Keap1 Regulates the Constitutive Expression of GST A1 during Differentiation of Caco-2 Cells[†]

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Received February 3, 2008; Revised Manuscript Received March 29, 2008

ABSTRACT: Kelch-like ECH-associated protein 1 (Keap1), a BTB-Kelch substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex, regulates the induction of the phase 2 enzymes, such as glutathione S-transferase (GST), by repressing the transcription factor Nrf2. It is known that, in the human gastrointestinal tract, both GST A1 and P1 are constitutively expressed as the major GST isozymes. In the present study, using the Keap1-overexpressing derivatives of Caco-2 cells, human carcinoma cell line of colonic origin, by stable transfection of wild type Keap1, we investigated the molecular mechanism underlying the constitutive expression of these GST isozymes during differentiation. It was revealed that the overexpression of Keap1 completely repressed the constitutive expression of GST A1, but not GST P1. In Keap1-overexpressed cells, dome formation disappeared, and the formation of the intact actin cytoskeletal organization at cell−cell contact sites and the recruitment of E-cadherin and β-catenin to adherens junctions were inhibited. The constitutive GST A1 expression in Caco-2 cells was repressed by disruption of E-cadherin-mediated cell−cell adhesion, suggesting the correlation between epithelial cell polarization and induction of the basal GST A1 expressions during Caco-2 differentiation. Keap1 overexpression indeed inhibited the activation of the small guanosine triphosphatase Rac1 on the formation of E-cadherin-mediated cell-cell adhesion. The transfection of V12Rac1, the constitutively active Rac1 mutant, into Keap1-overexpressed cells promoted the basal GST A1 expression, suggesting that Keap1 regulated the basal GST A1 expression during Caco-2 differentiation via Rac1 activation on the formation of E-cadherin-mediated cell−cell adhesion. The results of this study suggest the involvement of a novel Keap1-dependent signaling pathway for the induction of the constitutive GST A1 expression during epithelial cell differentiation.

Xenobiotic metabolizing enzymes play a major role in regulating the toxic, oxidative damaging, mutagenic, and neoplastic effects of chemical carcinogens. GSTs,[1] which are involved in phase II detoxification reactions, are a family of enzymes that catalyze the nucleophilic addition of the thiol of glutathione to a variety of electrophiles (EC 2.5.1.18) (*1*). Three major families of proteins that are widely distributed in nature, the cytosolic, mitochondrial and microsomal GST, exhibit glutathione transferase activity. In mammalian species, the cytosolic GST, based on amino acid sequence similarities, contains seven classes, designated Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta. At least 16 cytosolic GST subunits exist in the human. Human GST gene expression is known to be highly variable between tissues and between individuals, however, most tissues show specific patterns of isozyme expression (*2, 3*) that are conserved between individuals (*4, 5*). It has been reported (*4, 6*) that, in the gastrointestinal tract (stomach, duodenum, small intestine, and colon), GST P1, GST A1, and GST A2 are major components; GST P1 is expressed throughout the gastrointestinal tract and shows a decrease of expression from stomach to colon, and GST A1 and A2 are expressed at high levels in duodenum and small intestine and their expressions

[†] This work was supported by a research grant from the Ministry of Education, Culture, Sports, Science, and Technology and by the Center of Excellence (COE) Program in the 21st Century in Japan (K.U.), by a research grant from the Institute for Advance Research, Nagoya University (K.U.), and by the COE Program in the 21st Century in Japan (K.U.).

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[1] Abbreviations: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(2-amino-ethylether)tetraacetic acid; GST, glutathione S-transferase; Keap1, Kelch-like ECH-associated protein-1; Nrf2, nuclear factor-erythroid 2-related factor 2; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi800199z CCC: $40.75
Published on Web 05/14/2008

decrease from proximal to distal small intestine and are low in colon and stomach. The transcriptional activation of the phase 2 enzymes, such as GSTs, has been traced to a *cis*-acting transcriptional enhancer called an antioxidant response element (ARE) (*7*) or, alternatively, the electrophile response element (*8*). Nrf2, a member of the NF-E2 family of nuclear basic leucine zipper transcription factors, binds to the ARE and accelerates the transcription of cognate genes (*9*).

Kelch-like ECH-associated protein 1 (Keap1) is known to act as the principal regulator of Nrf2 (*10, 11*). Under basal conditions, Keap1 anchors Nrf2 in the cytoplasm, and targets it for ubiquitination and proteasome degradation, thereby repressing the ability of Nrf2 to induce phase 2 detoxification enzyme genes. Keap1 possesses two characteristic domains, the N-terminal broad complex, tramtrack, and bric-a-brac (BTB) domain and the double glycine repeat (DGR) domain, and belongs to the superfamily of BTB-Kelch proteins. Keap1 shares close similarity with *Drosophila* Kelch protein, which is essential for the formation of actin-rich intracellular bridges termed ring canals (*12*). Some Kelch-related proteins have been reported to colocalize with actin filaments through the kelch repeats, suggesting a biological role of the kelch repeats in the regulation and maintenance of the cytoskeleton (*13, 14*). Keap1, as a cytoskeletal protein, localizes in a variety of adhesion structures in tissues and cultured cell lines (*15*). It was hypothesized that Keap1 functions to bundle F-actin within these cell adhesion components, but the underlying mechanism still remains unclear (*16*).

Caco-2 cells, which is a well-characterized human carcinoma cell line of colonic origin, has served as a useful *in vitro* model to further ascertain the mechanisms contributing to gut cell differentiation (*17*). After reaching confluency, Caco-2 cells cease proliferation and spontaneously differentiate to a remarkable small intestinal enterocyte-like phenotype as noted by microvilli, dome formation, the presence of tight junctions and the expression of the differentiated enzymes, such as alkaline phosphatase and aminopeptidase (*18*). It has been reported that the expression of GST A1 and GST P1 increases during the proliferation and differentiation of Caco-2 cells (*17, 19, 20*). Bonnesen et al. (*21*) observed high levels of class α GSTs and identified mRNA for Nrf2 and small Maf protein in Caco-2 cells. In addition, due probably to the apparently high levels of GST, they could not demonstrate induction of GSTs by various chemopreventive phase 2 inducers, such as isothiocyanates and indoles, in Caco-2 cells (*21*). In the present study, using the Keap1-overexpressing derivatives of human carcinoma epithelial Caco-2 cells by stable transfection of wild type and dominant negative Keap1, we provide evidence that Keap1 controls the constitutive expression of GST A1 via E-cadherin-mediated cell−cell adhesion. The results of this study suggest the involvement of a novel Keap1-dependent signaling pathway for the induction of the constitutive GST A1 expression during epithelial differentiation.

MATERIALS AND METHODS

Materials. Anti-GST A1-1 rabbit polyclonal antibody and anti-GST P1-1 rabbit polyclonal antibody were obtained from Oxford Biomedical Research (Oxford, USA). Anti-Keap1 goat polyclonal antibody (E-20) and anti-actin rabbit polyclonal antibody (C-11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-E-cadherin and anti-β-catenin mouse monoclonal antibodies were obtained from BD Transduction Laboratories (Lexington, KY).

Plasmids. Full-length mouse Keap1 cDNA was subcloned into the pcDNA3 vector. Rac1 and V12Rac1 cDNA were subloned into the pcDNA3 or pEGFP-C1 vectors.

Cell Culture. Caco-2 cells, derived from a human colorectal carcinoma, were grown in Dulbecco's modified essential medium (SIGMA) supplemented with 10 mM HEPES (pH 7.4), 100 U/mL penicillin, 100 mM streptomycin, and 10% (v/v) heat-inactivated fetal calf serum at 37 °C in an atmosphere of 95% air and 5% CO_2. During culture, nondifferentiated cells were passaged upon reaching 80% confluence using 0.05% trypsin in phosphate-buffered saline (PBS) with 0.5 mM EDTA. For differentiation experiments, cells were plated into plastic dishes, at a density of 5300 cells/cm^2. Caco-2 cultures were allowed to undergo proliferation and differentiation and harvested on day −3, 0, 3, 7, 10, 14 and 18 postconfluence.

To generate Caco-2 cells stably expressing the wild-type or mutant form of Keap1, 1×10^6 Caco-2 cells were transfected with 2 μg of DNA, by using 4 μL of Lipofectamine 2000TM. Thereafter, stable transfectants were isolated by culture in selection medium containing 700 μg/mL G418 for 3 weeks. A single clone of the stably transfected cells was isolated and expanded. Several G418-resistant stable clones were maintained in regular growth medium containing 700 μg/mL G418.

Western Blot Analysis. The homogenates prepared from the cells were treated with SDS sample buffer and immediately boiled for 5 min. The protein concentrations were determined using the BCA protein assay kit (Pierce). The proteins separated by SDS−PAGE in the presence of 2-mercaptoethanol were electrotransferred onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences). To detect the immunoreactive proteins, we used ECL blotting reagents (Amersham Biosciences).

Reverse Transcription (RT)-PCR. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and spectrophotometrically quantified. Total RNAs were reverse-transcribed into cDNA and used for RT-PCR analysis (Qiagen Inc., Hilden, Germany). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard. The PCR products were separated on a 1% agarose gel, and the positive signals were quantified by densitometry analysis after staining with ethidium bromide. The primers were used as reported (*22, 23*). In short, a GST A1 PCR product was generated using primers GST A1-sense (5′-CCTGCCTTTGAAAAAGTCTTAAAG-3′) and GST A1-antisense (5′-AAGTTCCACCAGATGAATGTCA-3′). A GST P1 PCR product was generated using primers GST P1-sense (5′-CTCACTCAAAGCCTCCTGCCTAT-3′) and GST P1-antisense (5′-CAGGATGGTATTGGACTGG-TACAG-3′). A Keap1 PCR product was generated using primers Keap1-sense (5′-CAGAGGTGGTGGTGTTGCT-TAT-3′) and Keap1-antisense (5′-AGCTCGTTCATGAT-GCCAAAG-3′). A β-actin PCR product was generated using primers β-actin-sense (5′-AGAGATGGCCACGGCTGCTT-3′) and β-actin-antisense (5′-ATTTGCGGTGGACGATG-GAG-3′).

Immunofluorescence Microscopy. Cells were grown on coverslips and fixed for 30 min with 3.7% paraformaldehyde

in PBS, and then cells were permeabilized with −20 °C acetone (*24*). The fixed and permeabilized cells were incubated with PBS containing 2% BSA for 20 min, and then with primary antibody overnight. The cells were washed in PBS three times and incubated with FITC- or Cy3-conjugated second antibody. To detect actin filaments, cells were incubated with Alexa Fluor 594 or 488-conjugated phalloidin (Molecular Probes Inc.) for 30 min. The cells were rinsed with PBS, and covered with antifade solution. Images of the cellular immunofluorescence were acquired using a confocal laser scanning microscope (LSM5 PASCAL, Zeiss).

Subcellular Fractionation. The nuclear fraction was prepared as follows. Cells were washed three times with PBS on ice, scraped into PBS, and collected by centrifugation for 5 min at 500*g*. Cell pellets were incubated in 5 volumes of hypotonic buffer (10 mM HEPES, pH7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA) for 30 min on ice and lysed by homogenization with a loose-fitting pestle of a Dounce homogenizer. Nuclei were collected by centrifugation at 1000*g* for 5 min, and cell lysis was monitored by inspection under a microscope. The homogenization procedure was repeated three times until pure nuclear fractions were obtained. The final nuclear pellets were resuspended in nuclear extraction buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) and incubated on ice for 20 min. The nuclear extracts were clarified by centrifugation for 20 min at 14000*g*.

Extraction of the cell layers with Triton X-100 (TX-100) was prepared as follows. Cells were rinsed twice in cold PBS and solubilized in CSK buffer (50 mM NaC1, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% TX-100, 300 mM sucrose, and protease inhibitors) for 20 min at 4 °C on a rocking platform (*25*). The cells were scraped with a rubber policeman and sedimented in a microfuge for 20 min at 4 °C. The soluble supernatant was collected. The cell pellet was triturated in SDS buffer (15 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS), incubated at 100 °C for 10 min.

Inhibition of E-Cadherin-Mediated Cell−Cell Contacts. Confluent Caco-2 cells were serum-starved for 16 h in Dulbecco's modified Eagle's medium supplemented with 20 mM HEPES, 50 units/mL penicillin, and 50 mg/mL streptomycin (*26*). The adherens junctions were then disrupted by treatment with 4 mM EGTA at 37 °C. After the culture, total RNA was isolated from the cells and used for RT-PCR analysis.

Detection of GTP-Bound Rac1. Caco-2 cells on day 1 postconfluence were serum-starved for 16 h. E-cadherin-mediated cell−cell contacts were disrupted by treatment with 4 mM EGTA at 37 °C for 30 min. Thereafter, intercellular contacts were allowed to re-form in the presence of normal Ca^{2+}-containing medium ($CaCl_2 \sim 1.8$ mM) for 30−120 min at 37 °C. Rac1 activity was measured using the "Rac1 Activation Kit" (Stressgen Bioreagents Corporation, Victoria B.C., Canada). Briefly, whole-protein extracts were immunoprecipitated with protein binding domain of p21 activation kinase-1, which binds to activated forms of Rac1. The pulled-down active Rac1 was detected by Western blot analysis using a specific Rac1 antibody.

RESULTS

Effect of Keap1 Overexpression on Constitutive Expressions of GST A1 and GST P1 during Differentiation of Caco-2 Cells. When plated at subconfluent level, Caco-2 first proliferates toward confluence. This is followed by a postconfluent phase in which Caco-2 differentiates into a cell type with remarkable small intestinal enterocyte-like features. It has been reported that the expression of GST A1 and GST P1 increases during the proliferation and differentiation of Caco-2 cells (*17, 19, 20*). We first confirmed that protein and mRNA expression levels of GST A1 and GST P1 increased up to day 7 or 10 postconfluence and stayed elevated in Caco-2 cells plated into plastic dishes at a density of 5.3×10^3 cells/cm^2, which proliferated and reached confluence at seven days (Figure 1A). Despite the fact that, out of a total of 221 amino acids, there are only 11 differences between the GST A1 and GST A2 subunits (*27*), the GST A2 expression levels did not significantly change during differentiation (data not shown). In addition, Keap1 expression level during the differentiation of Caco-2 cells remained constant over time. Then, to characterize the role of Keap1-Nrf2 mechanism on GST expression, we established clones of stable transfectant of wild type Keap1, kWT cells, from Caco-2 cells (Figure 1B) and examined the constitutive expression of GST A1 and GST P1. Strikingly, although the GST P1 expression level did not change, the GST A1 protein and mRNA expression levels were repressed in the Keap1-overexpressed kWT cells (Figure 1C). These data suggest that the expression of GST A1 and GST P1 during differentiation might be regulated by distinct mechanisms and that Keap1 might be involved in the expression of GST A1, but not GST P1.

To examine if Nrf2 is involved in the constitutive expression of GST A1 during the Caco-2 differentiation, we determined the nuclear level of Nrf2. However, the Nrf2 level remained constant during incubation of the cells (Supporting Information, Figure S1, panel A). Consistently, the nuclear levels of Nrf2 in the mock transfected cells and kWT cells were unchanged (panel B). Furthermore, using the mutant Keap1, in which all cysteines except for those in the double glycine repeat are mutated to alanine, we established the cells overexpressing the mutant type of Keap1. As this Keap1 mutant cannot mediate the proteasomal degradation of Nrf2, its overexpression was expected to induce constitutive activation of Nrf2. Indeed, the nuclear Nrf2 protein level significantly increased (panel D), whereas the expression of GST A1, but not GST P1, was completely repressed in the mutant Keap1-overexpressing cells (panel E). The repression of GST A1 was also observed in the mRNA levels (panel F). These results suggest that there may be a distinct process of regulation of the GST A1 gene expression from the Nrf2-dependent mechanism during differentiation of the Caco-2 cells.

Effect of Keap1 Overexpression on Caco-2 Differentiation. On the other hand, we also observed that the repression of GST A1 by Keap1 overexpression was accompanied by the change in cell morphology. As shown in Figure 2A, the dome formation, which was observed with increasing number of days postconfluence in parent Caco-2 and mock transfected cells, the stable clone derived from Caco-2 cells transfected with the empty expression vector, disappeared in kWT cells. Most of the criteria for epithelial cell differentiation are cell polarization and gene expression. Since cell polarization could be assessed by dome formation, we evaluated the expressions of intestinal alkaline phosphatase and CDX2,

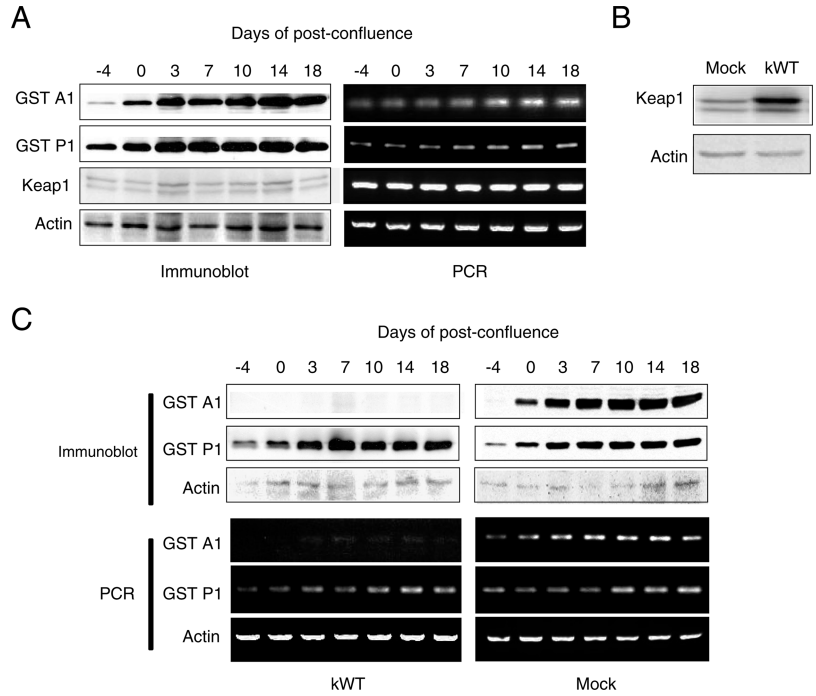

FIGURE 1: Overexpression of Keap1 in Caco-2 cells repressed the constitutive GST A1 expression during cell differentiation. (A) Parental Caco-2 cells were harvested at −3 day (60−70% confluence), 0 day (100% confluence), and 3, 7, 10,14 and 18 days postconfluence. Cell extracts were separated by 10% SDS−PAGE and proteins analyzed by Western blotting for expression of GST P1, GST A1 and Keap1. Semiquantitative RT-PCR was performed using total RNA isolated from Caco-2 cells during cell differentiation. (B) Western blots of a Keap1 for Keap1 wild type cDNA-transfected (kWT) and control vector-transfected (mock transfected) Caco-2 cells. (C) Both the stable clones were harvested at −3, 0, 3, 7, 10,14 and 18 days postconfluence. Cell extracts were separated by 10% SDS−PAGE and proteins analyzed by Western blotting for expression of GST P1, GST A1 and Keap1. Semiquantitative RT-PCR was performed using total RNA isolated from both the stable transfectants, kWT and mock transfected cells.

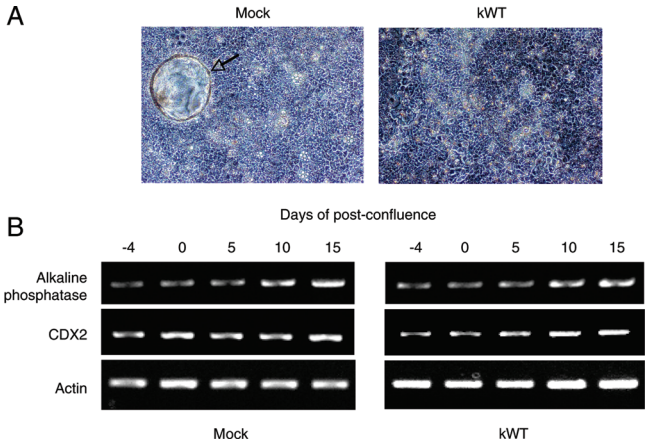

FIGURE 2: Overexpression of Keap1 in Caco-2 cells inhibits dome formation, but has no effect on the expression level of markers to study cell differentiation. (A) Phase-contrast images of mock transfected and kWT cells grown on plastic dishes at 10 days postconfluence. (B) Semiquantitative RT-PCR was performed using total RNA isolated from both clones at different stages of confluence (−2, 0, 6, and 15 days postconfluence).

well-established markers of Caco-2 differentiation. However, Keap1 overexpression had no effect on the expression levels of these enterocytic differentiation markers (Figure 2B). Thus, the GST A1 repression by Keap1 overexpression was associated with the changes in the cell morphology but not with the expression of differentiation markers.

Keap1 Overexpression Inhibits the Recruitment of E-Cadherin and β-Catenin to Adherens Junctions. The dome formation assesses the functional cell polarization (*28*). In epithelial cells, the cell polarization is determined by the formation of cell−cell adhesion, which is mediated through a junctional complex composed of adherens junction gap, desmosome, and tight junctions. Above all, the formation of E-cadherin molecules at the adherens junctions is the primary event, which organizes the formation of the other cell−cell junctions, to ensure the formation of an impermeable polarized epithelium (*29, 30*). Because Keap1 overexpression resulted in disappearance of the dome formation in Caco-2 cells (Figure 2A), it was speculated that Keap1 affected the formation of cell−cell adhesion. To examine the effect of Keap1 overexpression on the formation of cell−cell adhesion in Caco-2 cells, we first analyzed the localization of E-cadherin and β-catenin in mock transfected and kWT cells. In confluent mock transfected cells, both E-cadherin and β-catenin were mostly concentrated at the cell−cell contact sites, whereas their recruitment was no longer observed in kWT cells (Figure 3A), suggesting that Keap1 might inhibit the recruitment of E-cadherin and β-catenin into the cell−cell adhesion sites and affect the formation of E-cadherin-mediated cell−cell adhesion.

Epithelial remodeling was assessed using differential detergent solubility to determine the levels of E-cadherin and β-catenin associated with the cytoskeleton (*25, 31*). To further obtain evidence for the involvement of these adhesion molecules, the cell layers were fractionated into Triton X-100-soluble and -insoluble fractions and the compartmentalization of E-cadherin and β-catenin was analyzed by immunoblot analysis. Extraction of the cell layers with Triton X-100 produces a soluble fraction containing adherens junction proteins that were not tightly bound to the actin cytoskeleton. The remaining insoluble fraction, containing

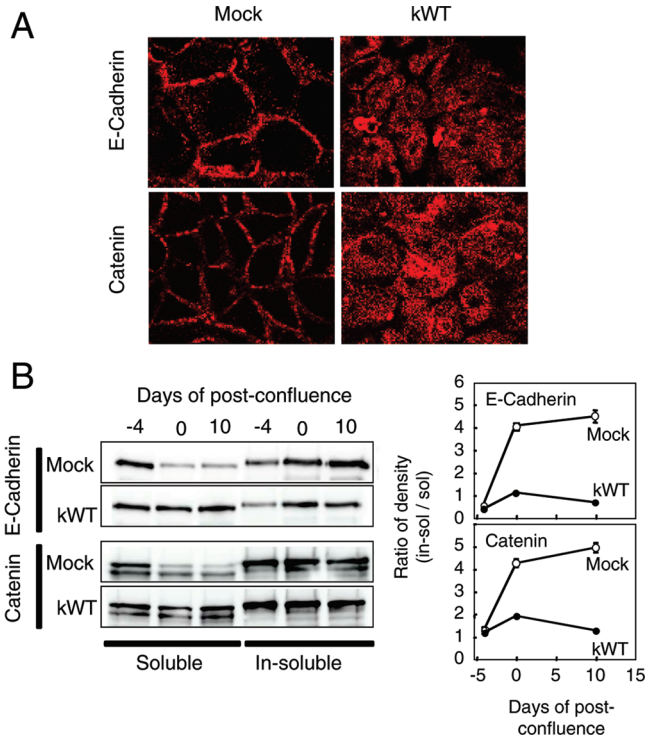

FIGURE 3: Effect of Keap1 overexpression on the recruitment of E-cadherin and β-catenin to adherens junctions. (A) Localization of E-cadherin or β-catenin in Keap1-overexpressing Caco-2 cells was examined by immunostaining. Mock transfected and kWT cells cultured on cover glass were fixed at confluence, doubly stained with E-cadherin or β-catenin, and analyzed by confocal microscopy. (B) Subcellular localization of E-cadherin and β-catenin during Caco-2 differentiation was examined by immunoblotting. The stable tranfectants at -4, 0 and 10 days postconfluence were extracted in buffers containing TX-100, generating TX-100-soluble (soluble) and -insoluble (insoluble) fractions. The TX-100-insoluble fraction was solubilized in 1% SDS, 100 °C. Both fractions were separated by 10% SDS−PAGE and proteins analyzed by Western blotting with anti-E-cadherin or anti-β-catenin antibody. The densities of the obtained bands were measured. The data are presented as the ratio of insoluble-to-soluble E-cadherin or β-catenin (means ± SE).

more tightly associated adherens junction proteins, is subsequently extracted with SDS buffer. Using these criteria, we examined the changes in the levels of E-cadherin and β-catenin in Triton X-100-soluble and -insoluble fractions during cell differentiation. Immunoblot analysis demonstrated that the levels of E-cadherin and β-catenin in Triton X-100-soluble and -insoluble fractions had no significant difference in both clones at day -4 postconfluence (Figure 3B). However, in mock transfected cells, the levels of E-cadherin and β-catenin in the soluble fractions were decreased in parallel with the increase in their amounts in the insoluble fractions up to day 10 postconfluence, whereas the levels of E-cadherin and β-catenin in both soluble and insoluble fractions were nearly unchanged in kWT cells. These data suggest that Keap1 affected the E-cadherin and β-catenin compartmentalization from soluble to insoluble Triton X-100 fraction that contains cytoskeletal proteins.

Keap1 Colocalizes with Actin Filaments at Cell−Cell Contact Sites and Is Involved in the Formation of the Actin Cytoskeletal Organization at Cell−Cell Contact Sites. The stable formation of E-cadherin-mediated cell−cell adhesion requires the formation of F-actin network. In addition, Keap1 shares close similarity with *Drosophila* Kelch protein, an

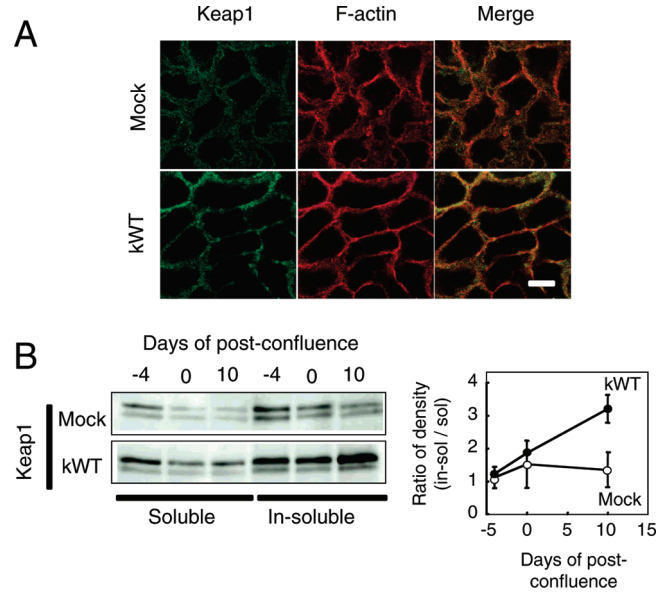

FIGURE 4: Keap1 colocalizes with the actin filament and contributes to the actin cytoskeletal organization at the cell−cell contact. (A) Localization of Keap1 was examined by immunostaining. Mock transfected and kWT cells cultured on cover glasses were fixed at confluence, doubly stained with Keap1 (green) and phalloidin (red), and analyzed by confocal microscopy. (B) Subcellular localization of Keap1 during Caco-2 differentiation was examined by immunoblotting. The stable tranfectants at -4, 0 and 10 days postconfluent were extracted in buffers containing TX-100, generating TX-100-soluble (soluble) and -insoluble (insoluble) fractions. The TX-100-insoluble fraction was solubilized in 1% SDS, 100 °C. Both fractions were separated by 10% SDS−PAGE and proteins analyzed by Western blotting with anti-Keap1 antibody. The densities of the obtained bands were measured. The data are presented as the ratio of insoluble-to-soluble Keap1 (means ± SE).

actin filament-cross-linking protein (*15, 16*). These findings suggest that Keap1 might colocalize with actin filament and affect the formation of intact actin cytoskeletal organization, leading to the changes in cell morphology. To verify this speculation, we examined the localization of Keap1 and the change in the actin cytoskeletal organization in confluent mock transfected and keap1-overexpressing kWT cells by immunocytochemistry. As shown in Figure 4A, Keap1 was doubly stained with actin filaments at lateral cell−cell contacts, but not detectable at the nuclei and basal regions, in both mock transfected and kWT cells. It was also observed that, although mock transfected cells formed both a higher order of F-actin organization forming a linear array of actin-staining and the peripheral thin actin bundles at cell−cell contact sites in lateral regions, the accumulation of thin actin bundles at cell−cell contact sites was reduced by Keap1 overexpression. To gain more insight into the association of Keap1 with the cytoskeleton during Caco-2 differentiation, we examined the changes in the Keap1 level in Triton X-100-soluble and -insoluble fractions. As shown in Figure 4B, a significant decrease in the Keap1 levels was observed in the insoluble fractions of mock transfected cells, whereas the Keap1 levels in kWT cells were nearly unchanged. These data suggest that Keap1 might dissociate from the actin filaments at cell−cell adhesion sites during differentiation and affect the formation of E-cadherin-mediated cell−cell adhesion through the actin cytoskeletal organization.

Actin Cytoskeletal Organization at E-Cadherin-Mediated Cell−Cell Adhesion Sites Is Involved in the Constitutive GST

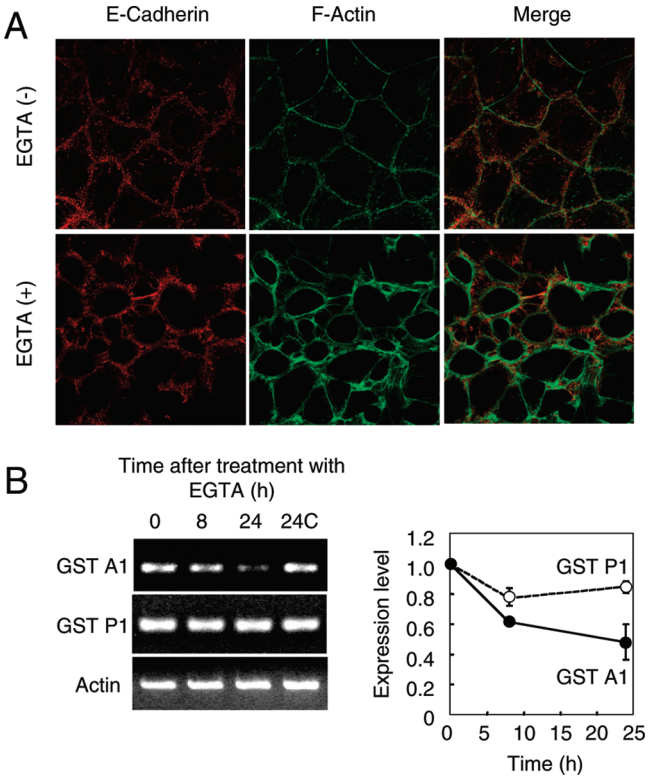

FIGURE 5: Actin cytoskeletal organization at E-cadherin-mediated cell−cell adhesion sites regulates the constitutive GST A1 expression in Caco-2 cells. (A) Caco-2 cells cultured on cover glass up to confluence were treated with EGTA (4 mM) and fixed, doubly stained with E-cadherin (red) and phalloidin (green), and analyzed by confocal microscopy. (B) Semiquantitative RT-PCR was performed using total RNA isolated from Caco-2 cells treated with EGTA (4 mM) at confluence for different time intervals as indicated. Quantitation of the GST mRNA levels after normalization for β-actin mRNA expression are shown as the means ± SE for three independent experiments.

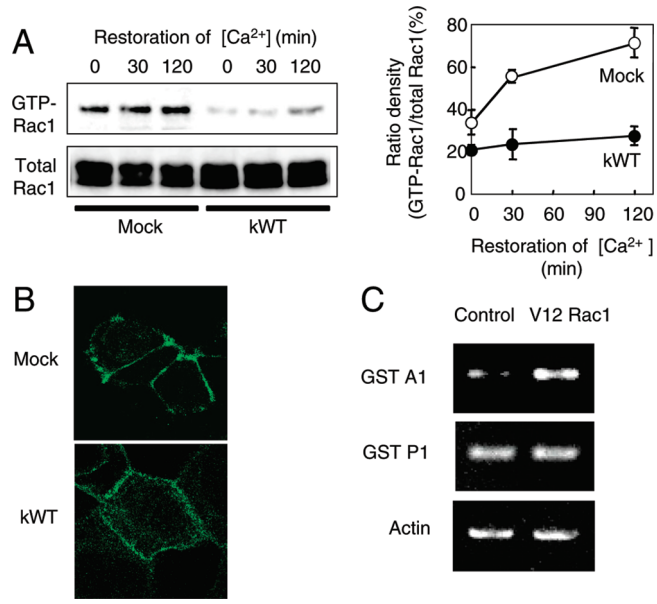

FIGURE 6: Keap1 overexpression inhibits the activation of Rac1 in the formation of E-cadherin mediated cell−cell contact. (A) In mock transfected and kWT cells, Rac1 activation during the formation of E-cadherin mediated cell−cell contact was measured. The cells at confluence were treated with 4 mM EDTA for 30 min. Subsequently, the cells treated with Ca^{2+}-containing medium for the indicated times were lysed with lysis buffer. The Rac1 activation was measured as described (see Materials and Methods). Data are presented as the ratio of GTP-bound Rac1-to-total Rac1 (means ± SE). (B) Localization of Rac1 in the formation of E-cadherin mediated cell−cell contact was examined by a calcium switch. Mock transfected and kWT cells were transfected with EGFP-Rac1. The cells at confluence were treated with 4 mM EDTA for 30 min. Subsequently, the cells treated with Ca^{2+}-containing medium for 120 min were fixed and analyzed by confocal microscopy to visualize Rac1. (C) Using total RNA isolated from kWT cells transiently transfected with V12Rac1 (constitutive active) or the empty vector, GST A1 and GST P1 mRNA expressions were analyzed by semiquantitative RT-PCR.

A1 Expression. We next investigated whether F-actin network formation in E-cadherin-mediated cell−cell adhesion is involved in the constitutive GSTA1 expression during Caco-2 differentiation. First, we examined the effect of EGTA, a Ca^{2+}-chelating agent, which disturbs the cell−cell adhesion through E-cadherin, on GSTA1 gene expression in Caco-2 cells. In the confluent Caco-2 cells treated with EGTA, actin cytoskeletal organization at cell−cell contact sites became disarranged (Figure 5A). Accompanied by these cytoskeletal alterations, we observed a significant decrease in the levels of GST A1 mRNA, whereas the Ca^{2+}-chelating agent did not exert any effect on the levels of GST P1 mRNA (Figure 5B). In addition, treatment of the Caco-2 cells with cytochalasin D, which inhibits the formation of cell−cell contacts via inhibition of the actin polymerization, also exerted similar effects on the expression of GST A1 and GST P1 (data not shown). These data strongly suggest that Keap1 regulates the constitutive GST A1 expression during Caco-2 differentiation via actin cytoskeletal organization at E-cadherin-mediated cell−cell adhesion sites.

Attenuation of Rac1 Activity in the Formation of E-Cadherin-Mediated Cell−Cell Adhesion by Keap1 Overexpression. It has been established that the small guanosine triphosphatase Rac1 is activated by E-cadherin-mediated cell−cell adhesion and is required for the accumulation of actin filaments, E-cadherin, and β-catenin at sites of cell−cell contact (*32*). Hence, we examined the changes in the levels of active Rac1 in confluent mock transfected and Keap1-overexpressing kWT cells in E-cadherin mediated cell−cell adhesion. In mock transfected cells, the amounts of active Rac1 rapidly increased up to 120 min after restoration of E-cadherin-mediated cell−cell adhesion, whereas Keap1 overexpression markedly suppressed the activation of Rac1 (Figure 6A). To examine the recruitment of Rac1 to the cell junction, EGFP-Rac1 was transfected into mock transfected and kWT cells in low calcium media and the localization of Rac1 was analyzed by conforcal microscopy. However, after the restoration of Ca^{2+}, EGFP-Rac1 was detected at sites of cell−cell contact in both clones (Figure 6B). These observations suggest that Keap1 may not affect the recruitment of Rac1 to the cell junction, but may participate in the activation of Rac1 during the formation of E-cadherin-mediated cell−cell adhesion.

To further establish the involvement of Rac1 in the constitutive expression of GST A1 in Caco-2 cells, we analyzed the effect of the transient overexpression of V12Rac1, the constitutively active mutant of Rac1, upon GST A1 gene expression in kWT cells. We confirmed the transfected V12Rac1 localized at cell−cell junctions in kWT cells (data not shown). The transient expression of V12Rac1 in kWT cells significantly increased the gene expression of GST A1, but not of GST P1 (Figure 6C). Thus, the

expression of GST A1 during differentiation of Caco-2 cells was confirmed to be regulated via the activation of Rac1 in the formation of E-cadherin-mediated cell−cell adhesion.

DISCUSSION

In the present study, using the Keap1-overexpressing derivatives of Caco-2 cells, we found that Keap1 was involved in the regulation of the constitutive GST A1 expression and the epithelial cell morphology. Following these findings, we investigated the molecular mechanism underlying the expression of GST A1 during Caco-2 cells differentiation and established that Keap1 regulated the GST A1 expression via Rac1 on the E-cadherin-mediated cell−cell adhesion. As far as we know, this is the first report that demonstrates the correlation between basal expression of phase 2 detoxification enzymes and actin cytoskeletal organization during cell differentiation.

It has been suggested that Nrf2 controls both constitutive and inducible expressions of ARE-driven genes, including GST A1 gene, through a dynamic pathway involving nucleocytoplasmic localization by Keap1 (*33*). The recent studies have shown that Keap1 knockdown upregulates the squamous differentiation genes in the squamous cells, resulting in the induction of the abnormal keratinization and cornification in the esophagus and forestomach of the mice, and that all of the Keap1-dependent phenotypes are reversed in Keap1-Nrf2 double mutants (*34*). These findings suggest the association of the epithelial cell differentiation with the Keap1-Nrf2 pathway. However, we observed in this study that the GST A1 expression was completely repressed by the overexpression of the wild type Keap1 (Figure 1C). This result and the observation that nuclear Nrf2 level remained constant despite the significant upregulation of GST A1 during proliferation and differentiation of Caco-2 cells (Supporting Information, Figure S1) suggest that there may be a distinct process of regulation of GST A1 gene expression from the Keap1-Nrf2-dependent mechanism during differentiation of Caco-2 cells. Stierum et al. (*20*) have proposed that the regulation of GST A1 gene expression in Caco-2 cells is related to a change in redox balance and controlled by activating protein (AP)-1 binding to ARE within the 5′ flanking region of the GST A1 gene. Both positive and negative regulatory regions have been identified in the GST A1 promoter, including several transcription factor recognition sites such as AP-1, AP-2, hepatic nuclear factor 1, a glucocorticoid-responsive element, and several, as yet uncharacterized, negative regulatory and enhancer elements (*1, 35–37*). Therefore, it is likely that multiple DNA elements and factors are involved in GST A1 gene expression during proliferation and differentiation of Caco-2 cells. Further studies on transcription factors interacting with the GST A1 promoter are required for elucidation of the specific expression of the gene in association with the gut epithelial differentiation.

On the other hand, consistent with the results that the overexpression of Keap1 completely repressed the constitutive expression of GST A1, the dome formation was not observed in Keap1-overexpressed Caco-2 cells (Figure 2). It is known that the dome formation reflects the functional cell polarization (*28*). It has also been shown that the formation of adherens junctions in the epithelial cells is critical for the decision of cell polarity and regulates the cell proliferation/differentiation transition (*38*). Cadherins contribute to the development of adherens junctions that start with the initial cell−cell contacts and belong to a large family of receptors that mediate intercellular adhesion by engaging in Ca^{2+}-dependent, homophilic, trans interactions (*38, 39*). E-Cadherin, primarily expressed in epithelia, is the prototype and best-characterized member of the cadherin family. The C-terminus of its cytoplasmic domain interacts with β-catenin, which itself binds α-catenin, an actin-binding protein, thereby providing a link between E-cadherin and the actin cytoskeleton. The connection of E-cadherin to the actin cytoskeleton is required for adherens junction formation (*40, 41*). Here we showed that Keap1 overexpression inhibited the recruitment of E-cadherin and β-catenin into the actin cytoskeleton at cell−cell adhesion sites (Figure 3). In addition, we also observed that the E-cadherin-mediated cell−cell adhesion was involved in the constitutive expression of GST A1 (Figure 5). These data support the idea that Keap1 might regulate both the GST A1 gene expression and the epithelial morphogenesis through the formation of E-cadherin-mediated cell−cell adhesion-dependent signaling mechanisms.

In the formation of E-cadherin-mediated cell−cell adhesion, E-cadherin, like the integrins, mediates outside-in and inside-out signaling. During the initial stages of cell−cell contact, as outside-in signaling, E-cadherin engagement initiates the activation of Rho family GTPases, actin network restructuring, and recruitment of tight junction proteins to the plasma membrane (*42, 43*). Expression of Rho mutants compromises apical−basal polarization and can disrupt the formation of both adherens and tight junctions (*44, 45*). It has also been shown that the perturbation of actin dynamics can disrupt E-cadherin-mediated adhesions (*46, 47*). Thus, the actin cytoskeletal organization and the activation of Rac1 at cell−cell contact sites are essential for the formation of E-cadherin-mediated cell−cell adhesion. Actin at cell−cell junctions is composed of two dynamic and functional populations, junctional actin and peripheral thin bundles (*48*). Although junctional actin stabilizes clustered cadherin receptors at cell−cell contacts, contraction of peripheral actin bundle, which is dependent on the activation of Rac1, is essential for an increase in the maximum height at the lateral domain during polarization (cuboidal morphology). In the present study, we observed that Keap1 overexpression resulted in the reduced accumulation of F-actin at cell−cell contact sites (Figure 4A) and the suppression of Rac1 activation in E-cadherin-mediated cell−cell contact (Figure 6A). Moreover, we showed that a significant decrease in the Keap1 levels was observed in the TX-100-insoluble fractions of mock transfected cells, whereas the Keap1 levels in kWT cells were nearly unchanged (Figure 4B). It can therefore be suggested that the dissociation of Keap1 from actin cytoskeletal organization at cell−cell contact sites might be involved in the inside-out signaling in the formation of E-cadherin-mediated cell−cell adhesion via regulation of the Rac1 activation. These data also suggest that Keap1 may be one of the components of the feedback loop (Figure 7): the organization of junctional actin by the intercellular interaction of E-cadherin (panel A); dissociation of Keap1 from junctional actin (panel B); (3) Rac1 activation (panel C); formation of actin network and establishment of E-cadherin-

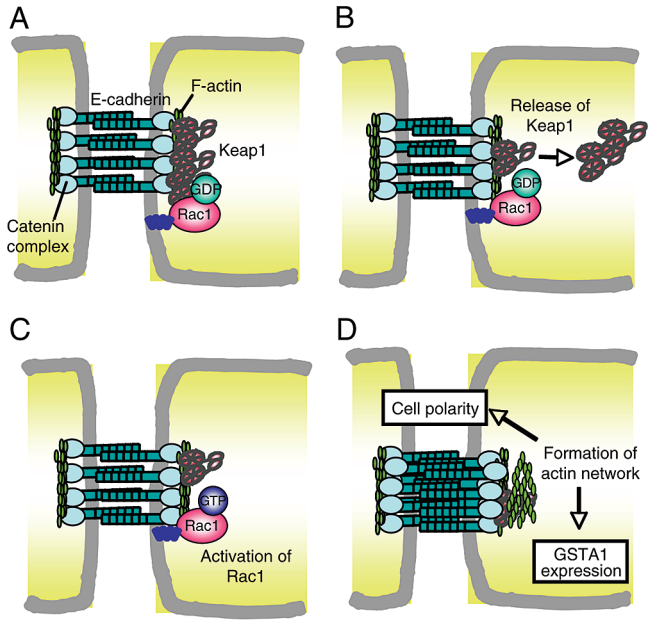

FIGURE 7: Model for the regulation of Keap1 in the constitutive GST A1 expression and the epithelial cell polarity via the formation of E-cadherin-mediated cell−cell adhesion. (A) E-cadherin-mediate intercellular adhesion by engaging in homophilic and trans interactions triggers the organization of the junctional actin. (B) Keap1 dissociates from actin filaments. (C) Rac1 is activated. (D) E-Cadherin-mediated cell−cell adhesion is established by inside-out signaling that the actin network is organized by activated Rac1.

mediated cell adhesion (panel D). A previous study has shown that *Drosophila* Kelch protein, with which Keap1 shares close similarity, is an actin filament-cross-linking protein and negatively regulates binding with F-actin by phosphorylation of its tyrosine residue at position 627 by Src protein-tyrosine kinase (*49*). Thus, it is likely that Keap1 may contribute to the organization of the junctional actin in the initial intercellular interaction of E-cadherin by tyrosine phosphorylation/dephosphorylation.

We also revealed that the activation of Rac1 in E-cadherin-mediated cell−cell adhesions might be involved in the constitutive GST A1 expression during Caco-2 cell differentiation (Figure 6). It has been speculated that there are at least two steps through which cell−cell adhesion activates Rac1: (i) Rac1 recruitment at sites of cell−cell contact; (ii) the conversion from the GDP-bound inactive form to the GTP-bound active form (*50*). The GDP-bound Rac1 is sequestered in the cytosol by Rho dissociation inhibitor (Rho GDI) before establishment of E-cadherin-mediated cell−cell adhesion. When cadherin-mediated homophilic interactions occurrs, GDP-bound Rac1 dissociates from Rho GDI, possibly through the action of Rho GDI displacement factors, and targets plasma membrane by its C-terminal CAAL motif or another regulatory mechanisms. GDP-bound Rac1 is then converted to GTP-bound Rac1 through the action of a certain Rac-guanine nucleotide exchange factor. Because Rac1 is required for the proper initiation of downstream signaling events and for the accumulation of actin filaments, E-cadherin, and β-catenin at sites of cell−cell contact (*32*), the direct signal cascade from Rac1 may simultaneously control the constitutive expression of GST A1 as well as the E-cadherin-mediated cell−cell adhesions during differentiation of Caco-2 cells (Figure 7).

In conclusion, using the Keap1-overexpressing derivatives of Caco-2 cells, we provide evidence that Keap1 controls the constitutive expression of GST A1 via the Rac1 activation on E-cadherin-mediated cell−cell adhesions. Based on the fact that the formation of E-cadherin-mediated cell−cell adhesion is essential for assembly of the junctional complexes, which represent barrier functions (*38*), it is hypothesized that Keap1-dependent regulation of the organization and dynamics of F-actin in epithelial morphology may also represent cellular protective functions. Further studies are necessary to appreciate the role of Keap1 in cellular protective responses and morphological cell differentiation.

ACKNOWLEDGMENT

We thank Dr. Kozo Kaibuchi of Nagoya University for providing pGEX-Rac1 and pEGFP-V12Rac1.

SUPPORTING INFORMATION AVAILABLE

Figure S1 depicting Western blotting and phase contrast image. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI800199Z