we investigated the altered characteristics of cell adhesion in these epithelial-mesenchymal transition (EMT)-like cells. Several extracellular matrix substrata were tested, and cell adhesion to fibronectin was found to be specifically increased in the CRT-overexpressing cells compared to controls. The expression of integrins was significantly up-regulated in subunits α5 and αV, resulting in an increase in the formation of complexes such as α5β1 and αVβ3. These integrins also contributed to the enhanced binding of fibronectin. In the CRT-overexpressing cells, the phosphorylation of Akt, a downstream target of integrin-linked kinase (ILK), was up-regulated on attachment to fibronectin or collagen IV. Integrin-associated signaling through ILK was also promoted on attachment to fibronectin, suggesting some of the correlation between ILK and Akt in the CRT-overexpressing cells. Furthermore, on treatment with 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester, a membrane-permeable Ca^{2+} chelator, the enhanced Akt signaling was suppressed with a concomitant decrease in the formation of complexes between integrins and ILK in the CRT-overexpressing cells. In conclusion, these findings demonstrate that CRT regulates cell-substratum adhesion by modulating integrin-associated signaling through altered Ca^{2+} homeostasis in the CRT-overexpressing EMT-like cells, suggesting a novel regulatory role for CRT in EMT. J. Cell. Biochem. 112: 2518–2528, 2011.

KEY WORDS: CALRETICULIN; INTEGRIN; INTEGRIN-LINKED KINASE; EPITHELIAL-MESENCHYMAL TRANSITION

The epithelial-mesenchymal transition (EMT) is a crucial process controlling the morphogenesis of multicellular organisms during embryogenesis, development, and diseased states such as malignancies, chronic inflammation, and tissue fibrosis [Hay, 1995; Thiery et al., 2009]. Epithelial cells are static and associate with neighboring cells to form well-organized cellular layers with distinct polarity. In the EMT, the epithelial cells change to a non-polarized mesenchymal phenotype with a high capacity for motility in the extracellular matrix. Conversely, the mesenchymal cells can regain a fully differentiated epithelial phenotype via the mesenchymal-epithelial transition (MET) under certain conditions. During the EMT, various changes to molecular markers are observed, including the increased expression of N-cadherin, vimentin, and fibronectin, nuclear locarization of β-catenin, and increased production of transcription factors such as Snail1, Snail2 (Slug), Twist, EF1/ZEB1, SIP1/ZEB2, and/or E47 that inhibit E-cadherin expression. Although a variety of signaling pathways, including TGF-β, receptor tyrosine kinases (i.e., HGF, FGF, and PDGF), Notch, and Wnt, are known to be involved in the EMT [Moustakas and Heldin, 2007], the overall mechanisms are yet to be fully clarified.

Calreticulin (CRT) is a Ca^{2+}-binding protein in the endoplasmic reticulum (ER), and has a variety of functions related to molecular chaperoning, calcium homeostasis, cell signaling, and so forth [Michalak et al., 2009]. Previously, we utilized kidney epithelia-derived Madin-Darby Canine Kidney (MDCK) cells to establish the cell lines overexpressing CRT by gene transfection [Hayashida et al., 2006]. The results showed apparent changes in the morphology of CRT-overexpressing MDCK cells, and destruction of the polarized

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epithelial cell phenotype. Furthermore, overexpression of CRT repressed E-cadherin gene expression through up-regulation of its repressor, Slug, via altered Ca^{2+} homeostasis [Hayashida et al., 2006]. These results suggested a novel function of CRT related to EMT-like changes of cellular phenotype.

In this study, we focused on cell adhesion and investigated how cell-substratum interactions are altered in the CRT-overexpressing EMT-like MDCK cells. The results showed that cell adhesion to fibronectin was apparently enhanced through up-regulation of the expression of integrins α5β1 and αVβ3. In the CRT-overexpressing cells, the formation of a complex between integrin β1 and integrin-linked kinase (ILK) was enhanced, and the integrin-associated signaling through ILK was also promoted on attachment to fibronectin. These findings suggest that altered cell adhesion and enhanced cell signaling related to integrins play an important role in the EMT-like phenotype of the cells overexpressing CRT.

MATERIALS AND METHODS

MATERIALS

Antibodies against CRT (SPA-600) and calnexin (CNX) (SPA-860) were purchased from Stressgen (Victoria, BC, Canada). Antibodies against integrins β1 (sc-6622) and α2 (sc-9089) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Glycogen synthase kinase (GSK) 3α/β-rapamysin fusion protein (No. 9237), and antibodies against ILK (clone 4G9, No. 3856), focal adhesion kinase (FAK) (No. 3285), Akt (No. 9272), phospho-Akt (Ser473) (No. 9271), phospho-Akt (Thr308) (No. 9275), and phospho-GSK-3α/β (Ser21/9) (No. 9331) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MAB374) and integrins (β1, MAB2000 and MAB2253Z; β3, AB1932; α3, MAB2290; α5, AB1928; αV, AB1930; and αVβ3, MAB1976Z) were purchased from Millipore Corporation (Billerica, MA). Peroxidase-conjugated secondary antibodies against IgG of rabbit, mouse, and goat were from Dako (Glostrup, Denmark). 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) was obtained from Dojindo (Kumamoto, Japan). Ionomycin was obtained from Wako Pure Chemicals (Osaka, Japan). Multiwell plates and dishes coated with various extracellular matrixes (ECM: rat collagen IV, rat collagen I, mouse laminin, and human fibronectin) (BD Biocoat) were from BD Bioscience (Bedford, MA). Multiwell plates coated with vitronectin (~0.2 μg/cm^2) were prepared with human vitronectin (BD Bioscience). Low cell binding (LCB) culture plates (2-methacryloyloxyethyl phosphoryl choline treated) were obtained from Nunc (Roskilde, Denmark). All other reagents used in this study were of high grade and obtained from Sigma-Aldrich Corporation (St. Louis, MO) and Wako Pure Chemicals.

CELL LINES AND CULTURE

MDCK cells were obtained from American Type Culture Collection (NBL-2). The CRT-overexpressing MDCK cell lines (MDCK-CRT1, CRT2) and the control cell line (MDCK-Cont) were established by introducing expression vectors for CRT-gene expression and the control, respectively, as described previously [Hayashida et al., 2006]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 U/ml of penicillin, 50 μg/ml of streptomycin, and 125 μg/ml of G418 at 37°C in an atmosphere of 5% CO_2 and 95% air. In the case of treatment with a Ca^{2+}-chelator, the cells were washed with Earle's balanced salt solution (EBSS; 26 mM $NaHCO_3$ [pH 7.4], 5.4 mM KCl, 116 mM NaCl, 5.5 mM glucose, and 0.8 mM $MgCl_2$), then treated with EBSS containing 10 μM BAPTA-AM. For the control medium, EBSS containing 2 mM $CaCl_2$ was used.

CELL ADHESION ASSAYS

The attachment of cultured cells was evaluated on specific substrates by measuring numbers of attached live cells photometrically after staining with crystal violet. First, the cells were harvested with 0.53 mM EDTA/0.05% trypsin, and the trypsin was inactivated with DMEM/10% fetal calf serum. Then the cells were washed twice with phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.2] and 0.15 M NaCl), and re-suspended in DMEM/1% bovine serum albumin (BSA). They were seeded onto 24-well plates pre-coated with ECMs, at a density of 2×10^4 cells per well in 0.5 ml of DMEM/1% BSA. After a period of incubation, the plates were washed three times with PBS containing Mg^{2+} and Ca^{2+}, and attached cells were fixed with 4% paraformaldehyde in PBS for 20 min. The plates were washed with PBS containing Mg^{2+} and Ca^{2+}, and the cells were stained with 1% crystal violet at room temperature for 10 min. The wells were washed three times with distilled water and air-dried. The stained cells were dissolved in 100 μl of 10% SDS and 0.1 M HCl, and cell numbers were estimated by measuring absorbance at 595 nm using a microplate reader. In some experiments, cells were pre-incubated with 30 μg/ml of adhesion-blocking antibodies (anti-integrin β1 or αVβ3 antibodies) in DMEM/1% BSA for 40 min before being seeded. In such cases, cells were seeded onto 48-well plates pre-coated with fibronectin, at a density of 1×10^4 cells per well in 0.2 ml of DMEM/1% BSA.

IMMUNOBLOT ANALYSIS

Cells were harvested and lysed in lysis buffer (Tris-buffered saline [TBS; 10 mM Tris–HCl (pH7.5) and 0.15 M NaCl] including 1% Nonidet P-40, and protease inhibitors [20 μM phenylmethylsulfonyl fluoride, 50 μM pepstatin, and 50 μM leupeptin]). The lysate was sonicated on ice for 15 min intermittently, and then solubilized samples were prepared after centrifugation at 10,000*g* for 10 min at 4°C. Protein samples were electrophoresed on 8% or 10% SDS–polyacrylamide gels and transferred onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore Corporation). The membrane was blocked with 5% skim milk in TBS containing 0.05% Tween 20, and incubated at 4°C overnight with the primary antibody in solution. The antibody was detected using a peroxidase-conjugated secondary antibody and Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation) as recommended by the manufacturer.

To examine the phosphorylation of proteins induced by cell adhesion to the ECM, cells were starved overnight in serum-free DMEM, then detached using 0.53 mM EDTA and 0.05% trypsin. Trypsin was inactivated with 0.1% trypsin inhibitor and washed twice with PBS, and resuspended in DMEM/1% BSA. The cells were

seeded onto plastic culture plates pre-coated with ECMs or LCB culture plates. After a period of incubation at 37°C, the cells were harvested, and protein samples were prepared by lysing the cells with RIPA buffer (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors and protein phosphatase inhibitors (10 mM NaF, 1 mM sodium vanadate). The detection of phosphorylated proteins was performed as stated above using specific antibodies.

IMMUNOPRECIPITATION

Cultured cells were harvested and lysed in the lysis buffer. The solubilized samples normalized for protein levels were subjected to immunoprecipitation to examine interactions between integrins. The samples were incubated with anti-integrin β1 or αVβ3 antibodies for 2 h at 4°C, and after Protein G-Sepharose beads (Thermo Scientific, Rockford, IL) were added, incubated overnight at 4°C. Beads were washed 4 times with the lysis buffer, and immunoprecipitated proteins were eluted in SDS sample buffer (1% SDS/10 mM dithiothreitol [DTT]) after incubation at 95°C for 5 min. Then the immunoprecipitates were analyzed by immunoblotting as described above.

IMMUNOFLUORESCENCE MICROSCOPY

Cells were grown on a cover glass for 2 days, fixed with 4% paraformaldehyde in PBS and permeabilized for 10 min with 1% Triton X-100 in PBS. The cells were then blocked with PBS containing 1% BSA, and stained with specific primary antibodies in solution. The primary antibodies were labeled with secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen Corporation, Carlsbad, CA) and visualized by laser scanning confocal microscopy (LSM5Pascal, Carl Zeiss Co., Jena, Germany), and analyzed using PASCAL analytic software.

KINASE ASSAY FOR ILK-ASSOCIATED COMPLEXES

ILK was immunoprecipitated from cell lysate using the anti-ILK antibody as described above. The ILK-related complexes bound to Protein-G Sepharose beads were washed three times with the lysis buffer, and once with kinase buffer (25 mM Tris–HCl [pH 7.5], 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium vanadate, and 10 mM $MgCl_2$). Then the complexes were incubated at room temperature for 30 min with 1 μg of GSK3α/β-rapamysin fusion protein (Cell Signaling Technology), a substrate for ILK-associated kinases [Delcommenne et al., 1998], in 80 μl of the kinase buffer containing 200 μM ATP. The kinase reaction was stopped by incubation with the SDS sample buffer at 95°C for 5 min. The ILK-related kinase activity was estimated by immunoblotting using the anti-phospho-GSK-3α/β (Ser21/9) antibody.

RESULTS

CELL ADHESION TO EXTRACELLULAR MATRIXES WAS ALTERED IN CRT-OVEREXPRESSING MDCK CELLS

In our previous study, canine renal epithelial MDCK cells were transfected with an expression vector for CRT cDNA to obtain cell lines overexpressing CRT [Hayashida et al., 2006]. Overexpression of CRT in MDCK cells caused an apparent morphological change with a fibroblastoid-like phenotype, loss of cell-cell contacts, and enhanced cellular migration, suggesting an EMT-like phenotype.

In the present study, we focused on cell-substrata interactions and their changes in the CRT-overexpressing EMT-like cells. Control and CRT-overexpressing cells were seeded on multiwell plates pre-coated with LCB material (i.e., 2-methacryloyloxyethyl phosphoryl choline) or extracellular matrixes (ECMs) (e.g., collagen I and IV, laminin, vitronectin, and fibronectin) then photographed (Fig. 1A), and the attachment was quantified by colorimetric assay as described in the Materials and Methods (Fig. 1B). The cells attached most rapidly to collagen IV, with the maximum values, obtained after 6 h, similar between control and CRT-overexpressing cells (Fig. 1B). Thus the results show no difference in attachment to collagen IV between control and CRT-overexpressing cells. Based on the results with collagen IV, the attachment to wells coated with the LCB material or fibronectin was quantified, and shown relative to the maximum value obtained with collagen IV after 6 h. No attachment to the LCB material was observed in control or CRT-overexpressing cells (Fig. 1A,B). In the case of fibronectin, extensive attachment occurred within 1 h among the CRT-overexpressing cells, but took another 2 h among the control cells. As shown in Figure 1B, with fibronectin, attachment values at 1 h were $22.8 \pm 6.9\%$ and $77.8 \pm 3.9\%$ for control and CRT-overexpressing cells, respectively. With laminin, attachment values at 1 h were $43.9 \pm 5.1\%$ and $67.7 \pm 3.9\%$, respectively. With vitronectin, attachment values at 1 h were $67.6 \pm 5.3\%$ and $96.7 \pm 4.1\%$, respectively. With collagen I, attachment values at 1 h were $68.6 \pm 3.7\%$ and $79.4 \pm 1.3\%$, respectively. In the cases of fibronectin, laminin, vitronectin and collagen I, the adhesion was enhanced more in CRT-overexpressing cells than in controls. The migration of control and CRT-overexpressing cells was also examined on culture plates coated with ECMs (Supplementary data Fig. S1). The distance migrated on the plates coated with collagen IV or fibronectin was greater for the CRT-overexpressing cells than control cells. The distance covered by CRT-overexpressing cells at 24 h was similar with collagen IV and fibronectin, suggesting no difference in preference for these substrates. However, in Figure 1, the difference at 1 h between control and CRT-overexpressing cells was most enhanced in the case with adhesion to fibronectin, suggesting a functional link between fibronectin-mediated adhesion and the EMT-like phenotype of the CRT-overexpressing cells.

EXPRESSION OF INTEGRINS AND RELATED MOLECULES WAS ALTERED IN CRT-OVEREXPRESSING MDCK CELLS

The increased adhesion to ECMs by CRT-overexpressing cells led us to investigate the cellular expression of integrins [Hynes, 2002; Takada et al., 2007], counter receptors for those ECMs, by immunoblotting. In Figure 2, the expression of integrin β1 was slightly enhanced and the mobility of the bands was also increased in CRT-overexpressing cells, compared to controls. The results also demonstrate that the level of some integrins (β3, α2, α5, and αV) increased, but that of integrin α3 decreased in the CRT-overexpressing cells, compared to controls such as parental and control vector-transfected cells. The transcriptional expression of integrins

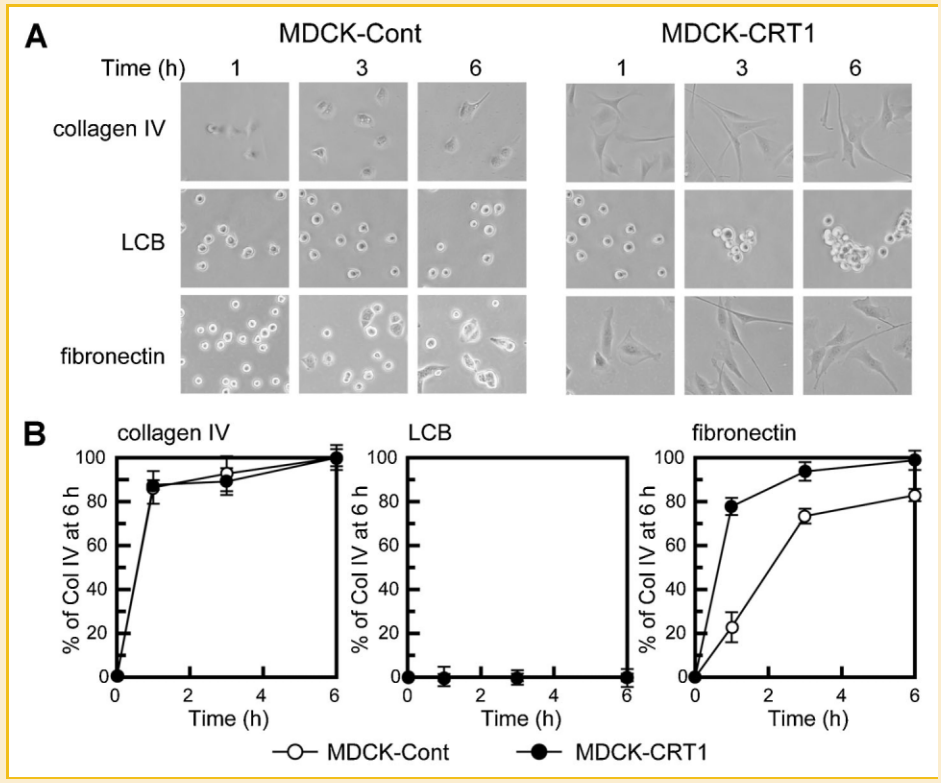

Fig. 1. Cell adhesion to extracellular matrixes is altered in CRT-overexpressing MDCK cells. (A) Control (MDCK-Cont) and CRT-overexpressing (MDCK-CRT1) cells were seeded on multiwell plates pre-coated with low cell binding (LCB) material or ECMs (collagen IV and fibronectin), then cultured for the periods indicated. The morphology of control and CRT-overexpressing cells was examined using phase-contrast microscopy, and photographed under magnification of 100×. (B) The cell attachment shown in (A) was quantified by colorimetric assay as described in the Materials and Methods. Col IV, collagen IV. Data are shown as the mean ± S.E., and represent three independent experiments.

was also examined by a reverse-transcriptional polymerase chain reaction in control and CRT-overexpressing cells (Supplementary Fig. S2). In the cases of integrin α5 and αV, the transcripts were apparently increased in the CRT-overexpressing cells, compared to controls. For the transcripts of α2, β3, and β1, however, no increase was apparent in the CRT-overexpressing cells. These results suggest that the altered expression of integrins was controlled not only by transcription but also by other factors including protein turnover and stability in the CRT-overexpressing cells. In the flow cytometric analysis, the expression levels of integrins α5 and αVβ3 were also higher in the CRT-overexpressing cells than controls (See Supplementary data Fig. S3), consistent with the results of immunoblotting in Figure 2. We also examined the expression of integrin-associated signaling molecules such as ILK and FAK. In CRT-overexpressing cells, the expression of ILK increased moderately, while that of FAK showed no significant change, compared to controls. Overexpression of CRT was also confirmed in the MDCK-CRT cells.

FORMATION OF α5β1 AND αVβ3 INTEGRIN HETERODIMERS ASSOCIATED WITH ILK WAS ENHANCED IN CRT-OVEREXPRESSING MDCK CELLS

Next, to investigate how the overexpression of CRT influenced the integrin complexes, integrins β1 and αVβ3 were immunoprecipitated from lysate of control and CRT-overexpressing cells, and the immunoprecipitates were characterized by immunoblotting using antibodies against other integrins and related proteins. In control cells, integrins α2 and α3 were mainly involved in the complexes with integrin β1 (Fig. 3A, left). In contrast, in CRT-overexpressing cells, a marked increase in α5, a decrease in α3, and a slight increase in α2 were observed in the complexes with β1. Among the integrins tested in this study, integrin α2β1 might influence the binding with collagens in CRT-overexpressing cells. However, there was no significant difference in the binding with collagen IV or I, a target of integrin α2β1, between control and CRT-overexpressing cells (Fig. 1). This suggests that, in control cells, the level of α2β1 may fully saturate the binding with collagens, and the up-regulated expression of α2 may not lead to further enhancement of the binding in the CRT-overexpressing cells. Thus we focused on other integrins, such as α5β1 and αVβ3, and their altered binding characteristics and signaling in the CRT-overexpressing cells. The increase in the α5β1 heterodimer is likely characteristic of CRT-overexpressing EMT-like cells (Figs. 3A and 4B), and is consistent with previous studies of mesenchymal cells [Zeisberg and Neilson, 2009]. In the immunoprecipitates with integrin β1, two bands were identified as integrin α5 in the CRT-overexpressing cells (Fig. 3A). Interestingly, the dominating lower band was not the same as the strong upper band in the total lysate of the cells. To investigate whether the lower-band form of α5 occurs on or in the cell, cell surface proteins were labeled with sulfo-N-hydroxysuccinimide-biotin, and the cell lysate samples were subjected to immunoprecipitation with the anti-β1 or α5 antibody, followed by blotting

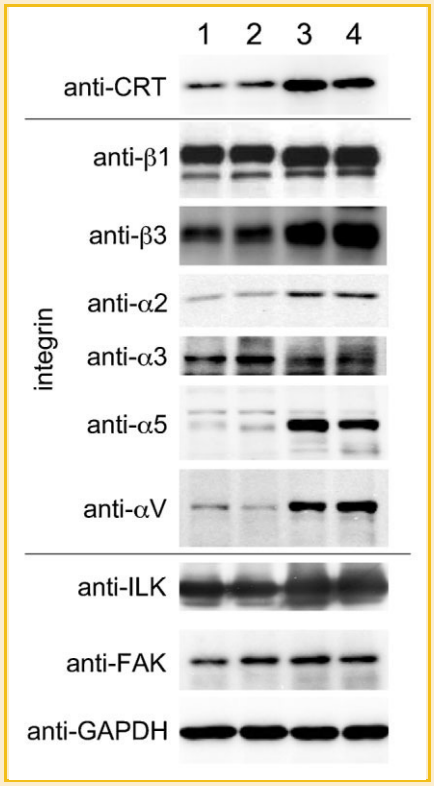

Fig. 2. Levels of CRT, integrins (β1, β3, α2, α3, α5, and αV), ILK, FAK, and GAPDH were estimated in control (MDCK parental and MDCK-Cont cells) and CRT gene-transfected MDCK (MDCK-CRT1 and CRT2) cells in an immunoblot analysis with specific antibodies as described in the methods. 1, MDCK; 2, MDCK-Cont; 3, MDCK-CRT1; 4, MDCK-CRT2. Data represent three independent experiments.

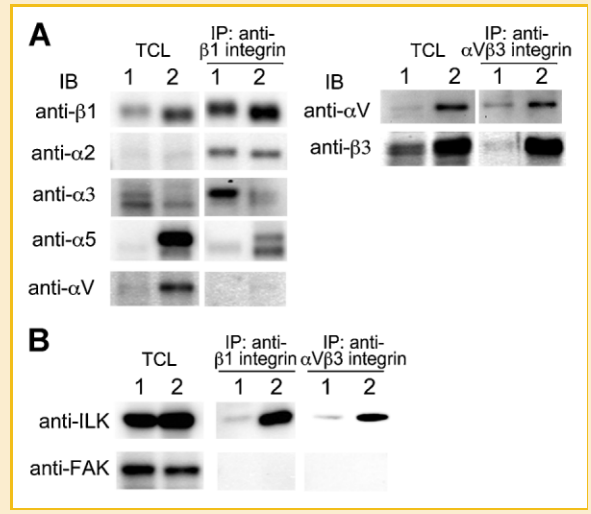

Fig. 3. The formation of complexes between α5β1 and αVβ3 integrin heterodimers and ILK is enhanced in CRT-overexpressing MDCK cells. (A) Integrin β1 and αVβ3 were immunoprecipitated with specific antibodies from lysate of MDCK-Cont and MDCK-CRT1 cells, and the immunoprecipitates were characterized by immunoblotting using antibodies against other integrins (β1, β3, α2, α3, α5, and αV). (B) Integrin β1 and the αVβ3 heterodimer were immunoprecipitated as described in (A), and the immunoprecipitates were characterized by immunoblotting using antibodies against ILK or FAK. 1, MDCK-Cont; 2, MDCK-CRT1; TCL, total cell lysate.

using peroxidase-conjugated avidin. We found that the lower-band form of α5 was dominantly biotinylated (data not shown), indicating that the mature form of the subunit was mainly associated with β1 on the cell surface. It is also worth noting that the transcriptional expression of integrin α5 was significantly up-regulated in the CRT-overexpressing cells, but most of the protein seems to occur in the immature form.

Interestingly, the amount of ILK co-precipitated with integrin β1 was apparently increased in CRT-overexpressing cells, compared to controls (Fig. 3B). In the immunofluorescence microscopic analysis, the colocalization of β1 and ILK was apparently increased in the peripheral areas of CRT-overexpressing cells, even though ILK broadly distributed in the cytoplasm in both control and CRT-overexpressing cells (Fig. 4C). Moreover, vinculin, a focal adhesion protein [Chen and Singer, 1982], was also colocalized with integrin α5 or ILK in the peripheral regions of the CRT-overexpressing cells (data not shown). These results suggest an increased colocalization of integrins α5β1 and ILK at focal adhesion and ruffle-like sites in the peripheral areas of CRT-overexpressing cells.

Levels of integrins αV and β3 were higher in CRT-overexpressing cells than control cells (Fig. 2 and Supplementary data Fig. S3), and an increase in the complex comprising αV and β3 was detected by immunoprecipitation and immunocytochemistry using a specific antibody for the αVβ3 heterodimer (Figs. 3A and 4D). The heterodimer was also immunoprecipitated with ILK (Fig. 3B). Next in the immunofluorescence microscopic analysis of FAK, the co-localization with integrin β1 or αVβ3 was increased at the periphery of CRT-overexpressing cells, compared to control cells (Supplementary data Fig. S4). However, FAK was not detected in the complexes with integrin β1 or αVβ3 in either cell line (Fig. 3B).

Both the α5β1 and αVβ3 heterodimers are receptors for fibronectin, and the effect of adhesion-blocking antibodies against integrin β1 or αVβ3 on the adhesion of CRT-overexpressing cells to plastic wells pre-coated with fibronectin was examined. After preincubation with the blocking antibody against integrin β1, the adhesion of CRT-overexpressing cells to fibronectin after 20 min was significantly suppressed to $67.2 \pm 5.1\%$ of the level for control IgG. However, less of an effect was observed with the antibody against αVβ3 ($97.2 \pm 2.2\%$ of the level for control IgG). These results suggest that the adhesion to fibronectin is mainly mediated via integrin β1 complexes at least in the early phase. Integrin αVβ3 is known as a ligand not only for fibronectin but also for vitronectin [Takada et al., 2007]. Therefore, the effects of adhesion-blocking antibodies against integrin αVβ3 and β1 on the adhesion of CRT-overexpressing cells to plastic wells pre-coated with vitronectin were examined. After preincubation with the blocking antibody against integrin αVβ3, the adhesion of CRT-overexpressing cells to vitronectin after 20 min was suppressed to $77.8 \pm 3.1\%$ of the level for control IgG. However, less of an effect was observed with the antibody against β1 ($96.3 \pm 6.5\%$ of the level for control IgG). These results suggest that the adhesion to vitronectin is mainly mediated via integrin αVβ3 complexes at least in the early phase. The intracellular distribution of CRT was also examined by immunoflu-

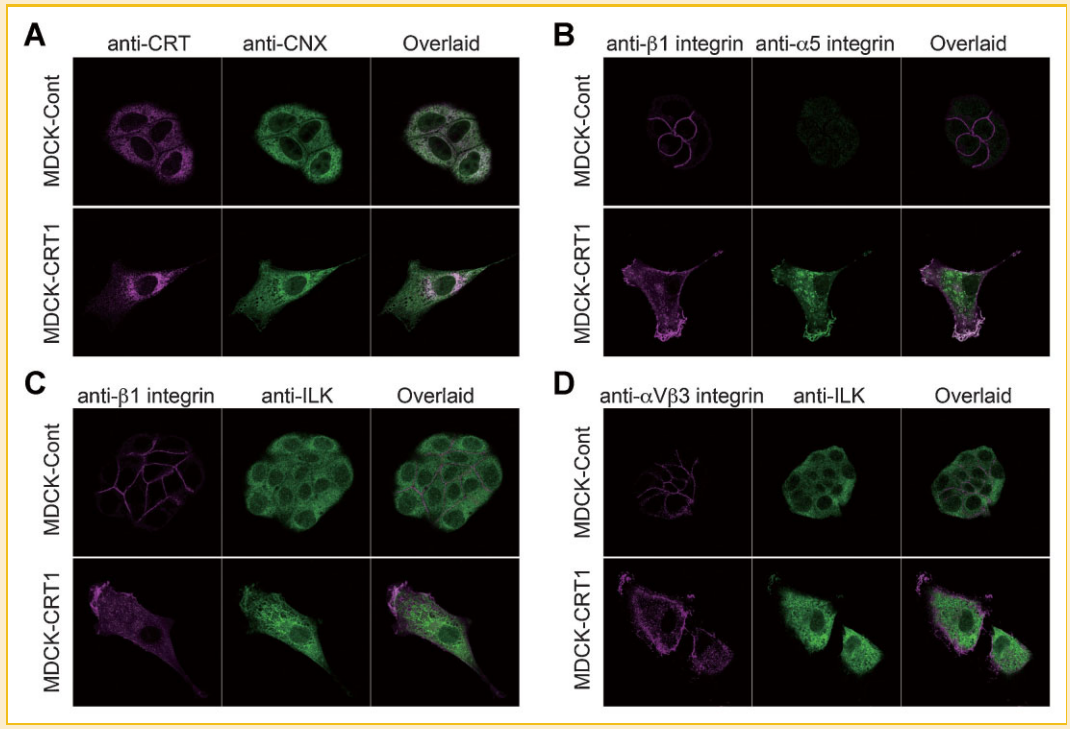

Fig. 4. Intracellular localization of CRT, integrins, and ILK in CRT-overexpressing MDCK cells. (A) The intracellular distribution of CRT (Magenta) and CNX (Green) was evaluated in MDCK-Cont and MDCK-CRT1 cells by immunofluorescence microscopy with specific antibodies as described in the methods (magnification 400×). (B) The intracellular distribution of integrin β1 (Magenta) and α5 (Green) was examined in MDCK-Cont and MDCK-CRT1 cells by immunofluorescence microscopy with specific antibodies. (C) The intracellular distribution of integrin β1 (Magenta) and ILK (Green) was evaluated in MDCK-Cont and MDCK-CRT1 cells by immunofluorescence microscopy with specific antibodies. (D) The intracellular distribution of the αVβ3 heterodimer (Magenta) and ILK (Green) was evaluated in MDCK-Cont and MDCK-CRT1 cells by immunofluorescence microscopy with specific antibodies. Data represent at least three independent experiments.

orescence microscopy in control and CRT-overexpressing cells. CRT was co-localized, to some extent, with CNX, an ER-resident membrane protein, and there was no detectable accumulation of CRT at cell adhesion sites or in peripheral ruffle-like areas in either cell line (Fig. 4A).

INTEGRIN/ILK-ASSOCIATED SIGNALING WAS ENHANCED IN CRT-OVEREXPRESSING MDCK CELLS

To investigate whether the enhanced formation of integrin/ILK complexes influences the downstream signaling in CRT-overexpressing cells, we focused on Akt, a downstream kinase of integrin-induced signaling through ILK [McDonald et al., 2008]. Control and CRT-overexpressing cells were seeded on culture plates pre-coated with or without ECMs (collagen IV and fibronectin) or the LCB material, then time-dependent changes in the phosphorylation status of Akt were examined by immunoblotting as described in the methods (Fig. 5A). In the CRT-overexpressing cells, the phosphorylation of Akt was apparently up-regulated, especially at Ser473, on attachment to both collagen IV and fibronectin, though it also increased gradually on attachment to the LCB material. In control cells, the phosphorylation was little enhanced by the attachment to ECMs or LCB, although a slight increase in phosphorylation at Ser473 was observed on attachment to collagen IV. The phosphorylation of Akt was up-regulated more at Ser473 than Thr308 in the CRT-overexpressing cells after the attachment to ECMs, consistent with the finding that the integrin/ILK-dependent phosphorylation of Akt occurs predominantly at Ser473 [Delcommenne et al., 1998]. Next, control and CRT-overexpressing cells were seeded on culture plates pre-coated with or without fibronectin or LCB, then ILK-associated kinase activity was investigated as described in the methods by immunoprecipitation of ILK followed by a kinase assay using GSK3α/β-fusion protein as a substrate. As shown in Figure 5B, ILK-associated kinase activity was apparently up-regulated in CRT-overexpressing cells treated with fibronectin, compared with controls.

FORMATION OF COMPLEX BETWEEN INTEGRIN β1 AND ILK WAS SUPPRESSED BY BAPTA-AM IN CRT-OVEREXPRESSING MDCK CELLS

Previously, we observed that in CRT-overexpressing MDCK cells, the expression of TRPV5, a Ca^{2+}-selective channel, was up-regulated and the basal level of Ca^{2+} influx from the extracellular space was also increased [Hayashida et al., 2006]. To investigate whether the increased influx of Ca^{2+} contributes to the enhanced formation of complexes between integrins and ILK, we examined the effect of BAPTA-AM, a cell-permeable Ca^{2+} chelator, on the complex of integrin β1 and ILK in the CRT-overexpressing cells. The cells were treated with EBSS containing 10 μM BAPTA-AM for the period

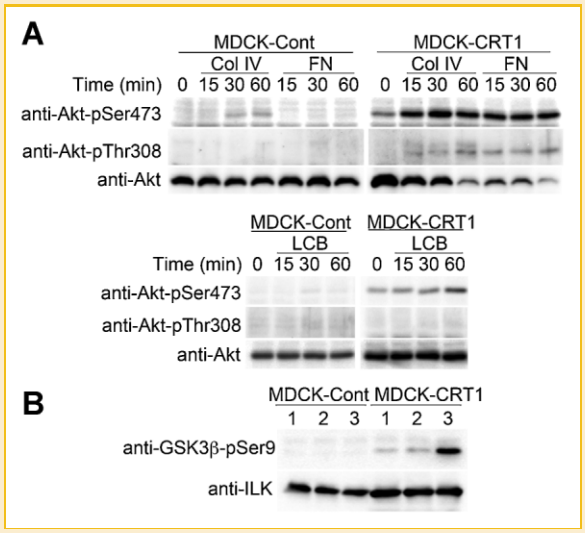

Fig. 5. ILK-associated signaling is enhanced by attachment to fibronectin in CRT-overexpressing MDCK cells. (A) MDCK-Cont and MDCK-CRT1 cells were seeded on culture plates pre-coated with the LCB material or ECMs (collagen IV and fibronectin), then incubated for the periods indicated. The time-dependent change in the phosphorylation state of Akt (Ser473 or Thr308) was examined by immunoblotting with specific antibodies as described in the methods. Col IV, collagen IV; FN, fibronectin. (B) MDCK-Cont and MDCK-CRT1 cells were seeded on culture plates pre-coated with the LCB material or fibronectin and incubated for 10 min, then ILK-associated kinase activity was investigated as described in the methods by immunoprecipitation of ILK followed by a kinase assay using GSK3α/β-fusion protein as a substrate. 1, no attachment; 2, 10 min after the attachment to LCB; 3, 10 min after the attachment to fibronectin. Data represent three independent experiments.

indicated (Fig. 6A) and the intracellular distribution of integrin β1 and ILK was examined by immunofluorescence microscopy as described in the methods. Initially, ILK was co-localized to some extent with integrin β1 at the periphery of the cell. However, after the treatment with BAPTA-AM, the signal for ILK decreased, through that for integrin β1 remained in the peripheral areas (arrowheads). The CRT-overexpressing cells were cultured with or without the control medium or EBSS containing 10 μM BAPTA-AM for 15 min, and the complex between integrin β1 and ILK was examined by immunoprecipitation with the anti-integrin β1 antibody, followed by an immunoblot analysis using specific antibodies (Fig. 6B). The band intensity was estimated densitometrically, and the rate of immunoprecipitated ILK is expressed as the relative intensity of the immunoprecipitated ILK/immunoprecipitated β1. The values from the cells treated with no agent, control medium, and BAPTA-AM were 0.37 ± 0.04, 0.34 ± 0.05, and $0.06 \pm 0.01^{*}$, respectively. Each value represents the mean ± S.E. for three experiments ($^{*}P < 0.01$ vs. no agent or control medium). The results showed that the amount of ILK associated with integrin β1 was decreased by the treatment with BAPTA-AM. Together, they demonstrate that the enhancement of the colocalization of integrin β1 and ILK was abolished by the chelation of cytoplasmic Ca^{2+} on treatment with BAPTA-AM, suggesting the enhanced formation of integrin/ILK complexes to be due to the up-regulated influx of Ca^{2+} in the CRT-overexpressing cells.

ENHANCED INTEGRIN/ILK-ASSOCIATED SIGNALING WAS SUPPRESSED BY BAPTA-AM IN CRT-OVEREXPRESSING MDCK CELLS

Next, to investigate whether the increased Ca^{2+} influx contributes to the enhanced integrin/ILK-associated signaling, we examined the effect of BAPTA-AM on the status of Akt and ILK-associated kinase activity in CRT-overexpressing cells. The basal state of phosphorylation at Ser473 of Akt was compared between control and CRT-overexpressing cells by immunoblotting (Fig. 6C). The results showed that the phosphorylation of Akt to be up-regulated in the CRT-overexpressing cells. The CRT-overexpressing cells were treated with control medium or EBSS containing 10 μM BAPTA-AM for the periods indicated in Figure 6D, and the phosphorylation of Akt was examined by immunoblotting. The level of phosphorylated Akt (Ser473) decreased after 15 min, although the total level of Akt was also slightly diminished. Next, the CRT-overexpressing cells were treated for 15 min with control medium or EBSS containing 10 μM BAPTA-AM, and ILK-associated kinase activity was examined as described in the methods (Fig. 6E). The kinase activity associated with ILK decreased on treatment with EBSS containing BAPTA-AM, compared to the control medium. These results demonstrate that, in the CRT-overexpressing cells, the up-regluted ILK-associated signaling was suppressed by the chelation of cytoplasmic Ca^{2+} on treatment with BAPTA-AM.

EFFECT OF IONOMYCIN ON INTEGRIN/ILK-ASSOCIATED SIGNALING IN MDCK CELLS

To investigate whether the increased influx of Ca^{2+} contributes to the enhanced formation of complexes between integrins and ILK, we examined the effect of ionomycin, a Ca^{2+} ionophore, on the complex of integrin β1 and ILK in control cells (MDCK-Cont). The cells were treated with 5 μM ionomycin for the period indicated (Fig. 7A) and the intracellular distribution of integrin β1 and ILK was examined by immunofluorescence microscopy as described in the methods. Initially, ILK was hardly co-localized with integrin β1 in the cell–cell border region. After the treatment with ionomycin, the signal for ILK co-localized with integrin β1 was slightly increased at the cell–cell border (arrowheads), but significant change was not observed. The control cells were treated with ionomycin or DMSO (a solvent for ionomycin) for 15 min, and the complex formed between integrin β1 and ILK was examined by immunoprecipitation with the anti-integrin β1 antibody, followed by an immunoblot analysis using specific antibodies (Fig. 7B). No significant difference between ionomycin and DMSO was observed in the amount of ILK associated with integrin β1 in the cells.

Next, we examined the effect of ionomycin on the activation status of Akt, a downstream molecule of ILK-associated signaling, in control cells. The control cells were treated with ionomycin for the periods indicated in Figure 7C, and the phosphorylation of Akt was examined by immunoblotting. The level of phosphorylated Akt (Ser473) increased in a time-dependent manner. Compared with the apparent increase in the level of phosphorylated Akt, the formation of a complex between integrin β1 and ILK was not so enhanced in the cells treated with ionomycin. The difference suggests other possible pathways to activate Akt signaling by ionomycin, although the precise mechanism is not yet clear. When the CRT-over-

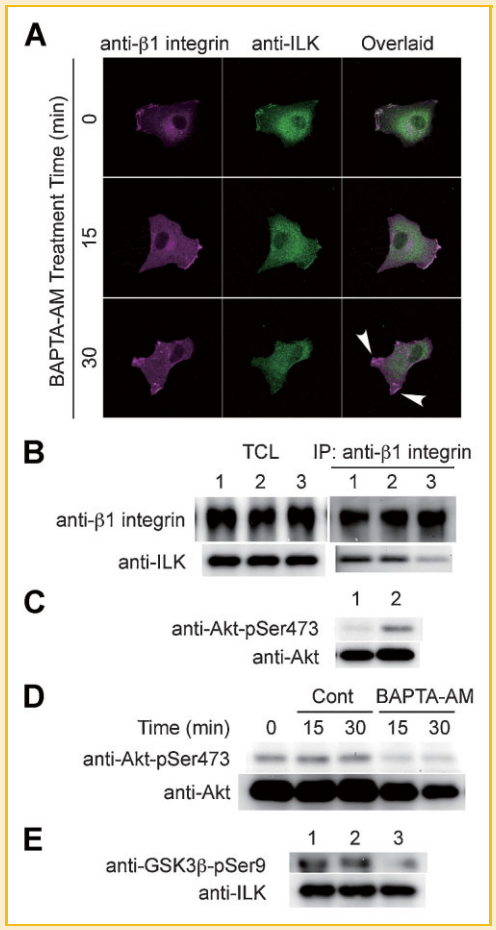

Fig. 6. BAPTA-AM suppresses the formation of a complex between integrin β1 and ILK to downregulate the ILK-associated signaling in CRT-overexpressing MDCK cells. (A) MDCK-CRT1 cells were treated with EBSS containing 10 μM BAPTA-AM for the periods indicated, and the cellular distribution of integrin β1 (Magenta) and ILK (Green) was examined by immunofluorescence microscopy as described in the methods using specific antibodies (magnification 400×). (B) MDCK-CRT1 cells were treated for 15 min with control medium or EBSS containing 10 μM BAPTA-AM, and integrin β1 was immunoprecipitated from each cell lysate by specific antibodies, and the total cell lysate (left) and immunoprecipitates (right) were examined by immunoblotting using antibodies against integrin β1 and ILK. 1, no treatment; 2, control; 3, BAPTA-AM; TCL, total cell lysate. (C) The basal level of Akt phosphorylated at Ser473 was examined by immunoblotting using specific antibodies in MDCK-Cont and MDCK-CRT1 cells. The total amount of Akt protein in each sample is also shown. 1, MDCK-Cont; 2, MDCK-CRT1. (D) MDCK-CRT1 cells were treated with control medium or EBSS containing 10 μM BAPTA-AM for the periods indicated, and the amount of Akt phosphorylated at Ser473 was examined by immunoblotting. The total amount of Akt protein in each sample is also shown. (E) MDCK-CRT cells were treated for 15 min with control medium or EBSS containing 10 μM BAPTA-AM, and the ILK-associated kinase activity was estimated as described in the methods by detecting phosphorylated GSKα/β-fusion protein in an immunoblot analysis. The total amount of ILK protein immunoprecipitated by specific antibodies in each sample is also shown. 1, no treatment; 2, control; 3, BAPTA-AM. Data represent three independent experiments.

expressing cells were treated with ionomycin, neither further enhancement of the formation of a complex between integrin β1 and ILK nor enhanced phosphorylation of AKT was observed (data not shown).

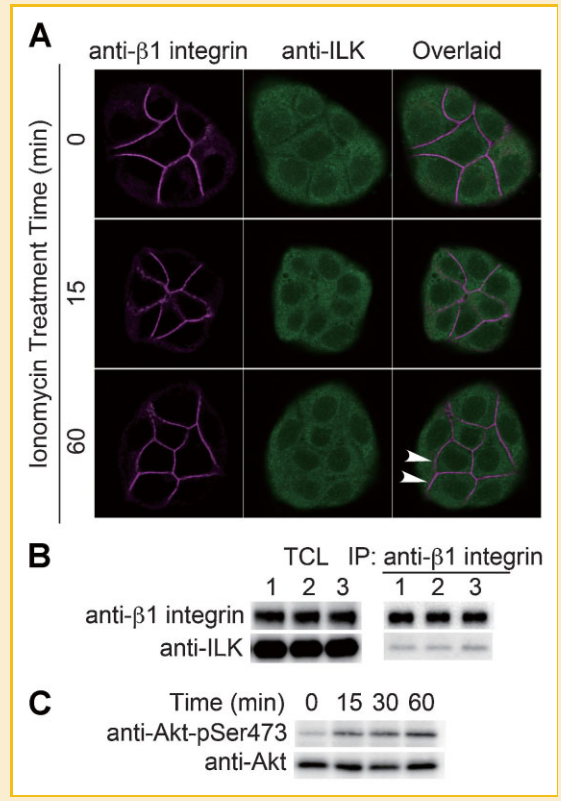

Fig. 7. Effect of ionomycin on the interaction between integrin β1 and ILK in control MDCK cells. (A) MDCK-Cont cells were treated with ionomycin (5 μM) for the periods indicated, and the cellular distribution of integrin β1 (Magenta) and ILK (Green) was examined by immunofluorescence microscopy as described in the methods using specific antibodies (magnification 400×). (B) MDCK-Cont cells were cultured for 15 min with ionomycin (5 μM) or DMSO (0.1%), integrin β1 was immunoprecipitated from each cell lysate by specific antibodies, and the total cell lysate (left) and immunoprecipitates (right) were examined by immunoblotting using antibodies against integrin β1 and ILK. 1, no treatment; 2, DMSO; 3, ionomycin; TCL, total cell lysate. (C) MDCK-Cont cells were treated with ionomycin (5 μM) for the periods indicated, and the amount of Akt phosphorylated at Ser473 was examined by immunoblotting. The total amount of Akt protein in each sample is also shown.

Taken together, in the control cells with an increase in cytoplasmic Ca^{2+} on treatment with ionomycin, phosphorylation of Akt was up-regulated, although formation of the complex of integrin β1 and ILK was not enhanced.

DISCUSSION

Previously, we reported that overexpression of CRT through gene transfection caused EMT-like changes in MDCK cells [Hayashida et al., 2006]. In that study, we found that overexpression of CRT suppresses E-cadherin expression by up-regulating the expression of Slug, a pivotal repressor of the E-cadherin gene via altered Ca^{2+} homeostasis [Hayashida et al., 2006]. In the present study, we further examined CRT-overexpressing EMT-like MDCK cells, in terms of adhesion to extracellular matrix substrata such as fibronectin and collagen IV. We found that CRT-overexpressing EMT-like MDCK cells prefer to bind fibronectin through the α5β1 heterodimer.

In EMT, integrins play important roles in cell migration through enhanced cell–substratum interaction [Hynes, 2002; Liu, 2010]. In addition, integrin-induced signaling molecules, such as FAK and ILK, have pivotal functions in EMT [Li et al., 2003, 2009; Thiery and Sleeman, 2006]. FAK signaling is activated during the TGF-β-induced EMT, and the activation is required for the EMT [Bailey and Liu, 2008; Cicchini et al., 2008]. In this study, the colocalization of FAK and integrins β1 or αVβ3 was increased in the peripheral areas of CRT-overexpressing cells (Supplementary data Fig. S4). The activation status of FAK was also examined, and the basal level of active FAK phosphorylated at Tyr397 was found to be higher in CRT-overexpressing cells than in controls (Supplementary data Fig. S5). When detached cells were seeded on culture plates coated with collagen IV, fibronectin, or LCB, the level of phosphorylated FAK was decreased with a concomitant decrease of the total level of FAK in the CRT-overexpressing cells attached to fibronectin (Supplementary data Fig. S5). In contrast, the phosphorylation of Akt, a downstream kinase of integrin signaling, was clearly up-regulated in the cells on attachment to collagen IV or fibronectin (Fig. 5A and Supplementary data Fig. S5). These results suggest that some of the proteolytic process is specifically promoted with FAK in the CRT-overexpressing cells under the conditions attached to fibronectin. But less of an effect was observed on those in the control cells. FAK is degraded by several proteases such as calpain [Cooray et al., 1996] or caspases [Wen et al., 1997]. Recently, it was suggested that calpain-mediated proteolysis of FAK plays a functional role in the regulation of adhesion dynamics in motile cells [Chan et al., 2010]. In our previous studies, we showed that Ca^{2+}-calpain pathway is involved in the activation of caspase-12 in CRT-overexpressing myocardiac cells under oxidative stress [Ihara et al., 2006]. These findings suggest some of the linkage between the enhanced proteolysis of FAK and altered Ca^{2+} signaling in the CRT-overexpressing cells on attachment to ECMs, although further investigation is required to clarify the mechanism. These results suggest that the altered FAK signaling is involved in the EMT-like characteristics of the CRT-overexpressing cells. However, when the integrin complexes were immunoprecipitated using the antibody against integrin β1 or αVβ3, FAK protein was not detected in the complexes from the control or CRT-overexpressing cells (Fig. 3B). In contrast, ILK was detected in complexes from both cells, and the amount of ILK associated with integrins was apparently increased in the CRT-overexpressing cells (Fig. 3B). These results strongly suggest that ILK has functional relevance to CRT-induced EMT in the cells. Thus we focused the attention more on the ILK-associated signaling in CRT-overexpressing EMT-like cells.

ILK is a multifunctional intracellular mediator for cell–extracellular matrix interactions, and functions in a variety of cellular processes, such as proliferation, differentiation, survival, migration, mitosis, and so forth [McDonald et al., 2008]. ILK was first discovered as integrin β1-binding protein [Hannigan et al., 1996], and connects integrins (e.g., β1 and β3) with the actin cytoskeleton through various molecules, including PINCH, parvin, ILKAP, SPARC, and T-cadherin [McDonald et al., 2008]. ILK has also been shown to possess serine/threonine kinase activity [Hannigan et al., 1996] and to phosphorylate signaling proteins such as Akt and GSK3β in mammalian cells [Delcommenne et al., 1998; McDonald et al., 2008]. Although the kinase activity of ILK leading to Akt signaling plays a pivotal role in EMT [Somasiri et al., 2001; Grille et al., 2003; Li et al., 2009], it has been reported that ILK might be a pseudokinase with no kinase activity itself [Fukuda et al., 2009; Lange et al., 2009]. In this study, enhanced colocalization between integrins (β1 and β3) and ILK was observed at peripheral cell adhesion and ruffle-like sites in the CRT-overexpressing cells (Fig. 4C,D). In addition, when detached cells were seeded on culture plates coated with collagen IV or fibronectin, the phosphorylation of Akt was found to be up-regulated in CRT-overexpressing cells, compared with controls (Fig. 5A). ILK-associated kinase activity to phosphorylate GSK3β was also highly induced by cell attachment to fibronectin in CRT-overexpressing cells (Fig. 5B). Furthermore, we found that BAPTA-AM, a membrane-permeable Ca^{2+} chelator, significantly suppressed the formation of complexes between integrin β1 and ILK in the CRT-overexpressing cells (Fig. 6). On treatment with BAPTA-AM, the phosphorylation of Akt was suppressed after 15 min. These results suggest the cytoplasmic Ca^{2+}-dependent enhancement of the formation of complexes between integrins and ILK to be consistent with the up-regulated cytoplasmic Ca^{2+} influx in the CRT-overexpressing cells [Hayashida et al., 2006].

In contrast, when control MDCK cells were treated with ionomycin, a Ca^{2+} ionophore, the formation of complexes between integrin β1 and ILK was little influenced, although the level of phosphorylated Akt was apparently increased (Fig. 7). The results suggest that, in the control cells, the increase in cytoplasmic Ca^{2+} with ionomycin could stimulate the phosphorylation of Akt through some of the alternative pathways other than the integrin/ILK pathway. Furthermore, when detached control cells were pre-incubated in DMEM/1% BSA with or without ionomycin, and seeded on culture plates coated with collagen IV or LCB, the level of phosphorylated Akt was little influenced, and no enhancement was observed with ionomycin (Supplementary data Fig. S6). The results suggest that, in the control cells, the enhancing effect of ionomycin on the phosphorylation of Akt shown in Figure 7C is caused not only by the increase in cytoplasmic Ca^{2+} but also by other factors such as the fetal calf serum in culture medium or completion of the cell-substratum interaction. On the other hand, when detached CRT-overexpressing cells were seeded on culture plates coated with ECMs, the phosphorylation of Akt was apparently activated even in the absence of the serum in the medium (Fig. 5A). The basal level of phosphorylated Akt was suppressed by the decrease in cytoplasmic Ca^{2+} with BAPTA-AM in the CRT-overexpressing cells cultured in the normal medium containing the serum (Fig. 6D). These results suggest that the regulation of Akt signaling seems quite different between control and CRT-overexpressing cells. Thus the composition of the complexes related to integrin β1 seems quite different between control and CRT-overexpressing cells (Fig. 3A), and this also suggests that some specific component may be required in part to respond to the Ca^{2+}-triggered regulation of the integrin/ILK-associated signaling in the cell. It was demonstrated that Ca^{2+}-related signaling is triggered upon direct interaction between integrins and CRT [Coppolino et al., 1997; Kwon et al., 2000]. However, CRT was neither detected in the integrin/ILK complex (data not shown), nor localized with integrins β1 and β3 at

peripheral cell adhesion and ruffle-like sites in CRT-overexpressing MDCK cells (Fig. 4A). Collectively, these findings suggest that the Ca^{2+}-triggered regulation of the intracellular localization and formation of integrin/ILK complexes plays an important role in the EMT-like phenotype of CRT-overexpressing cells. However, it is not known whether Ca^{2+} modulators, such as BAPTA-AM and ionomycin directly affect the cellular signaling initialized by integrins. It is also noteworthy that the precise mechanism by which integrins directly trigger the ILK-associated signaling to activate Akt is not clear in the CRT-overexpressing cells. To clarify the mechanism for Ca^{2+}-triggered regulation of the integrin/ILK-associated signaling, further investigation is required.

Is it possible that altered Ca^{2+}-homeostasis plays a functional role in causing EMT in the cell? Although it is not fully understood whether EMT depends on Ca^{2+}-triggered mechanisms, several studies have demonstrated the involvement of Ca^{2+}-homeostasis in the regulation of EMT. Aldehni et al. [2009] found that bestrophin1, a component of Ca^{2+}-dependent Cl^- channels, promotes EMT in renal collecting duct cells. In PC Cl3 thyroid cells, an EMT-like phenotype was induced by treatment with thapsigargin or tunicamycin through ER stress [Ulianich et al., 2008]. Thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase, induces ER stress through lack of Ca^{2+} in the ER, and also causes a rise in the level of cytoplasmic Ca^{2+} via a store-operated Ca^{2+} influx [Thastrup et al., 1990], suggesting a link between altered Ca^{2+} homeostasis and EMT in the cells. A signaling pathway related to Ca^{2+} may also play a pivotal functional role in EMT. Integrin/ILK signaling is likely to be enhanced in CRT-overexpressing MDCK cells via altered Ca^{2+} homeostasis to induce or maintain the EMT-like phenotype. Papp et al. [2007] also reported that, in mouse embryonic fibroblasts, CRT regulated focal contact through both c-Src and calmodulin/CaMKII pathways by altering the amount of Ca^{2+} in the ER. Although it is not clear whether the CRT-overexpressing MDCK cells are identical to primary fibroblasts or general fibroblastic cell lines, it would be of interest to investigate overall alterations of cell signaling related with the EMT-like phenotype of CRT-overexpressing cells.

What might be the pathological or physiological function of CRT in the kidney? In the developing metanephric kidney, the expression of CRT was significantly up-regulated during MET [Plisov et al., 2000]. Actually, in adult kidney tissues, CRT protein is moderately expressed [Waisman et al., 1985; Michalak et al., 2009]. These findings indicate that CRT expression is required for the function of epithelial cells in the kidney. However, in kidney epithelial MDCK cells, EMT-like transformation was induced by overexpression of CRT through gene transfection [Hayashida et al., 2006]. Furthermore, in a rat model of renal fibrosis caused by unilateral ureteric obstruction, a significant up-regulation of CRT expression was found at the protein level in fibrotic tissue, suggesting CRT to be a novel fibrosis-associated EMT marker in kidney fibrosis [Kypreou et al., 2008]. The authors suggested a causative role for CRT in the EMT of kidney fibrosis, because CRT expression was up-regulated by TGF-β, a well-known inducer of EMT, in kidney-derived HK cells [Kypreou et al., 2008]. These findings suggest that an appropriate range may be determined for the expression of CRT to maintain the epithelial phenotype in kidney cells. Therefore it would be of interest to examine how CRT functions in the regulation of EMT/MET in a variety of different tissues, organs, or cell types.

In conclusion, we have shown that integrin-dependent adhesion and signaling were significantly altered in CRT-overexpressing EMT-like cells, compared with parental epithelial MDCK cells. In the CRT-overexpressing MDCK cells, altered Ca^{2+} homeostasis plays a pivotal role in the enhancement of the formation of integrin/ILK complexes and ILK-associated signaling, and may result in maintenance of the EMT phenotype.

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