The Keap1 BTB/POZ Dimerization Function Is Required to Sequester Nrf2 in Cytoplasm*

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Transactivation of phase II detoxification enzymes and antioxidant proteins is mediated by the Cap’N’Collar transcription factor, Nrf2, which is sequestered in the cytoplasm by the actin-binding protein Keap1. Mutation of a conserved serine (S104A) within the Keap1 BTB/POZ domain disrupts Keap1 dimerization and eliminates the ability of Keap1 to sequester Nrf2 in the cytoplasm and repress Nrf2 transactivation. Disruption of endogenous Keap1 dimerization using BTB/POZ dominant negative proteins also inhibits the ability of Keap1 to retain Nrf2 in the cytoplasm. Exposure to an electrophilic agent that induces Nrf2 release and nuclear translocation disrupts formation of a Keap1 complex in vivo. Collectively, these data support the conclusion that Keap1 dimerization is required for Nrf2 sequestration and transcriptional repression. Furthermore, exposure to inducing agents disrupts the Keap1 dimerization function and results in Nrf2 release.

Cells respond to certain types of oxidative or electrophilic insults by coordinately up-regulating the expression of various cellular protective and antioxidant genes. Among the genes up-regulated are those encoding the phase II detoxification enzymes, such as NAD(P)H quinone reductase (NQO1), glutathione S-transferase, heme oxygenase-1, and epoxide hydrolase as well as genes involved in cellular redox maintenance, including the catalytic (GCLC) and modulatory (GCLM) subunits of glutamate cysteine ligase (GCL). GCL catalyzes the first and rate-limiting step in the de novo synthesis of glutathione, a critical non-protein thiol involved in detoxification of xenobiotics, electrophiles, free radicals, and reactive oxygen species. Coordinate induction of many of the enzymes in this protective battery is transcriptionally mediated through antioxidant response elements (AREs) (also called electrophile response elements (EpREs)) present in the promoters of the respective genes (1–5). Antioxidant response elements share a common core sequence, 5’-G(A/T)G(A/G)NNNGG(A)/A)-3’, which bears a remarkable resemblance to Maf recognition elements (MAREs), originally identified as binding sites for the Cap’N’Collar protein NF-E2 within the promoter of the β-globin gene (6, 7). When dimerized with certain b-zip transcription factors, members of the Cap’N’Collar transcription factor family (NF-E2, Nrf1, Nrf2) are able to bind and transactivate ARE or ARE-like sequences. Although members of the small Maf family of proteins (Maf F, Maf G, and Maf K) as well as members of the Jun family (c-Jun, JunD) have been implicated as potential binding partners for Cap’N’Collar proteins, specific dimerization partners for Nrf2 in the context of AREs have not been clearly defined (8–21).

Overexpression of Nrf2 up-regulates expression of ARE-containing genes, demonstrating that Nrf2 is a major determinant in the ARE-mediated induction of genes encoding several of the Phase II detoxification enzymes and both GCL subunits. Constitutive and induced expression of several ARE-containing genes, including glutathione S-transferase, NQO1, GCLC, and GCLM are reduced in Nrf2 knock-out mice (22–29). Nrf2 null mice are more susceptible to the carcinogenic effects of benzo(a)pyrene than are wild-type mice and are less responsive to the chemoprotective effects of Oltipraz, a chemopreventive agent that functions in part via ARE induction (30). By virtue of its central role in the transactivation of detoxification genes involved in cellular responses to oxidative or electrophilic stresses, Nrf2 activation is a critical target for cancer chemoprevention and other protective strategies (31, 32).

Attempts to identify Nrf2 binding partners by yeast two-hybrid screens resulted in the identification of Keap1, a homologue of the Drosophila Kelch protein (33). Subsequent experiments demonstrated that Keap1 normally sequesters Nrf2 in the cytoplasm. After an oxidative/electrophilic stress or stimulus, Nrf2 is liberated from Keap1, whereupon it translocates to the nucleus and, in association with dimerization partners, binds ARE sequences to induce expression of the battery of genes that contribute to the protective response (33). Although the nature of the molecular event(s) that signals dissociation of the Keap1-Nrf2 complex is unknown, it has been hypothesized that thiol modulation of one or more reactive cysteine(s) in Keap1 might result in a conformational change that releases Nrf2 (33). Alternatively, other post-translational mechanisms such as phosphorylation of Nrf2 or Keap1 by kinases activated by oxidative or electrophilic stress might trigger Nrf2 release from Keap1.

Members of the Kelch family, including Keap1, contain an N-terminal BTB/POZ domain and a series of 4–7 C-terminal Kelch repeats (34–37). BTB/POZ domains mediate protein dimerization, whereas Kelch repeats have been implicated in binding to the actin cytoskeleton and the formation of multi-protein complexes (35–40). An alignment of multiple BTB/POZ domains from various proteins with Keap1 revealed the presence of a highly conserved serine residue corresponding to Ser-104. Mutation of the homologous residue in the PLZF transcription factor (S56A) inhibited PLZF activity, suggesting the integrity of the BTB/POZ domain is critical to maintain protein stability.

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function. We performed a similar mutational analysis of this conserved serine residue in Keap1 to determine the role of the BTB/POZ domain in Keap1 regulation of Nrf2. The Keap1-S104A mutant was deficient at homodimerization, failed to sequester Nrf2 in the cytoplasm, and was incapable of repressing Nrf2 transactivation of a GCLM-ARE reporter gene in vivo. Wild-type Keap1 formed a high molecular weight complex when overexpressed in HepG2 cells, whereas the Keap1-S104A mutant was unable to form this complex. Exposure of transfected cells to the pro-oxidant pyrrolidinedithiocarbamate (PDTC), disrupted formation of the high molecular weight complex. Collectively, the evidence suggests that the BTB/POZ dimerization function of Keap1 is required for Nrf2 cytoplasmic sequestration and that disruption of endogenous Keap1 dimers in vivo is involved in Nrf2 release.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids—The cDNA for mouse Keap1 (pEF-mKeap1) was kindly provided by Drs. Yamamoto and Itoh (University of Tsukuba, Tskuba, Japan) (33). p3XFLAG-Keap1 was constructed by digesting pEF-mKeap1 with HindIII/EcoRI and ligating the 2-kb fragment in-frame at the HindIII/EcoRI sites of p3XFLAGCMV.10 (Sigma). Generation of the GCLM-luciferase reporter that contains ~1.9 kb of promoter, the GCLM promoter upstream of the luciferase reporter gene has been described previously (42). The cDNA for Nrf2 was provided by Dr. Etsuro Itoh and recloned into pCIneo to obtain pCIneo-Nrf2 (18). pEGFP-Nrf2 was generated by blunt ligation of a ~2.3-kb BspHI/Smal fragment of pCIneo-Nrf2 into the SmaI site in the multiple cloning region of pEGFP-C1 (CLONTECH). pEF-Keap1 and p3XFLAG-Keap1 proteins were cloned into vectors containing T7 transcription and Translation System (Promega). pCIneo-Keap1 proteins were cloned by digesting pEF-Keap1 with EcoRI, and ligating the ~2-kb fragment into pCIneo at the EcoRI site. pGEM-ZF(-FLAG-Keap1 was created by first digesting p3XFLAG-Keap1 with BglII followed by ligation of the ~2.8-kb fragment containing 3XFLAG-Keap1, and the poly(A) tail into the pGEM-ZF(-) (Promega). For TnT T7 Quick Coupled Transcription and Translation System (Promega), pCIneo-Keap1 was digested by digesting pEF-Keap1 with EcoRI, and ligating the ~2-kb fragment into pCIneo at the EcoRI site. pGEM-ZF(-)FLAG-Keap1 was created by first digesting p3XFLAG-Keap1 with BglII followed by ligation of the ~2.8-kb fragment containing 3XFLAG-Keap1 (Promega). pCIneo-Keap1 proteins were cloned into vectors containing T7 transcription and Translation System (Promega) was initiated with 2 μg of each cDNA and 20 μCi of [35S]methionine (Amersham Biosciences) as described in the manufacturer’s instructions. Products were incubated at 30 °C for 1.5 h before the addition of 1 μl of anti-FLAG M2 monoclonal antibody (Sigma). After samples had been incubated overnight at 4 °C, Immune precipitates were washed 3 times before the addition of 4X SDS loading buffer and separation on 7% polyacrylamide gels. Proteins were transcribed to polystyrene diurethane diisocyanate membranes and blotted for FLAG using a 1:4000 dilution of FLAG M2 monoclonal antibody (Sigma) in blocking buffer (5% nonfat dry milk, Tris-buffered saline with 0.1% Tween 20). The membranes were developed, stripped, and re-probed with anti-GFP antibody as described above.

In Vitro Homodimerization Assay—TnT T7 Quick Coupled Transcription and Translation (Promega) was initiated with 2 μg of each cDNA. Products were incubated at 30 °C for 1.5 h before the addition of 1 μl of anti-FLAG M2 monoclonal antibody (Sigma). After samples had been incubated overnight at 4 °C, Immune precipitates were washed twice and separated on 7% polyacrylamide gels. The gels were fixed in 40% H2O2, 50% methanol, 10% acetic acid for 0.5 h before being exposed to a phosphorimaging screen overnight. To study homodimerization of proteins in vitro, the two cDNAs of interest (pCIneo-Keap1 and pGEMZf7(FLAG-Keap1) were co-translated in the same TnT lysate and then immunoprecipitated by the addition of anti-FLAG antibody. A 5-μl aliquot of the TnT reaction was taken before the addition of anti-FLAG antibody and combined with 15 μl of 4X SDS sample buffer (these samples are called input in Fig. 5, A and B). The input samples were boiled and stored at −20 °C until the immunoprecipitation steps were complete, and all samples were analyzed by SDS-PAGE as described above.

In Vivo Homodimerization Assay—HepG2 cells were transfected with p3XFLAG-Keap1-wt or -S104A. 48 h after transfection, cells were exposed to 100 μM PDTC or double-distilled H2O for 3 h. Cells were collected, resuspended in PBS, and divided equally. Half of the sample was boiled, and analyzed by denaturing Western blotting using the FLAG antibody and α-tubulin. The remaining half of the sample was lysed in buffer C (20 mM Hepes (pH 7.9), 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA supplemented with protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, and leupeptin) and sodium orthovandandate. Lysates were centrifuged for 10 min at 4 °C to pellet insoluble material. The cell extracts were then pre-cleared 2 times by the addition of protein G-Sepharose (Amersham Biosciences) for 1 h at 4 °C. Samples were incubated with GFP antibody (Molecular Probes) for 1–2 h at 4 °C before the addition of protein G-Sepharose. Samples were inverted continuously overnight at 4 °C. Immunoprecipitates were washed 3 times before the addition of 4X SDS loading buffer and separation on 7% polyacrylamide gels. Proteins were transcribed to polystyrene diurethane diisocyanate membranes and blotted for FLAG using a 1:4000 dilution of FLAG M2 monoclonal antibody (Sigma) in blocking buffer (5% nonfat dry milk, Tris-buffered saline with 0.1% Tween 20). The membranes were developed, stripped, and re-probed with anti-GFP antibody as described above.
lysed in 4× SDS loading buffer, and analyzed by denaturing Western blotting using the FLAG antibody. Procedures for Western blotting were the same as described above.

RESULTS

The conserved serine residue 104 in the Keap1 BTB domain was mutated to an alanine (Fig. 1A), and the ability of this Keap1-S104A mutant to repress Nrf2 transactivation of a GCLM/luciferase transgene was evaluated in HepG2 cells. The GCLM/luciferase transgene contains an ARE sequence previously shown to be responsible for mediating Nrf2 binding and gene induction (14, 18, 42). Co-transfection of the GCLM/luciferase reporter and pClneo-Nrf2 into HepG2 cells resulted in an ∼10-fold increase in luciferase expression compared with cells co-transfected with GCLM/luciferase and an empty pCIneo vector (Fig. 1B). Co-transfection of a wild-type Keap1 expression vector with pClneo-Nrf2 reduced Nrf2 transactivation of the luciferase transgene by ∼80%. When the Keap1-S104A mutant cDNA was co-transfected with Nrf2, expression of the GCLM/luciferase reporter gene was significantly increased from that detected in HepG2 cells transfected with Nrf2 and wild-type Keap1 (p < 0.05). Wild-type and mutant FLAG-tagged Keap1 proteins were expressed to similar levels, suggesting that the functional alterations associated with the S104A mutation did not reflect differences in translation efficiency or protein stability (Fig. 1C).

To determine whether the Keap-S104A mutants altered Nrf2 localization, we monitored the distribution of a GFP-Nrf2 fusion protein in HepG2 cells co-transfected with cDNAs for wild-type or mutant Keap1. As expected, GFP fluorescence was concentrated in the nucleus in cells transfected with GFP-Nrf2 alone (Fig. 2A). In contrast, GFP-Nrf2 was distributed throughout the cytoplasm in cells co-transfected with GFP-Keap1 and wild-type Keap1 (Fig. 2B). Interestingly, when Keap1-S104A was co-transfected with GFP-Nrf2, the fluorescent pattern was

FIG. 1. Effect of S104A mutation on Keap1 repression of Nrf2 transactivation of GCLM-luciferase expression. A, BTB/POZ domains from multiple proteins were aligned, and conserved serine residue is indicated. Also shown is a diagram of the domain structure of Keap1. B, HepG2 cells were transiently transfected with 1.0 μg of pClneo-Nrf2, 0.5 μg of GCLM-luciferase transgene, and 50 ng of pEF-Keap1 as indicated. The total amount of DNA transfected was held constant by the addition of empty vector. Data are represented as the mean ± S.E. of three independent determinations. C, extracts were collected from HepG2 cells transfected with p3XFLAG-Keap1 cDNAs. Equal amounts of protein were separated on 7% polyacrylamide gels and analyzed by Western blotting with FLAG antibody.
similar to that detected in cells transfected with GFP-Nrf2 alone (Fig. 2C), indicating that the mutant protein was unable to sequester Nrf2 in the cytoplasm.

GFP-Nrf2 was also detected by Western analysis of nuclear extracts prepared from HepG2 cells transfected with GFP-Nrf2 alone (Fig. 3A). The amount of GFP-Nrf2 detected in the nucleus of cells co-transfected with wild-type Keap1 was reduced, consistent with the results of the fluorescence localization studies (Fig. 2). However, when HepG2 cells were co-transfected with GFP-Nrf2 and the Keap1-S104A mutant, GFP-Nrf2 was detected in the nucleus at levels comparable with that observed in the nuclei of cells transfected with GFP-Nrf2 alone (Fig. 3A).

The influence of the S104A mutation on Keap1/Nrf2 interaction was further examined by co-immunoprecipitation experiments using extracts prepared from cells co-transfected with vectors expressing GFP-Nrf2 and wild-type or mutant FLAG-tagged Keap1. The addition of the FLAG epitope to the N terminus of Keap1 did not alter the function of the wild-type protein in terms of its ability to bind and sequester Nrf2 (data not shown). Cell extracts were immunoprecipitated with a GFP-specific antibody and analyzed for the presence of FLAG-tagged Keap1 by Western analysis. A single ~66-kDa band corresponding to the FLAG-tagged Keap1 protein was detected in extracts from each experimental group. However, the intensity of the Keap1 band was reduced in cells transfected with the Keap1-S104A mutant relative to that detected in cells expressing wild-type Keap1 (Fig. 3B, top panel). Blotting for GFP revealed that similar levels of GFP-Nrf2 were immunoprecipitated in each group (Fig. 3B, bottom panel).

The subcellular localization of exogenous Keap1 in HepG2 cells transfected with the FLAG-tagged Keap1 expression vector was examined by confocal microscopy. Transfected cells were also stained with rhodamine-phalloidin to examine FLAG-Keap1 localization in relation to the actin cytoskeleton. Fig. 4 illustrates that wild-type FLAG-Keap1 and the mutant FLAG-Keap1 protein co-localized with the actin cytoskeleton, indicating that the S104A mutation does not influence the relationship between Keap1 and actin.

The serine residue corresponding to position 104 in Keap1 is highly conserved in almost all BTB/POZ domains identified to date (43, 44). Because the BTB/POZ domain has been shown to be an obligate dimerization motif (34, 40), we hypothesized that the functional defects associated with the S104A mutation reflect alterations in the dimerization function of the Keap1 BTB/POZ domain. To test this hypothesis, the ability of Keap1 to form homodimers was examined using wild-type and mutant Keap1 proteins translated in vitro. FLAG-tagged and untagged Keap1 proteins migrated differently on SDS-polyacrylamide gels, permitting easy discrimination of tagged and untagged protein (Fig. 5B, lanes 1 and 2). As expected, when untagged
Keap1 proteins were incubated with the anti-FLAG antibody, very little Keap1 was detected in the immunoprecipitates (Fig. 5A, lanes 2 and 4). In contrast, FLAG-tagged Keap1 proteins were specifically recognized and immunoprecipitated upon incubation with the anti-FLAG antibody (Fig. 5A, lanes 6 and 8). Consequently, the presence of any untagged Keap1 protein in immunoprecipitates derived from mixtures of the two proteins is indicative of an association between FLAG-tagged and untagged Keap1 proteins.

When a mixture of tagged and untagged Keap1-wt proteins (lane 3, Fig. 5B) was immunoprecipitated with the anti-FLAG antibody, both tagged and untagged Keap1 proteins were detected in the precipitate (lane 4, Fig. 5B). In contrast, when tagged and untagged Keap1-S104A mutants were co-translated and then immunoprecipitated with the FLAG antibody, very little untagged Keap1-S104A was co-precipitated (lane 6 versus lane 4, Fig. 5B). These results suggest that the S104A mutation decreases the efficiency of Keap1 dimerization. Similar dimerization deficiency was detected between untagged Keap1-S104A and wild-type FLAG-Keap1 (lane 7 versus lane 8). The wild-type Keap1 protein formed two complexes when the proteins were separated on native gels (Fig. 5C), and this property was not altered by fusion of the FLAG tag to Keap1.

However, only the faster migrating complex was detected in preparations of in vitro translated S104A Keap1 protein, suggesting that mutation of the BTB/POZ domain impairs higher order complex formation.

The correlation between inefficient dimerization and loss of Nrf2 sequestration observed with Keap1-S104A mutants suggested that the ability of Keap1 to dimerize might be an essential element in Nrf2 sequestration. To evaluate whether Keap1 dimerization might be involved in Nrf2 regulation in vivo, we transfected HepG2 cells with FLAG-Keap1 expression vectors. The transfected cells were then exposed to PDTC, an agent known to induce Nrf2 nuclear localization and ARE transactivation (18, 45). After PDTC exposure, the cells were harvested and divided equally for Western blot analysis under native (Fig. 6A) and denaturing conditions (Fig. 6B).

Under non-denaturing conditions, a prominent band was detected in extracts collected from cells overexpressing the FLAG-Keap1-wt protein. The migration of the FLAG-Keap1 complex in vivo was similar to the pattern observed in vitro, suggesting the complex represents FLAG-Keap1 dimer formation (data not shown). PDTC treatment caused a decrease in the intensity of this band (Fig. 6A). Exposure to phenethyl isothiocyanate (PEITC), another ARE-inducing agent, also decreased the intensity of the Keap1 complex (data not shown), suggesting multiple oxidative/electrophilic agents are capable of disrupting Keap1 dimerization in vivo. In HepG2 cells expressing the FLAG-Keap1-S104A mutant protein, formation of the Keap1-containing complex was greatly reduced, consistent with the deficiency of Keap1-S104A homodimer formation detected in vitro (Fig. 5B).

Neither PDTC treatment nor overexpression of the Keap1-S104A mutant protein was accompanied by a corresponding appearance of any other Keap1-containing bands on the non-denaturing gels even though equivalent amounts of FLAG-Keap1 were detected in each group by Western analysis on SDS-PAGE gels (Fig. 6B). In an attempt to account for the difference, the insoluble material remaining after the native protein extraction (i.e., from Fig. 6A) was subsequently denatured and analyzed by Western blotting with the anti-FLAG antibody (Fig. 6C). FLAG-tagged Keap1 was detected in extracts from each group, but the level detected in the extracts from PDTC-treated groups was consistently higher than that detected in extracts from untreated controls, suggesting that oxidant exposure alters Keap1 conformation, rendering the complex less soluble in the native extraction buffer.

Our previous work demonstrated that PDTC induction of GCLM requires activation of both Erk and p38 mitogen-activated protein kinase pathways prior to Nrf2 binding at the ARE sequence (45). To investigate the involvement of both Erk and p38 in the formation and disruption of the Keap1 complex in vivo, HepG2 cells were transiently transfected with FLAG-tagged wild-type Keap1. The cells were then incubated with either the Erk pathway inhibitor, PD98059, or the p38 inhibitor, SB202190, before PDTC exposure. The effect of Erk and p38 inhibition on the formation or disruption of the FLAG-Keap1-containing complex was monitored by non-denaturing Western blot analysis as just described. Although PD98059 effectively inhibited Erk2 activation (Fig. 6F), disruption of the Keap1-containing complex after PDTC exposure was unaltered (Fig. 6D). Similarly, exposure of transfected cells to 25 μM SB202190 (a dose previously shown to decrease Nrf2 binding to the GCLM ARE sequence (45)) did not affect Keap1 complex disruption by PDTC exposure, indicating that PDTC dissociation of the Keap1 complex in vivo occurs via an Erk2- and p38-independent mechanism.

Collectively, the data suggest that Keap1 dimerization is required for Keap1-Nrf2 association. If correct, then disruption...
of the dimerization potential of endogenous Keap1 in vivo would be expected to result in Nrf2 liberation and increased expression of the Nrf2-responsive GCLM/luciferase reporter gene. To test this hypothesis, we generated truncated Keap1 proteins (amino acids 1–247) by deleting the C-terminal Kelch repeat domains from the corresponding Keap1-wt and Keap1-S104A proteins to generate BTB/POZ-wt and BTB/POZ-S104A. Both in vitro translated BTB/POZ proteins migrated on denaturing polyacrylamide gels with an apparent molecular mass of ~30 kDa (Fig. 7A). However, bands corresponding to BTB/POZ dimers were only detected on native gels with the dimerization-proficient BTB/POZ-wt proteins. Like their full-length counterparts, the BTB/POZ-S104A proteins failed to dimerize in vitro (Fig. 7B).

The cDNA for each truncated protein was co-transfected into HepG2 cells along with the GCLM/luciferase reporter transgene. Transfection of a full-length wt-Keap1 cDNA inhibited expression of the reporter gene as shown previously (33). Transfection of the BTB/POZ-wt expression vector resulted in a significant increase (p = 0.02) in reporter gene expression, indicative of the release of endogenous Nrf2 (i.e. they function as dominant negatives) (Fig. 7C). Luciferase expression was not influenced by transfection of the dimerization-deficient BTB/POZ-S104A mutant.

**DISCUSSION**

Recent studies demonstrate that Nrf2, a member of the Cap’N’Collar family of transcription factors, plays a central role in the basal expression and ARE-dependent induction of a battery of detoxification and antioxidant enzymes, including NQO1, glutathione S-transferase, GCLM, and GCLC (24, 28, 29, 46). Furthermore, Nrf2-deficient mice have been demonstrated to be more susceptible to certain cancers and are unresponsive to chemopreventive agents, implicating Nrf2 as an important susceptibility factor and identifying the Nrf2 activation pathway as a potential target for improved chemoprevention strategies (28, 30–32).

Although Nrf2 involvement in ARE-dependent gene expression is now well established, the mechanism(s) and signal(s) involved in Nrf2 regulation are as yet poorly defined. Itoh et al. (33) demonstrated that Nrf2 is normally excluded from the nucleus by association with the Kelch-related protein, Keap1. In response to oxidants or electrophiles, Nrf2 is liberated from Keap1, whereupon it translocates to the nucleus, heterodimerizes with an unidentified b-zip partner, and binds to ARE sequences in the promoter of responsive genes. Considering the significance of Nrf2 in the activation of gene expression after exposure to oxidative or electrophilic agents, a better understanding of the nature and dynamics of the Keap1-Nrf2 interaction represents an important research objective.

Like the Drosophila Kelch protein, Keap1 belongs to a family of proteins having a BTB/POZ domain near the N terminus and Kelch repeats at the C terminus (47). The Kelch repeats are involved in binding to actin (35, 36, 48) and other cytoskeletal elements, whereas the BTB/POZ domain mediates dimerization as well as the formation of higher order macromolecular complexes (34, 38, 40, 49). Itoh et al. (33) determined that Keap1 and Nrf2 associate via interactions between the Kelch and Neh2 domains of the two proteins, respectively. Presumably, the Kelch region of the Keap1 protein is also responsible for anchoring the complex to the actin cytoskeleton, but specific molecular components of the association have not been reported to date.

The serine corresponding to position 104 in the Keap1 protein is evolutionarily conserved across species in proteins from both the BTB/POZ-Kelch family and members of the related BTB/POZ-containing zinc finger transcription factor family (41, 43, 44). Mutation of this serine, which is located within the monomer core region of the BTB/POZ domain, has been shown to disrupt BTB/POZ dimerization potential. Mutation of the corresponding serine to alanine (S56A) in the promyelocytic leukemia zinc finger (PLZF) transcription factor impaired protein function, suggesting that the BTB/POZ domain plays an important role in PLZF activity (43). We introduced a similar mutation at the corresponding residue in the Keap1 BTB/POZ domain (S104A) to assess the role of BTB/POZ domain in mediating Keap1 protein function.

The Keap1-S104A mutant failed to repress Nrf2 activation of a battery of antioxidants and detoxification enzymes, as shown previously (33). The cDNA for each truncated protein was co-transfected into HepG2 cells along with the GCLM/luciferase reporter transgene. Transfection of a full-length wt-Keap1 cDNA inhibited expression of the reporter gene as shown previously (33). Transfection of the BTB/POZ-wt expression vector resulted in a significant increase (p = 0.02) in reporter gene expression, indicative of the release of endogenous Nrf2 (i.e. they function as dominant negatives) (Fig. 7C). Luciferase expression was not influenced by transfection of the dimerization-deficient BTB/POZ-S104A mutant.
BTB/POZ peptides were translated in vitro in the presence of [35S]methionine and separated on denaturing polyacrylamide gels (A) or non-denaturing polyacrylamide gels (B). C, HepG2 cells were co-transfected with GCLM luciferase transgene and 50 ng of pCIneo, full-length Keap-wt, BTB/POZ-wt, or BTB/POZ-S104A as indicated in the figure. Luciferase activity is reported as the mean ± S.E. of five determinations.

Fig. 7. Overexpression of truncated Keap1 BTB/POZ-containing proteins disrupts endogenous Keap1-Nrf2 interaction. cDNAs encoding only the first 247 amino acids were generated for Keap1-wt and Keap1-S104A as described under “Experimental Procedures.” The BTB/POZ peptides were translated in vitro in the presence of [35S]methionine and separated on denaturing polyacrylamide gels (A) or non-denaturing polyacrylamide gels (B). C, HepG2 cells were co-transfected with GCLM luciferase transgene and 50 ng of pCIneo, full-length Keap-wt, BTB/POZ-wt, or BTB/POZ-S104A as indicated in the figure. Luciferase activity is reported as the mean ± S.E. of five determinations.

The BTB/POZ domain is an obligate dimerization motif that mediates protein-protein interactions (34, 40, 49, 51). We demonstrate that wild-type Keap1 formed dimers in vitro (Fig. 5B); however, the S104A Keap1 mutant was unable to efficiently homodimerize or heterodimerize with a wild-type Keap1 partner. Similarly, when the in vitro translated products were examined on non-denaturing gels, two bands were detected in the wild-type preparations, whereas only the faster migrating band was evident in the S104A mutant proteins (Fig. 5C). Again, the evidence suggests that the S104A mutation within the BTB/POZ domain impairs the ability of the mutant protein to form dimeric complexes.

A slow migrating complex containing FLAG-tagged Keap1 protein was detected on non-denaturing gels of whole cell lysates prepared from transfectants overexpressing wild-type Keap1 proteins. This band was absent or greatly diminished in similar preparations from cells expressing the dimerization-deficient S104A mutant protein. The reduction in the prominence of this band in vivo in Keap1-S104A-transfected cells correlated with the inefficient dimerization of Keap1-S104A proteins generated in vitro. Interestingly, the prominence of this Keap1 complex was also greatly reduced in transfectants treated with PDTC (Fig. 6A) or PEITC (data not shown), agents that are known to result in Nrf2 release from Keap1 and induction of ARE-containing genes (18, 52). This treatment also resulted in the appearance of a faster migrating band containing a reciprocal amount of FLAG tag in the insoluble fraction of each preparation, suggesting that PDTC treatment induced a conformational change of the Keap1-containing complex, decreasing its solubility in the native extraction buffer. These observations lend credence to the hypothesis that Keap1-dependent dimerization is involved in Nrf2 sequestration in the cytoplasm and that alteration in Keap1 dimerization potential either by S104A mutation or exposure to an inducing agent is involved in Nrf2 release. The fact that the dimerization-competent wild-type BTB/POZ truncated protein was capable of functioning in a dominant negative fashion to affect Nrf2 release (presumably by competing with endogenous Keap1 proteins and thereby disrupting endogenous Keap1 dimerization) whereas the corresponding dimerization-competent truncated protein containing the S104A mutation failed to do so also supports the conclusion that Keap1 dimerization plays a critical role in Nrf2 sequestration and release in vivo.

A working model of the Keap1-Nrf2 interaction incorporating data from multiple sources is depicted in Fig. 8. In the simplest possible model consistent with existing data, Keap1 binds to the actin cytoskeleton via the Kelch repeats in the C terminus of the protein and dimerizes with a second Keap1 molecule through their respective BTB/POZ domains. Nrf2 is then an-
Nrf2 Cytoplasmic Retention Requires Keap1 Dimerization

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