Calreticulin (CRT) is a multifunctional Ca$^{2+}$-binding molecular chaperone in the endoplasmic reticulum. In mammals, the expression level of CRT differs markedly in a variety of organs and tissues, suggesting that CRT plays a specific role in each cell type. In the present study, we focused on CRT functions in the kidney, where overall expression of CRT is quite low, and established CRT-overexpressing kidney epithelial cell-derived Madin-Darby canine kidney cells by gene transfection. We demonstrated that, in CRT-overexpressing cells, the morphology was apparently changed, and the original polarized epithelial cell phenotype was destroyed. Furthermore, CRT-overexpressing cells showed enhanced migration through Matrigel-coated Boyden chamber wells, compared with controls. E-cadherin expression was significantly suppressed at the protein and transcriptional levels in CRT-overexpressing cells compared with controls. On the other hand, the expression of mesenchymal protein markers, such as N-cadherin and fibronectin, was up-regulated. We also found that the expression of Slug, a repressor of the E-cadherin promoter, was up-regulated by overexpression of CRT through altered Ca$^{2+}$ homeostasis, and this led to enhanced binding of Slug to the E-box element in the E-cadherin promoter. Thus, we conclude that CRT regulates the epithelial-mesenchymal transition-like change of cellular phenotype by modulating the Slug/E-cadherin pathway through altered Ca$^{2+}$ homeostasis in cells, suggesting a novel function of CRT in cell-cell interaction of epithelial cells.

Calreticulin (CRT) is a multifunctional Ca$^{2+}$-binding molecular chaperone in the endoplasmic reticulum (ER) (1) and known to influence many biological processes, such as the regulation of Ca$^{2+}$ homeostasis (2), intercellular or intracellular signaling (3, 4), gene expression (5), glycoprotein folding (6), and nuclear transport (7). The biological significance of CRT was revealed by the finding that CRT-deficient mice die in the embryonic stage due to impaired development of cardiac and neural tissues (8, 9). CRT is expressed in rat embryos, especially in the heart, but its expression is significantly suppressed after birth (10). On the other hand, CRT-overexpressing transgenic mice are born alive, but suffer a complete heart block and sudden death after birth (11). We also found that overexpression of CRT enhanced sensitivity to apoptosis in myocardial H9c2 cells undergoing differentiation in response to retinoic acid (12) or in cells exposed to stress caused by hydrogen peroxide (13), suggesting the importance of CRT in the pathophysiology of myocardial cells. These findings indicate that CRT expression plays a vital role in the development and physiology of cardiac cells.

Despite its general importance in cell physiology, CRT is differentially expressed in various organs and tissues in mammals, showing a characteristic expression pattern. For example, CRT levels are low in the kidney and heart, compared with the pancreas and liver, in both bovine and rat tissues (14, 15). This characteristic distribution of CRT suggests specific functions in each organ or tissue.

In this study, we focused on the function of CRT in kidney epithelial cells, because CRT levels are quite low in these cells compared with other cell types such as liver cells (14). To investigate the functional effects of CRT overexpression in kidney epithelial cells, we chose Madin-Darby canine kidney (MDCK) cells to establish stable CRT-overexpressing cell lines by gene transfection. MDCK cells are derived from canine kidney and have a well polarized epithelial cell phenotype, maintaining the normal characteristics and functions of renal efferent duct epithelial cells (16). The results showed apparent changes in the morphology of CRT-overexpressing MDCK cells and destruction of the polarized epithelial cell phenotype. Furthermore, overexpression of CRT repressed E-cadherin gene expression through up-regulation of its repressor, Slug, via altered Ca$^{2+}$ homeostasis in MDCK cells. The results suggest a novel function of CRT related to an epithelial-mesenchymal transition-like change of cellular phenotype.
Calreticulin Regulates the Adhesion of MDCK Cells

EXPERIMENTAL PROCEDURES

Materials—Antibodies against CRT, calnexin (CNX), binding protein (BiP), and ERp57 were purchased from Stressgen (Victoria, BC, Canada). Mouse antibodies against E-cadherin and fibronectin were obtained from BD Biosciences, and rabbit antibodies against β-catenin and pan-cadherin were obtained from Sigma. Antibodies against Slug, SIP1/ZEB2, transient receptor potential vanilloid receptor (TRPV) 5, TRPV6, and polycystin 2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon (Temecula, CA). Anti-Cu,Zn-superoxide dismutase antibody was kindly provided by Dr. K. Suzuki (Hyogo College of Medicine, Japan). Peroxidase-conjugated secondary antibodies against IgG of rabbit, mouse, and goat were from Sigma. Antibodies against Cu,Zn-superoxide dismutase antibody were purchased from Chemicon (Temecula, CA). Anti-Cu,Zn-superoxide dismutase antibody was kindly provided by Dr. K. Suzuki (Hyogo College of Medicine, Japan).

Cell Lines and Culture—MDCK cells were obtained from American Type Culture Collection (NBL-2). The expression vector for mouse CRT cDNA was constructed as described previously (12). Expression vectors for CRT-gene expression and the control were introduced into MDCK cells using Lipofectamine 2000 reagent (Invitrogen) in accordance with the instructions provided by the manufacturer. Stable cell lines were generated after selection with 500 µg/ml G418.

Two independent clones expressing high levels of CRT protein were isolated from CRT gene transfecteds and used in the study. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C in an atmosphere of 5% CO2 and 95% air.

Subcellular Fractionation—Cultured cells were harvested and homogenized with homogenization buffer (10 mM Heps, pH 7.0, 0.25 M sucrose, 2 mM EGTA, and protease inhibitors (20 µM phenylmethylsulfonyl fluoride, 50 µM pepstatin, and 50 µM leupeptin)) by using a homogenizer of the Potter-Elvehjem type. Subcellular fractionation was performed at 4 °C according to the method of Hogeboom (17) with a modification. The homogenates were centrifuged at 2,000 × g for 10 min, and the post-nuclear supernatant was again centrifuged at 8,000 × g for 20 min at 4 °C. The post-lysosome supernatant was ultracentrifuged at 55,000 × g for 2 h at 4 °C in a Beckman SW41TI rotor (Beckman Instruments). The resulting supernatant contains the soluble cytosolic fraction, and the microsomal pellet represents the ER membrane and lumen proteins as well as Golgi membranes. The pellet was dissolved in lysis buffer (20 mM Tris-HCl (pH 7.2), 130 mM NaCl, and 1% Nonidet P-40, including protease inhibitors), and used as a microsomal fraction.

Immunoblot Analysis—Cells were harvested and lysed in the lysis buffer. The lysate was sonicated on ice for 10 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 7.5% or 10% SDS-polyacrylamide gels and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS, 10 mM Tris-HCl (pH 7.5) and 0.15 M NaCl) and incubated at room temperature for 2 h with the primary antibody in TBS containing 0.05% Tween 20. The blots were coupled with peroxidase-conjugated secondary antibodies, washed, and then developed using the ECL detection kit (Amersham Biosciences) according to the instructions recommended by the manufacturer.

Immunofluorescence Microscopy—Cells (5 × 104 per ml) were grown on Lab Tek chamber slides (Nalgene Nunc International, Naperville, IL) for 24 h. They were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2) and permeabilized for 10 min with PBS containing 1% Triton X-100. The cells were then blocked with 1% bovine serum albumin in PBS, incubated with the antibody for 1 h, and washed with PBS containing 1% bovine serum albumin.

The immunoreactive primary antibodies were visualized with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulins (Cappel), anti-mouse immunoglobulins (Dako), or rhodamine-conjugated anti-rabbit immunoglobulins (Cappel). After being washed, stained cells were mounted in the Vectashield medium, visualized under a Carl Zeiss LSM5 microscope (Carl Zeiss, Jena, Germany), and analyzed using PASCAL analytic software.

Invasion Assays—A cell invasion assay was carried out using Boyden chambers (Transwell chambers) as described previously (18) with a slight modification. In brief, Transwell chambers, equipped with 8-µm Matrigel®-coated filters (24-well format, BD Biosciences), were rehydrated, and suspensions of 1 × 105 cells in 200 µl of Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum were plated in the upper compartment of the chamber. Serum-free medium (800 µl) was placed in the lower compartment. After 24 h at 37 °C, noninvasive cells on the upper surface of the filters were removed completely by wiping with a cotton swab. The filters were then fixed with 4% paraformaldehyde in PBS and stained with 0.1% crystal violet. Cells on the lower surface were photographed under a microscope (magnification ×100), and enumerated. The data were expressed as the mean ± S.D. of assays performed in triplicate for each filter.

Cell Proliferation Assays—Cell proliferation assays were performed as described previously (12). The proliferation of cultured cells was evaluated by measuring attached live cells photometrically after staining with crystal violet. Cells were seeded onto 96-well plates at a density of 3000 cells per well in 100 µl of medium. After culturing for 5 days, cells were fixed with 4% paraformaldehyde in PBS, washed three times with 1% crystal violet and cell numbers were estimated by measuring the absorbance at 570 nm using a microplate reader.

Reverse Transcription-PCR Analysis—Total RNA was isolated from cultured cells (i.e. MDCK, NIH3T3, PC3, and LNCaP) or rat tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany). A specific system for the amplification of mRNA was used: an mRNA-selective PCR kit (AMV, TaKaRa Biomedicals, Shiga, Japan). One microgram of total RNA extracted from cells was used as a template. PCR products were obtained after 30–35 cycles of amplification with an annealing temperature of 55–65 °C. The primer sequences used were as
following: canine E-cadherin (GenBank™ accession number XM_536807, fragment size 497 bp), forward primer (5'-GGC ATT CTC AGA ATG ATC CTC GC-3'), and reverse primer (5'-CCA TAT AGC TCC AGC AGC AGC TCC-3'); mouse Snail (M95604, 258 bp), forward primer (5'-GGA TCT TCT CCT GGT ACC CTA GGT GCC GGC-3'), and reverse primer (5'-CTT TGG CCA CCG AGA GCC TGG CCA GCT GC-3'); canine Slug (XM_544069, 454 bp), forward primer (5'-CAG CTC (G/A)GG AGC (G/A)TA CAG CCC C-3') reverse primer (5'-TAA CCA GCC GTG TCT GGA AAA GGC C-3'); mouse 8EF1 (NM011546, 570 bp), forward primer (5'-GCT CCC GTG GAA GTG ACC TAT CCA GGA A-3'), and reverse primer (5'-GCA CAC CAC CCT GAG GAG AAC TGG TTG C-3'); mouse SIP1 (AF033116, 401 bp), forward primer (5'-GCT AGC ACC ATA CCC AGG ACG-3'), and reverse primer (5'-TCT CGG CCG AGT GCA GCC-3'); mouse E12/E47 (BC006860, 620 bp), forward primer (5'-AGT GAC CTC CGT GAC TTC ATG ATG TTC CCG CT-3'), and reverse primer (5'-GGG TGC GCT GGG G-3'); human TWIST (BC036704, 436 bp), forward primer (5'-CTG AGC ACC AAC AGC GAG GAA GA-3'), and reverse primer (5'-CTG GTA GAA GTC GAT GTG CT-3'); canis GAPDH (NM_001003142, 979 bp), forward primer (5'-GGT CGG AGT CAA CGG ATT TGG C-3'), and reverse primer (5'-CAT GTA GAC GAT GTC GAC CAC CAC C-3'); rat TRP2 (NM_053787, 419 bp) primer (5'-CCG AGG ATC CCA GAT CCG GGG G-3'), and reverse primer (5'-CGC AGC ACC ACC ATG GCC ACC AGC G-3'). The PCR products were subcloned into pCRII (Invitrogen). The E-box C probe, 5'-CCT TGG CCA CCG AGA GCC TGG CCA GCT GC-3'; reverse primer (5'-TAA CCA GCC GTG TCT GGA AAA GGC C-3'); mouse 8EF1 (NM011546, 570 bp), forward primer (5'-GCT CCC GTG GAA GTG ACC TAT CCA GGA A-3'), and reverse primer (5'-GCA CAC CAC CCT GAG GAG AAC TGG TTG C-3'); mouse SIP1 (AF033116, 401 bp), forward primer (5'-GCT AGC ACC ATA CCC AGG ACG-3'), and reverse primer (5'-TCT CGG CCG AGT GCA GCC-3'); mouse E12/E47 (BC006860, 620 bp), forward primer (5'-AGT GAC CTC CGT GAC TTC ATG ATG TTC CCG CT-3'), and reverse primer (5'-GGG TGC GCT GGG G-3'); human TWIST (BC036704, 436 bp), forward primer (5'-CTG AGC ACC AAC AGC GAG GAA GA-3'), and reverse primer (5'-CTG GTA GAA GTC GAT GTG CT-3'); canis GAPDH (NM_001003142, 979 bp), forward primer (5'-GGT CGG AGT CAA CGG ATT TGG C-3'), and reverse primer (5'-CAT GTA GAC GAT GTC GAC CAC CAC C-3'); rat TRP2 (NM_053787, 419 bp) primer (5'-CCG AGG ATC CCA GAT CCG GGG G-3'), and reverse primer (5'-CGC AGC ACC ACC ATG GCC ACC AGC G-3'). The PCR products were subcloned into pCRII (Invitrogen). The nucleotide sequences were confirmed by sequencing with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). Oligonucleotides used were as follows: E-box A probe, 5'-CCG CCC GCC GCA GGT GTA GCA GCC GCA G-3'; E-box B probe, 5'-CTC CGG GCT CAC CGT GCC GCC GCA C-3'; and E-box C probe, 5'-GGG GCT GCG GCC GCC ACC TGT CAT TC-3' (bold letters indicate consensus sequences for the E-box). Binding reactions were carried out in 15 μl of reaction mixture (25 mM Tris-HCl (pH 7.0), 6.25 mM MgCl₂, 0.5 mM dithiothreitol, and 10% glycerol) containing 10 μg of nuclear extract and 25 ng of labeled oligonucleotides. For the supershift assay, anti-Slug and anti-SIP1 antibodies were added to the reaction mixture during the 30-min binding reaction.

**Promoter Reporter Assays**—The promoter of human E-cadherin (−178 to +66 bp; GenBank™ accession number AF34545) was isolated from the genomic DNA of A549 cells and amplified by PCR using Pfu turbo DNA polymerase (Stratagene). The primer sequences used were as follows (20): forward primer (5'-ACT CCA GCC TAG AGG GTC A-3') and reverse primer (5'-TGG AGC GGG CTT GAG TCT-3'). The PCR product was subcloned into pGL3-Basic vector (KpnI-PstI site, Promega, Madison, WI). PCR-based site-directed mutagenesis was used for the generation of reporter gene constructs with E-box mutations by using a QuikChange site-directed mutagenesis kit (Stratagene), resulting in a mutation in the E-box element from 5'-CANNTG-3' to 5'-AANNTA-3' (sense strand). Each vector was transiently transfected into MDCK cells using Lipofectamine 2000 (Invitrogen) as described above. Twenty-four hours after the transfection, luciferase activities were assayed with cellular extracts using a Dual Luciferase Reporter Assay System (Promega) and were normalized to pRL activity.

**Assays for Release and Uptake of Ca²⁺ in the Cell**—For the 45Ca²⁺ release assay, cells were cultured for 48 h with medium containing 54Ca²⁺ (1 μCi/ml). After a wash with Ca²⁺-free Earle’s balanced salt solution (EBSS, Invitrogen) containing 3 mM EGTA, cells were detached from the culture plates with trypsinization buffer (0.25% trypsin and 0.02% EDTA in EBSS), and the cell suspensions were preincubated in Ca²⁺-free EBSS at 37 °C for 3 min, and sequentially stimulated with thapsigargin (0.1 μM), ionomycin (2 μM), and monensin (2 μM). The cell suspensions were collected 5 min after the addition of each reagent and centrifuged. The radioactivity released from the cells was measured in the supernatant. Cell pellets were lysed, and protein amounts were determined using a BCA assay kit (Pierce). 45Ca²⁺ release was expressed as the cpm subtracted from those recovered in the preceding collection, and normalized to the protein in the corresponding cell pellets. The uptake of Ca²⁺ was measured radiometrically using the Millipore filtration technique as described previously (13) with a slight modification. The cells were washed with 45Ca²⁺ uptake buffer, which consisted of EBSS supplemented with 0.1 mM CaCl₂, and cultured for specific periods in 45Ca²⁺ uptake buffer containing 54Ca²⁺ (5 μCi/ml). Cells were detached from the culture plates by trypsination buffer, and the cell suspension was filtered through a 0.45-μm nitrocellulose filter (Bio-Rad) under vacuum. The filters were rinsed twice with 0.5 ml of washing buffer (10 mM Heps (pH 7.4), 150 mM KCl, 2 mM EGTA, and 2.5 mM MgCl₂). The 45Ca²⁺ uptake was calculated by measuring the radioactivity and standardized using protein concentrations.

**Measurement of Cytoplasmic-free Ca²⁺**—The cytoplasmic free Ca²⁺ concentration, [Ca²⁺]cyt, was measured with a dual-excitation wavelength fluorescence microscope using Fura-2. Cultured cells on quartz-bottom dishes were loaded with 5 μM Fura-2 tetra(acetoxymethyl)ester (Fura-2-AM, Dojindo, Kumamoto, Japan) for 20 min in EBSS with 2 mM CaCl₂ in the pres-
ence of 0.01% pluronic acid F-127. After four washes with EBSS, Fura-2 fluorescence was determined at 37 °C using an IX71 inverted research microscope (Olympus, Tokyo, Japan) and a FURA ratiometric imaging system operating at an emission wavelength of 505 nm with an excitation wavelength of 340 and 380 nm. The FURA ratiometric imaging system includes filters (Chroma Technology Corp., Rockingham, VT) switched by filter wheels (Sutter Instrument Company, Novato, CA) and a MicroMax camera (Roper Scientific, Tucson, AZ) controlled by SlideBook software. To measure the change in $[Ca^{2+}]_i$ during store-operated Ca$^{2+}$ influx, Fura-2-labeled cells were washed with Ca$^{2+}$-free EBSS, then stimulated with thapsigargin (5 μM) followed by re-addition of Ca$^{2+}$ (2 mM). Stimulated calcium release was calculated as the change in the excitation ratio from baseline integrated over 800 s of stimulation with thapsigargin or Ca$^{2+}$. The maximal signal ($R_{\text{max}}$) was obtained by adding ionomycin at a final concentration of 4 μM. The minimal signal ($R_{\text{min}}$) was then obtained by adding EGTA at a final concentration of 10 mM, followed by Tris-free base to a final concentration of 30 mM, to increase the pH to 8.3. $R$ is the ratio ($F_1/F_2$) of the fluorescence of Ex 340 nm, Em 505 nm ($F_1$) to that of Ex 380 nm, Em 505 nm ($F_2$). The actual calcium concentration was calculated as $K_d \times (R - R_{\text{min}})/(R_{\text{max}} - R) \times Sf2/Sb2$ with the $K_d$ equal to 224 nM (21). Sf2/Sb2 is the ratio of Fura-2 fluorescence at 380 nm in Ca$^{2+}$-free and Ca$^{2+}$-replete medium, respectively.

RESULTS

Overexpression of CRT Causes Morphological Change in MDCK Cells—Canine renal epithelial MDCK cells were transfected with the expression vector for CRT cDNA to obtain cell lines overexpressing CRT (MDCK-CRT1 and -CRT2). The expression level of CRT was examined by immunoblot analysis with specific antibodies as described under “Experimental Procedures.” Fig. 1A shows that the expression of CRT was increased in the overexpressers to 3-fold the levels in the parental (MDCK-WT) and mock-transfected MDCK (MDCK-Control) cells. The transfection had no apparent effect on the expression of molecular chaperones in the ER, such as BiP, ERp57, and cytosolic GAPDH. However, the expression of CNX, another membrane-bound ER homologue of CRT, showed a slight decrease in the CRT-overexpressing cells. Cell morphology was examined in control and CRT gene-transfected cells using specific antibodies as described under “Experimental Procedures.” Fig. 1B shows that the expression of CRT was increased in the overexpressors to ~3-fold the levels in the parental (MDCK-WT) and mock-transfected MDCK (MDCK-Control) cells. The transfection had no apparent effect on

![FIGURE 1. Effect of CRT overexpression on cell morphology in MDCK cells transfected with the CRT gene. A, expression levels of CRT, CNX, BiP, ERp57, and GAPDH were estimated in parental (MDCK-WT), mock vector-transfected (MDCK-Control), and CRT gene-transfected MDCK (MDCK-CRT1 and -CRT2) cells using immunoblot analysis with specific antibodies as described under “Experimental Procedures.” Data represent three independent experiments. B, cell morphology of control and CRT gene-transfected cells was examined using phase-contrast microscopy and photographed under a magnification of ×100.](image-url)
phenotype and loss of cell-cell contacts, although there was no remarkable morphological change in mock-transfected cells compared with parental cells. Intracellular localization of CRT was characterized by immunofluorescence microscopy in control and CRT-overexpressing cells (Fig. 2A). Under conditions in which cellular membranes were permeabilized by Triton X-100, strong immunoreactivity for CRT showed a perinuclear localization and a vesicular pattern in CRT-overexpressing cells, although the immunoreactive signal was weak in controls. No significant increase in the cell surface expression of CRT was observed in the CRT-overexpressing cells under conditions without Triton X-100 treatment. To investigate whether the cytosolic localization of CRT was increased in the gene-transfected cells, control and MDCK-CRT1 cells were lysed and fractionated by centrifugation to separate cytosolic and microsomal fractions as described under “Experimental Procedures.” As shown in Fig. 2B, overexpressed CRT was present in the microsomal but not cytosolic fraction. CNX and Cu,Zn-superoxide dismutase were detected as marker proteins for microsomes and the cytosol, respectively. Similar results were also obtained with MDCK-CRT2 cells (data not shown). Together, these results indicate that overexpression of CRT did not influence the localization of CRT in the ER of MDCK cells.

**Overexpression of CRT Enhances Cellular Migration in MDCK Cells**—To investigate whether the altered morphology in CRT-overexpressing cells affected cellular functions, a cell invasion assay was performed using a modified Boyden chamber as described under “Experimental Procedures.” Cells were seeded on Transwell filters that had been coated with extracellular matrix components, including laminin, fibronectin, and proteoglycans (i.e. Matrigel®-coated polycarbonate membrane). After 24 h, the numbers of cells that had migrated through the filters were estimated by enumerating stained cells. There was a significant increase in cell motility through a Matrigel®-coated polycarbonate membrane in CRT-overexpressing cells compared with controls (Fig. 3, A and B). To investigate whether the enhanced migration of CRT-overexpressing cells was due to an increase in cell growth, cell proliferation was examined in control and CRT gene-transfected cells as described under “Experimental Procedures.” However,
the results showed that cell growth was suppressed in CRT-overexpressing cells compared with controls (Fig. 3C). These results indicate that the increase in migration of CRT-overexpressing cells was not simply due to an increase in cell growth, implicating other mechanisms related to cell movement, such as cell adhesion and cell de-attachment.

Overexpression of CRT Suppresses E-cadherin Expression—
E-cadherin plays an important role in cell-cell interaction in MDCK cells, and a loss of E-cadherin is closely associated with enhanced migration of epithelial cells via a mechanism known as epithelial-mesenchymal transition (EMT) (24). In addition, mesenchymal protein markers (such as N-cadherin and fibronectin) are expressed in EMT (25). To investigate whether overexpression of CRT affected the expression of E-cadherin and EMT-related proteins, an immunoblot analysis was carried out to examine the expression level of EMT-related proteins, such as E-cadherin, N-cadherin, β-catenin, fibronectin, and vinculin, in control and CRT-overexpressing cells (Fig. 4A). The results showed that the expression level of E-cadherin was apparently decreased in CRT-overexpressing cells compared with control cells. The expression of β-catenin, a cytoplasmic signaling molecule associated with E-cadherin, was slightly decreased in CRT-overexpressing cells. In contrast, expression of N-cadherin, fibronectin, and vinculin was apparently induced in CRT-overexpressing cells. The intracellular localization of E-cadherin was characterized by immunofluorescence microscopy (Fig. 4B). In controls, immunoreactivity for E-cadherin was strong and mainly located at cell-cell contact regions. However, the signal was apparently diminished in CRT-overexpressing cells. These results were consistent with the data from the immunoblot analysis (Fig. 4A). Taken together, these results indicate that overexpression of CRT caused a decrease in E-cadherin, and an increase in N-cadherin, fibronectin, and vinculin in MDCK cells, resulting in a gain of migratory characteristics in the cells. This also suggests that CRT expression might be involved in the regulatory mechanism of EMT in the conversion of early stage tumors into invasive malignancies.
Expression of Slug, a Repressor of the E-cadherin Gene, Is Upregulated in CRT-overexpressing MDCK Cells

To investigate whether E-cadherin expression is down-regulated in CRT-overexpressing cells at the transcriptional level, we examined the E-cadherin mRNA level by Northern blot analysis in control and CRT-overexpressing cells. As shown in Fig. 5, the expression of Slug mRNA was apparently up-regulated in CRT-overexpressing cells. The level of SIP1 mRNA was also slightly increased in the gene-transfected cells compared with the controls. On the other hand, there was little difference in the expression levels of Snail and Twist and no expression of δEF1 and E12/E47 in control and CRT-overexpressing cells. Therefore, we focused on the functions of Slug and SIP1 in the mechanism of E-cadherin gene suppression in CRT-overexpressing cells.

Slug Binds to the E-box Element in the E-cadherin Gene Promoter to Repress Gene Expression in CRT-overexpressing MDCK Cells—Slug and SIP1 block E-cadherin transcription by binding to specific DNA sequences (5′-CANNTG) called E-boxes (27). As shown in Fig. 6A, in the canine E-cadherin gene promoter, there are two E-box sites located from −78 to −73 (designated as E-box A) and from −28 to −23 (designated as E-box B), and downstream of the transcription start site there is another 5′-CANNTG sequence positioned from +22 to +27 (designated as E-box C) (23, 28, 29). To investigate whether Slug or SIP1 could interact with E-boxes in the E-cadherin gene promoter, EMSA was performed with nuclear extracts from control (MDCK-Control) and CRT-overexpressing (MDCK-CRT1) cells using 32P-labeled oligonucleotides designed for each E-box (Fig. 6B). In the case of E-box A, a major band appeared with the extracts from CRT-overexpressing cells but not with controls. The shifted band was not affected by the presence of antibodies against Slug and SIP1, suggesting that Slug and SIP1 had no correlation with E-cadherin gene repression. In the case of E-box B, a major band also appeared with extracts from CRT-overexpressing cells but not with control extracts. The shifted band was not affected by the anti-SIP1 antibody but disappeared with addition of the anti-Slug antibody, suggesting that Slug was involved in repression of the E-cadherin gene in CRT-CRT1 cells.
Calreticulin Regulates the Adhesion of MDCK Cells

In previous reports, overexpression of CRT led to an increase in the intracellular store of Ca\(^{2+}\) (1). CRT also appears to modulate store-operated Ca\(^{2+}\) influx (1, 30). We also reported that overexpression of CRT influences Ca\(^{2+}\) homeostasis in myocardial H9c2 cells under various stressful conditions (12, 13). However, the mechanism by which overexpression of CRT influenced Ca\(^{2+}\) homeostasis was not clear in MDCK cells. To investigate whether the intracellular store of Ca\(^{2+}\) was affected by overexpression of CRT, intracellular Ca\(^{2+}\) pools were characterized in control and CRT-overexpressing cells. After 48 h of loading with \(^{45}\)Ca\(^{2+}\), the cells were washed and resuspended in Ca\(^{2+}\)-free buffer. Unidirectional fluxes to the extracellular medium after stimulation with several Ca\(^{2+}\) modulators were then measured as described under “Experimental Procedures.” Thapsigargin (an inhibitor of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase), ionomycin (a Ca\(^{2+}\) ionophore), and monensin (another ionophore affecting acidic stores) were used to stimulate the cellular Ca\(^{2+}\) pools (Fig. 7A). The results showed that cellular Ca\(^{2+}\) levels were apparently increased mainly in the thapsigargin-sensitive Ca\(^{2+}\) pools of CRT-overexpressing cells, compared with controls, suggesting that Ca\(^{2+}\) stores in the ER were increased in CRT-overexpressing cells.

Next, to investigate whether the intracellular Ca\(^{2+}\) homeostasis was affected by overexpression of CRT, the cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and response of [Ca\(^{2+}\)], to thapsigargin were examined in control and CRT-overexpressing cells using Fura2-AM as described under “Experimental Procedures.” The release of Ca\(^{2+}\) from ER stores by thapsigargin was measured in the absence of extracellular Ca\(^{2+}\), and the influx of Ca\(^{2+}\) from the extracellular space was measured by re-adding Ca\(^{2+}\) to the external medium. As shown in Fig. 7 (B and C), the basal level of [Ca\(^{2+}\)], was slightly increased in CRT-overexpressing cells, compared with controls. Thapsigargin induced a greater increase of [Ca\(^{2+}\)], in CRT-overexpressing cells than in the controls, indicating that substantially more Ca\(^{2+}\) was released from Ca\(^{2+}\) stores in CRT-overexpressing cells. Furthermore, the Ca\(^{2+}\) influx-induced increase of [Ca\(^{2+}\)], when Ca\(^{2+}\) was re-added was also more extensive in CRT-overexpressing cells than the controls. These results suggest that the level of [Ca\(^{2+}\)], was relatively high in CRT-overexpressing cells compared with controls. However, it was reported that store-
operated Ca\(^{2+}\) entry was suppressed by overexpression of CRT in several cell types (30) and was inconsistent with the present results of CRT-overexpressing MDCK cells.

To further investigate why the influx of Ca\(^{2+}\) was enhanced in CRT-overexpressing cells, we examined the expression of epithelial Ca\(^{2+}\) channels in control and CRT-overexpressing cells. In Fig. 8A, the expression of epithelial Ca\(^{2+}\) channels related to cellular Ca\(^{2+}\) uptake, such as TRPV5, TRPV6 (31), and polycystin 2 (32), was examined by immunoblot analysis using specific antibodies in control and CRT-overexpressing cells. The expression of TRPV5 was stronger in the CRT-overexpressing cells. In contrast, TRPV6 was not detected in either of the cells. Polycystin 2 was similarly expressed in both control and CRT-overexpressing cells. In Fig. 8B, the expression of TRPV5 was also examined by immunofluorescence microscopy. The immunoreactive signals for TRPV5 showed a perinuclear vesicular distribution and were abundant in CRT-overexpressing cells compared with less signals in control cells. In Fig. 8C, transcriptional levels of TRPV5 and -6 were examined in control and CRT-overexpressing cells by RT-PCR using specific primers as described under “Experimental Procedures.” The results showed that the transcriptional expression of TRPV5 was detected in CRT-overexpressing cells but not in control cells under the experimental conditions. The expression of TRPV6 was not detected in either control or CRT-overexpressing cells. For positive controls, RNAs from rat kidney and prostate were used for RT-PCR to detect TRPV5 and -6, respectively (33). Together, these results indicate that the expression of TRPV5 is apparently up-regulated in CRT-overexpressing cells compared with controls, suggesting a functional link with the up-regulation of Ca\(^{2+}\) influx in...
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Overexpression of CRT Down-regulates E-cadherin Gene Expression through Alteration of \( \text{Ca}^{2+} \) Homeostasis—To determine whether the increase in [\( \text{Ca}^{2+} \)] was part of the causative mechanism for E-cadherin gene repression in the gene-transfected cells, E-cadherin expression was examined in control cells treated with thapsigargin (5 \( \mu \)M) or ionomycin (1 \( \mu \)M) to increase [\( \text{Ca}^{2+} \)]. RT-PCR analysis was performed to examine E-cadherin expression at the mRNA level. As shown in Fig. 9A, the mRNA levels of E-cadherin were decreased in both cases with thapsigargin and ionomycin in a time-dependent manner. The expression of E-cadherin was also examined by immunofluorescence microscopy after a 4-h treatment with thapsigargin or ionomycin (Fig. 9B). The results showed that the immunoreactive signal for E-cadherin was diminished in both cases with thapsigargin and ionomycin, compared with untreated controls. To investigate further the effect of increased [\( \text{Ca}^{2+} \)], on the repressors of the E-cadherin gene, we examined mRNA levels of Slug by RT-PCR analysis in control cells treated with or without thapsigargin or ionomycin for 1 h. Expression of Slug was increased in both cases with thapsigargin and ionomycin, compared with untreated controls (Fig. 9C). The expression of Slug was also examined by immunofluorescence microscopy in control cells after 4 h of treatment with thapsigargin or ionomycin (Fig. 9D). The results showed that the immunoreactive signal for Slug was increased in both cases with thapsigargin and ionomycin, compared with untreated controls. In the cells treated with thapsigargin or ionomycin, increased signals for Slug were detected in the cytoplasm and nucleus and were similar with the signals in the CRT-overexpressing cells (MDCK-CRT1). Taken together, these results indicate that the rise in [\( \text{Ca}^{2+} \)], increased the expression of Slug and decreased the expression of E-cadherin, suggesting a \( \text{Ca}^{2+} \)-dependent repression of E-cadherin through Slug in MDCK cells.

Next, to further investigate whether the repression was caused by an rise in [\( \text{Ca}^{2+} \)], in CRT-overexpressing cells, the effect of BAPTA-AM, a cell-permeable \( \text{Ca}^{2+} \) chelator that reduces intracellular \( \text{Ca}^{2+} \) levels, on the expression of E-cad-
In addition, the binding ability of E-box elements of the E-cadherin promoter was also examined by EMSA in control cells treated with or without thapsigargin (5 μM) or ionomycin (1 μM) for 2 h (Fig. 11A). The results showed that a positive gel-shift band appeared in response to the treatment with both thapsigargin and ionomycin in the case of E-box B, but not E-boxes A or C. The gel-shift band induced by thapsigargin or ionomycin was similar to that seen in the case of nuclear extracts from CRT-overexpressing cells (MDCK-CRT1). Next, to confirm the Ca^{2+}-dependent up-regulation of E-cadherin gene expression, CRT-overexpressing cells were treated for 2 h with BAPTA-AM (10 μM), and then the binding ability of E-box elements of the E-cadherin promoter was examined by EMSA (Fig. 11B). The results showed that the protein-binding ability of E-box B apparently decreased in MDCK-CRT1 cells treated with BAPTA-AM, although that of E-box A also slightly decreased with BAPTA-AM. There was no influence of BAPTA-AM on the binding ability of E-box C. Similar results were also obtained with MDCK-CRT2 cells (data not shown). Taken together, these results indicate that overexpression of CRT in MDCK cells causes an increase in [Ca^{2+}]), which up-regulates Slug expression, resulting in suppression of E-cadherin gene expression via E-box B in the gene promoter.

**DISCUSSION**

In this study, we have shown that overexpression of CRT repressed E-cadherin gene expression in MDCK cells, leading to alteration of cell characteristics such as morphology and motility. E-cadherin, a Ca^{2+}-dependent transmembrane glycoprotein, plays a key role in the maintenance of intercellular adhesion, regulation of tissue morphogenesis, and cell polarity in epithelial cells, and disruption of E-cadherin expression or function causes invasion and metastasis (34–37). Therefore, E-cadherin is thought to be an invasion suppressor (38, 39). E-cadherin-mediated cell-cell adhesion plays a critical role in early embryonic development through a mechanism known as EMT (24). EMT also determines progression in epithelial tumors, occurring concomitantly with the acquisition of migratory properties followed by down-regulation of E-cadherin expression (40, 41). In CRT-overexpressing MDCK cells, the morphology changed from a polarized, epithelial phenotype to a highly motile fibroblastoid-like, mesenchymal phenotype. In addition to a decrease in E-cadherin expression, the expression level of mesenchymal markers, such as N-cadherin and...
fibronectin, was apparently up-regulated in the gene-transfected cells. The conversion of the cadherin class is observed in EMT in general (42–44). These results strongly suggest that overexpression of CRT could cause EMT-like changes in MDCK cells.

Recently, it has been reported that EMT is caused by several transcriptional repressors of E-cadherin, such as the zinc finger factors (i.e. Snail (22, 23), Slug (45, 46), E12/E47 (27, 50), Twist (51, 52)). The zinc finger factors have been described to directly repress transcription of the E-cadherin gene by binding to E-boxes (consisting of the sequence 5′-CANNTG) in the proximal E-cadherin promoter (23, 28, 29, 53), and E-boxes are the recognition sites of the basic helix-loop-helix family (54). Bolos et al. (46) reported that stable expression of Slug in MDCK cells leads to full repression of E-cadherin at the transcriptional level and triggers a complete EMT, although the binding affinity of Slug to the E-box is lower than that of Snail and E12/E47. Slug is also responsible for repression of the E-cadherin gene through binding to E-box C in human breast cancer cells (29). On the other hand, Snail is also a repressor of E-cadherin expression in various epithelial tumor cells (23). In SIP1-expressing human mammary cancer cells, mutation of E-box B (designated as E2-box 3) restored the E-cadherin promoter activity similarly to mutation of E-Box A (designated as E2-box1) (28). These studies indicate that the regulatory system for repression of the E-cadherin gene is complex, suggesting that the specific repressors could work in a cell type-dependent manner (29). In this study, we showed that overexpression of CRT induced the expression of Slug and that this enhanced the binding of Slug to E-box B in the E-cadherin promoter to repress E-cadherin expression in MDCK cells.

In addition to transcriptional repression, several molecular mechanisms that down-regulate E-cadherin function have been reported, including gene mutation (55), promoter methylation (56), and post-translational modification of the cadherin-catenin complex (57). Indeed, we observed that, in CRT-overexpressing cells, the promoter activity of E-cadherin was suppressed to ~50% of the control level, despite extensive suppression of E-cadherin gene expression. Therefore, other regulatory mechanisms as stated above may also be involved in E-cadherin gene repression in CRT-overexpressing cells, suggesting that further investigation is required.
CRT modulates cell adhesiveness via the expression of N-cadherin and vinculin and affects Wnt signaling pathways in L-fibroblasts (58, 59). In that study, the authors found that CRT overexpression increased cell adhesion with a concomitant increase of N-cadherin and vinculin in fibroblasts. On the other hand, we found that overexpression of CRT in MDCK cells caused a loss of cell-cell interaction and induced cell migration through the Matrigel® (Figs. 1B, 3A, and 3B). In terms of the effect of overexpressed CRT on cell-cell interaction, the reason for the discrepancy is not yet clear. However, it is noteworthy that primary fibroblasts show a mesenchymal phenotype with less expression of E-cadherin, compared with the levels in the epithelial cell-like MDCK cells (23). This suggests that the basal mechanisms for cell adhesion and motility differ between these cell types. Despite the discrepancy in cell morphology, the expression of N-cadherin and vinculin was induced by overexpression of CRT in both fibroblasts (59) and MDCK cells (this study), indicating that the present result does not necessarily contradict previous findings using CRT-overexpressing fibroblasts, in respect of the expression of N-cadherin and vinculin (58, 59). Very recently, Afshar et al. (60) reported that cellular migration and binding to collagen type V were apparently suppressed in embryonic fibroblasts from CRT knock-out mice, indicating that the cellular level of CRT is important for the regulation of cell motility. Cell surface expression of CRT has been reported in several cell types (3). In addition, Goicoechea et al. (61) reported that cell surface CRT functions as a receptor for thrombospondin to contribute to focal-adhesion disassembly of the cell. Although it is not clear how cell surface CRT contributes to thrombospondin-mediated signaling and regulation for cell migration, no significant expression of CRT was observed on the surface of CRT-overexpressing MDCK cells. Taken together, these findings suggest that

FIGURE 10. BAPTA-AM, a Ca²⁺ chelator, up-regulated the expression of E-cadherin and down-regulated the expression of Slug in CRT-overexpressing cells. A, MDCK-CRT1 cells were treated with BAPTA-AM (10 μM) for the periods indicated, and the level of E-cadherin expression was examined by RT-PCR analysis using specific primers. The RT-PCR for E-cadherin was also shown in untreated MDCK-Control cells. B, MDCK-CRT1 cells were treated for 8 h with BAPTA-AM as described in A, and the expression of E-cadherin was examined by immunofluorescence microscopy by using the anti-E-cadherin antibody. Data represent three independent experiments. C, MDCK-CRT1 cells were treated with BAPTA-AM as described in A, and the level of Slug expression was examined by RT-PCR analysis using specific primers. The RT-PCR for Slug was also conducted in untreated MDCK-Control cells. D, MDCK-CRT1 cells were treated for 8 h with BAPTA-AM as described in A, and the expression of Slug was examined by immunofluorescence microscopy using the anti-Slug antibody. Data represent three independent experiments.
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CRT plays a vital role in the regulation of cell-cell interaction and cell motility in a variety of cell types.

E-cadherin appears to be an important target molecule in the mechanism influenced by CRT. To investigate the mechanism whereby CRT up-regulates the expression of Slug to repress the E-cadherin gene, we focused on Ca\(^{2+}\) homeostasis influenced by CRT overexpression in MDCK cells, because CRT plays an important role in the regulation of Ca\(^{2+}\) homeostasis in the ER (1). We found that thapsigargin-sensitive intracellular Ca\(^{2+}\) stores in the ER were apparently increased in CRT-overexpressing cells, compared with controls. This was consistent with previous reports concerning the intracellular Ca\(^{2+}\) stores in cells overexpressing CRT (13, 30, 62, 63). On the other hand, it has been reported that CRT modulates store-operated Ca\(^{2+}\) influx (1, 30, 64). In previous studies, decreased Ca\(^{2+}\) influx was observed in stable CRT-overexpressing cells (13, 30, 63), but not in cells transiently transfected with the CRT gene (65). In the present study, when the cells in Ca\(^{2+}\)-free medium were stimulated with thapsigargin, the level of [Ca\(^{2+}\)]\(_i\) was apparently elevated more in CRT-overexpressing cells than controls. Then store-operated Ca\(^{2+}\) influx was examined by re-adding Ca\(^{2+}\) to the external medium, and the results showed that the [Ca\(^{2+}\)]\(_i\) was significantly elevated in CRT-overexpressing cells compared with controls. This was unexpected and inconsistent with previous findings of the suppressive effect of CRT overexpression on store-operated Ca\(^{2+}\) influx (13, 30, 63). To explain why the Ca\(^{2+}\) influx was up-regulated by overexpressed CRT, the expression of several epithelial Ca\(^{2+}\) channels (i.e. TRPV5, TRPV6, and Polycystin 2) was examined in control and CRT-overexpressing cells. The results showed that, in CRT-overexpressing cells, the expression of TRPV5 was apparently up-regulated and the basal level of Ca\(^{2+}\) influx from external medium was also increased. TRPV5 and TRPV6 are highly Ca\(^{2+}\)-selective epithelial channels in the TRP cation channel superfamily, and they function to (re) absorb Ca\(^{2+}\) in the epithelial cells of specific organs, such as kidney, intestine, testis, esophagus, ileum, and colon (31). The causative relation between overexpressed TRPV5 and increased Ca\(^{2+}\) influx seen in CRT-overexpressing cells seemed to be consistent with previous findings in MDCK cells stably transfected with the TRPV5 gene (66). Recently, Pigozzi et al. (67) has reported that a change in Ca\(^{2+}\) stores in the ER influenced the gene regulation of some TRP channels, including TRPC1, TRPC3, and TRPV6 in a prostate cancer cell line, suggesting that the expression of these TRP channels may be regulated by alteration of cellular Ca\(^{2+}\) homeostasis. However, in the present study, it is not clear how the expression of TRPV5 is specifically up-regulated in the CRT-overexpressing MDCK cells. Taken together, these results suggest that stable overexpression of CRT in MDCK cells may cause an imbalance of Ca\(^{2+}\) homeostasis, and this leads to the up-regulation of TRPV5, resulting in the increase in the basal level of Ca\(^{2+}\) uptake. On the other hand, in previous reports, levels of [Ca\(^{2+}\)]\(_i\) in the resting state were not significantly different between control and CRT-overexpressing cells (13, 30, 63). Thus, the up-regulation of the [Ca\(^{2+}\)]\(_i\) state in CRT-overexpressing MDCK cells seems to be unusual compared with other cell types, and further investigation is required to clarify the mechanism involved.

To our knowledge, whether the expression of Slug is controlled in a Ca\(^{2+}\)-mediated manner has not been investigated. In this study, we showed that, in MDCK cells, the transcription of Slug was up-regulated by thapsigargin or ionomycin, which increased [Ca\(^{2+}\)]\(_i\) (Fig. 9), leading to an increase in intensity of the gel-shift band of the protein-DNA complex of E-box B in the E-cadherin promoter (Fig. 11A). These results were consistent with the finding that E-cadherin expression was suppressed through the up-regulation of Slug expression in MDCK cells by treatment with thapsigargin or ionomycin. Conversely, in CRT-overexpressing cells treated with BAPTA-AM, the expression of Slug was suppressed and the expression of E-cadherin was increased. This indicates that the altered regulation of E-cadherin expression through Slug was dependent on an increased [Ca\(^{2+}\)]\(_i\) in CRT-overexpressing cells. Collectively, these results strongly support the notion that overexpression of CRT suppresses E-cadherin expression by up-regulating Slug expression.
through the increased level of Ca\(^{2+}\). Zhao et al. (68) reported that the Slug gene is a downstream target of the transcription factor MyoD, and is up-regulated by MyoD through the E-boxes in its promoter. Furthermore, a Ca\(^{2+}\)-responsive E-box element, CaRE2, was identified to function in the transcription of the brain-derived neurotrophic factor gene in neurons (69), suggesting a possible regulation of the Ca\(^{2+}\)-induced up-regulation of E-box elements in the Slug gene. In terms of these findings, further investigation is required to clarify how the Slug gene is up-regulated by the increased [Ca\(^{2+}\)], in CRT-overexpressing cells.

In terms of the relationship between CRT and cancer, proteomic analysis has revealed a new functional role of CRT for the early diagnosis of cancers. CRT is proposed as a new tumor marker of bladder cancer (70) and autoantibodies to CRT isoforms have utility for the early diagnosis of pancreatic cancer (71). These reactions are not indicative of malignant properties of CRT but, rather, are markers of immunogenicity and anti-cancer responses (72, 73). On the other hand, another report demonstrated that CRT is overexpressed in the nuclear matrix in hepatocellular carcinoma, compared with normal liver tissue, suggesting a relationship between overexpressed CRT and malignant transformation (74). In this study, we found that overexpression of CRT conferred migratory ability on epithelial-like MDCK cells. To gain invasive ability is a characteristic of malignancy, although the increase of invasiveness induced by CRT does not directly indicate that CRT is an oncogenic factor. MDCK cells transfected with an expression vector for the Snail gene, a zinc finger transcription factor like Slug, gained invasive and angiogenic properties through the repression of the E-cadherin gene and behaved like carcinomas (75). However, cell growth of Snail-overexpressing MDCK cells is less than that of control cells. These results are compatible with our present findings. In addition, a recent report indicated that Snail overexpression in MDCK cells up-regulated the matrix metalloproteinase-9 (MMP-9) gene, suggesting some contribution to cell invasiveness (76).

Taken together, these findings suggest that CRT has a potential function to increase cell invasion by suppressing E-cadherin gene expression through zinc finger transcription factors like Slug, resulting in a contribution to the establishment of invasive characteristics in malignancies. To test this hypothesis, further investigations, including studies in vivo, will be required.

In conclusion, in the present study, we demonstrated a novel function of CRT in cell adhesion and motility of the epithelium. The enhanced cell invasiveness mediated through E-cadherin gene repression was regulated by the gene repressor, Slug, via altered Ca\(^{2+}\) homeostasis caused by overexpression of CRT in epithelial MDCK cells.

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