Molecular Evolution of Keap1

TWO Keap1 MOLECULES WITH DISTINCTIVE INTERVENING REGION STRUCTURES ARE CONSERVED AMONG FISH

Received for publication, October 22, 2007, and in revised form, December 4, 2007 Published, JBC Papers in Press, December 5, 2007, DOI 10.1074/jbc.M708702200

Li Li†, Makoto Kobayashi‡§, Hiroshi Kaneko†, Yaeko Nakajima-Takagi†§, Yuko Nakayama‡, and Masayuki Yamamoto‡§

From the †Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan. Tel.: 81-22-754-1744; Fax: 81-22-754-1745.

‡ Institute of Basic Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan.

§ Environmental Response Project, Japan Science and Technology Agency, and the §Institute of Basic Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan.

Keap1 is a BTB-Kelch-type substrate adaptor protein of the Cul3-dependent ubiquitin ligase complex. Keap1 facilitates the degradation of Nrf2, a transcription factor regulating the inducible expression of many cytoprotective genes. Through comparative genome analyses, we found that amino acid residues composing the pocket of Keap1 that interacts with Nrf2 are highly conserved among Keap1 orthologs and related proteins in all vertebrates and in certain invertebrates, including flies and mosquitoes. The interaction between Nrf2 and Keap1 appears to be widely preserved in vertebrates. Similarly, cysteine residues corresponding to Cys-273 and Cys-288 in the intervening region of mouse Keap1, which are essential for the repression of Nrf2 activity in cultured cells, are conserved among Keap1 orthologs in vertebrates and invertebrates, except fish. We found that fish have two types of Keap1, Keap1a and Keap1b. To our surprise, Keap1a and Keap1b contain the cysteine residue corresponding to Cys-288 and Cys-273, respectively. In our analysis of zebrafish Keap1a and Keap1b activities, both Keap1a and Keap1b were able to facilitate the degradation of Nrf2 protein and repress Nrf2-mediated target gene activation. Individual mutation of either residual cysteine residue in Keap1a and Keap1b disrupted the ability of Keap1 to repress Nrf2, indicating that the presence of either Cys-273 or Cys-288 is sufficient for fish Keap1 molecules to fully function. These results provide an important insight into the means by which Keap1 cysteines act as sensors of electrophiles and oxidants.

The transcription factor Nrf2 induces the expression of phase 2 detoxifying and antioxidant proteins in response to electrophilic insults (1). These induced proteins contribute to the prevention of oxidative damage and chemically induced cancer in animals. The importance of Nrf2 in this induction and the resulting chemoprevention has been demonstrated by a number of experiments using Nrf2-deficient mice (2). The electrophile response is regulated through a cis-acting element called the antioxidant- or electrophile-responsive element within the regulatory region of each gene (3). Nrf2 binds to the antioxidant/electrophile-responsive element sequence as a heterodimeric complex with small Maf proteins through a basic region leucine zipper domain (4). Under normal homeostatic conditions, Nrf2 protein is targeted for proteasomal degradation and has a short half-life. This degradation is positively controlled by Keap1, a member of the BTB (Broad complex/Tramtrack/Bric-a-brac)-Kelch protein family (5, 6). Keap1 binds to Nrf2 and promotes its degradation as a substrate-specific adaptor protein for the Cul3 ubiquitin ligase complex (7). When oxidative/electrophilic stress signals disrupt the Nrf2-Keap1-Cul3 complex, ubiquitination of Nrf2 is blocked, and Nrf2 becomes stable (8). Consequently, the expression of a battery of cytoprotective genes is induced as Nrf2 accumulates in the nucleus.

Keap1 is composed of three major domains: a BTB domain, a double glycine repeat (DGR) domain, and an intervening region (IVR) domain (1). The BTB domain functions to dimerize Keap1 (9), whereas the DGR domain serves as a binding site for Nrf2 (5) and actin (10). Our group (11) and Hannink and co-workers (12) have determined the crystal structure of the Keap1 DGR domain and identified its interface with Nrf2. Involvement of the Keap1 IVR domain in the ubiquitination of Nrf2 has been demonstrated (8, 13). In cultured cells, mutation of Cys-273 or Cys-288 in the IVR domain to alanine or serine reduces Keap1-dependent ubiquitination and increases Nrf2 stability, suggesting that these residues are crucial for the Nrf2-repressing activity of Keap1 (13–15).

We previously isolated homolog genes of Nrf2 and Keap1 in zebrafish and established that the Nrf2-dependent induction of cytoprotective genes is conserved among vertebrates (16, 17). We thus speculated that the Nrf2-Keap1 system of cytoprotec-
Molecular Evolution of Keap1

Isolation of cDNA—A partial cDNA fragment encoding zebrafish Keap1b was prepared by PCR using specific primers designed based on genomic DNA information. A zebrafish Keap1b was prepared by PCR using specific primers.

Radiation Hybrid Mapping—Radiation hybrid mapping using panel LN54 was performed as described by Hukriede et al. (19) using specific primers for each Keap1 gene. The sequences of each primer were as follows: keap1a, 5'-AGGATTTCTCCCGCCATTTGTG and 5'-CTTCCGGAGTTGCTGGTGAAC; and keap1b, 5'-AGGATTTCTCCCGCCATTTGTG and 5'-AGGATTTCTCCCGCCATTTGTG.

Plasmid Construction—The plasmid pCS2keap1b was constructed by subcloning the open reading frame of zebrafish keap1b into the BamHI and XbaI sites of the vector pCS2. To construct pS3keap1aC, cDNA encoding the C-terminal region (amino acids 353–601) containing the 3'-untranslated region of zebrafish keap1a was inserted into the NotI and Sall sites of the vector pSPORT1.

Molecular Evolution of Keap1

Expression Analysis—Zebrafish embryos and larvae were obtained by natural mating. All experiments were carried out using a wild-type AB strain. The expression of keap1a, keap1b, and gstp1 genes was analyzed by reverse transcription (RT)-PCR and whole mount in situ hybridization. For RT-PCR analysis, total RNA was prepared from adult tissues or the whole bodies of embryos and larvae using QIAzol (Qiagen). First-strand cDNA was synthesized by incubation at 25 °C for 15 min and at 42 °C for 45 min with murine leukemia virus reverse transcriptase (SuperScript II, Invitrogen) and random hexamer oligonucleotide primers. From the 20–μl first-strand reaction, 0.025–0.1 was used for PCR using the following primers: keap1a, 5'-ATGATATGTCCAAGAAAGAAG and 5'-TCATGAGGAAATTCGACGAG; keap1b, 5'-ACGGAGTTGAAGGCCGAG and 5'-ACCTGGGCTGAAGTTCATG; and gstp1, 5'-CTAGGAGAGGTTGAAACGCAC and 5'-GGCAGTTGACCAGGAG.

Microinjection of Zebrafish Embryos—Synthetic capped RNA was made with an SP6 mMESSAGE mMACHINE in vitro transcription kit (Ambion) using linearized DNA of the pCS2 derivatives described above. For expression in whole bodies, RNA was injected into yolk at the one-cell stage using an IM300 microinjector (Narishige). GFP expression was examined under the GFP Plus filter (480 nm excitation, 505 nm emission) of a MZFLIII microscope (Leica) equipped with a 600CL-CU digital camera (Pixera).
Molecular Evolution of Keap1

Identification of the Second Keap1 in Zebrafish—By virtue of recent progress in the zebrafish genome project, we came across a novel Keap1-related gene that shows a higher similarity to mammalian Keap1 than previously reported zebrafish Keap1 (16). A partial cDNA was isolated by RT-PCR using specific primers whose design was based on genomic DNA information. We screened a zebrafish cDNA λ-phage library using this partial cDNA as a probe and isolated a full-length cDNA clone.

We refer to this gene as keap1b, and the previous keap1 was renamed keap1a. The deduced amino acid sequence of the Keap1b cDNA product showed 81 and 78% identities to the BTB and DGR domains, respectively, of mouse Keap1 protein (Fig. 1A). These values are quite high compared with those of Keap1a, whose identities to the BTB and DGR domains are only 49 and 55%, respectively. We mapped both Keap1 genes using an LNS4 hybrid panel (19) and found that keap1a and keap1b are localized on zebrafish chromosomes 2 and 6, respectively. The latest information from the zebrafish genome project supported these mapped sites and further demonstrated that synteny was found between keap1b and the human KEAP1 locus on chromosome 19p13.2 (supplemental Fig. 1).

Neh2 is the domain in Nrf2 that interacts with the DGR domain in Keap1 (5). Within the Neh2 domain, we found that the motifs ETGE and DLG are critical for the interaction with Keap1 (16, 23). Recently, we identified the region of the Keap1 DGR domain responsible for binding to the ETGE and DLG motifs by structural analysis of the mouse Keap1 protein (11, 24). The amino acid residues important for binding to the ETGE motif have been recognized as Ser-363, Arg-380, Asn-382, Arg-383, Arg-415, Arg-483, Ser-508, Ser-555, Tyr-572, Phe-478 (Fig. 1B, white characters highlighted in black). Mutation analyses of mouse and human Keap1 proteins have demonstrated that Tyr-334, Gly-364, Gly-430, His-436, and Phe-478 (Fig. 1B, white characters highlighted in gray), in addition to Arg-380, Asn-382, Arg-415, Arg-483, Tyr-525, and Tyr-572, are critical for inhibiting Nrf2 activity (11, 12). Interestingly, all these residues, except Asn-382 and Tyr-572, are conserved in both zebrafish Keap1a and Keap1b, suggesting that both proteins can interact with Nrf2. Indeed, zebrafish Keap1a has been shown to interact with Nrf2 and to inhibit its activity (16). Although Mayven is the protein with the highest homology to Keap1 in the DGR domain among mouse BTB-Kelch proteins (25), it possesses only 2 of the 13 critical Nrf2-interacting residues in mouse Keap1 (Fig. 1B, mK). This case is similar to that of KLHL20 and KLHL5, two other Keap1-related proteins (supplemental Table 1). These results suggest that the activity of Nrf2 is regulated by two Keap1 proteins, Keap1a and Keap1b, in zebrafish and by a single Keap1 protein in mouse, which may be the only BTB-Kelch protein that can facilitate Nrf2 degradation. Here, we propose to define Keap1 as a BTB-Kelch protein carrying the evolutionarily conserved Nrf2-interacting surface.

Unlike Keap1, we could not find a second Nrf2 gene in the zebrafish genome data base. Nrf2 is a member of the CNC
(Cap 'n' collar) protein family, whose members are NF-E2 p45, Nrf1, Nrf2, Nrf3, Bach1, and Bach2 (1). Among them, genetic loci of mammalian NF-E2 p45, Nrf1, Nrf2, and Nrf3 genes have been mapped close to those of HoxC, HoxB, HoxD, and HoxA, respectively (34). Interestingly, the zebrafish genome has two copies of HoxA, HoxB, and HoxC clusters, but only one HoxD cluster (35). We assume that the second Nrf2 gene in zebrafish had been lost together with the second HoxD cluster during evolution.

Keap1 Is Present in Vertebrates and in Some Invertebrates—To identify the range of species in which Keap1 is present, we searched the Ensemble and DDBJ/GenBank™/EBI Data Bank for Keap1-related proteins. As well as in mammals, Keap1 genes were found in chicken, frogs (Xenopus laevis and Xenopus tropicalis), fugu, Tetraodon nigroviridis, medaka fish, stickleback, ascidians (Ciona intestinalis and Ciona savignyi), mosquitoes (Aedes aegypti and Anopheles gambiae), and Drosophila.

A phylogenetic tree based on the amino acid sequences of their DGR domains classified the Keap1 proteins into five subgroups: 1) vertebrate Keap1, 2) fish Keap1a, 3) fish Keap1b, 4) ascidian, and 5) invertebrate Keap1 (Fig. 1C). No Keap1-related genes were found in nematode or yeast. We noted that all these Keap1 proteins carry 13 critical Nrf2-interacting residues, with the exceptions of Asn-382 and Tyr-572 for fish Keap1a and Tyr-525 for invertebrate Keap1 (supplemental Table 1). The results suggest that Keap1 regulates Nrf2-related proteins in these organisms in a manner similar to that in mammals. Keap1a and Keap1b are conserved among fish, but not in other vertebrates, signifying that both proteins are essential to the fish Nrf2-Keap1 system. Keap1b rather than Keap1a may represent the ortholog of vertebrate Keap1 because conserved synteny was observed between human KEAP1 and fish keap1 loci (supplemental Fig. 1). No synteny was found between human Keap1 and fish Keap1a genes or with ascidian or invertebrate Keap1. This implies that Keap1b may be the proper homolog of vertebrate Keap1.

Keap1a and Keap1b Repress Nrf2 Activity Despite Their Lack of a Cysteine Residue Corresponding to Mouse Keap1 Cys-273 and Cys-288, Respectively—All fish Keap1a and Keap1b lack a cysteine residue corresponding to Cys-273 and Cys-288, respectively, whereas both these cysteines are conserved even in ascidian and invertebrate Keap1 proteins (Fig. 2. This finding was surprising because both Cys-273 and Cys-288 in the IVR were demonstrated to be crucial for the Nrf2-repressing activity of mouse Keap1 (13–15). To elucidate whether zebrafish Keap1a and Keap1b can repress the inducible function of Nrf2, we tested the extent of their repression on the Nrf2-mediated inducible expression of the endogenous gstp1 gene in zebrafish embryos. The gstp1 gene encodes a Pi class glutathione S-transferase and is strongly induced in both electrophile-treated larvae and Nrf2-overexpressing embryos (16, 26). Its promoter contains an evolutionarily conserved antioxidant/electrophile-responsive element sequence that is critical for both Nrf2 binding and promoter activity (26). In vitro-synthesized zebrafish Keap1a or Keap1b mRNA (200 pg) was coinjected with Nrf2 mRNA (100 pg) into zebrafish embryos at the one-cell stage (Fig. 3A). At midgastrula, gstp1 expression was analyzed by whole-mount in situ hybridization analysis. Nrf2-induced expression of gstp1 was reduced by co-overex-
Molecular Evolution of Keap1

expression of either Keap1a or Keap1b (Fig. 3B), indicating that both Keap1a and Keap1b possess the ability to repress Nrf2 activity. To confirm this, we used FLAG-tagged Keap1 proteins to standardize the protein expression level of each Keap1 by immunoblotting (supplemental Fig. 2). Seventy-five pg of Keap1a mRNA and 200 pg of Keap1b mRNA expressed similar amounts of Keap1 proteins in zebrafish embryos. Only full-length proteins were overexpressed in embryos. The FLAG-tagged constructs were used to compare the Nrf2 repression activity of Keap1a and Keap1b by real-time RT-PCR analyses (Fig. 3C). Sixty pg of Nrf2 mRNA were co-injected with various amounts of Keap1a or Keap1b mRNA (Fig. 3C). The dose effects of Keap1 mRNA on Nrf2 repression were similar between Keap1a and Keap1b, suggesting that the activities of Keap1a and Keap1b to repress Nrf2 activity are comparable, at least in zebrafish embryos.

Both Keap1 Proteins Promote Nrf2 Degradation—Mouse Keap1 has been shown to promote the degradation of Nrf2 as a substrate-specific adaptor protein for the Cul3 ubiquitin ligase complex (7). To elucidate whether zebrafish Keap1 proteins also promote Nrf2 degradation, we examined the effects of Keap1 co-overexpression on the level of Nrf2 protein. FLAG-tagged Nrf2 protein overexpressed in zebrafish embryos by mRNA injection was detectable by whole mount immunostaining using anti-FLAG antibody. A, immunostaining analysis of overexpressed Nrf2 protein. FLAG-tagged Nrf2 (FL-Nrf2) and/or Keap1 mRNA was injected into embryos at the one-cell stage. After 8 h, the stability of FLAG-tagged Nrf2 protein was analyzed by immunostaining using anti-FLAG antibody. B, expression analysis of Nrf2-GFP fusion protein. mRNA encoding a fusion protein comprising the N-terminal half of Nrf2 protein and enhanced GFP (eGFP) protein connected by two copies of SV40 nuclear localizing signal (NLS) was injected with or without mRNA encoding Keap1 proteins into one-cell stage embryos, and GFP expression was analyzed after 6 h.

zebrafish Nrf2 was used to construct the GFP fusion protein because this region corresponds to the mouse Nrf2 protein that was shown to be sufficient for Keap1-dependent degradation in both cultured cells and mouse intestine (27). GFP expression was observed in Nrf2NTnGFP-overexpressing embryos, whereas GFP expression was dramatically lower when either Keap1a or Keap1b was co-overexpressed (Nrf2NTnGFP, 53.7%, n = 93; Nrf2NTnGFP + Keap1a, 0%, n = 132; Nrf2NTnGFP + Keap1b, 0%, n = 73; no injection, 0%, n = 100) (Fig. 4B). These results demonstrate that both Keap1a and Keap1b repress Nrf2 activity by facilitating its degradation, as is the case for mouse Keap1.

Keap1a and Keap1b Can Form Homodimers and Heterodimers—We previously found that coexpression of C273A and C288A mutant proteins of mouse Keap1 substantially restores repressor activity, whereas each Keap1 mutant alone lacks repressor activity (15). This observation implies that C273A and C288A form a heterodimer, and the simultaneous presence of Cys-273 on one monomer and Cys-288 on the other is sufficient for the repressor activity. Similarly, it is possible that overexpressed Keap1a or Keap1b in zebrafish embryos forms a heterodimer with endogenous Keap1 proteins to share cysteine residues in the same complex. To assess this possibility, we carried out pulldown analysis using in vitro translated Keap1a and Keap1b proteins with FLAG and HA tags (Fig. 5). Tagged Keap1 proteins were mixed and pulled down with anti-HA beads. Precipitated proteins were analyzed by immunoblotting using anti-FLAG and anti-HA antibodies. FLAG-tagged Keap1a protein coprecipitated with both HA-tagged Keap1a and Keap1b. Similarly, FLAG-tagged Keap1b protein was pulled down with HA-tagged Keap1a and Keap1b. These results demonstrate that Keap1a and Keap1b can form both homodimers and heterodimers.
Keap1a and Keap1b Genes are Coexpressed in Many Tissues—

Keap1a and Keap1b require simultaneous expression to function as heterodimers. To provide insight into the roles of Keap1a and Keap1b in vivo, we examined the tissue distribution of Keap1 mRNA in adult fish (Fig. 6A). Total RNA fractions were prepared from various tissues of 10-month-old zebrafish males and analyzed by RT-PCR. The amount of cDNA was standardized by the expression level of ef1α/H9251. Although both keap1a and keap1b were expressed ubiquitously, the expression of keap1b was relatively abundant in brain and scarce in gut. We also examined the expression levels of the zebrafish Keap1 genes during the embryonic and larval stages (Fig. 6B). RT-PCR analyses demonstrated that keap1b was expressed at every stage tested and at similar levels, whereas keap1a expression was quite low during the embryonic stages and started to increase around the time of hatching (2.5 days). Spatial expression profiles of zebrafish Keap1 genes were assessed at the embryonic stages by whole mount in situ hybridization analysis (Fig. 6C). Both genes were expressed ubiquitously in the whole body, although some specific regions, such as lens (arrow), expressed keap1a more strongly than others. Overall, these observations suggest that keap1a and keap1b are coexpressed in many cells.

Cysteine Residues corresponding to Cys-273 and Cys-288 in Mouse Keap1 Are Important for the Nrf2-repressing Activity of Keap1a and Keap1b—

The critical cysteine residues in Keap1a and Keap1b must be important for repressing Nrf2 if these two proteins function as heterodimers. To verify this, point mutations were introduced in these cysteines, and the ability to repress Nrf2 was analyzed. In this work, we refer to the cysteine residues in the IVR domain as IVR cysteines (ICs) to ease comparison among the corresponding cysteines of various Keap1 proteins (see Fig. 2). Cysteine residues corresponding to Cys-273 and Cys-288 in mouse Keap1 are called IC6 and IC7. We introduced Cys-to-Ser point mutations in IC7 of Keap1a and in IC6 of Keap1b and examined the effects of these mutations on Nrf2-repressing activity (Fig. 7A). We used FLAG-tagged Keap1 proteins to standardize the protein expression level of each Keap1 by immunoblotting. Mutations in Keap1a IC7 and Keap1b IC6 strongly abolished the Nrf2-repressing activity (Fig. 7B). IC7 in Keap1a and IC6 in Keap1b are thus essential for the repression of Nrf2 activity.

DISCUSSION

This is the first work referring to the evolutionary aspects of Keap1, as well as to its definition. Stogios and Privé (28) predicted that more than 53 members of the BTB-Kelch protein
family exist in human. Some of them, such as Mayven, KLHL20, and KLHL5, show relatively high similarity to Keap1. For example, Mayven has a DGR with the highest amino acid sequence identity (44%) to that of Keap1 among the mouse members of the BTB–Kelch family. This value is close to that between zebrafish Keap1a and mouse Keap1 (55%). However, mouse Mayven shares only 2 of the 13 critical amino acid residues of mouse Keap1, which were shown to form the interaction surface for Nrf2 (supplemental Table 1). In contrast, zebrafish Keap1a shares 11 of them. This indicates that Mayven cannot bind to Nrf2 and is inactive in repressing the function of Nrf2. Indeed, Mayven was not able to repress Nrf2 activity in cultured cells, even when its BTB and IVR domains were swapped with those of mouse Keap1.\(^3\) We anticipate that Keap1 is the only BTB–Kelch protein that regulates Nrf2 activity.

We recently proposed “the hinge and latch model” for the interaction between Nrf2 and Keap1 and the induction of cellular defense enzymes (24, 29–31). Keap1 dimer recruits its substrate Nrf2 by binding to the evolutionarily conserved DLG and ETGE motifs within the Neh2 domain of Nrf2 (16, 23). The structural plasticity of its Neh2 domain allows Nrf2 to link two Keap1 molecules in tandem on either side of the central Neh2 α-helix that exists between the DLG and ETGE motifs, thereby presenting the lysines for ubiquitin-protein isopeptide ligase-catalyzed ubiquitination (29). These lysine residues were shown to be important for Nrf2 degradation (32). In this work, we have shown that the domain interacting with both the DLG and ETGE motifs is highly conserved among various Keap1 proteins, even in invertebrate Keap1, suggesting that the hinge and latch model may also be conserved. It is plausible that the DLG and ETGE motifs are also conserved among vertebrate and some invertebrate species. Indeed, high conservation of these two motifs was observed by comparative genome analysis (supplemental Fig. 3). Of six CNC proteins, only Nrf1 and Nrf2 possess the DLG and ETGE motifs. In ascidian, mosquito, and fly, only one Nrf1/2-related protein exists that has both DLG and ETGE motifs. The QDxDLG and DXETGE sequences of the DLG and ETGE motifs, respectively, are the only perfectly conserved amino acid sequences in the Neh2 domain of these Nrf1/2-related proteins (supplemental Fig. 3, *white characters* highlighted in *black*). Lysine residues also exist between these two motifs in every protein (supplemental Fig. 3, *red characters*). So, it seems that the DLG and ETGE motifs are quite important for Nrf1/2-related proteins and that Keap1 proteins are important regulators of these proteins, even in invertebrates.

The second topic of this work covers functional Keap1 proteins lacking either IC6 or IC7. The finding is inconsistent with those we (15) and others (13, 14) observed in cultured cells, that both IC6 and IC7 are indispensable for mouse Keap1 activity. There are two explanations for this contradiction. First, in Keap1a (lacking IC6) mRNA-injected embryos, it is possible that exogenous Keap1a can heterodimerize with endogenous Keap1b. Likewise, in Keap1b (lacking IC7) mRNA-injected embryos, exogenous Keap1b may heterodimerize with endogenous Keap1a. This hypothesis is plausible because we found previously that coexpression of C273A and C288A mutant proteins of mouse Keap1 leads to the substantial restoration of repressor activity (15). Moreover, zebrafish Keap1a and Keap1b can form heterodimers, and both genes are coexpressed in many cells. However, it was curious to discover that the Nrf2-repressing activities of overexpressed Keap1a and Keap1b were comparable in embryos, in which *keap1b* was dominantly expressed judged on RT-PCR analysis (see Figs. 3 and 6). Similarly, the mRNA expression of *keap1b* was undetectable in adult gut, where *keap1a* was dominantly expressed (see Fig. 6). The second idea is that the ubiquitin ligase machinery may differ in structure between fish and mammals, such that the effectual structure for Keap1 activity may also be distinctive.

According to this idea, the tertiary structure of the IVR domain is more important than the presence or absence of each cysteine residue. This is contradictory to the zinc binding model proposed by Dinkova-Kostova et al. (33). They demonstrated that Keap1 is a zinc-containing protein and that alanine substitutions of both Cys-273 and Cys-288 reduces the binding affinity between Keap1 and zinc to 1/20, and they suggested that these two cysteine residues participate in the binding to zinc. At present, it is difficult to adopt a proper hypothesis from these and other theories. In this context, it will be of interest to know whether Keap1a and Keap1b bind zinc in zebrafish embryos. Furthermore, the crystal structures of the IVR domains of various Keap1 proteins should be determined in the future.

Acknowledgments—We thank M. Eguchi, T. Kinoshita, H. Niu, T. Shimokoube, Y. Terasita, and Y. Wada for help in fish maintenance; R. Ide, F. Katsuo, T. Suzuki, M. Takeuchi, and T. Tsujita for help and discussion; and A. Kobayashi, H. Motohashi, T. O’Connor, and K. Tong for critical reading of the manuscript.

REFERENCES

1. Kobayashi, M., and Yamamoto, M. (2006) Adv. Enzyme Regul. 46, 113–140
2. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) Biochem. Biophys. Res. Commun. 236, 313–322
3. Kobayashi, M., and Yamamoto, M. (2005) *Antioxid. Redox Signal.* 7, 385–394
4. Katsuo, F., Motohashi, H., Ishii, T., Aburatani, H., Engel, J. D., and Yamamoto, M. (2005) Mol. Cell. Biol. 25, 8044–8051
5. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Genes Dev. 13, 76–86
6. Wakabayashi, N., Itoh, K., Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., Imakado, S., Kotsuji, T., Otsuka, F., Roop, D. R., Harada, T., Engel, J. D., and Yamamoto, M. (2003) Nat. Genet. 35, 238–245
7. Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., and Yamamoto, M. (2004) Mol. Cell. Biol. 24, 7130–7139
8. Kobayashi, A., Kang, M. I., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., and Yamamoto, M. (2006) Mol. Cell. Biol. 26, 221–229
9. Zipper, L. M., and Mulcahy, R. T. (2002) *J. Biol. Chem.* 277, 36544–36552
10. Kang, M. I., Kobayashi, A., Wakabayashi, N., Kim, S. G., and Yamamoto, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2046–2051
11. Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsuji, M., Kang, M. I., Kobayashi, A., Yokoyama, S., and Yamamoto, M. (2006) Mol. Cell 21, 689–700
12. Lo, S. C., Li, X., Henzl, M. T., Beamer, L. J., and Hannink, M. (2006) *EMBO J.* 25, 3605–3617

\(^3\) M. Kang, A. Kobayashi, and M. Yamamoto, unpublished data.
