Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes

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Transcription factor Nrf2 regulates basal and inducible expression of phase 2 proteins that protect animal cells against the toxic effects of electrophiles and oxidants. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, a multidomain, cysteine-rich protein that is bound to the actin cytoskeleton. Keap1 acts both as a repressor of the Nrf2 transactivation and as a sensor of phase 2 inducers. Electrophiles and oxidants disrupt the Keap1–Nrf2 complex, resulting in nuclear accumulation of Nrf2, where it enhances the transcription of phase 2 genes via a common upstream regulatory element, the antioxidant response element. Reporter cotransfection–transactivation analyses with a series of Keap1 deletion mutants revealed that in the absence of the double glycine repeat domain Keap1 does not bind to Nrf2. In addition, deletion of either the intervening region or the C-terminal region also abolished the ability of Keap1 to sequester Nrf2, indicating that all of these domains contribute to the repressor activity of Keap1. Immunocytochemical and immunoprecipitation analyses demonstrated that Keap1 associates with actin filaments in the cytoplasm through its double glycine repeat domain. Importantly, disruption of the actin cytoskeleton promotes nuclear entry of an Nrf2 reporter protein. The actin cytoskeleton therefore provides scaffolding that is essential for the function of Keap1, which is the sensor for oxidative and electrophilic stress.

Oxidative and electrophilic stresses provoke physiological responses that induce the expression of various cytoprotective genes (1). Recently, the transcription factor Nrf2 (2) or ECH (3) was identified as the major regulator of the cytoprotective genes encoding phase 2 detoxication and antioxidant enzymes (4, 5). Nrf2, a basic region–leucine zipper (b-Zip) transcription factor (6) contains the N-terminal Neh2 domain, which is conserved between human Nrf2 (2) and chicken ECH (3). Biochemical analyses further revealed that the Neh2 domain serves as a negative regulatory domain of Nrf2 transcriptional activity, and we subsequently isolated a protein, Keap1, as an Neh2-associated protein (7).

Keap1 shares close similarity with *Drosophila* Kelch protein, which is essential for the formation of actin-rich intracellular bridges termed ring canals (8). These proteins have two common characteristic domains, i.e., the BTB (Broad complex, Tramtrack, and Bric a Brac)/POZ (poxvirus and zinc finger) and double glycine repeat (DGR or Kelch repeats) domains at the N- and C-terminal regions (NTR and CTR), respectively. The BTB domain of Keap1 has been examined in a transfection assay and was shown to be important for Keap1 function (9). The DGR domain comprises six repeats of the Kelch motif, and according to the x-ray structural analysis of galactose oxidase, which is a protein containing a Kelch motif, Kelch repeats form β-propeller structures (10). Importantly, many Kelch-related proteins colocalize with actin filaments through the Kelch repeats, suggesting a biological role of the DGR domain in the regulation and maintenance of the cytoskeleton (11, 12).

The association of Nrf2 with Keap1 has been examined (7). In the absence of electrophiles or oxidants, Nrf2 localizes in the cytoplasm in association with Keap1. On exposure to these inducers, however, Keap1 liberates Nrf2, allowing it to translocate to the nucleus and transactivate cytoprotective genes. Germline Nrf2-deficient mice have significantly reduced inducible and/or basal level expression of phase 2 and antioxidant enzymes compared with wild-type mice (4, 5). Deficient expression of cytoprotective enzymes renders mice highly sensitive to carcinogens and oxidative stresses, demonstrating that Nrf2 plays major roles in the defense systems against chemical carcinogenesis and acute drug intoxication (reviewed in refs. 1 and 13).

We also generated germline Keap1-deficient mice (14). Although homozygous *Keap1* mutant newborns appeared normal, they all died within 3 weeks after birth. Detailed postmortem analyses revealed severe hyperkeratosis in the esophagus and forestomach of these mutants. We found that the Keap1–Nrf2 pathway also regulates a subset of genes induced in squamous cell epithelia in response to mechanical stress. Importantly, all of the Keap1-dependent phenotypes were reversed in *Keap1–Nrf2* combined null-mutant mice, indicating that the Keap1 deficiency caused Nrf2 to accumulate constitutively accumulate in the nucleus. These results thus establish that the Keap1–Nrf2 system is an essential regulatory pathway that controls the cellular response to oxidative and xenobiotic stresses.

These *in vivo* examinations led us to address the next important question: how signals from oxidants and electrophiles are transmitted to the Keap1–Nrf2 system. Because the only common chemical property of phase 2 inducers is their ability to react with sulfhydryl groups, it has been proposed that the inducers may react with cysteine residues of a sensor protein (15). Indeed, Keap1 contains 25 cysteine residues, some of which have the characteristics of reactive cysteine. Phase 2 inducers react with sulfhydryl groups of Keap1, resulting in the disruption of the Keap1–Nrf2 complex (23). Hence, we envisage that Keap1 may function as one of the stress sensors in eukaryotes. Here, we describe the molecular mechanisms whereby Keap1 regulates Nrf2 activity under unstressed conditions. We identified five domains of Keap1 that may have discrete functions. These domains are referred to as N-terminal region (NTR), BTB, intervening region (IVR), DGR, and C-terminal region (CTR). In closer structure–function analyses of these domains, we found that Keap1 interacts with the actin filaments through DGR and that this interaction is crucial for Keap1 activity. We also found

Abbreviations: ARE, antioxidant response element; BTB, Broad complex, Tramtrack, and Bric-a-Brac; CTR, C-terminal region; DGR, double glycine repeat; IVR, intervening region; NTR, N-terminal region.

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that both IVR and CTR are essential for Keap1 to retain Nrf2 in the cytoplasm. Taken together, these data demonstrate that the Keap1–Nrf2 system provides a unique biological regulatory mechanism, formed through interaction with the actin filament network.

Experimental Procedures

Plasmid Construction. Full-length mouse Keap1 cDNA was subcloned into pcDNA3 (Invitrogen) vector (pcDNA-mKeap1). Keap1 deletion mutants were generated by inserting appropriate PCR-amplified cDNA fragments into the pcDNA3 vector. Information on the primers is available on request. These mutants were named ΔNTR (amino acids 1–60 deleted), ΔBTB (amino acids 61–179 deleted), ΔIVR (amino acids 192–308 deleted), ΔDGR (amino acids 315–598 deleted), and ΔCTR (amino acids 599–624 deleted). Structures of all constructs were verified by DNA sequencing.

Transfection Experiments and Luciferase Assay. Transfection experiments were performed as described (7) by using Lipofectamine plus reagents (Invitrogen). Luciferase assay was performed by using the Dual-Luciferase reporter assay system (Promega). Expression plasmids of Keap1 deletion mutants and Nrf2 were transfected into NIH 3T3 cells along with pNQO1 (nicotinamide quinone oxidoreductase 1)-ARE (antioxidant response element) reporter plasmid and pRL-TK as a control. pNQO1-ARE plasmid contains a single ARE and was used to measure the transactivation activity of Nrf2.

Laser Confocal Scanning Microscopy. A mouse Keap1 cDNA fragment was inserted into pCAGGS vector (pCAGGS-mKeap1; ref. 17). The resultant plasmid was injected into fertilized eggs and mouse embryonic fibroblasts (MEF) were prepared from transgene-positive 14.5-day-old embryos. Subcellular localization of Keap1 and actin was examined by immunohistochemical staining with laser confocal microscope (Leica). Anti-Keap1 antibodies were raised in rabbits by a standard method by using oligopeptides against the N and C termini of Keap1 individually.

Immunohistochemical Staining. Expression plasmids of Neh2-GFP and Keap1 deletion mutants were transfected into NIH 3T3 cells grown on slides. Cells were washed and fixed 36 h after transfection as described (7). Actin filament disruption experiments were modified from previous methods (18–21). In brief, cells were incubated with cytochalasin B (6 μM), swinholide A (50 nM), or colchicines (1 μM) for several periods of time as described in the figure legends. Cells were washed with PBS, blocked with 2% goat serum, and treated with anti-Keap1 antibody (100-fold dilution). Cells were then treated with goat anti-rabbit IgG conjugated with tetramethylrhodamine B isothiocyanate (TRITC, Zymed), 4′,6-diamidino-2-phenylindole (DAPI, 200 ng/ml), and Texas red-X phalloidin (200 units/ml, Molecular Probes). After washing with PBS, a drop of fluorescent mounting medium (DAKO) was placed on the slides.

Immunoprecipitation Analysis. 293T cells expressing Keap1 deletion mutants and Flag-Nrf2 were grown on culture dishes. Cells were harvested with Harlow buffer (50 mM Tris·HCl, pH 7.5/1% Nonidet P-40/20 mM EDTA/50 mM NaF) supplemented with protease inhibitors (Roche Diagnostics). Cell extracts were first cleared with protein G Sepharose and incubated with ANTI-FLAG M2 affinity gel (Sigma) or anti-actin (C-2) mouse monoclonal IgG (Santa Cruz Biotechnology) bound to protein G Sepharose. The immunocomplexes were washed five times with Harlow solution and subjected to immunoblot analysis.

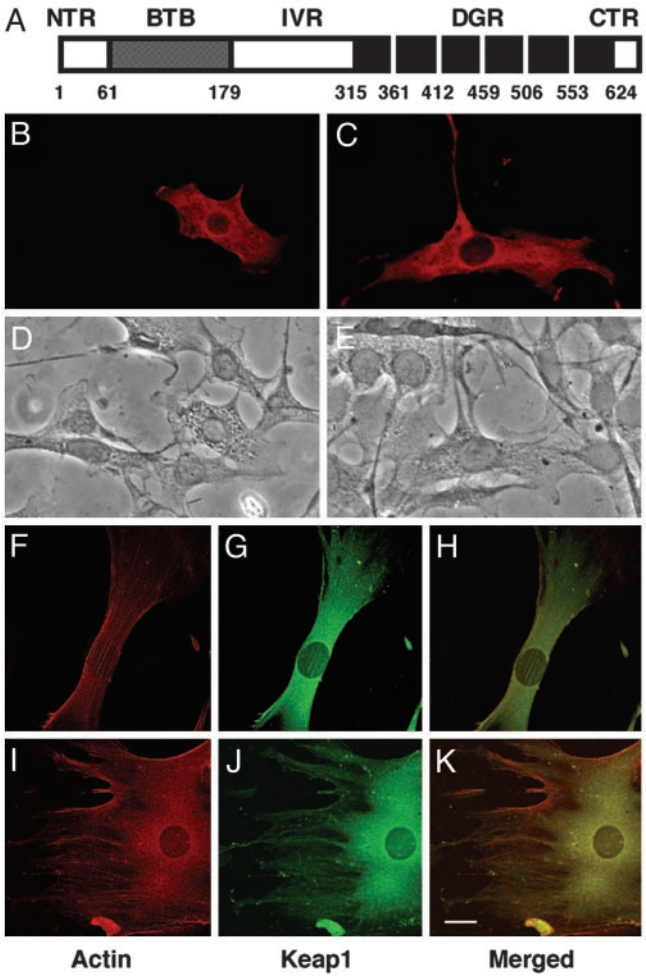

Fig. 1. Keap1 colocalizes with actin filaments in the cytoplasm. (*A*) Schematic presentation of Keap1 based on Swiss-Prot, using the Sanger Center Database. We assigned five domains within Keap1: NTR, BTB, IVR, DGR, and CTR. (*B–E*) Cytoplasmic localization of Keap1 in NIH 3T3 cells. Keap1 was expressed in NIH 3T3 cells, and subcellular localization of Keap1 was detected immunohistochemically by two anti-Keap1 antibodies against the N- and C-terminal ends of Keap1 (*B* and *C*, respectively). Bright-field microscopic images for *B* and *C* are shown in *D* and *E*, respectively. (*F–K*) colocalization of Keap1 and actin filaments in MEF derived from transgenic mouse embryos expressing Keap1. Subcellular localization of actin filaments (*F* and *I*) and Keap1 (*G* and *J*) are visualized by staining with phalloidin conjugated with Texas red and anti-Keap1 antibody, respectively. *H* and *K* show merged signals. Fluorescence was recorded by confocal microscopy. (Scale bar, 40 μm.)

Results

Keap1 Functions as an Actin-Binding Protein. To clarify the molecular mechanisms of Keap1 function, we first investigated the functional domains of Keap1. Comparison of the amino acid sequences of mouse, rat, and human (KIAA0132) Keap1 proteins shows that their sequences are highly conserved (>94%) among these species (data not shown). Pfam database (q9z2x8, mouse Keap1) analyses indicate that Keap1 protein consists of five characteristic domains: NTR, BTB/POZ, IVR, DGR, and CTR (Fig. 1*A*). The DGR structure also exists in other Kelch-related proteins, and some of them, such as Mayven (22), have been reported to interact with actin filaments through DGR. These data led us to examine whether Keap1 might act as an actin-binding protein.

We examined the colocalization of Keap1 with actin filaments in the cytoplasm. We raised two anti-Keap1 antibodies, which

recognize either the N-terminal or the C-terminal end regions of Keap1. Keap1 expression plasmid was transfected into NIH 3T3 cells, and the subcellular localization of Keap1 was monitored by immunocytochemical staining with anti-Keap1 antibodies. Both anti-N terminus antibody (Fig. 1 *B* and *D*) and anti-C terminus antibody (*C* and *E*) recognized Keap1 as a cytoplasmic factor. The localization of the signals suggests a fiber-based distribution of Keap1.

The subcellular localization of Keap1 was examined by confocal microscopy. Because the expression level of endogenous Keap1 was below the detection limit of the antibodies, for this analysis we prepared transgenic mice that express Keap1 at relatively high levels under the regulation of the CAGGS promoter (17). We assumed that overexpression of Keap1 in transgenic mouse embryos would reflect the physiological localization of Keap1 more closely than overexpression in cultured cells. Overexpression of Keap1 in transgenic mice did not affect the development or growth of the mice (data not shown). Immunostaining of mouse embryonic fibroblasts (MEF) derived from the transgenic embryos with the mixture of anti-Keap1 antibodies is shown in Fig. 1 (*F–K*) along with the staining of actin filaments with phalloidin conjugated with Texas red. Keap1 was localized in the perinuclear region and showed a fibrous pattern (*G* and *J*); and expression of Keap1 appeared to overlap that of the actin filaments (*F* and *I*). When we merged the two staining patterns, they overlapped markedly (*H* and *K*). The overlapping image (yellow) is more pronounced in the perinuclear region than in the region beneath the plasma membrane. Thus, these data suggest that Keap1 may bind directly to the actin filaments or cytoskeleton in the cytoplasm.

Direct Association of Keap1 with Actin Through DGR. To address whether Keap1 and actin filaments interact directly, we performed an immunoprecipitation analysis by using whole-cell extracts of 293T cells expressing a series of Keap1 deletion mutants (Fig. 2*A*). Precipitates obtained by anti-actin antibody were subjected to immunoblot analysis with anti-Keap1 antibodies. As shown in Fig. 2*B*, Keap1 was detected in the complex precipitated by the anti-actin antibody (*Upper*, lane 1), indicating that Keap1 and actin filaments interact directly.

To identify the surface of Keap1 interacting with actin, we carried out similar analyses with a series of Keap1 deletion mutants. The anti-actin antibody precipitated ΔNTR, ΔBTB, ΔIVR, and ΔCTR mutant proteins (Fig. 2*B*, lanes 2–5) but not the ΔDGR mutant protein (lane 7). Immunoblotting analysis with the anti-Keap1 antibodies indicated the presence of ΔDGR Keap1 protein as well as other mutant proteins in the whole-cell extracts (Fig. 2*B Lower*). These results demonstrate that DGR is the domain primarily responsible for the interaction of Keap1 with actin filaments.

Keap1 Requires Actin Filaments as Scaffolding. The results described above indicate that Keap1 retains Nrf2 in the cytoplasm under unstressed conditions. To elucidate whether the Keap1 activity requires actin filaments as scaffolding, we disrupted the actin cytoskeleton and examined the effect on the subcellular localization of Nrf2. NIH 3T3 cells were treated with cytochalasin B or swinholide A, which inhibit polymerization of actin filaments, and stained with phalloidin. Because prolonged treatment of NIH 3T3 cells (>12 h) with these compounds induced cell death (data not shown), we treated the cells with these reagents for <3 h and analyzed the effects. As a negative control, we also used colchicine, a specific inhibitor of microtubule polymerization. As shown in Fig. 3, treatment of NIH 3T3 cells with cytochalasin B or swinholide A disrupted the actin filament network effectively within 3 h (Fig. 3 *A–C*), whereas that with colchicine did not (Fig. 3*D*).

We then examined the effect of actin disruption on localization of Neh2-GFP containing GFP fused to the Neh2 domain in NIH 3T3 cells. Because we previously established that the Neh2 domain is the interactive interface of Nrf2 with Keap1 (7), we used this fusion protein as a reporter for the expression site of Nrf2. The subcellular localization of Neh2-GFP and Keap1 were monitored by the green fluorescence of GFP and immunostaining with anti-Keap1 antibodies, respectively. Whereas Neh2-GFP was localized exclusively in the cytoplasm in the presence of Keap1 (Fig. 3 *E* and *F*, 0 h), treatment with cytochalasin B resulted in an ≈5-fold increase in nuclear translocation of Neh2-GFP within 1 h. Additional incubation of the cells for 2 and 3 h with these reagents did not further enhance the entry of Neh2-GFP into the nucleus. The results are summarized in Fig. 3*F*. Subcellular localization of Neh2-GFP and Keap1 after treatment with swinholide A showed essentially similar profiles (Fig. 3*F*). In contrast, treatment with colchicine did not affect significantly the subcellular localization of Neh2-GFP (Fig. 3*F*, black bars). These results establish that disruption of the actin filament network releases Neh2-GFP from Keap1, resulting in entry of Neh2-GFP into the nucleus, thereby supporting our contention that Keap1 requires the actin cytoskeleton as a scaffold to sequester Nrf2 efficiently in the cytoplasm.

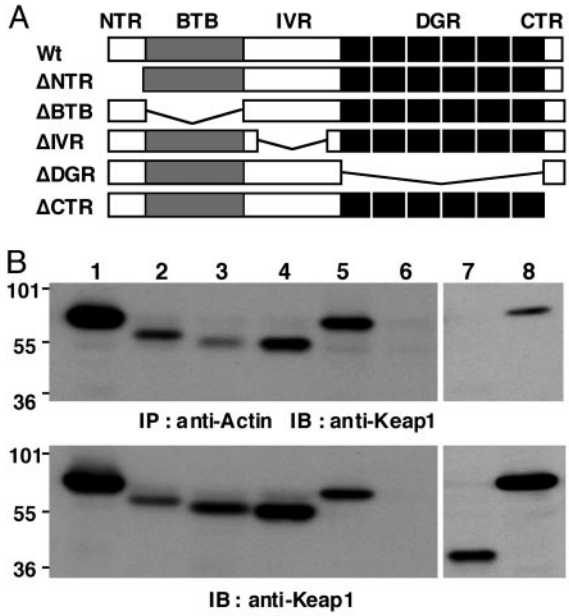

Fig. 2. Keap1 interacts with actin filaments through the DGR domain. (*A*) Schematic presentation of the structure of Keap1 deletion mutants. (*B*) Immunoprecipitation with whole-cell extracts of 293T cells expressing deletion mutants of Keap1. Immunoprecipitates (IP) obtained by anti-actin antibody were subjected to immunoblot analysis (IB) with anti-Keap1 antibody (*Upper*). The expression level of Keap1 deletion mutants was verified by immunoblot analysis (*Lower*). Analysis with wild-type Keap1-transfected cell lysates (lanes 1 and 8) as well as cell lysates transfected with Keap1 mutant ΔNTR (lane 2), ΔBTB (lane 3), ΔIVR (lane 4), ΔCTR (lane 5), and ΔDGR (lane 7) are shown. Lane 6 is loaded with cell extract expressing Nrf2 but not Keap1. Two anti-Keap1 antibodies were used: one against CTR (lanes 1–6) and the other against NTR (lanes 7 and 8).

Identification of a New Function of CTR. We examined the domain function of Keap1 further by expressing Keap1 deletion mutants (see Fig. 2*A*) and Neh2-GFP. Immunocytochemical staining with anti-Keap1 antibodies showed that all deletion mutants of Keap1 were localized in the cytoplasm (Fig. 4). In addition, wild-type Keap1 as well as ΔNTR, ΔBTB, and ΔIVR mutants localized Neh2-GFP exclusively in the cytoplasm, whereas ΔDGR and ΔCTR mutants of Keap1 did not. These results

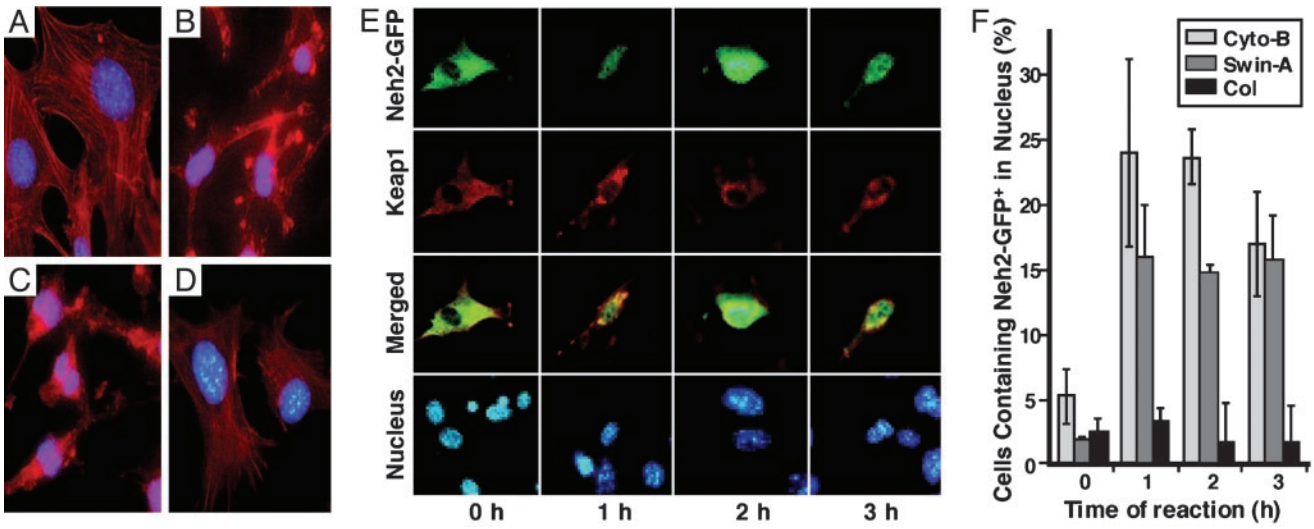

Fig. 3. Disruption of actin filaments triggers nuclear transport of Neh2-GFP. (*A*) NIH 3T3 cells were stained with phalloidin conjugated with Texas red and 4′,6-diamidino-2-phenylindole (DAPI) after addition of DMSO (*A*; a vehicle control) or cytoskeletal filament disruptors, cytochalasin B (*B*), swinholide A (*C*), or colchicine (*D*). (*E*) subcellular localization of Neh2-GFP and Keap1 after treatment with cytochalasin B. Cells (4×10^3) were transfected with expression plasmids of Keap1 (0.2 μg) and Neh2-GFP, a reporter protein of Nrf2 (0.8 μg). The latter is a fusion protein of Neh2 domain and GFP. Localization of these proteins was examined by fluorescence microscopy with use of GFP fluorescence and anti-Keap1 antibody, respectively (first and second rows). Merged images of Neh2-GFP and Keap1 signals are shown in the third row. Nuclei are shown with DAPI staining (fourth row). (Original magnification, ×400.) (*F*) nuclear transport of Neh2-GFP 3 h after the addition of cytochalasin B (Cyto-B), swinholide A (Swin-A), and colchicine (Col). Shown is the percentage of cells expressing Neh2-GFP in nucleus among the total transfected cells. The average and standard errors represent three independent transfection experiments.

suggest that in addition to DGR, CTR is also critical for Keap1 to retain Nrf2 in the cytoplasm.

DGR, but Not CTR, Directly Associates with Nrf2. We examined the direct interaction of each domain of Keap1 with Nrf2 by immunoprecipitation. Whole-cell extracts of 293T cells expressing a series of Keap1 deletion mutants and Flag-tagged Nrf2 were subjected to immunoprecipitation analysis by using anti-Flag antibody and then immunoblot analysis with anti-Keap1 antibodies. Consistent with the results in Fig. 4, deletion of DGR completely abolished the association of Keap1 with Nrf2 (Fig. 5*A*, lane 8). The amount of Nrf2 in the whole-cell extracts was monitored by immunoblot analysis with anti-Nrf2 antibody (Fig. 5*B*). Thus, DGR appears to be indispensable for interaction with both Nrf2 and actin filaments.

Surprisingly, the ΔCTR mutant interacted with Nrf2 in this immunoprecipitation analysis (Fig. 5*A*, lane 7), although this mutant did not retain Neh2-GFP in the cytoplasm (Fig. 4, ΔCTR). One plausible explanation for this discrepancy is that the CTR domain may modulate the conformation of DGR *in vivo* and thus regulate the interaction between Keap1 and Nrf2.

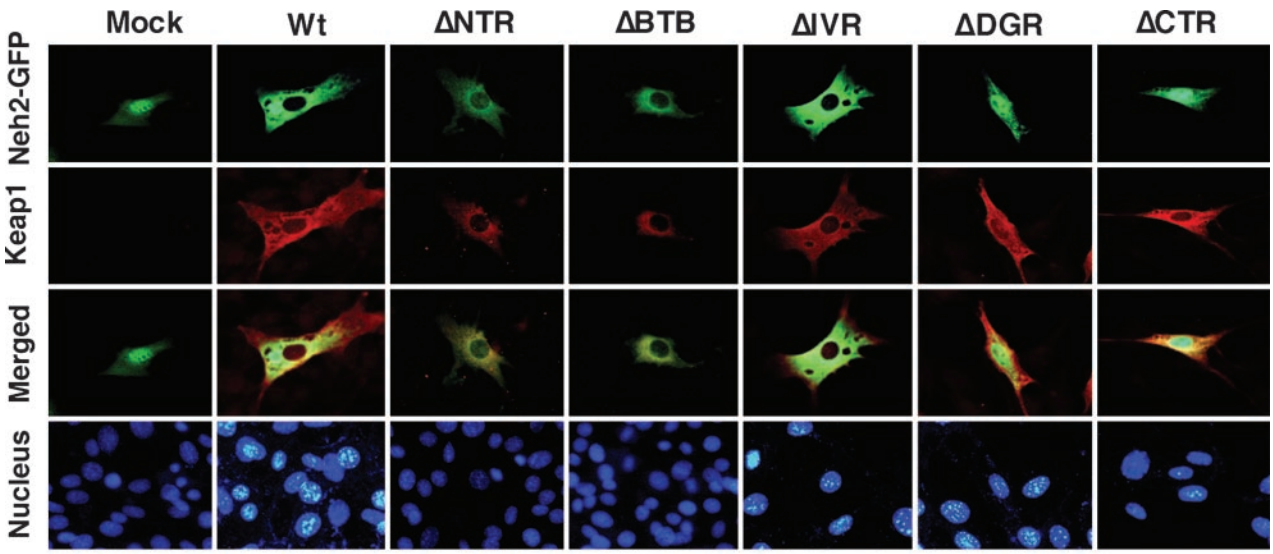

Fig. 4. CTR contributes to Keap1 activity retaining Neh2-GFP in cytoplasm. Subcellular localization of Neh2-GFP was examined in the presence of Keap1 deletion mutants. Transfection was performed as described in the legend to Fig. 3. Localization of Neh2-GFP and Keap1 mutant proteins was examined by fluorescence microscopy (first and second rows). Merged signals of both Neh2-GFP and Keap1 are shown in the third row. Nuclei are shown with DAPI staining (shown as Nucleus; fourth row). (Original magnification, ×400.)

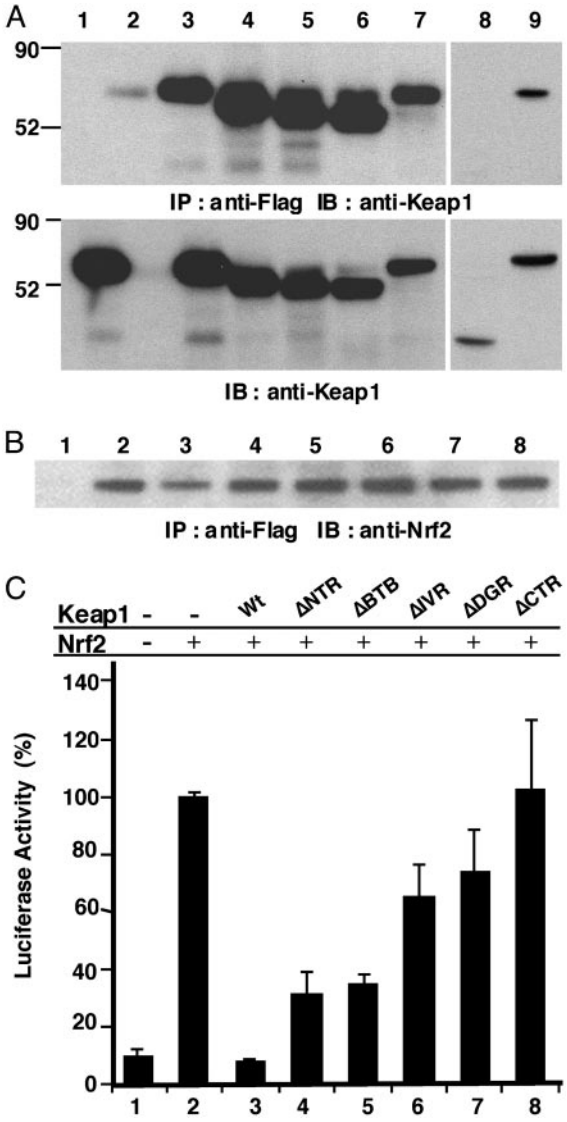

Fig. 5. IVR and CTR are both essential for Keap1 repression of Nrf2. (*A*) DGR of Keap1 directly associates with Nrf2. Whole-cell extracts prepared from 293T cells cotransfected with expression plasmids of various Keap1 deletion mutants (2 μg) and Flag-tagged Nrf2 (2 μg) were subjected to immunoprecipitation (IP). Immunoprecipitates obtained by anti-Flag antibody were subjected to immunoblot analysis (IB) with anti-Keap1 antibodies (*Upper*). The expression level of Keap1 deletion mutants was verified by immunoblot analysis (*Lower*). Analysis of cell lysates cotransfected with Nrf2 and wild-type Keap1 (lanes 3 and 9) as well as cell lysates cotransfected with Nrf2 and Keap1 ΔNTR (lane 4), ΔBTB (lane 5), ΔIVR (lane 6), ΔCTR (lane 7), or ΔDGR (lane 8) mutants are shown. Lane 1 is loaded with cell extract expressing only Keap1, and lane 2 is loaded with Nrf2 only. Anti-Keap1 CTR antibody was used in lanes 1–7, and anti-Keap1 NTR antibody was used for lanes 8 and 9. (*B*) Expression level of Nrf2 in immunoprecipitates was monitored by immunoblot analysis with anti-Nrf2 antibody. Analysis of cell lysates cotransfected with Nrf2 and wild-type Keap1 (lane 3) as well as cell lysates cotransfected with Nrf2 and Keap1 ΔNTR (lane 4), ΔBTB (lane 5), ΔIVR (lane 6), ΔCTR (lane 7), or ΔDGR (lane 8) mutants are shown. Lane 1 is loaded with cell extract expressing only Keap1, and lane 2 is loaded with Nrf2 only. (*C*) Three domains (DGR, CTR, and IVR) are crucial for the Keap1 activity. Expression plasmids of Nrf2 (90 ng) and various Keap1 deletion mutants (shown in the figure; 10 ng) were transfected into NIH 3T3 cells (2×10^4) along with a reporter plasmid, pNQO1-ARE (50 ng). Assays were performed in triplicate.

DGR and CTR Are both Indispensable to Suppress the Transactivation Activity of Nrf2. We then examined the ability of various Keap1 mutants to repress the transactivation activity of Nrf2 (Fig. 5*C*). Plasmids expressing Keap1 deletion mutants were cotransfected with plasmids expressing Nrf2 into NIH 3T3 cells along with the reporter plasmid (pNQO1-ARE) containing a single Nrf2-binding site. The luciferase activity attained by transfection of Nrf2 alone was set to 100% and used to normalize the relative activity in the presence of Keap1 mutants. Immunoblot analysis verified similar expression levels of each mutant protein (data not shown). Whereas Nrf2 activated the reporter gene expression >10-fold over the basal expression, simultaneous expression of Keap1 almost completely abolished this activation (compare lanes 1–3 in Fig. 5*C*). Transfection with ΔNTR and ΔBTB also markedly repressed the Nrf2 activity (Fig. 5*C*, lanes 4 and 5).

In contrast, deletion of the DGR and CTR from Keap1 almost abolished Keap1 activity in repressing transactivation of Nrf2 (Fig. 5*C*, lanes 7 and 8). Because ΔDGR and ΔCTR could not entrap Neh2-GFP in the cytoplasm (Fig. 4, ΔDGR and ΔCTR), these data suggest that both DGR and CTR are indispensable for Keap1 activity. Interestingly, deletion of IVR also affects the repressor activity of Keap1 (Fig. 5*C*, lane 6). This was an unexpected observation, because IVR does not interact directly with Nrf2 or actin (Figs. 2*B* and 5*A*). Four cysteine residues in IVR were recently shown to be highly reactive with electrophiles (16), and it was proposed that some of them act as sensors for electrophilic stimuli that regulate the association of Keap1 and Nrf2. The present result further supports our hypothesis that the cysteine residues in IVR are essential for Keap1 to repress the transactivation activity of Nrf2 (16).

Discussion

We investigated in this study how a cytoplasmic protein Keap1 regulates Nrf2 activity. We found that Keap1 binds to the actin cytoskeleton and traps Nrf2, thereby preventing the nuclear translocation of this transcription factor. Whereas several Kelch-related proteins are known to colocalize with actin filaments, the physiological significance of the actin binding has not been well characterized (11). This study therefore provides the first convincing evidence that the direct interaction between Keap1 and the actin cytoskeleton contributes to the regulatory activity of Keap1. The present analyses further indicated that the DGR domain of Keap1 interacts primarily and directly with Nrf2, and the CTR and IVR domains also contribute to the ability of Keap1 to retain Nrf2 in the cytoplasm. Structure–function analyses of the Keap1–Nrf2 system provide plausible molecular understanding of how Keap1 functions as a sensor for inducing this signal pathway.

Keap1 colocalizes with the actin cytoskeleton and is abundantly distributed in the perinuclear region of the cytoplasm. This localization profile of Keap1 suggests three biological roles for Keap1. First, the perinuclear localization may allow Keap1 to entrap effectively Nrf2 protein synthesized *de novo* as it migrates into the nucleus. Second, because phase 1 enzymes that initially metabolize xenobiotics are usually localized on the cytoplasmic surface of the endoplasmic reticulum (23), Keap1 has easy access to the highly reactive phase 1 products by selecting actin filaments as a scaffold. Third, recently we and other groups found that Nrf2 is degraded rapidly and efficiently with the 26S proteasome under unstressed conditions (24–27). Because the proteasome is also known to colocalize with the actin filaments and intermediate filaments (28), we envisage that Keap1 may transfer the newly synthesized Nrf2 to the proteasome localized nearby, resulting in the rapid turnover of Nrf2.

CTR consists of 26 amino acid residues, and the primary structure is not well conserved among the other Kelch-related β-propeller proteins. CTR has one reactive cysteine (Cys-613), which binds dexamethasone mesylate (16). These results suggest a unique function of Keap1 CTR among Kelch family proteins. In the structure–function analysis of Keap1, CTR was shown to be essential for Keap1 repression of Nrf2. However, deletion of

CTR did not affect Keap1 interaction with Nrf2 in the immunoprecipitation analysis (Fig. 5*A*). One plausible explanation for this discrepancy is that CTR may act indirectly to modulate DGR activity. In contrast to the present results, it was recently reported that the presence of either DGR or CTR is sufficient for Keap1 to retain Nrf2 (29). In our experiments, however, the ΔDGR mutant possessing CTR could not repress Nrf2 activity at all, indicating that DGR is absolutely required for the Keap1 retention of Nrf2 in the cytoplasm. Zhang and Hannink (30) recently reported that 15 amino acid residues from C terminus of Keap1 are not required for the Keap1 activity, whereas in our experiments, ΔCTR (26-aa deletion) could not repress Nrf2 activity, indicating that CTR is required for the Keap1 activity.

Zipper and Mulcahy recently reported that Keap1 forms a homodimeric complex through the BTB domain (9). Keap1 dimerization was suggested to be an important step for sequestration of Nrf2 in the cytoplasm and the Ser-104 residue in the BTB domain appeared to be critical for Keap1 self-association. In contrast, we found that ΔBTB-Keap1 effectively repressed Nrf2 transactivation activity (Fig. 5*C*, lane 5). Thus, deletion of the BTB domain did not impair Keap1 activity in our transfection analysis. We surmise that this discrepancy may be due to differences in the experimental conditions. Whereas the BTB domain is not the direct binding interface, it is possible that this domain may modulate the function of the DGR domain, as is the case for CTR and IVR domains.

Transcriptional regulation through the actin cytoskeleton seems to be unique to the Keap1–Nrf2 system. In this regard, it is noteworthy that Cubitus interruptus (Ci) of *Drosophila*, which is a transcription factor under the Hedgehog signal pathway (31), may have some similarity to the Keap1–Nrf2 system. In the absence of ligand Hedgehog, Ci is tethered to microtubules through forming a complex with Fused, Cos2, and Su(fu) proteins. On Hedgehog binding to the receptor Patched, an inhibitor protein Smoothened is released and it liberates Ci from microtubules. The microtubule association seems to be essential for Ci, because Slimb associated with microtubules modifies Ci to a transcriptional repressor through cleavage. Because Keap1-mediated tethering of Nrf2 to the actin cytoskeleton provokes degradation of Nrf2 (26, 27), the actin cytoskeleton seems to provide a scaffold for protein modification and degradation in the Keap1–Nrf2 system.

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