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Cancer Chemoprevention Mechanisms Mediated Through the Keap1–Nrf2 Pathway

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Abstract

The cap'n'collar (CNC) bZIP transcription factor Nrf2 controls expression of genes for antioxidant enzymes, metal-binding proteins, drug-metabolising enzymes, drug transporters, and molecular chaperones. Many chemicals that protect against carcinogenesis induce Nrf2-target genes. These compounds are all thiol-reactive and stimulate an adaptive response to redox stress in cells. Such agents induce the expression of genes that posses an antioxidant response element (ARE) in their regulatory regions. Under normal homeostatic conditions, Nrf2 activity is restricted through a Keap1-dependent ubiquitylation by Cul3–Rbx1, which targets the CNC-bZIP transcription factor for proteasomal degradation. However, as the substrate adaptor function of Keap1 is redox-sensitive, Nrf2 protein evades ubiquitylation by Cul3–Rbx1 when cells are treated with chemopreventive agents. As a consequence, Nrf2 accumulates in the nucleus where it heterodimerizes with small Maf proteins and transactivates genes regulated through an ARE. In this review, we describe synthetic compounds and phytochemicals from edible plants that induce Nrf2-target genes. We also discuss evidence for the existence of different classes of ARE (a 16-bp 5′-TMAnnRTGABnnnGCR-3′ versus an 11-bp 5′-RTGABnnnGCR-3′, with or without the embedded activator protein 1-binding site 5′-TGASTCA-3′), species differences in the ARE-gene battery, and the identity of critical Cys residues in Keap1 required for de-repression of Nrf2 by chemopreventive agents. *Antioxid. Redox Signal.* 13, 1713–1748.

Introduction

THE ABILITY OF XENOBIOTICS to inhibit or arrest chemical carcinogenesis has been recognized for many years, as evidenced by the fact that this research area was first reviewed in 1966 (320). Compounds possessing such properties have been referred to as anti-carcinogens and chemoprotective agents, but are now most frequently referred to as cancer chemopreventive agents (96, 288). They have been subdivided into blocking agents or suppressing agents, based on the stage during the development of neoplastic disease at which they are effective (323). Blocking agents prevent carcinogens from damaging DNA by inhibiting the activation of carcinogenic compounds, enhancing the detoxification of activated carcinogens, trapping reactive intermediates, or increasing DNA repair (326). By contrast, suppressing agents are able to retard or reverse the development of neoplastic disease after exposure to mutagenic compounds. The mechanisms by which suppressing agents act include stimulation of cellular differentiation, inhibition of activated oncogenes, compensation for defects in tumor suppressor genes, antagonism of cell proliferation, activation of apoptosis, and inhibition of angiogenesis (326).

When first coined, the designations blocking and suppressing agents were helpful in an experimental sense: blocking agents had to be administered either prior to or during exposure to a chemical carcinogen in order to prevent tumorigenesis, whereas suppressing agents could be administered after exposure to a chemical carcinogen and still be effective. However, the usefulness of these terms is limited because many chemopreventive agents fall into both categories. For example, dietary isothiocyanates not only inhibit cytochrome P450 (CYP) isoenzymes involved in the activation of carcinogens (41), induce genes for drug-metabolizing enzymes that inactivate carcinogens (122), and increase intracellular glutathione levels to scavenge free radicals (88), but at higher concentrations they can also stimulate apoptosis (232), affect G_2/M-phase cell cycle arrest (86, 282), inhibit histone deacetylase (90, 237), and inhibit activator protein-1 (AP1) transcription factor (52). These diverse effects suggest isothiocyanates could be classed as both blocking and suppressing agents.

Faced with the pleiotropic effects of many cancer chemopreventive agents, it is probably worthwhile classifying them according to their molecular targets. Promising chemoprevention targets include the estrogen receptor, retinoid X receptor, peroxisome proliferator-activated receptor-γ (PPARγ),

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vitamin D receptor, nuclear factor-κB (NF-κB), prostaglandin receptors, and cyclooxygenase-2 (289). The E3 ubiquitin ligase substrate adaptor Kelch-like ECH-associated protein 1 (Keap1), which negatively regulates nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) (138), represents another target that is inhibited by numerous chemopreventive compounds. This review describes the molecular mechanisms by which antagonism of Keap1, and thus activation of transcription factor Nrf2, contributes to cancer chemoprevention through induction of a battery of cytoprotective genes that are each regulated *via* an antioxidant response element (ARE).

Chemopreventive Blocking Agents Upregulate Cytoprotective Genes

Induction of genes for drug-metabolizing enzymes

Synthetic phenolic antioxidants have been thoroughly investigated from a health safety perspective because they have been employed as food preservatives for over 40 years (40). As a consequence of such studies, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin, EQ) were among the first chemopreventive agents, already present in the human diet or the food of domestic animals, shown to induce enzymes involved in drug metabolism (reviewed in (109), with structures shown in Fig. 1). Subsequently, the dithiolethione oltipraz, developed originally as an antischistosomal drug, was intensively investigated as a cancer chemopreventive agent (156), as was 4-methylsulfinylbutyl isothiocyanate (sulforaphane), because humans are exposed to it as a consequence of eating cruciferous vegetables (345). Studies of phenolic antioxidants, oltipraz and sulforaphane, have provided important insights into biochemical events that accompany cellular resistance to chemical carcinogens. More recently, triterpenoids based around oleanolic acid, such as 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazolide (CDDO-Im), have attracted considerable attention as promising chemopreventive agents, and their use in intervention studies has supported the hypothesis that changes in the metabolism of carcinogens coupled with activation of anti-inflammatory pathways are important in cancer prevention (187, 189).

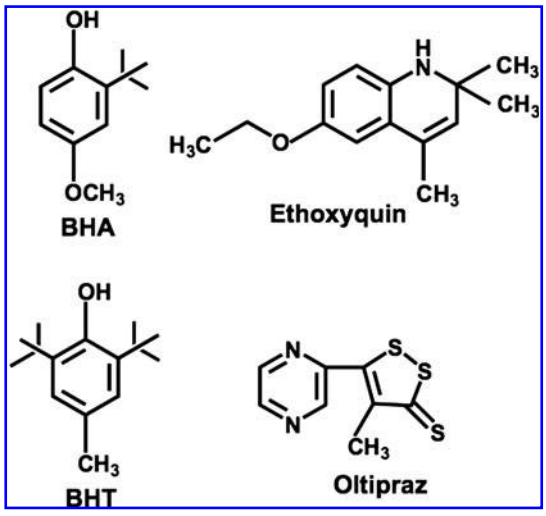

FIG. 1. Prototypic chemopreventive agents. The structures of butylated hydroxyanisole (BHA), ethoxyquin, butylated hydroxytoluene (BHT). and oltipraz are shown.

It was first reported in 1973 that rats fed diets containing BHT were resistant to the carcinogenic aromatic amines *N*-2-acetylaminofluorene and *N*-hydroxy-*N*-2-acetylaminofluorene (306). This resistance coincided with a decrease in the ability of the aromatic amines to form DNA adducts in rats fed a BHT-containing diet along with a concomitant increased ability of such animals to excrete the aromatic amines as glucuronide conjugates in the urine (95), presumably because of an increase in hepatic activity of UDP-glucuronosyl transferase (UGT). Increases in rat hepatic UGT activity were not only affected by BHT but were also observed following feeding with BHA (273). Between 1978 and 1980, studies conducted in the laboratories of Ernest Bueding and Paul Talalay at Johns Hopkins University showed that treatment of mice with BHA led to a marked increase in hepatic microsomal epoxide hydrolase (EPH1) (31), glutathione *S*-transferase (GST) (15, 16), and NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme activities (17). Few of the early studies addressed the question of whether the increases in UGT, EPH1, GST, or NQO1 activities produced by BHT and BHA were due to induction of gene expression, rather than allosteric activation of the enzymes. However, in 1983, two-dimensional gel electrophoresis was employed to show that levels of GST protein (*i.e.*, GT-8.7 and GT-9.3) were substantially increased in murine liver by BHA (249), and subsequently Western blot analysis confirmed that this was due to increases in class Alpha, Mu, and Pi transferases (208, 209). Following the isolation of cDNA clones for class Alpha and class Mu GSTs, it was demonstrated that feeding mice diets containing BHA produced large increases in mRNA for these enzymes (250).

The majority of chemoprevention studies have used model substrates to monitor drug-metabolizing enzymes, and many fail to make a link between enzyme induction and resistance to specific carcinogens. However, an exception to this generalization has been provided by the body of work into the basis of chemoprevention in the rat against aflatoxin B_1 (AFB_1), a mycotoxin produced by *Aspergillus flavus* that is a potent hepatocarcinogen. Like many genotoxic xenobiotics, AFB_1 requires metabolic activation from a pro-carcinogen to an ultimate carcinogen, through its biotransformation by a CYP isoenzyme(s) to an 8,9-*exo* epoxide that reacts with N^7-guanine in DNA (64, 165). Pretreatment of rats with diets containing EQ prior to exposure to the mycotoxin can confer substantial protection against hepatocarcinogenesis (30). Using an HPLC-based assay, collaborative work between Don Neal, at the MRC Toxicology Unit, Carshalton, and Roland Wolf, at the University of Edinburgh, showed that treatment of rats with EQ resulted in an increased hepatic conversion of AFB_1 to AFM_1 and AFQ_1, relative to the production of the ultimate carcinogen AFB_1-8,9-epoxide (204). Subsequently, the increased production of AFM_1 and AFQ_1 was shown to coincide with induction of CYP 1A2, 2B1/2 and 3A in rat liver microsomes (205), suggesting that these phase I drug-metabolizing enzymes catalyze the hydroxylation of AFB_1; see Figure 2 for an outline of AFB_1 metabolism.

The same assay that was used to examine AFB_1 metabolism by CYP isoenzymes also allowed an EQ-inducible GST subunit, GSTA5 (originally called GST Yc_2), to be discovered in

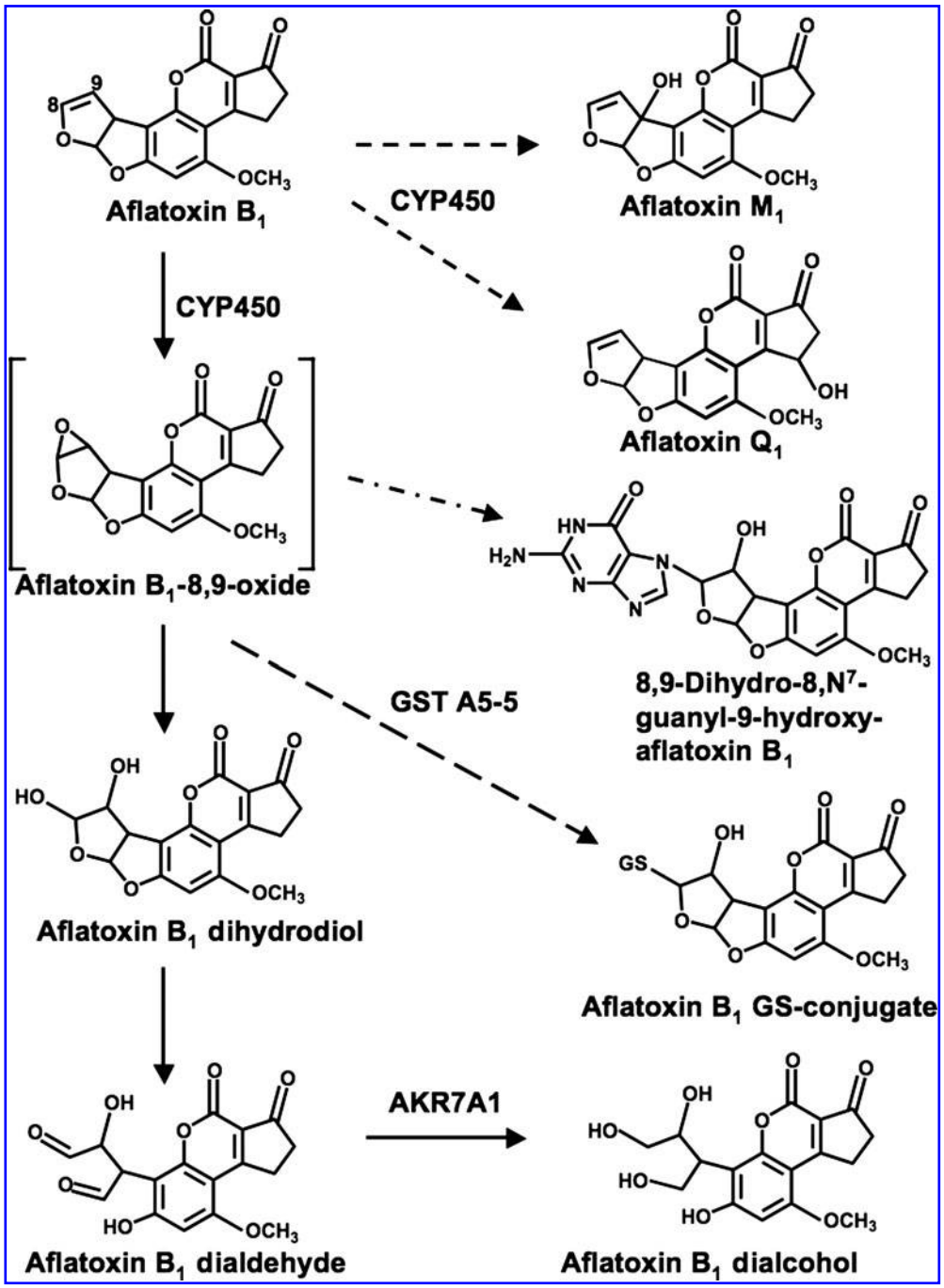

FIG. 2. Metabolism of aflatoxin B_1. The hepatocarcinogen can be metabolized in the liver by cytochromes P450 to AFB_1-8,9-epoxide, which can react with DNA to form 8,9-dihydro-8,N^7-guanyl-9-hydroxyaflatoxin B_1. Alternatively, AFB_1 can be hydroxylated by cytochrome P450 to less toxic metabolites such as AFM_1 or AFQ_1. Once formed, the 8,9-epoxide can be conjugated with GSH, a reaction catalyzed by GSTs containing the A5 subunit, and excreted *via* the mercapturic acid pathway. In addition, the 8,9-epoxide may hydrolyze to form a dihydrodiol and subsequently rearrange to a dialdehyde, which is reduced by AKR7A1 to a dialcohol. Induction of AKR7A1, CYP and GSTA5 by chemopreventive agents diminishes the amount of AFB_1 that forms adducts with DNA.

rat liver that catalyzes the conjugation of GSH with AFB_1-8,9-epoxide (102). When the cDNA encoding rat GSTA5 was cloned and expressed in bacteria (105), it was found to have a very substantially lower K_m for AFB_1-8,9-epoxide and a much higher k_2/K ratio, a measure of enzyme efficiency, than other transferases (142). Indeed, it has been estimated that the specific activity of heterologously expressed rat GST A5-5 for AFB_1-8,9-epoxide (*i.e.*, 30.8 nmol/min/mg protein) is about 200-fold higher than that of heterologously expressed rat GST A3-3 for AFB_1-8,9-epoxide (0.17 nmol/min/mg protein), which is the most closely related rat enzyme to GST A5-5 (107). The hypothesis that induction of *GSTA5* in rat liver, rather than induction of another transferase subunit, confers resistance against AFB_1 genotoxicity is supported by data

obtained from comparative biology studies. In particular, the mouse is intrinsically resistant to AFB_1, and this has been attributed to the fact that it constitutively expresses substantial levels of a class Alpha Gsta3 subunit in the liver that shares 91% identity with rat GSTA5 and exhibits high activity towards the 8,9-epoxide (15.0 nmol/min/mg protein) (103, 105). Heterologous expression of the murine Gsta3 subunit in hamster V79 cells that stably expressed CYP2B1, and could therefore generate AFB_1-8,9-epoxide within the cell, conferred ~5-fold resistance against AFB_1 cytotoxicity and decreased the amount of AFB_1 that was recovered bound covalently to DNA to ~30% following exposure to a standard dose of the mycotoxin (80). Most compellingly, either depletion of GSH (218) or disruption of the *Gsta3* gene renders mice highly sensitive to acute hepatotoxicity caused by AFB_1 and to the formation of AFB_1-DNA adducts (134); *Gsta3*$^{-/-}$ mice were found to exhibit a remarkable 100-fold greater sensitivity to covalent modification of DNA by AFB_1.

At the same time that rat GSTA5 was being characterized, a previously unrecognized highly inducible aldo-keto reductase (AKR) 7A1 was identified (initially called aflatoxin B_1 aldehyde reductase, AFAR) from the livers of rats fed on an EQ-containing diet that produced a unique AFB_1 metabolite (68, 104, 144). AKR7A1 is a dimeric enzyme that catalyzes the reduction of a dialdehyde metabolite of the mycotoxin (97), and it was originally postulated that the reductase would diminish the formation of AFB_1-protein adducts through a Schiff base mechanism rather than provide protection against formation of AFB_1-DNA adducts (104, 144). From overexpression of AKR7A1 in human lymphoblastoid cells, as well as in monkey kidney COS7 cells, it has been reported that the reductase does indeed protect against both the formation of AFB_1-protein adducts and the cytotoxic effects of AFB_1-dialdehyde *ex vivo* (22). However, whilst overexpression of AKR7A1 in transgenic rats decreased formation of AFB_1-protein adducts *in vivo*, its overexpression did not diminish acute hepatotoxicity or the occurrence of preneoplastic foci (267). It has therefore been proposed that the ability of AKR7A1 to protect against AFB_1 cytotoxicity may depend on the dose of the mycotoxin (69).

When taken together, the studies outlined above show that the metabolism of AFB_1 in the rat can be profoundly altered by the dietary administration of EQ through induction of CYP1A2, CYP2B1, CYP3A, GSTA5, and AKR7A1, though it seems likely that GSTA5 is more important than AKR7A1 in protecting the rat against AFB_1 hepatocarcinogenesis.

As was the case with EQ, it was similarly found that pretreatment of rats with BHA, BHT, oltipraz, or CDDO-Im confers resistance against AFB_1 hepatocarcinogenesis (141, 155, 328, 336). In these cases, protection against the mycotoxin was associated primarily with changes in the expression of GSTA5 and AKR7A1, rather than CYP isoenzymes. The phytochemical coumarin, present in legumes, that also protects against AFB_1 hepatocarcinogenesis, has similarly been found to be a potent inducer of GSTA5 and AKR7A1, and whilst it does not induce CYP1A, CYP2B, and CYP4A, it upregulates CYP3A modestly (108, 152).

Induction of genes for antioxidant proteins

Through their investigations into the antimutagenic effects of BHA, Batzinger *et al.* (14) discovered that feeding mice diets containing phenolic antioxidants produced an increase in the nonprotein thiol content, comprising primarily reduced glutathione (GSH), of the small intestine, liver, kidney, and lung. The demonstration that BHA induced expression of the catalytic subunit of glutamate-cysteine ligase (*i.e.,* GCLC, also previously called γ-glutamylcysteine synthetase heavy subunit), the enzyme that catalyzes the rate-limiting step in GSH biosynthesis, was made later by Dave Eaton and colleagues at the University of Washington (25). Also, *tert*-butyl-1,4-hydroquinone (tBHQ), a metabolite of BHA that is itself oxidized to *tert*-butyl-1,4-benzoquinone, the compound that is ultimately thought to be responsible for gene induction by the phenolic antioxidant (109, 318), can induce both GCLC and the regulatory GCLM subunit (195). Predating the discovery that BHA and tBHQ induce GCLC, it had also been reported that treatment of rats with BHA, EQ or oltipraz significantly increased glutathione reductase (GSR) and glucose-6-phosphate dehydrogenase (G6PD) enzyme activities (154). Importantly, GSR converts oxidized glutathione (GSSG) to GSH whereas G6PD generates NADPH; but interestingly, GSR requires NADPH as a cofactor. These data provided evidence that chemopreventive agents coordinately increase the antioxidant capacity of the cell. Moreover, they suggested that there might be a fundamental link between the induction of certain drug-metabolizing enzymes and the upregulation of mechanisms that facilitate glutathione homeostasis.

The ability of BHA, BHT, and EQ to induce the expression of GST and other cytoprotective genes is not a unique feature of phenolic antioxidants but is also a property exhibited by dithiolethiones (155). Such changes were not limited to GSH metabolism as dithiolethione and various other chemopreventive agents were found to induce heme oxygenase 1 (HMOX1) (255), the enzyme that converts heme to biliverdin, which is then metabolized to the antioxidant bilirubin by biliverdin reductase. By using subtractive hybridization, Primiano *et al.* (256) discovered the ferritin heavy (FTH) and light (FTL) chains to be induced by 1,2-dithiole-3-thione (a congener of oltipraz), an observation indicating that chemopreventive agents can augment the ability of the cell to sequester iron and presumably prevent Fenton-type reactions. Notably, induction of ferritin occurs coordinately with induction of HMOX1, and this level of orchestration is especially important in view of the fact that iron is released during degradation of heme by the oxygenase (311). Tom Kensler and his collaborators at Johns Hopkins University also discovered prostaglandin reductase 1 (PTGR1), frequently called leukotriene B_4 12-hydroxydehydrogenase or NAD(P)H-dependent alkenal/one oxidoreductase, to be a dithiolethione-inducible gene in rat liver (51, 257). These findings therefore serve to emphasize that chemopreventive agents do not merely induce drug-metabolizing enzymes, but that they increase the expression of various proteins with broad antioxidant activities.

The studies described briefly above provided the basis for the widely accepted hypothesis that BHA, BHT, EQ, and dithiolethiones protect against carcinogenesis by inducing not only phase I and phase II drug-metabolizing enzymes but also endogenous antioxidant systems in a fashion that optimizes the capacity of the host to detoxify carcinogens, to limit the formation of reactive oxygen species (ROS), and to prevent the secondary metabolites formed by ROS, such as the α,β-unsaturated carbonyls 4-hydroxynonenal and acrolein, from damaging DNA.

Properties of Chemopreventive Agents That Induce Detoxication and Antioxidant Genes

Chemical signature of inducing agents

Induction of Nqo1 enzyme activity in mouse Hepa1c1c7 hepatoma cells has been employed extensively to screen potential chemopreventive agents (74, 258). Use of this assay has revealed that a remarkably large number of xenobiotics can increase quinone reductase activity: inducers have been grouped into ten distinct chemical classes, namely, Michael acceptors (olefins or acetylenes conjugated with electron-withdrawing groups), oxidizable diphenols and diamines, conjugated polyenes, hydroperoxides, trivalent arsenicals, heavy metals, isothiocyanates, dithiocarbamates, dithiolethiones, and vicinal dimercaptans. Talalay and his colleagues appreciated that despite this astonishing structural diversity, all inducers possess the ability to react with sulfhydryl groups by oxido-reduction, alkylation, or thiol–disulfide interchange (297). Although somewhat controversial at the time, these workers hypothesized that gene induction was not mediated by a classic receptor mechanism, but rather that gene induction was under the control of an intracellular 'sensor' that responded to a chemical signal; this hypothesis was strengthened by the findings that many inducers are GST substrates (287), and inducer potency parallels reactivity with sulfhydryl agents (55). Based on these observations, it was postulated that the 'sensor' would contain reactive cysteine residue(s) that might be modified by inducing agents (106, 297). Some inducers are able to react directly with thiol groups in proteins or in GSH, whilst others need to be metabolized first. For example, isothiocyanates can interact with GSH directly, whereas BHA, BHT, and EQ require to be metabolized to electrophilic quinones or α,β-unsaturated carbonyl-containing compounds in order to serve as inducing agents (109, 318). In the case of oltipraz, it appears that the dithiolethione is subject to reductive cleavage resulting in the generation of a superoxide anion that is responsible for gene induction (118).

Naturally occurring isothiocyanates and epithionitriles serve as inducing agents

Isothiocyanates are encountered in the human diet during the consumption of cruciferous vegetables as glucosinolate hydrolysis products, generated by the β-D-thioglucosidase enzyme myrosinase (EC 3.2.1.147) (23, 111). Glucosinolates are composed of a β-D-thioglucose group, a sulfonated oxime moiety, and a highly variable side-chain that is derived from amino acids. The type of glucosinolates, and their abundance, varies considerably in different cruciferous vegetables: for example, broccoli contains substantial amounts of glucoraphanin and glucoiberin, whereas cabbage contains glucoiberin, sinigrin, and glucobrassicin. Myrosinase is present in specialized cells within the vegetable and when released, during chewing of the plant, it hydrolyses the β-glucosyl bond in glucosinolates, resulting in the liberation of glucose and the generation of an unstable thiohydroxamate-*O*-sulfonate that contains a variable amino acid-derived side-chain (72). Once formed, the thiohydroxamate-*O*-sulfonates readily undergo a 'Lossen' type rearrangement to release sulfate and, in so doing, yield a variety of indoles, thiocyanates, isothiocyanates, and nitriles. The hydrolysis products generated by myrosinase are dependent on the glucosinolate and the reaction conditions. Although mammalian tissues do not contain myrosinase, hydrolysis of glucosinolates takes place in mammals by a combination of the actions of endogenous plant myrosinases and those in the microflora of the gastrointestinal tract. When the plant myrosinase activity is destroyed (*e.g.*, by boiling), glucosinolate hydrolysis still occurs in healthy human subjects, but it can be completely abolished by antibiotic treatment or mechanical bowel cleansing (275).

Following the recognition that many inducers of Nqo1 in Hepa1c1c7 cells are thiol-reactive, the quinone reductase bioassay was used to identify sulforaphane from broccoli (*Brassica oleracea* var. *italica*) extracts, along with synthetic isothiocyanate-containing analogues, as possible chemopreventive agents (252, 345, 348). Many years later, sulforaphane still remains one of the most potent phytochemical inducers known to date. In the ensuing years after its discovery, sulforaphane or sulforaphane-rich broccoli extracts have been demonstrated to protect against tumor formation in the following animal models: *I,* mammary carcinogenesis in rats initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) (346); *II,* gastric carcinogenesis in mice produced by benzo[*a*]pyrene (BP) (73); *III,* intestinal polyps in mice that are genetically predisposed to multiple intestinal neoplasia (Apc^{Min} mice) (123, 230, 278); *IV,* lung adenocarcinomas in mice treated with the tobacco carcinogens BP and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (42); *V,* skin carcinogenesis in mice produced by DMBA/12-*O*-tetradecanoylphorbol 13-acetate (TPA) (91); *VI,* skin carcinogenesis in mice caused by ultraviolet (UV) irradiation (60); *VII,* pancreatic tumor formation in Syrian hamsters treated with *N*-nitroso-bis(2-oxopropyl)amine (173), and *VIII,* bladder carcinogenesis in rats treated with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (228). Curiously, the isothiocyanates were among the first compounds with documented chemopreventive activity; Lee Wattenberg (321, 322, 324, 325), who was a pioneer in this area, demonstrated that benzyl-, phenyl-, and phenethyl- isothiocyanate inhibited the carcinogenic effects of polycyclic aromatic hydrocarbons (see Fig. 3 for structures).

Glucosinolates are abundant in young sprouts of broccoli and are particularly effective at inducing Nqo1 activity in Hepa1c1c7 cells (71). Based on the notion that seeds also contain large amounts of glucosinolates, and therefore ought to exert powerful chemopreventive properties, we have fed mice with diets containing crushed broccoli seeds (15% by weight) and found significant induction of Nqo1, Gclc, Gsta3, and Gstm1 in stomach, small intestine, and liver (214). Aqueous extracts from cabbage and Brussels sprouts seeds also induce *NQO1* gene expression in rat liver RL34 cells (M. O. Kelleher, N. Thomas and J.D. Hayes, unpublished results).

In the case of alkenyl glucosinolates such as sinigrin, with a terminal double-bond in their side-chains, hydrolysis by myrosinase at pH 7 results in the production of 2-propenyl isothiocyanate [also called allyl isothiocyanate]. Uniquely however, these alkenyl glucosinolates yield epithionitriles, such as 1-cyano-2,3-epithiopropane (Fig. 3), when they are hydrolyzed by myrosinase at pH 4 in the presence of both epithiospecifier protein and ferrous ions (23, 111). It has been known for many years that 1-cyano-2,3-epithiopropane is the major hydrolysis product from sinigrin in cabbage (178), but the ability of this epithionitrile to induce cytoprotective genes has only relatively recently been demonstrated (151). Further *in vivo* work is required to establish the utility of epithionitriles as chemopreventive agents.

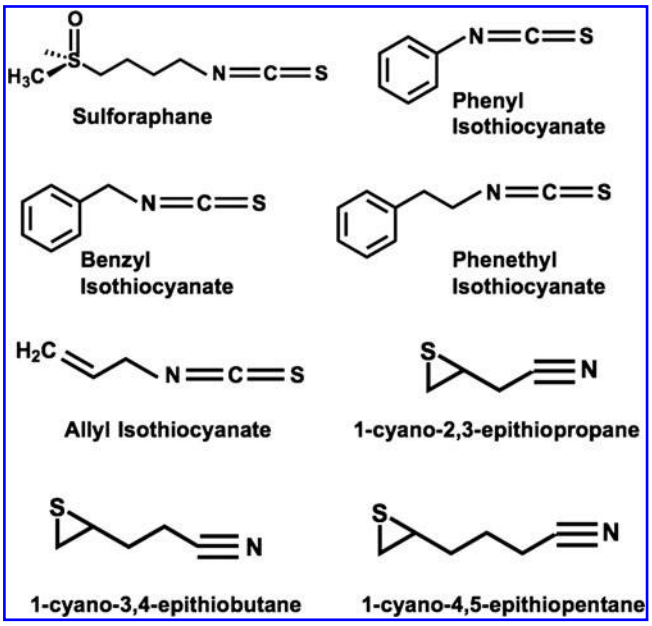

FIG. 3. Glucosinolate breakdown products that induce *NQO1*. The structures of isothiocyanates and epithionitriles that upregulate drug-metabolizing enzymes are shown.

Michael reaction acceptors, diphenols, and triterpenoids as inducing agents

In addition to isothiocyanates and epithionitriles, many other compounds that induce NQO1, such as carotenoids, curcumin, flavonoids, indoles, organosulfides, and polyphenols, are present in edible plants (Table 1), strongly suggesting that dietary habits influence the expression of genes that protect against carcinogenesis (57). Indeed, this hypothesis is supported by epidemiological data indicating that an inverse relationship exists between the consumption of vegetables and fruit, and the incidence of bladder, colon, gastric, and lung cancer; the evidence that isothiocyanates inhibit carcinogenesis appears to be more persuasive than the evidence for other phytochemicals (193, 225, 226, 307, 351). The structures of some of the better-characterized natural inducing agents are shown in Figure 4. Several of these natural products have been used as platforms for the synthesis of highly potent synthetic derivatives. In common with their parent molecules, these synthetic compounds have been first shown to induce *Nqo1* and subsequently to prevent tumor development in animal models, and *vice versa*.

The Michael-acceptor containing fumaric acid, found in shepherd's purse *(Capsella bursa-pastoris)*, is protective against chemically-induced carcinogenesis in rat liver (171, 172), and in mouse forestomach and lung (170). Dietary dimethyl fumarate increases tissue levels of cytosolic GST and NQO1 activities in mice and rats (286). In humans, fumaric acid salts and esters are already used as therapeutic agents, for example, ferrous fumarate for iron deficiency (356) and alkyl esters for psoriasis (5, 238). Dimethyl fumarate reduces the formation of new inflammatory lesions in clinical trials of patients with relapsing-remitting multiple sclerosis (145, 274). The double Michael acceptor curcumin [1,7-bis(3-methoxy-4-hydroxyphenyl)-1,6-heptadiene-3,5-dione] from turmeric (*Curcuma longa*), the principal coloring and flavoring agent of curry, induces *Nqo1* (54) and inhibits tumor development in several animal models of skin, liver, oral, stomach, intestinal, and colon carcinogenesis (see 101 and 294 for comprehensive reviews). Curcumin is remarkably nontoxic and is well tolerated by humans at doses up to 12 g/day (179). Curiously, despite the apparently poor bioavailability of curcumin, its effects are not limited to the gastrointestinal tract, but occur in many organs, including the brain (334). As of March 2010, curcumin has been or is currently part of forty-four different clinical trials targeting various disease conditions, including patients with multiple myeloma, pancreatic cancer, colon cancer, myelodysplastic syndromes, as well as psoriasis, and Alzheimer's disease (www.clinicaltrials.gov).

One of the first phytochemicals shown to induce *Nqo1* in Hepa1c1c7 cells, albeit weakly, was coumarin (48), which is present in leguminous plants. More than a decade later, its hydroxylated derivative, 3-hydroxycoumarin, was found to be >300-fold more potent at increasing Nqo1 activity, whereas 3-acetylcoumarin was only ~2-fold more potent than the parent compound, implying that a hydroxyl substitution at the 3-position improves inducer potency dramatically (53). Incorporation of coumarin into the diet was also effective at inducing both NQO1 and GST activities in rat liver and mouse small intestine (152, 210). Furthermore, coumarin significantly inhibited tumor growth in several liver, prostate, and mammary cancer models in the rat, and was especially effective when given prior to administration of the carcinogen (79, 152, 207, 310).

The oxidizable diphenol carnosol from rosemary (*Rosmarinus officinalis*) protects against the development of: *I,* skin papillomas in mice produced by DMBA/TPA treatment (126); *II,* mammary carcinogenesis in rats produced by DMBA (283); and *III,* intestinal adenoma formation in Apc^{Min} mice (219). Quercetin, which contains both Michael acceptor and catechol functionalities, is a flavonoid abundant in many plants that are common components of the human diet, for example, apples, onions, and green and black tea. Quercetin is an Nqo1 inducer and effectively prevents chemically induced carcinogenesis in many animal models (reviewed in 229). In humans, epidemiological studies have revealed an association between quercetin intake and lower incidence of lung cancer (117). Caffeic acid phenethyl ester, found in honey, is another oxidizable diphenol/Michael acceptor that induces detoxication and antioxidant genes (12) and has demonstrated antitumor activities (reviewed in 303).

The quinone methide triterpene celastrol from the traditional Chinese medicinal plant known as "Thunder of God Vine" (*Tripterygium wilfordii*) is an inducer of *Nqo1* (A.T. Dinkova–Kostova and P. Talalay, unpublished observations). Celastrol inhibits tumor growth of human glioma and prostate xenografts in mice (127, 335, 354), and prevents osteolytic bone metastasis in the rat (129). The Michael-acceptor bearing synthetic derivatives of the naturally occurring triterpenes, oleanolic acid and betulinic acid, are exceedingly potent inducers of Nqo1 (59, 188) and inhibitors of tumor formation and development in several animal models. The triterpenoid CDDO-methyl ester (CDDO-Me) delayed the development of ER-negative mammary tumors in MMTV-neu mice (191); the structures of triterpenoids are shown in Figure 5. Furthermore, in the same model, treatment of tumors that had already been established with CDDO-Me arrested their growth ~90%. Similarly, CDDO-Me, CDDO-ethylamide, and CDDO-methyl amide all reduced the number, size, and severity of the histo-

Table 1. Cancer Chemopreventive Agents that Induce Drug-Metabolizing and Antioxidant Enzymes

Class of chemical	*Compound*	*Source*
Carotenoids	β-carotene	Mangoes, carrots, etc
	Lycopene	Tomatoes
Curcumin and analogs	Curcumin	Tumeric
	Dibenzoylmethane	Synthetic
	Salicylcurcuminoid	Synthetic
	Yakuchinone B	*Zingiberaceae*
Cyclic lactones	α-Angelicalactone	*Archangelica officinalis*
	Coumarin	Leguminosae spp.
Diterpenes	Cafestol	Green coffee beans
	Kahweol	Green coffee beans
Dithiolethiones	Oltipraz	Synthetic
Epithionitriles	1-Cyano-2,3-epithiopropane	Brussels sprouts, cabbages
	1-Cyano-3,4-epithiobutane	Kale
Flavonoids	β-naphthoflavone	Synthetic
	Fisetin	Acacia, mangoes
	Kaempferol	Apples, broccoli, tea
	Quercetin	Apples, capers
Indoles	Indole-3-acetonitrile	Brussels sprouts, cabbages
	Indole-3-carbinol	Brussels sprouts, cabbages
Isothiocyanates	Allyl ITC	Brussels sprouts
	Benzyl ITC	Garden cress
	Eugenol	Cloves, cinnamon
	6-methylsulfinylhexyl ITC	Wasabi
	Phenethyl ITC	Turnips, watercress
	Sulforaphane	Broccoli
Organosulfides	Allyl methyl disulfide	Garlic
	Diallyl disulfide	Garlic
	Diallyl sulfide	Garlic
Phenols	Butylated hydroxyanisole	Synthetic
	Butylated hydroxytoluene	Synthetic
	Caffeic acid	Lignin-containing plants
	Ellagic acid	Grapes, strawberries
	Ethoxyquin	Synthetic
	Ferulic acid	Apples, cabbages, plums

The list of chemicals compiled is based on information presented in References 106 and 108.
ITC, isothiocyanate.

pathology of vinyl carbamate-initiated lung tumors in A/J mice (189, 190). CDDO-Im is highly effective (100 times more potent than oltipraz) at inhibiting AFB_1 hepatocarcinogenesis in the rat (336). Topical applications of nanomol quantities of the oleanane dicyanotriterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile (TP-225) decreased skin tumor multiplicity in UV-irradiated SKH-1 hairless mice (62). Oral administration of a related acetylenic tricyclic bis-(cyano enone), TBE-31, significantly reduced the formation of AFB_1-DNA adducts and decreased the size and number of preneoplastic hepatic lesions produced by the mycotoxin in rats by >90% (192). Two synthetic triterpenoids [*i.e.*, CDDO (bardoxolone) and CDDO-Me], are currently in six clinical trials, targeting several pathologies, including hepatic dysfunction, chronic kidney disease, diabetic nephropathy, lymphoid malignancies, and advanced metastatic or unresectable solid tumors.

The ARE Directs Gene Induction by Chemopreventive Agents

Characterization of the antioxidant response element

Those genes that are transcriptionally activated by chemopreventive agents are co-induced through an ARE present in their promoters. Cecil Pickett and his colleagues at Merck Frosst, Montreal, first identified this *cis*-element as a sequence in the regulatory region of rat *GSTA2* that controlled both basal and inducible gene expression (269). As the enhancer was found using β-naphthoflavone (β-NF), it was initially referred to as a β-NF-responsive element. At around the same time, the research group of Violet Daniel at the Weizmann Institute, Israel, identified the same *cis*-element in the mouse *Gsta1* gene promoter and called it an electrophile responsive element (EpRE) because it was responsible for induction of a reporter gene by tBHQ, dimethyl fumarate and *trans*-4-phenyl-3-buten-2-one (83). Upon further examination, the rat *GSTA2*-ARE was also found to be activated by tBHQ, as well as di-phenols that can redox-cycle, and because it responded to pro-oxidants it was given the name ARE. Following mutational analysis of a 41-bp region in the promoter of rat *GSTA2*, which encompassed the ARE, it was proposed that the 'core' enhancer sequence is 5′-TGACnnnGC-3′ (with essential nucleotides in capitals, and where the letter 'n' represents any nucleotide) (270). At around the same time, closely similar elements that could respond to either BHA or tBHQ and β-NF were found in the promoter of rat and human *NQO1* (77, 140). Although not originally characterized as a

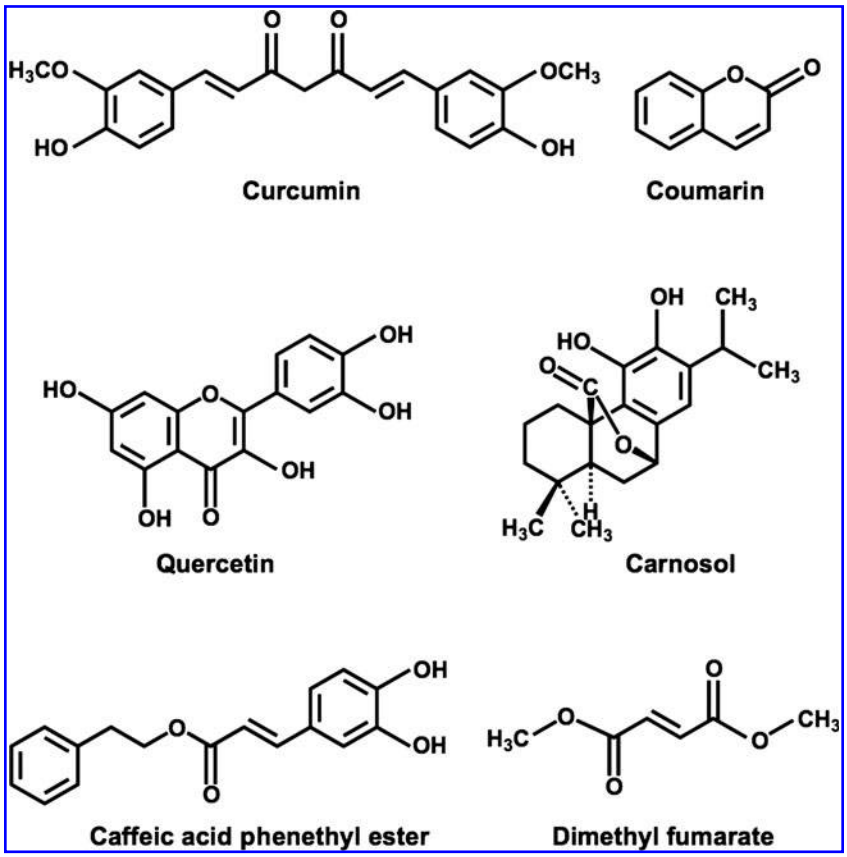

FIG. 4. Phytochemicals in edible plants that induce Nqo1 enzyme activity. The structures of curcumin, carnosol, dimethyl fumarate, quercetin, caffeic acid phenethyl ester, and coumarin are shown.

drug-inducible gene, but rather as a gene upregulated during hepatic preneoplasia, the regulatory region of rat *GSTP1* was shown to contain a *cis*-element designated glutathione transferase P enhancer I (GPEI) that is responsible for increased expression of the gene during liver carcinogenesis (244). The GPEI resembles an ARE and was subsequently found to respond to tBHQ (78).

Nucleotides situated immediately 5′ to the 'core' ARE were shown by deletion analysis to diminish both basal and inducible expression of the reporter gene (270). Consistent with the view that flanking sequences attenuate the activity of the 9-bp 'core' ARE, Wasserman and Fahl (319) proposed that the fully functional enhancer be extended to 20 bp in length. Specifically, by aligning the AREs from rat *GSTA2*, mouse *Gsta1*, rat *NQO1*, human *NQO1,* and rat *GSTP1*, they suggested an ARE consensus sequence could be represented by 5′-TMAnnRTGAYnnnGCRwwww-3′ (where M = A or C; R = A or G; Y = C or T; and W = A or T), with the original 'core' enhancer sequence between nucleotides 7 and 15 shown underlined. Point mutations introduced into a 41-bp region of the mouse *Gsta1* promoter demonstrated that the 5′ tri-nucleotide 'TMA' motif in the ARE is necessary for induction (319). However, subsequent point mutations across the entire ARE in the mouse *Nqo1* promoter demonstrated that the 3′ tetra-nucleotide 'wwww' motif in the enhancer is required for neither basal nor inducible gene expression (239). Thus, the minimal length of the extended ARE appears to be 16 bp, and amongst the genes examined by Wasserman and Fahl (319) can be represented by the consensus sequence 5′-TMAnnRTGAYnnnGCR-3′. Interestingly, point mutations across the entire murine *Nqo1*-ARE also revealed the importance of nucleotides previously thought to be redundant. Within the 5′-TMAnnRTGAYnnnGCR-3′ consensus, five bases are represented as 'n'. However, mutations introduced at two of these positions in the *Nqo1*-ARE 5′-TcA*C*aG**TgAGt*C*gg*C***A-3′, shown underlined and italicized (with the 'core' enhancer in bold and nonessential bases in lower case) completely abolished induction (239). Also, two guanines in the 'core' of the mouse *Nqo1*-ARE that would have been predicted to be essential for function, 5′-TcACaG**T*g*AGtC*gg*C**A-3′ (shown underlined and italicized) were in fact found to be dispensable (239). Thus the analysis of the *Nqo1* regulatory region suggests that AREs in different gene promoters might be more malleable than was originally supposed (239).

Poor conservation between AREs in different genes

In Table 2, a list of ARE enhancers in the regulatory regions of genes for antioxidant, metal-binding, and detoxication proteins, described in the literature, reveals substantial diversity amongst the elements in different genes (13, 38, 46, 70, 77, 84, 100, 116, 140, 143, 153, 162, 163, 200, 217, 227, 239, 241, 245, 268, 270, 272, 285, 305, 312, 341). Within the 9-bp 'core' sequence, a number of AREs contain a G nucleotide at position 4, such as that in the promoter of *Nqo1*, rather than a C or T, and therefore it might be more accurate to depict the 'core' consensus as 5′-TGABnnnGC-3′ (where B = C, or G, or T). This alignment also shows that the 5′ tri-nucleotide 'TMA' motif is not particularly well conserved in different ARE enhancers, whereas the 'core' sequence is better conserved.

It is noteworthy that because of apparent redundancy within the 'core' 5′-TGABnnnGC-3′ sequence (indicated by

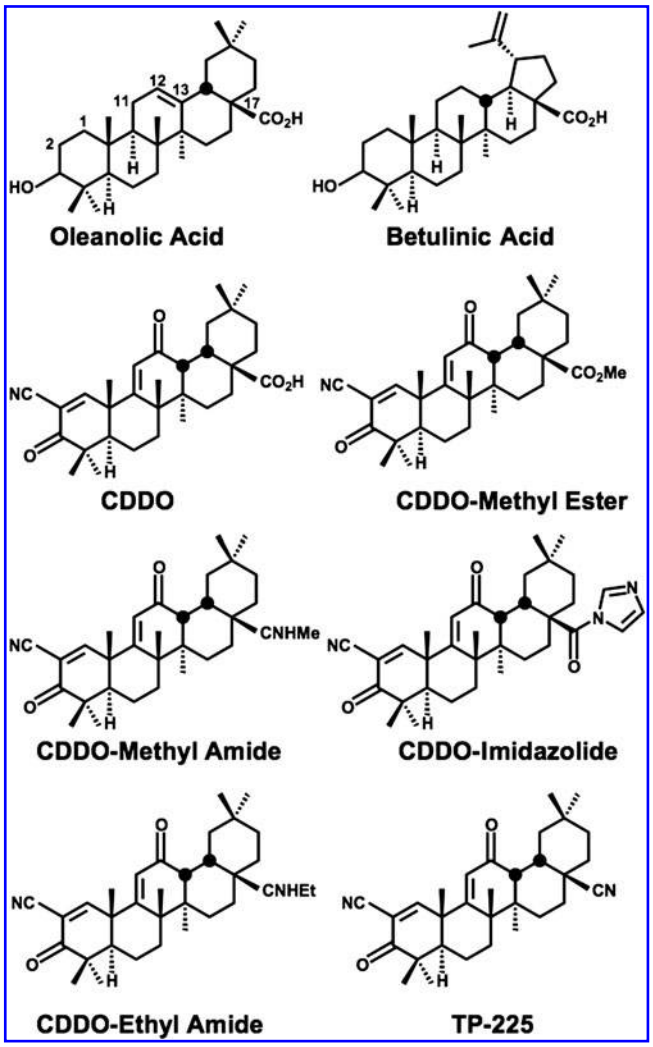

FIG. 5. Triterpenoids used in chemoprevention research. The structures of the naturally occurring oleanolic and betulinic acid, and their Michael-acceptor-containing synthetic derivatives CDDO, CDDO-methyl ester, CDDO-methyl amide, CDDO-imidazolide, CDDO-methyl amide, and TP-225 are shown.

'nnn'), a subset of AREs exists that also incorporate the AP1 DNA-binding sequence 5′-TGASTCA-3′ (where S = C or G) (7). This is an important observation because these ARE/AP1 enhancers ought to be capable of recruiting homodimers of the Jun family members or heterodimers between Jun and Fos proteins.

The variable context, and content, of an ARE suggests the following four distinct classes of enhancer may exist: class 1, an extended 16-bp ARE with the 'TMA' motif plus an embedded AP1-binding site; class 2, an extended 16-bp ARE with the 'TMA' motif without an embedded AP1 site; class 3, a minimal 11-bp ARE plus an embedded AP1-binding site; class 4, a minimal 11-bp ARE without an embedded AP1 site. Examination of the sequences presented in Table 2 reveals that this type of nomenclature is not as clear-cut as first might appear. Nevertheless, the AREs could be grouped as follows: class 1, are found in mouse *Ftl*, human *FTL*, rat *SRXN1*, human *AKR1C2* and human *NQO1*; class 2, are found in mouse *Gsta1* and rat *GSTA2*; class 3, are found in mouse *Mt2*; class 4, are found in mouse *multidrug resistance-associated protein 2* (*Mrp2*). Recognition that certain AREs contain AP1-binding sites, whereas other do not, allows possible gene-specific heterogeneity in basal and inducible expression.

The diversity amongst AREs has three consequences. First, the mutational analyses of *Gsta1*-ARE and *Nqo1*-ARE suggest that the 16-bp class 1 and class 2 enhancers with the 5′ trinucleotide 'TMA' motif will be more highly responsive to chemopreventive agents than those grouped as class 3 or class 4. Second, the dual nature of the class 1 and class 3 enhancers suggests they may respond to additional stressors such as UV radiation and TPA that are mediated by AP1. Third, the extended 16-bp AREs may be less likely to be negatively regulated by various basic-region leucine zipper (bZIP) transcription factors (see below for details).

The heme oxygenase 1 promoter contains multiple AREs

HMOX1 has been regarded as a quintessential stress response protein; in addition to its marked induction by dithiolethiones and phytochemicals such as carnosol, chalcones, curcumin, epigallocatechin-3-gallate, and rosolic acid (6, 12, 81, 206), it can also be strongly upregulated by arsenite, cadmium, cobalt, heme, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, H_2O_2, and UV irradiation (4, 11, 92, 93, 160). It might therefore have been anticipated that induction of *HMOX1* by chemopreventive agents, and many of the phytochemicals, would occur through AREs in its regulatory region. Molecular cloning and bioinformatics have shown the promoter of mouse *Hmox1* and human *HMOX1* both contain numerous sequence-related *cis*-elements, a number of which are clustered around −4.0 kb and −9.0 kb (2, 3, 265, 292). In the mouse, the regions of the promoter around −4.0 kb and −9.0 kb have been called enhancer 1 (E1) and enhancer 2 (E2), respectively, and contain multiple stress response elements (StREs). As shown in Table 3, the majority of the StREs in *HMOX1* contain the 'core' ARE sequence with an embedded AP1 site, though the 5′ adjacent 'TMA' motif is poorly conserved. The magnitude of induction of *heme oxygenase 1* is likely to be due to the presence of numerous AREs in its regulatory region.

Relationship between the ARE and Maf recognition elements

The StREs in *Hmox1* have been likened to musculoaponeurotic fibrosarcoma (Maf) protein recognition elements (MAREs), which have been described in the promoters of human genes for β-globin, porphobilinogen deaminase, and thromboxane synthase (49, 215, 233). The 13-bp MARE is a palindromic 5′-TGCTGASTCAGCA-3′ sequence (where S = C or G) (147, 159, 164, 174, 332). Whilst obvious similarities exist between StREs and MAREs in the region equivalent to the 9-bp 'core' ARE sequence, the six 5′ nucleotides in the extended 16-bp ARE (*i.e.,* TMAnnR) differ from the three 5′ nucleotides in the MARE (*i.e.,* TGC). This comparison raises the interesting point that among authentic 16-bp AREs the tri-nucleotide 'TGC' sequence between bases 4 and 6 is under-represented. For example amongst the enhancers listed in Table 2, none contain the 'TGC' sequence: only the *Gsta1*-EpRE and *GSTA2*-ARE contain a T at position 4, only *GCLM*-ARE-(var), *GPX2*-ARE-1, *Gsta1*-EpRE, *GSTA2*-ARE, *Mrp2*-ARE-1 contain a G at

Table 2. Antioxidant Response Elements in the Promoters of Genes that Are Induced by Chemopreventive Agents

Function	Species	Gene	Element	Orientation	Sequence	Position of 5′ T in 'core' from TSS	Reference
Antioxidant enzymes	Human	*GCLC*	ARE-4/AP1	reverse	**TC**ccc**GTGAC**tca**GCG**	−3118	227
	Human	*GCLM*	EpRE	reverse	ag**A**ca**ATGAC**taa**GCA**	−291 (ATG)	217
			ARE (var)	forward	**TAA**cg**GT**t**AC**gaa**GCA**	−330 (ATG)	70
	Human	*GPX2*	ARE-1	reverse	c**CA**gg**ATGAC**tta**GCA**	−76	13
			ARE-2	reverse	gt**A**ca**GTGA**gagg**GCA**	−387	13
	Mouse	*Gsr1*	ARE-1	reverse	**TC**gcc**GTGAC**taa**GCA**	−35	100
			ARE-2	reverse	**TCA**ca**GTGAC**caa**GCG**	−804	100
	Human	*PRDX1*	EpRE-1	forward	**T**gtaac**TGA**atca**GC**c	−3429 (ATG)	163
			EpRE-2	forward	**T**tctcc**TG**c**C**tca**GC**c	−4322 (ATG)	163
	Human	*PRDX6*	ARE	forward	g**CA**ac**GTGAC**cga**GC**c	−349 (ATG)	38
	Mouse	*Slc7a11*	EpRE-2	reverse	c**CA**gct**TGA**gaaa**GCG**	−440 (ATG)	272
	Rat	*SRXN1*	ARE-1/AP1	forward	**TCA**ccc**TGA**gtca**GCG**	−247	285
	Human	*TRX*	ARE/AP1	forward	**TCA**cc**GT**t**AC**tca**GCA**	−416	162
	Human	*TXNRD1*	ARE	reverse	**TCA**ga**ATGAC**aaa**GCA**	−301	268
Metal-binding proteins	Mouse	*Fth1*	FER1	forward	c**C**tcc**ATGAC**aaa**GCA**	−4076	305
			AP1/NF-E2	reverse	c**CA**cc**GTGAC**tca**GCA**	−4023	305
	Mouse	*Ftl1*	EpRE	forward	**TCA**gc**GTGAC**tca**GCA**	−1118	116
	Human	*FTL*	MARE/ARE	forward	**TCA**gc**ATGAC**tca**GCA**	−1565 (ATG)	116
	Mouse	*Mt1*	ARE	forward	ggcgc**GTGAC**tat**GCG**	−69	46
	Human	*MT1B*	ARE	reverse	g**A**gca**GTGAC**ctg**GC**c	−99	46
	Mouse	*Mt2*	ARE/AP1	forward	ggggt**GTGAC**tca**GCG**	−214	46
Detoxication proteins	Mouse	*Akr1b3*	ARE-1	forward	gg**A**gc**ATGAC**cca**GCA**	−925	241
	Human	*AKR1C2*	ARE	reverse	**TCA**gg**GTGAC**tca**GCA**	−5522	200
	Mouse	*Gsta1*	EpRE	forward	**TAA**tg**GTGAC**aaa**GCA**	−728	84
	Rat	*GSTA2*	ARE	forward	**TAA**tg**GTGAC**aaa**GCA**	−696	270
	Mouse	*Gsta3*	ARE	forward	c**A**ggc**ATGAC**att**GCA**	−147	143
	Rat	*GSTP1*	GPEI/AP1	forward	**TCA**ct**ATGAT**tca**GCA**	−2528	245
	Human	*MGST1*	EpRE	forward	a**CA**tc**GTGAC**aaa**GCA**	−499	153
	Mouse	*Mrp2*	ARE-1	forward	ctggg**ATGAC**ata**GCA**	−94	312
	Mouse	*Nqo1*	ARE	forward	**TCA**ca**GTGA**gtcg**GCA**	−435	239
	Rat	*NQO1*	ARE	forward	**TCA**ca**GTGAC**ttg**GCA**	−421	77
	Human	*NQO1*	ARE/AP1	forward	**TCA**ca**GTGAC**tca**GCA**	−463	140
	Human	*UGT1A1*	ARE	forward	a**AA**cccg**GAC**ttg**GC**c	−3296	341
			ARE 'core'		**TGAC**nnn**GC**		270
			ARE 'consensus'		**TMA**nn**RTGAY**nnn**GCR**		319
			AP1-binding site		**TGAS**tca		7

The sequences shown aligned are from the genes for antioxidant, metal-binding, and detoxication proteins. The nucleotides in bold capital letters are those that share identity with the extended 16-bp ARE consensus sequence (319). The 5′ upstream region (*i.e.*, 10 kb) of each gene was identified and derived using a combination of previously published data and assembled genome database for human, mouse, and rat, which are available through the University of California Santa Cruz (Genome) world-wide website. The promoter region was confirmed, based on the presence of potential promoter regulatory elements, including the TATA box and barbiturate response elements. The positions of AREs are presented with reference to the transcriptional start site (TSS), assigned based on the position of the TATA box that is usually situated approximately 25–35 bp upstream of the TSS. In the absence of a TATA box, the position of AREs is shown relative to the ATG initiation codon; such instances are indicated with ATG being placed in parenthesis. The 16-bp ARE consensus is proposed as 5′-TMAnnRTGABnnnGCR-3′ (where M = A or C; R = A or G; B = C, or G, or T).

position 5, and only *PRDX1*-EpRE-1, *PRDX1*-EpRE-2, *SRXN1*-ARE-1, and *UGT1A1*-ARE contain a C at position 6.

Transcription Factors Involved in Gene Induction by Chemopreventive Agents *via* the ARE

Nrf2 controls basal and inducible expression of ARE-driven genes

Following discovery of the ARE, a number of years elapsed before the transcription factor that mediates chemoprevention was identified. In fact, between 1991 and 1995, intense debate took place concerning whether the ARE, or EpRE, was regulated by AP1 (18, 254, 340). Possibly the most telling piece of information that emerged from this period was that induction of ARE-driven gene activity was observed in mouse F9 embryonal carcinoma cells following transfection of a reporter construct, despite the fact that this cell line apparently lacks AP1 activity (245, 254).

As the ARE resembles a nuclear factor-erythroid 2 (NF-E2) binding site, Venugopal and Jaiswal (308) tested whether members of the cap'n'collar (CNC) family of bZIP transcription factors are responsible for induction of ARE-driven genes

TABLE 3. THE *HEME OXYGENASE* 1 GENE PROMOTER CONTAINS MULTIPLE STRESS RESPONSE ELEMENTS THAT REPRESENT ARE SEQUENCES WITH EMBEDDED AP1 SITES

Function	*Species*	*Gene*	*Element*	*Orientation*	*Sequence*	*5′ T in 'core' ARE from ATG* initiation codon
Stress response	Mouse	*Hmox1*				
			E1, StRE	reverse	ga**gAccGTGAG**cga**GCA**	−126
			E1, StRE	reverse	cac**Ac**ac**TGAC**ttg**GC**t	−3318
			E1, StRE	reverse	tg**a**T**ggATGAC**cct**GC**c	−3547
			E1, StRE	forward	agcTt**cc****TGAG**gct**GC**c	−3612
			E1, StRE	reverse	ca**gAggGTGAC**tca**GCA**	−3990
			E1, StRE	reverse	cc**aAccATGAC**aca**GCA**	−4042
			E1, StRE	reverse	ga**aA**t**cA**ca**AC**tca**GCA**	−4090
			E2, StRE	forward	g**cc****Agc**c**TGAC**tct**GC**c	−6069
			E2, StRE	forward	t**cc**Taac**TGAC**tca**GC**c	−7426
			E2, StRE	reverse	c**ca**g**gc**G**TGAC**taa**GC**t	−8709
			E2, StRE	reverse	g**gaAccATGAC**tca**GCG**	−9734
			E2, StRE	reverse	g**ggAccGTGAC**tca**GCG**	−9763
			E2, StRE	reverse	c**ggAcc**t**TGAC**tca**GCA**	−9791
			E2, StRE	forward	t**g**cgaa**GTGAG**caa**GC**t	−9878
Stress response	Human	*HMOX1*				
			ARE	reverse	cgc**A**t**g**c**TGAT**tca**GC**c	−3231
			ARE	reverse	ga**gA**a**g**c**TGA**ggag**GCA**	−3995
			ARE	reverse	gc**a**ct**gGTGAC**tca**GCA**	−4009
			ARE	reverse	cc**aA**a**cATGAC**gca**GCA**	−4073
			ARE	reverse	gg**g**tca**GTGAC**tcgc**CA**	−4186
			ARE	forward	tg**gA**atc**TGAG**tga**GC**c	−5520
			ARE	reverse	cctttt**cATGAT**tca**GC**c	−6047
			ARE	reverse	ttctg**gATGAT**tct**GCA**	−6080
			ARE	reverse	ga**aA**a**cGTGAC**aag**GCA**	−7184
			ARE	reverse	ta**gA**c**cGTGAC**tca**GCG**	−9059
			ARE	reverse	gg**gA**c**cGTGAC**tca**GCG**	−9088
			ARE	reverse	gg**g**gc**gGTGAC**tta**GCG**	−9117
			ARE	reverse	gg**ga**c**cGTGAC**tca**GCA**	−9146
			ARE	forward	gcctg**gGTGAC**aga**GCA**	−9579
			ARE	forward	gcccc**c**t**TGAG**gca**GC**t	−11779
			ARE 'consensus'		**TMA**nn**RTGAY**nnn**GCR**	
			AP1-binding site		**TGAS** tca	

The *cis*-acting StRE sequences from the 5′ 10-kb upstream region of mouse *Hmox1* and human *HMOX1* have been obtained as described in Table 2. Some of the StREs flanking mouse *Hmox1* have been described by Alam and his colleagues (2, 92, 93), and some of the AREs in human *HMOX1* have been listed by Reichard *et al.* (265). The nucleotides in bold capital letters are those that share identity with the extended 16-bp ARE consensus sequence (319). The 5′ upstream region of mouse *Hmox1* has been reported to contain E1 and E2 enhancer sequences; for the purpose of classification, the position of E1 was allocated to the proximal 5-kb of the upstream region, whereas the position of E2 was considered to reside between 5 kb and 10 kb from the ATG initiation codon.

by chemopreventive agents. They examined Nrf1 and Nrf2 because unlike NF-E2 p45 these factors are widely expressed in nonhemopoietic cells that support gene induction by chemopreventive agents. Using transient transfection experiments, Venugopal and Jaiswal found that both CNC-bZIP proteins could mediate induction of an ARE-driven reporter construct by tBHQ and β-NF (308). Importantly, Nrf2 appeared to be more effective in this regard than Nrf1. A cartoon showing the predicted domain structure of Nrf1, Nrf2, and Nrf3, based on bioinformatics (136, 148, 347, 349), is shown in Figure 6; on the basis of sequence comparisons between human Nrf1, human Nrf2, mouse NF-E2 p45, and chicken erythroid-derived protein with CNC homology (ECH), it was proposed that mammalian Nrf2 proteins comprise six domains, designated Nrf2-ECH homology (Neh) 1–6, with Neh1 representing the CNC-bZIP domain (136).

Definitive proof that Nrf2 is required for induction by BHA came from a ground-breaking study by Masayuki Yamamoto and his colleagues at the University of Tsukuba, Japan, in which they demonstrated induction of *Nqo1* and *Gst* gene expression by BHA was impaired in *Nrf2*$^{-/-}$ mice (137). The Nrf2 null mouse showed a substantial reduction in the basal levels of Nqo1, and class Alpha and Mu Gst subunits, in the liver and small intestine (33, 109, 210, 260). Furthermore, induction of many of these detoxication enzymes by BHA, EQ, coumarin, dithiolethione, and oltipraz was greatly diminished in the mutant mouse. Whilst the majority of ARE-driven genes displayed diminished basal expression in *Nrf2*$^{-/-}$ mice, it was noted that a number of genes, such as *Gsta1* and/or *Gsta2*, *Gsta4,* and *Gstm1*, were still induced by BHA and EQ in the knockout animals. Thus, although the normal homeostatic levels of these transferases were significantly lower in the mutant mice than wild-type mice, they were still found to be inducible by phenolic antioxidants, albeit from a lower baseline (33, 210). This effect was most noticeable in the small intestine, where *Gsta1* and/or *Gsta2*, *Gsta4,* and *Gstm1* were also inducible by coumarin and coffee-specific diterpenes, as well as by BHA and EQ (114, 137, 210). It seems likely that

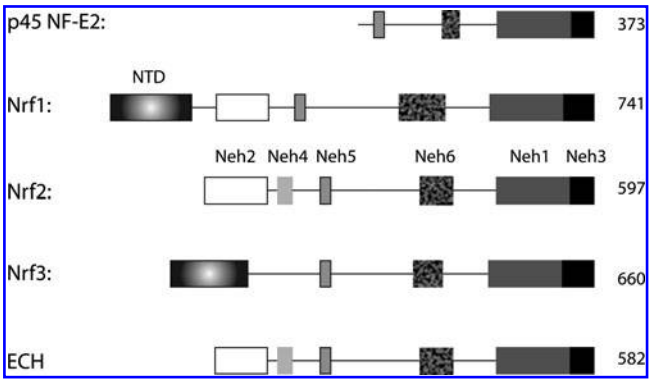

FIG. 6. The domain structures of CNC transcription factors. The location of the Neh1–Neh6 domains in mammalian Nrf2, and the positions of similar domains in NF-E2 p45, Nrf1, and Nrf3, are depicted in the cartoon. The domain structure of Nrf2 was proposed following its comparison with chicken ECH, which is also presented for completeness. The position of the N-terminal domain (NTD), which is only found in Nrf1 and Nrf3, is also shown. The number of amino acids each CNC–bZIP factor comprises is indicated on the *right-hand side*.

Nrf1 mediates at least a portion of the residual induction of these class Alpha and class Mu transferases in *Nrf2*$^{-/-}$ mice because it is capable of mediating induction of an ARE-driven reporter gene by tBHQ (308, 350). In addition, it is possible that in the absence of Nrf2, certain chemopreventive agents can activate other families of transcription factors. This probably applies to agents that can generate ROS during their biotransformation, either by redox-cycling or by the uncoupling of CYP isoenzymes. Possible candidates include AP1 and NF-κB because it has been proposed that whereas activation of Nrf2 enables cells to adapt to low levels of oxidants, activation of AP1 and NF-κB provides a separate tier of defense against moderate levels of ROS; this has been referred to as 'the hierarchical oxidative stress model' (330).

Credence is given to the notion that in the absence of Nrf2, phenolic antioxidants may be able to induce gene expression through stimulation of AP1 activity, because treatment of human liver HepG2 cells with 150 μM BHA or 100 μM BHT for between 3 and 6 h has been reported to produce a robust increase in mRNA for cJun and cFos (37). Consistent with this idea, electrophoretic mobility-shift assays have also shown that treatment of HepG2 cells with 30 μM tBHQ or 50 μM β-NF for between 3 and 24 h increases AP1 DNA-binding activity (1). Moreover, treatment of HepG2 cells with 100 μM tBHQ for 20 h increased substantially the levels of cFos and cJun proteins (1). Caution has to be exercised in the interpretation of these results because the doses of phenolic antioxidants that have been used are relatively high, and it remains unclear whether cJun/cFos might transactivate only genes regulated through dual ARE/AP1 enhancers (class 1 and 3) and not genes with AREs lacking an embedded AP1 site (class 2 and 4).

Dimerization partners for Nrf2

It is widely accepted that Nrf2 is principally responsible for the basal and inducible expression of ARE-driven genes. However, Nrf2 binds DNA as a heterodimer, and there has been much discussion about its dimerization partner. The most compelling evidence suggests that Nrf2 binds to AREs as a heterodimer with small Maf proteins, of which there are three, MafF, MafG, and MafK. These factors contain a bZIP domain and an adjacent extended homology region, but no transactivation domain (Fig. 7). In particular, electrophoretic mobility-shift assays and chromatin immunoprecipitation analyses have revealed that small Maf proteins can bind ARE sequences (234, 239). Thus, small Maf proteins bind AREs as heterodimers with Nrf2, whereas they bind MAREs as homodimers. Consistent with this proposal, knockout of all small Maf proteins in mouse embryonic fibroblasts (MEFs) largely abolished induction of *Fth, Gclc, Gclm, Gsta4, Hmox1, Nqo1,* and *thioredoxin reductase 1* (*Txnrd1*) by diethylmaleate (150), a model glutathione depleting agent that induces NQO1 and GST *in vivo* (106, 152). Furthermore, in genetic rescue experiments, the overexpression of keratin 6 in keratinocytes from *Keap1*$^{-/-}$ mice, which occurred as a consequence of Nrf2 being constitutively active, was abolished in compound knockout mice in which genes for Keap1 and small Maf proteins were disrupted (224).

It has been proposed that cJun influences the expression of a number of ARE-driven genes (94, 99, 182, 304, 309). It is not, however, clear whether Nrf2 can form a heterodimer with cJun, whether cJun can only activate ARE-driven gene expression in instances where an AP1-binding site co-exists within the enhancer (*i.e.,* class 1 and 3 AREs), whether the dose response of gene induction mediated by cJun differs from that mediated by Nrf2, and whether the timing of induction mediated by cJun differs from that mediated by Nrf2. These questions need to be addressed before the contribution of cJun to induction of ARE-driven genes can be stated with certainty.

Modulation of Nrf2 activity by other nuclear proteins

The Neh4 and Neh5 domains of Nrf2 bind cAMP response element-binding protein (CREB)-binding protein (CBP), also called p300, which possesses intrinsic histone acetyltransferase activity (148). It is therefore thought that CBP serves as a co-activator for Nrf2, allowing chromatin to relax around the promoters of ARE-driven genes, thereby facilitating the recruitment of RNA polymerase II (196).

Studies of a number of ARE-driven genes suggest that the activity of Nrf2 is influenced by Brahma-related gene 1 (BRG1), a subunit of SWI2/SNF2-like chromatin-remodeling complexes, in a DNA context-specific fashion. Zhang *et al.* (344) have shown that Nrf2 recruits BRG1 to the promoters of *HMOX1* and *NQO1* primarily through its Neh4 and Neh5 domains. The Nrf2-directed recruitment of BRG1 to the

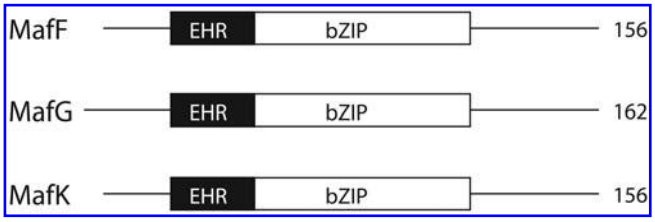

FIG. 7. The domain structures of small Maf proteins. The organization of the bZIP domain and extended homology region (EHR) of MafF, MafG, and MafK is shown.

HMOX1 promoter during treatment with diethylmaleate led to increased binding of RNA polymerase II and increased gene expression. The ability of BRG1 to increase Nrf2-mediated induction of *HMOX1* by diethylmaleate was attributed to the existence of a sequence of 30 GT repeats between nucleotides −200 and −260 from the transcriptional start site that might form a Z-DNA structure with the assistance of BRG1 (344). By contrast, while it was shown that Nrf2 also directed the recruitment of BRG1 to the promoter of *NQO1*, this did not result in increased gene expression; presumably because the *NQO1* promoter lacks a GT repeat sequence capable of forming a Z-DNA structure. Curiously, whilst knockdown of BRG1 diminished induction of *HMOX1* by diethylmaleate, and had no effect on induction of *NQO1, GCLC,* or *GCLM*, it resulted in increased induction of *AKR1C1*. From the knockdown experiments, it has been concluded that in the case of the *HMOX1* gene BRG1 augments the activity of Nrf2, but in the case of *AKR1C1* it inhibits the activity of the CNC-bZIP factor (344). At present, the mechanism by which BRG1 represses *AKR1C1* expression is not known.

Antagonism of Nrf2 activity by other transcription factors

Although poorly understood, it is clear that gene expression through the ARE can be negatively regulated by a number of transcription factors. In most of the cases that have been described, it is uncertain whether repression of ARE-driven genes is a general phenomenon, pertinent to all members of the gene battery, or whether it is restricted to just a subset of genes. The promoter of *MafG* contains an ARE and it, as well as the other small Maf proteins, are inducible by various thiol-reactive agents (149, 220). It is possible that the increased expression in these factors contributes to the downregulation of ARE-driven genes that is observed a few hours after the onset of redox stress, though as mentioned above, small Maf homodimers would be expected to negatively regulate 13-bp MAREs rather than AREs. Alternatively, it is possible that because Nrf2 protein rapidly accumulates following treatment with tBHQ, β-NF, or sulforaphane (211, 235), it is necessary to synthesize more small Maf protein simply to serve as a dimerization partner for the CNC-bZIP protein.

Disruption of the *cFos* gene in the mouse has been reported to increase expression of *Nqo1* and *Gsta1* and/or *Gsta2* (327). Among the organs examined, overexpression of *Nqo1* and *Gsta1* and/or *Gsta2* was most obvious in kidney. Presumably transcription factor cFos negatively controls Nqo1 and class Alpha Gst, but it is not clear whether its knockout alters the expression of all ARE-driven genes, or only certain members of the ARE-gene battery. Unfortunately, it is uncertain whether the effect of cFos knockout on the expression of *Nqo1* and class Alpha *Gst* is direct or indirect. Further work is required to clarify the mechanism by which cFos negatively controls the expression of detoxication genes.

The bric-à-brac, tramtrack, and broad complex and CNC homology 1 (Bach1) protein is a potent repressor of *heme oxygenase 1* expression. It binds StREs (*i.e.,* AREs) in the promoter of mouse and human *HMOX1* as a heterodimer with small Maf proteins. Knockout of Bach1 in the mouse has been shown to cause a marked increase in expression of *Hmox1* (292). Furthermore, knockdown of Bach1 in human HaCaT keratinocytes has been found to affect a profound ~150-fold overexpression of *HMOX1* following transfection with siRNA, but most importantly a similar increase in other ARE-driven genes was not observed (202); interestingly, the low basal expression of *HMOX1* in keratinocytes is partly due to its repression by high constitutive levels of HMOX2 protein (352). These experiments suggest that Bach1 represents the principal negative regulator of *HMOX1*, and that its effect is dominant over Nrf2 and BRG1. Thus, unless Bach1 is displaced from binding AREs in the *HMOX1* promoter, chemopreventive agents are likely to stimulate a relatively low level of induction. The unique level of repression of *HMOX1* by Bach1 appears to be due to the large number of AREs in its regulatory region, which allow the recruitment of multiple Bach1-small Maf heterodimers. In turn, it has been postulated that individual Bach1 proteins bound to different AREs in the *HMOX1* promoter are able to interact physically through their BTB domains, thereby preventing Nrf2 from gaining access to the promoter (63).

The GPEI element in the regulatory region of rat *GSTP1* partially overlaps with a binding site for CCAAT enhancer binding protein (C/EBP) (with the C/EBP consensus binding site underlined, and the 'core' ARE in bold italics, in the enlarged ARE sequence 5′-TCACTA***TGATTCAGC***AAC-3′). In the liver, C/EBPα is highly expressed and is responsible for keeping genes quiescent under normal circumstances. However, during hepatocarcinogenesis, C/EBPα is downregulated, and presumably the reduction in its level is responsible for the derepression of *GSTP1* that is observed in rat hepatic preneoplastic foci (132). Chemopreventive agents such as BHA, EQ, β-NF, and coumarin can stimulate a marked increase in GSTP1 protein in normal rat liver (152, 279), and it therefore seems probable that Nrf2 can effectively compete with C/EBPα for binding to the GPEI element. Many other genes, such as class Alpha *GST, NQO1,* and *EPH1* are induced in rat hepatic preneoplastic foci (76). Moreover, GSH levels are also augmented in such lesions, presumably through upregulation of GCLC and GCLM. It therefore seems plausible that C/EBPα contributes to the repression in liver of certain other ARE-driven genes besides just rat *GSTP1*.

A number of other factors have been reported to antagonize Nrf2. These include activating transcription factor (ATF) 3, estrogen receptor (ER) α, short form estrogen-related receptor (SFERR) β, PPARγ, and retinoic acid receptor (RAR) α, all of which have been reported to inhibit Nrf2 through forming a complex with the CNC-bZIP protein (8, 27, 133, 317, 353). In a separate mechanism, NF-κB/p65 has been shown to antagonize Nrf2 activity by depriving the CNC-bZIP factor of CBP (194). Also, during ROS-induced DNA damage that is sufficiently severe to stimulate apoptosis, p53 has been reported to trans-repress Nrf2 activity by interacting with ARE-containing promoters and thus, at least in the case of the gene for the x-CT subunit of the cystine/glutamate exchange transport system (SLC7A11), interferes somehow with the assembly of the basal transcriptional machinery (75). It is not clear whether ATF3, ERα, SFERRβ, PPARγ, RARα NF-κB, or p53 attenuate the actions of chemopreventive agents, but it is noteworthy that vitamin A deficiency is capable of upregulating ARE-driven gene expression, presumably because it prevents inhibition of Nrf2 by RARα (317).

Regulation of Nrf2 Activity by Control of Its Protein Stability

The Neh2 domain of Nrf2 represents a redox-sensitive degron

The activity of Nrf2 is negatively controlled under normal homeostatic conditions through physical interactions between its N-terminal Neh2 domain and the Kelch-repeat domain of Keap1 (138). In a seminal study by the research group led by Masayuki Yamamoto, it was demonstrated by gene knockout experiments that under normal homeostatic conditions Keap1 is the principal repressor of Nrf2. Thus, in livers from 10-day old *Keap1*$^{-/-}$ mice, Nrf2 protein was found to be present in higher amounts than in livers from wild-type mice of a similar age (313). Also, livers from the mutant mice contained higher levels of mRNA for Nqo1, and Gstp1 and/or Gstp2 than the wild-type mice. Consistent with these observations, MEFs from *Keap1*$^{-/-}$ mice expressed higher levels of Gclc and Nqo1 than did *Keap1*$^{+/+}$ fibroblasts, and neither were induced upon treatment with diethylmaleate.

As shown in Figure 8, Keap1 comprises an N-terminal region (NTR), a bric-à-brac, tramtrack and broad complex (BTB) domain, an intervening region (IVR), a Kelch-repeat domain, and a C-terminal region (CTR). The BTB domain in Keap1 allows it to form a homodimer (355), and the Kelch-repeat domain, along with the CTR, forms a six-bladed β-propeller that serves as a protein-docking site (185, 199, 247). Single particle electron microscopy has revealed that the two BTB domains in dimeric Keap1 form a 'Y-shaped' forked-stem structure that is attached to the two drum-shaped six-bladed β-propellers formed by the Kelch-repeat and CTR domains (242). Most interestingly, the electron microscopy data suggest that the IVR domain is not a free helical linker between the BTB and Kelch-repeat domains, as had been widely supposed, but rather much of it is wrapped around the β-propeller (242). As a consequence, the distance between the dimerization domain and the two β-propellers is much shorter than would be the case if the IVR did not interact with the Kelch-repeat domain.

As described above, dimeric Keap1 contains two β-propeller protein-docking sites. In turn, Nrf2 contains two separate sequences within its Neh2 domain, a low-affinity 'DLG' motif, between amino acids 29–31, and a high-affinity 'ETGE' motif, between amino acids 79–82, with which it binds to the two Kelch-repeat domains in dimeric Keap1 (213, 300). The 'DLG' and 'ETGE' motifs each dock onto different subunits of the dimeric Keap1 protein through electrostatic interactions with a cluster of basic amino acids (Arg-380, Arg-415, and Arg-483) and polar residues (Tyr-334, Ser-363, Asn-382, Ser-508, Gln-530, Ser-555, and Ser-602) situated at the bottom surface of the Kelch-repeat domain (199, 247). Several models have been advanced by various research groups to account for the repression of Nrf2 by Keap1, such as 'anchoring of Nrf2 in the cytoplasm', 'antagonism of Nrf2 nuclear-cytoplasmic shuttling' and 'repression of *Nrf2* gene induction', but the dominant mechanism appears to entail destabilization of the Nrf2 protein through its two-site interaction with Keap1 (for a review of mechanisms, see Reference 112).

Under normal unstressed conditions, the amount of Nrf2 protein is restricted because it is rapidly ubiquitylated and degraded by the 26S proteasome. The ubiquitylation of Nrf2 occurs principally through its Neh2 domain, and if this is deleted, or if either the 'DLG' or 'ETGE' motifs are mutated, the resulting mutant CNC–bZIP protein is substantially more active and longer-lived than the wild-type protein (138, 211, 213); the Neh2 domain therefore contains a redox-dependent degron (211). In homeostatic cells, Keap1 mediates the rapid turnover of Nrf2 by virtue of the fact that it acts as a substrate adaptor protein for the Cul3–Rbx1 E3 ubiquitin ligase (45, 85, 166, 342). Most importantly, the ability of Keap1 to act as a substrate adaptor for Cul3–Rbx1 is redox sensitive and is inhibited by thiol-reactive compounds such as *tert*-butyl-1,4-benzoquinone (generated from tBHQ) and sulforaphane. Thus, in the absence of redox stress, Keap1 recruits Nrf2 to the Cul3–Rbx1 complex, allowing polyubiquitylation of the CNC–bZIP factor to occur at Lys-44, Lys-50, Lys-52, Lys-53, Lys-56, Lys-64, and Lys-68 that lie between 'DLG' and 'ETGE' motifs (Fig. 9). However, upon treatment with a chemopreventive agent, Keap1 is inactivated, the half-life of Nrf2 increases, and the transcription factor accumulates in the nucleus of the cell where it induces target genes. The fact that ubiquitylation of the Neh2 domain of Nrf2 occurs only under normal homeostatic conditions explains why it has been called a redox-sensitive degron.

Keap1 can itself interact with the ubiquitin- and LC3-binding protein sequestosome-1 (SQSTM1), also commonly called p62 (139, 169); in the mouse this protein is sometimes called A170, and in the rat it has been referred to as ZIP (89). Since SQSTM1/p62 serves as a cargo receptor for autophagic degradation, it is possible that the level of Keap1 might be controlled by autophagy (139, 223). Moreover, SQSTM1/p62 is a scaffold protein for a number of kinases (222), and therefore, should Keap1 be controlled by autophagy, this process could be regulated by various signalling pathways.

Activation of Nrf2 by proteins that block the Neh2 degron function

The ability of the 'DLG' and 'ETGE' motifs in the Neh2 domain of Nrf2 to dock onto the two Kelch-repeat and CTR domains of Keap1 can be antagonized by other protein–protein interactions, and this appears to be sufficient to induce ARE-driven gene expression. The first example of this type of regulation of the Keap1-Nrf2 pathway by other proteins was provided by Donna Zhang and colleagues at the University of Arizona who reported that cyclin-dependent kinase p21, which is regulated by the p53 tumour suppressor protein, is capable of interacting with the 'DLG' motif of Nrf2, through a

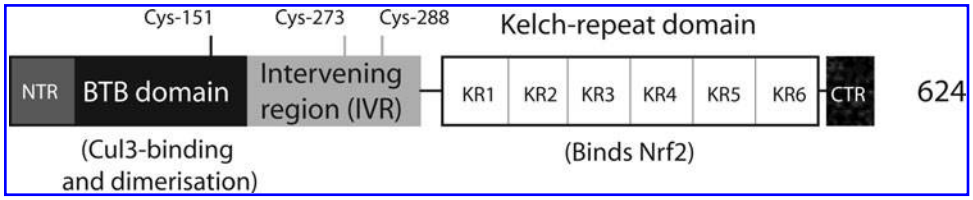

FIG. 8. Structure of Keap1. The position of the N-terminal region (NTR), BTB domain, IVR, the six Kelch-repeat domains, and C-terminal region (CTR) are shown, as is the location of Cys-151, Cys-273, and Cys-288.

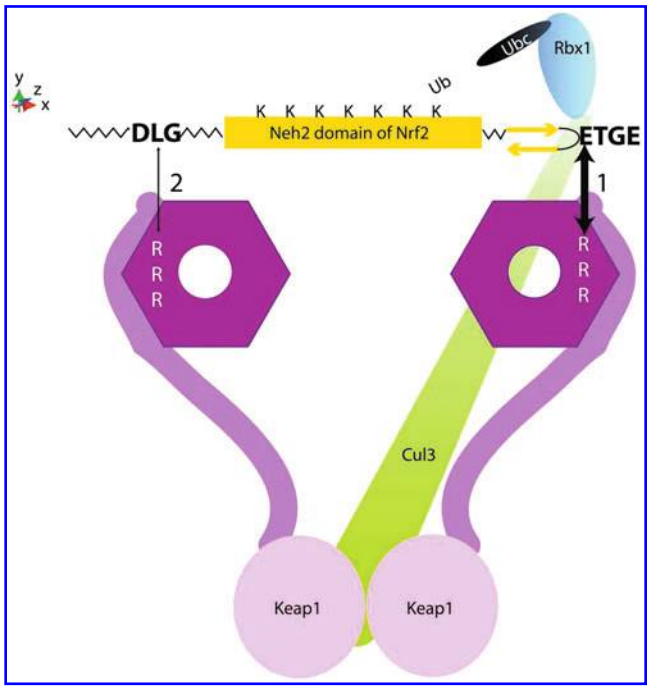

FIG. 9. Ubiquitylation of Nrf2 through the $BC_3B^{Keap1/Keap1}$ complex. A model depicting the complex formed between Nrf2, Keap1, Cul3 and Rbx1 is shown. Within an individual Keap1 subunit, the six-bladed β-propeller formed by the Kelch-repeat and CTR domains is depicted as a hexagon that is partially enveloped by the IVR, as proposed by Ogura *et al.* (242). Two spheres at the bottom of the cartoon depict the BTB domain in Keap1, which is responsible for its dimerization and recruitment of Cul3 to the complex. The high affinity interaction between the ETGE motif in the Neh2 domain of Nrf2 and basic amino acids on the surface of a β-propeller (shown as 'RRR') in one subunit of dimeric Keap1 is indicated at the *top right as a large double-headed arrow against an Arabic numeral 1.* The low affinity interaction between the DLG motif in the Neh2 domain of Nrf2 and the β-propeller in the other subunit of dimeric Keap1 is indicated at the *top left as a relatively slim double-headed arrow against an Arabic numeral 2.* The Rbx1 subunit, which associates with Cul3 and is responsible for the ligation of ubiquitin to the lysine residues in Nrf2 that lie between the DLG and ETGE motifs, is shown at the *top right of the cartoon.* (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

basic 'KRR' tripeptide between amino acids 154–156 (34). Most interestingly from a cancer chemoprevention perspective, these authors found that the levels of Nrf2 and the Nqo1 enzyme in the livers from $p21^{-/-}$ mice were lower than in the livers of $p21^{+/+}$ mice. Furthermore, 12 h after an i.p. injection with 350 mg/kg BHA, the levels of both Nrf2 and Nqo1 were lower in the livers of $p21^{-/-}$ mice than wild-type animals (34). It has therefore been proposed that p53 can activate ARE-driven anti-apoptotic genes by increasing p21 protein levels and stabilizing Nrf2 (301). Notably, it is recognized that p53 is activated by various redox stressors, such as diquat and paraquat (98), suggesting that increases in the level of p21 protein may antagonize Keap1 and thereby contribute to Nrf2-mediated induction of ARE-driven genes more frequently than is currently recognized.

A second way in which the degron function of Neh2 can be inhibited is by other proteins competing with Nrf2 for docking onto the β-propeller of Keap1. Examples of such proteins include prothymosin α, phosphoglycerate mutase family member PGAM5, IκB kinase β (IKKβ), and SQSTM1/p62 (139, 146, 169, 180, 198). In human PGAM5, the sequence ESGE (between amino acids 79 and 82), in human IKKβ, the sequence ETGE (between amino acids 36 and 39), and in SQSTM1/p62, the sequence STGE (between amino acids 349 and 352) are probably responsible for binding to the Kelch-repeat domain of Keap1. Whilst it is unclear whether upregulation of PGAM5 or IKKβ can increase Nrf2 activity, it is noteworthy that knockout of Atg7, which negatively controls SQSTM1/p62, can markedly increase Nrf2 activity (169). Most interestingly, *SQSTM1/p62* is an Nrf2 target gene (139). Thus, activation of Nrf2 induces SQSTM1/p62 expression, causing antagonism of Keap1. This therefore produces a positive feedback loop that will probably result in sustained induction of the ARE-gene battery beyond the period of time that the chemopreventive agent exists in the cell.

The Neh6 domain of Nrf2 represents a redox-insensitive degron

Even when cells are treated with chemopreventive agents, and the substrate adaptor activity of Keap1 is inhibited, Nrf2 is not a long-lived protein. Cyclohexamide-chase experiments have revealed that the Neh6 domain of Nrf2, which in the mouse protein lies between amino acids 329–379, controls its stability under redox stress conditions; it therefore contains a redox-insensitive degron (212). Deletion analyses have suggested that the region in mouse Nrf2 that is responsible for its redox-independent turnover can be narrowed down to amino acids 329–339, but the mechanism involved has yet to be elucidated. It is noteworthy that even when both the DLG motif in Neh2 and residues 329–339 in Neh6 have been removed from mouse Nrf2, the CNC-bZIP protein is not stable (212). It therefore appears that several redox-insensitive degrons exist in Nrf2.

Regulation of Nrf2 by alternative mechanisms

Evidence has been presented that Nrf2 is phosphorylated by PKC, CK2, ERK2, ERK5, GSK3, JNK1, PERK, and PI3K following treatment with various stressors (reviewed in Reference 112). It however remains unclear which of these is most important and, in particular, whether any are dominant over Keap1 when cells are subjected to specific types of stress (*e.g.*, endoplasmic reticulum stress). It is clear from knockout and knockdown experiments that downregulation of Keap1 is sufficient to activate Nrf2-mediated gene expression (50, 313), as is inhibition of Keap1 by upregulation of SQSTM1/p62 (139, 169). It is however unclear whether stimulation of signal transduction pathways can overcome repression of Nrf2 by Keap1. Significantly, mutation of all the MAP kinase sites in Nrf2 appears to have little impact on its activity (293). It therefore appears unlikely that phosphorylation of Nrf2 alters the activity of its Neh2 redox-sensitive degron. However, the possibility remains that phosphorylation of Nrf2 controls the activity of its redox-insensitive degrons, such as Neh6, and this warrants investigation.

He and Ma have reported that several of the Cys residues in Nrf2 can be modified by arsenic (113) but it is not known

whether chemopreventive agents might similarly modify cysteines in the CNC–bZIP protein. Evidence has also been presented that the translation of Nrf2 can be increased by H_2O_2 and sulforaphane through the presence of an inhibitory element within an internal ribosome entry site in the 5′ untranslated region of its mRNA (184), and this represents a further mechanism that might be activated by chemopreventive agents.

Identity of Nrf2-Target Genes

ARE-driven genes regulated by Nrf2 in rodents

Initial experiments to identify Nrf2-target genes in mouse macrophages, liver, and small intestine employed a candidate approach (33, 109, 135, 137, 210); these showed that basal and inducible expression of Gclc, Gclm, class Alpha, Mu and Pi Gst subunits, Hmox1, Nqo1, Prdx1 (also called Msp23), the cystine membrane transporter Scl7a11 (also called x-CT), and SQSTM1/p62 (also called A170) were regulated by the CNC–bZIP protein. Thereafter, microarray analyses were performed by a number of research groups to identify Nrf2-target genes. The first of these examined gene expression in the small intestine of wild-type and mutant mice treated with sulforaphane and led to the unexpected discovery that enzymes involved in the generation of NADPH, G6pd, malic enzyme (Me1), and 6-phosphogluconate dehydrogenase (Pgd), are inducible by the isothiocyanate in an Nrf2-dependent manner (299). Subsequently, microarray experiments were used to examine gene expression in livers and/or small intestine from *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice treated with BHA, CDDO-Im, curcumin, dithiolethiones, phenethyl isothiocyanate, or sulforaphane (124, 125, 177, 231, 276, 277, 336). As an alternative approach, hepatic Nrf2-target genes have been sought by identifying upregulated genes in liver-specific Keap1 null mice (243, 246, 337). Besides the small intestine and the liver, identification of Nrf2-target genes has also been carried out in lungs of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice, where the effects of cigarette smoke and hyperoxia have been investigated (35, 262).

Expression profiling has revealed that Nrf2 dominantly regulates about 100 genes in the mouse, though the expression of possibly as many as 250 genes is influenced by the CNC–bZIP factor. Approximately one-third of the Nrf2-target genes are involved in maintaining cellular redox, binding metals, detoxifying xenobiotics and otherwise contributing to adaptive stress responses (Table 4A). Among these, genes such as *Fth, Gsta1, Gsta3, Hmox1,* and *Nqo1* are now considered highly likely to be regulated directly by Nrf2 because they have been found to contain functional AREs in their promoter regions (2, 84, 116, 143, 239); *Gclc* and *Gclm* were similarly regarded as likely to be regulated directly by the CNC–bZIP factor because their human orthologues were known to contain AREs (70, 217, 227). The microarray experiments showed that the expression of a large number of phase I enzymes, including aldehyde dehydrogenase, aldehyde reductase, carbonyl reductase (Cbr1), carboxyl esterase, and Cyp isoenzymes, that were not known to contain AREs in their gene promoters, are dependent on Nrf2. Surprisingly, as many as two-thirds of the Nrf2-target genes are not involved in detoxication or antioxidant functions, but many of them would nevertheless be regarded as protective. These include inflammation and immunity proteins, and also chaperones.

Collaborative work between the laboratories of Tom Kensler and Masayuki Yamamoto resulted in the production of one of the largest Nrf2-dependent gene sets from mouse liver that has been published to date, and in this organ a substantial number of proteasome-associated proteins were found to be inducible by dithiolethiones in an Nrf2-dependent manner (177). These workers therefore proposed that Nrf2 assists cell survival by regulating the removal of oxidized proteins (176). Consistent with the view that Nrf2 helps eliminate damaged macromolecules from the cell, several research groups have reported that the CNC–bZIP protein controls expression of the adaptor protein SQSTM1/p62 (135, 139, 169, 197, 262), which is a cargo receptor for autophagic degradation and is involved in forming protein aggregates that can ultimately be removed from the cell by lysosomal degradation (see References 21, 128, 248, 329).

Although Nrf2-null rats have not been described, microarray experiments have been performed to identify genes in rat liver that are induced by sulforaphane (122). This approach has revealed that AKR7A1, CYP3A3, CYP3A9, CYP2B19, class Alpha GSTs, MT1, MT2, and UGT were induced by the isothiocyanate. It is gratifying to note that a significant number of the genes induced in rat liver by sulforaphane have also been found to be induced by chemopreventive agents in the mouse.

ARE-driven genes regulated by Nrf2 in the human

Whilst many researchers have used microarray analysis to examine gene induction by chemopreventive agents in the mouse, this technology has not been widely used to study human tissues (see Table 4B). Following treatment of human IMR-32 neuroblastoma cells with tBHQ, the most inducible genes identified included those for AKR1C1, GCLM, HMOX1, NQO1, and ME1, though a significant increase in mRNA for TXNRD1 was also observed (183). In human HepG2 liver cells and Caco-2 colon cells that had been treated with sulforaphane, increases in mRNA for MT1, MT2, NQO1, and TXNRD1 were reported (302, 338). Similarly, when primary prostate epithelial cells isolated from a patient with benign prostatic hyperplasia were exposed to sulforaphane, pronounced increases in mRNA for GCLC, GCLM, TXNRD1, FTH1, FTL, AKR1C1, AKR1C2, AKR1C4, NQO1, and PTGR1 were observed (32). Especially striking was the magnitude of upregulation of AKR1C family genes, ranging from 12- to 16-fold. The increased expression of this oxidoreductase family is reassuring as members had been found previously to be highly inducible in many cell lines including colon Caco-2, HT-29, and LS-174, keratinocyte HaCaT, liver HepG2, and mammary MCF-7 (24, 28, 39, 50, 200, 316).

In a notable *in vivo* study, gastric mucosa biopsy samples were taken from human volunteers both before and 6 h after they had consumed broccoli soup containing a known dose of isothiocyanate (87). Subsequent microarray analysis of the biopsy samples revealed that consumption of broccoli soup resulted in between 1.5- and 3.0-fold increases in the levels of mRNA for AKR1C1, AKR1C2, CBR1, GCLM, PTGR1, and TXNRD1. Determination of the NQO1 enzyme activity in skin punch biopsies of healthy human volunteers revealed that, despite large inter-individual variations in basal activity levels, quinone reductase activity was increased ~2-fold 24 h after application of a sulforaphane-rich broccoli extract; three repeated applications, at 24-h intervals, led to even greater elevations, with increases of ~4.5-fold being observed (61).

In a separate series of experiments, Nrf2 was activated genetically in human HaCaT keratinocytes by knockdown of Keap1 in order to avoid possible confounding side effects caused by activating other transcription factors that can occur using chemopreventive agents (50, 202). Whole-genome microarray analysis of gene expression in the HaCaT cells revealed that knockdown of Keap1 resulted in ~20 mRNAs being increased >2.0-fold, the majority of which can be classed as encoding either detoxication or antioxidant enzymes. The mRNA for the phase I drug-metabolizing enzymes AKR1B10, AKR1C1, AKR1C2, and AKR1C3 were increased to the greatest extent, between 12- and 16-fold, whilst NQO1 was induced about 4.0-fold (202). By contrast, the mRNAs for the antioxidant proteins GCLC, GCLM, HMOX1, sulfiredoxin (SRXN1), and TXNRD1, along with the cystine/glutamate transporter SLC7A11, were induced between 2.0- and 4.8-fold, the mRNA for the iron-binding protein FTL was increased 2.7-fold, and mRNAs for enzymes involved in the generation of NADPH, G6PD, ME1 and PGD, were induced 1.8- to 2.9-fold following knockdown of Keap1.

An important caveat to the Keap1 knockdown experiments is that in humans (but not mice) the BTB-Kelch protein represses activation of the canonical NF-κB pathway by targeting IκB kinase β (IKKβ) for Cul3-Rbx1 ubiquitylation (180). While it cannot be stated with complete certainty that none of the genes that are upregulated upon Keap1 knockdown might be attributable to increased NF-κB activity, rather than increased Nrf2 activity, it should be noted that double knockdown of Keap1 plus Nrf2 abolished induction of AKR1B10, AKR1C1, NQO1, GCLC, GCLM, HMOX1, SRXN1, and TXNRD1 (202). This proviso, that inhibition of Keap1 might activate NF-κB, also applies to the use of chemopreventive agents too, and this is an issue that needs to be considered when interpreting human chemoprevention data. It seems possible that inactivation of Keap1 by chemopreventive agents might increase NF-κB activity, but only when IKKβ has already been activated by tumor necrosis factor α, interleukin 1, or lipopolysaccharide (251).

Species differences in the ARE-gene battery

Although relatively few human tissues and cell lines have been examined, it is clear that most of the antioxidant genes that are regulated by Nrf2 in the mouse are similarly regulated in the human. However, comparison between genes for drug-metabolizing enzymes that are regulated by Nrf2 in the mouse and human suggests significant differences exist between the two species. Whilst Nrf2 mediates induction of cytosolic class Alpha, Mu, and Pi Gst subunits, as well as the microsomal Mgst3 protein, in murine small intestine, liver, and lung, the question of whether human Gst subunits are inducible is not firmly established. Part of the reason for this lack of certainty is because few studies have been reported, and in those cases where induction of human *GST* genes have been described, the involvement of AREs and Nrf2 in the process is unclear. For example, among human cytosolic and mitochondrial transferase genes, only the upstream regulatory region of *GSTP1* has been reported to contain the potential ARE sequence 5′-GCGCCG***TGACTCAGC***A-3′ (with the 'core' in bold italics), between −75 and −60 bp from the transcriptional start site. While *GSTP1* has been demonstrated to be upregulated in drug-resistant human mammary VCREM cells through an AP1-dependent mechanism (216), it is not known if Nrf2 controls the basal and/or inducible expression of this gene in other cells. Amongst human membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) family members, only the microsomal *MGST1* gene has been shown to contain a functional ARE (153). The MGST1 gene is only modestly induced in human prostate LNCaP, MDA PCa 2B, and PC3 cells (26), though knockdown of Nrf2 in human lung A549 and H460 cell lines has been found to reduce MGST1 mRNA levels by between 58%–75% (280). It is interesting to note that knockdown of Nrf2 in A549 and H460 cells also diminished the levels of mRNA for the cytosolic GSTM4 by 50%–65% (280), though it is not currently known if the promoter of the gene for this transferase contains an ARE.

Using Northern blot analysis to study gene induction in primary human hepatocytes, Fabrice Morel and André Guillouzo and their colleagues showed marked induction (possibly ~10-fold) of GSTA1 and/or GSTA2 mRNA following treatment with sulforaphane or oltipraz (203, 221). In IMR-32 cells, Li *et al*. (183) reported that GSTM3 was induced by tBHQ, and in MCF-10A cells Steiner *et al.* (291) reported that GSTP1 was induced by genistein. Also, in a study of human volunteers who were given escalating doses of sulforaphane in a broccoli sprout homogenate, ~2-fold increases in mRNA for GSTM1 and GSTP1 were reported in cells from the upper airways collected by nasal lavage (266). It should be noted however that Nrf2 has not been shown to mediate the induction of these genes for cytosolic GST.

By contrast with the modest increases in GST observed in human cell lines and in human volunteers, members of the AKR1B and AKR1C families appear to be highly inducible in humans, but are not so obviously induced in mice. For example, Bonnesen *et al.* (24) showed that AKR1C1 and/or AKR1C2 were induced 10-fold in LS-174 and Caco-2 cells by tBHQ and sulforaphane under conditions in which no induction of GSTA1, GSTA2, or GSTP1 was observed. Moreover, the human *AKR1C2* gene promoter contains a functional ARE (200).

The investigations reported to date are limited and more human *in vivo* studies, as well as further characterization of human primary cells, are required before it can be stated with certainty how the human and mouse ARE-gene batteries compare. This type of information is important to allow the selection of suitable biomarkers for the response to chemopreventive agents in human intervention studies.

Association between Nrf2 Activity and Cell Proliferation and Its Relevance to Chemoprevention

It is interesting to note that in certain tissues, activation of Nrf2 appears to increase cell growth. Many reports indicate that administration of BHA, BHT, or ethoxyquin to rodents causes hepatomegaly (47, 253, 339). While these studies do not prove that chemopreventive agents cause liver growth through activation of Nrf2, more recent observations in cells with mutant *Keap1* lend weight to this hypothesis. For example, global knockout of Keap1 in mice results in hyperkeratosis of the esophagus and forestomach (313). Moreover, human lung A549 adenocarcinoma cells, which contain a

Table 4. Groupings of Nrf2-Target Genes

(A) Mouse. Microarray data describing Nrf2-target genes in mouse liver, lung, small intestine, and fibroblasts are summarized.

Function	*Gene*	*Organ/cells*	*References*
Antioxidant proteins	*Gclc*	small intestine, liver, lung, MEFs	35, 150, 177, 243, 246, 262, 277, 299, 336, 337
	Gclm	small intestine, liver, lung, MEFs	35, 150, 177, 262, 299, 336
	Gpx2	liver, lung	35, 243, 246, 262
	Gsr1	small intestine, liver, lung, MEFs	35, 124, 150, 177, 262, 299, 336, 337,
	Prdx1 (Msp23)	liver, lung, MEFs, macrophages	135, 150, 177, 262
	Prdx6	lung	35
	Slc7a11 (x-CT)	lung, macrophages	135, 262
	Srxn1	liver	246
	Trx	liver	177, 336
	Txnip	liver	277
	Txnrd1	liver, lung, MEFs	35, 124, 150, 177, 246, 262, 277, 336
NADPH-generating enzymes	*Me1*	small intestine, liver, lung	35, 124, 125, 299
	G6pd	small intestine, lung	35, 262, 299
	Pgd	small intestine, liver, lung	246, 299, 337
Metal-binding	*Fth*	MEFs	150
	Ftl (chain 1 and/or 2)	small intestine, liver, lung	35, 124, 125, 262, 299, 336
	Mt1	MEFs	150
	Mt2	MEFs	150
Drug-metabolizing enzymes and drug transporters	*Aldh3a1*	small intestine, lung	35, 262, 299
	Akr1a	small intestine, liver	177, 299
	Akr1b7	liver	124
	Akr1b8	small intestine, liver, lung	35, 124, 262, 299
	Akr1c19	liver	246
	Cbr1	small intestine, liver	177, 246, 299
	Cbr3	small intestine, liver	124, 177, 243, 246, 277
	Cyp2a4	liver, lung	35, 177, 262
	Cyp2b9	liver	243, 337
	Cyp2b13	liver	243
	Cyp2c39	liver	177
	Cyp4a10	liver	124, 177
	Cyp4a14	liver	124, 177
	Cyp39a1	liver	124, 246
	Eph1	small intestine, liver	124, 177, 299, 336
	Fmo1	liver	177
	Fmo2	small intestine, liver	231, 243
	Fmo3	liver	243, 246, 337
	Gsta1	small intestine, liver, lung	33, 262, 299
	Gsta2	small intestine, liver, lung	33, 35, 124, 177, 243, 246, 262, 299, 336
	Gsta3	small intestine, lung	35, 262, 277, 299
	Gsta4	small intestine, liver, lung, MEFs	35, 124, 150, 177, 277, 336
	Gstm1	small intestine, liver, lung	33, 35, 124, 125, 177, 277, 262, 299, 336
	Gstm2	small intestine, liver, lung	33, 35, 177, 299, 336, 337
	Gstm3	liver	33, 124, 125, 177, 231, 243, 246, 336
	Gstm4	liver	33, 243, 246, 336, 337
	Gstm5	liver, lung	35, 124
	Gstm6	liver	243, 336
	Gsto1	liver	336
	Gstp1/Gstp2	liver, lung	33, 35, 177, 262, 336
	Gstt1	liver, lung	262, 336
	Gstt2	liver	177, 336
	Gstt3	liver	125, 337
	Mgst2	small intestine	299
	Mgst3	small intestine, liver	177, 243, 299, 336
	Mrp4 (*Abcc4*)	liver	337
	Mrp5 (*Abcc5*)	liver	243, 337
	Mrp12 (*Abcc12*)	liver	243
	Nqo1	small intestine, liver, lung	35, 124, 177, 243, 246, 262, 299, 336, 337
	Ptgr1	small intestine	299
	Sult3a1	liver	124, 243
	Ugt2b5	small intestine, liver	177, 277, 299
Stress-response proteins	*Gadd45g*	lung	262
	Hmox1	liver, lung, MEFs, macrophages	35, 124, 135, 150, 231, 246, 262
	Hspa1a	small intestine, liver, MEFs	124, 125, 150, 277
	Hsp40 (DnaJ)	small intestine, liver, lung	35, 124, 177, 262, 299
	Hsp70	liver	124

TABLE 4. (CONTINUED)

(B) Human. Microarray data for Nrf2-target genes in human tissue and cell lines.

Function	*Gene*	*Organ/cells*	*References*
Antioxidant enzymes	*GCLC*	HaCaT, prostate epithelial cells	32, 202
	GCLM	HaCaT, IMR-32, prostate epithelial cells, gastric mucosa	32, 87, 183, 202
	GPX2	Caco-2	13
	GSR	HaCaT, IMR-32	183, 202
	SLC7A11	HaCaT, prostate epithelial cells, gastric mucosa	32, 87, 202
	SRXN1	HaCaT, prostate epithelial cells	32, 202
	TXNRD1	HaCaT, IMR-32, Caco-2, prostate epithelial cells, gastric mucosa	32, 87, 183, 202, 302
NADPH-generating enzymes	*G6PD*	HaCaT, prostate epithelial cells	32, 202
	ME1	HaCaT, IMR-32	183, 202
	PGD	HaCaT, prostate epithelial cells, gastic mucosa	32, 87, 202
Metal-binding proteins	*FTH1*	prostate epithelial cells	32
	FTL	HaCaT, HepG2, prostate epithelial cells	32, 116, 202
	MT1	HepG2	338
	MT2	HepG2	338
Drug-metabolizing enzymes and drug transporters	*AKR1B1*	HaCaT	202
	AKR1B10	HaCaT	202
	AKR1C1	HaCaT, IMR-32, prostate epithelial cells, gastric mucosa	32, 87, 183, 202
	AKR1C2	HaCaT, prostate epithelial cells, gastric mucosa	32, 87, 202
	AKR1C3	HaCaT	202
	AKR1C4	prostate epithelial cells	32
	CBR1	gastric mucosa	87
	GSTM3	IMR-32	183
	MRP2	Caco-2	302
	NQO1	HaCaT, IMR-32, Caco-2, prostate epithelial	32, 183, 202, 302
	PTGR1	HaCaT, prostate epithelial cells, gastric mucosa	32, 87, 202
Stress-response proteins	*GADD45*	IMR-32, Caco-2	183, 302
	HMOX1	HaCaT, IMR-32	183, 202
	HSP40	IMR-32, prostate epithelial cells	32, 183
	HSP70	IMR-32, gastric mucosa	87, 183

somatic mutation in *Keap1* that renders it nonfunctional, proliferate strongly, but when Nrf2 is knocked down their growth is reduced dramatically (119, 280). It has also been noted that liver regeneration is impaired in $Nrf2^{-/-}$ mice following partial hepatectomy (19), an observation that is consistent with the notion that Nrf2 influences cell proliferation.

A number of mechanisms have been proposed to explain the influence that Nrf2 has on cell growth. The partial hepatectomy experiments, mentioned above, led to the proposal that Nrf2 increases cell proliferation by altering ROS levels, which in turn influences insulin/insulin-like growth factor signaling (19). Based on studies of primary alveolar epithelial cells from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice, Reddy *et al.* (264) have similarly suggested that the influence exerted by the CNC–bZIP factor on ROS levels is responsible for alterations in cell growth. In this case, the impairment of cell cycle progression in $Nrf2^{-/-}$ alveolar epithelial cells occurred principally at the G_2/M-phase of the cell cycle, and was associated with a reduction in the phosphorylation of Akt (264). Interestingly, supplementation of the $Nrf2^{-/-}$ epithelial cells with GSH restored Akt phosphorylation and overcame the G_2/M cell cycle arrest. Transient knockdown of the constitutively active Nrf2 protein in human A549 cells, using siRNA, has been found to cause growth arrest at G_1 in the cell cycle, and this was associated with a decrease in the phosphorylation of the retinoblastoma protein (119). From experiments in A549 cells, it was proposed that high constitutive expression of the ARE-gene battery attenuates ROS levels, and that this is responsible for hyperphosphorylation of retinoblastoma protein. Taken together, these reports suggest that Nrf2 influences cell growth primarily through the upregulation of antioxidant proteins. It can therefore be envisaged that supra-normal Nrf2 activity could be detrimental because excessive amounts of GSH, Trx, NADPH, NADH, and antioxidant proteins may suppress ROS levels and favor cell proliferation; for a review of the role of ROS in cell cycling, see (29).

In addition to controlling redox status, it has also been found that Nrf2 regulates growth factors, growth factor receptors, and integrins (263), and therefore upregulation of the CNC–bZIP factor may activate a number of proliferative pathways. It has recently been found that Nrf2 controls the expression of components of the Notch1 signalling pathway, including *Hes-1, Herp1, Herp2,* and *Nrarp* (315). At present it is uncertain what significance, if any, induction of the Notch1 signaling pathway has in cancer chemoprevention, but it clearly influences liver growth. The possibility that chemopreventive agents may simulate cell proliferation is cause for some concern. It is possible that sustained administration of excessive doses of chemopreventive agents will have undesired proliferative side effects (110, 158).

Table 5. Modification of Cys residues in Keap1

		Electrophile								
Amino acid	*Domain*	*Dex-mes*	*BMCC*	*IAB***	*IAB***	*SFN****	*SFN****	*Xanthohumol*	*Isoliquir*	*10-Shog*
Cys-12	NTR	−	−	−	−	+	−	−	−	±
Cys-13	NTR	−	−	−	−	+	−	−	−	±
Cys-23*	NTR	−	−	−	−	−	−	−	++	±
Cys-38*	NTR	−	−	−	−	+	−	±	−	±
Cys-77	BTB	−	+++	−	−	++	−	±	++	−
Cys-151*	BTB	−	−	−	+++	−	+++	+++	+++	+++
Cys-171	BTB	−	−	−	−	+	−	−	−	−
Cys-196	IVR	−	+++	+++	−	±	−	−	++	−
Cys-226*	IVR	−	−	+	−	++	+	−	+++	+
Cys-241*	IVR	−	−	+++	−	−	−	−	−	++
Cys-249	IVR	−	+	−	−	++	−	−	±	++
Cys-257	IVR	+++	−	++	+	+	+	−	−	+++
Cys-273*	IVR	+++	−	−	+	−	±	−	−	±
Cys-288*	IVR	+++	−	+++	+++	−	+++	−	−	−
Cys-297*	IVR	+++	−	−	+++	−	+	−	−	−
Cys-319*	KR1, β1	−	−	+	++	−	+++	+++	++	++
Cys-368	KR2, β1	−	+++	−	−	+	−	−	±	+++
Cys-395	KR2, β3	−	−	−	−	−	−	−	+	−
Cys-406	KR2, β4	−	−	−	−	−	−	−	−	−
Cys-434	KR3, β2 - β3	−	−	−	−	−	++	+	±	++
Cys-489	KR4, β3	−	+++	−	−	+++	−	+	+	+
Cys-513	KR5, β1	−	−	−	−	++	−	−	++	−
Cys-518	KR5, before β2	−	−	−	−	++	−	−	++	−
Cys-583	KR6, β3	−	−	−	−	+++	−	−	+++	−
Cys-613*	CTR	−	−	−	+	−	++	+++	++	++
Cys-622	CTR	−	−	−	−	−	−	−	−	++
Cys-624	CTR	−	−	−	−	+++	−	−	−	++
Reference		56	121	121	65	120	66	201	201	201

The position and location of Cys residues in mouse and human Keap1 that form adducts with dexamethasone mesylate (Dex-mes), 1-biotinamido-4-(4-[maleimidoethyl-cyclohexane]-carboxamido)butane (BMCC), N-iodoacetyl-N-biotinylhexylenediamine (IAB), sulforaphane (SFN), xanthohumol, isoliquiritigenin (Isoliquir), and 10-shogaol (10-Shog) are listed; note that mouse Keap1 lacks Cys-12 and Cys-13 found in human Keap1. An asterisk (*) indicates those Cys residues that are likely to be more reactive by virtue of the fact they are situated adjacent to basic amino acids (20, 271, 284). The location of each of the cysteines with respect to the domain structure shown in Figure 8 is indicated in the second column. The data are taken from References 56, 65, 66, 120, 121, 201, as indicated at the bottom of the table. **Note that two separate groups studied IAB. ***Note that two separate groups studied SFN.

Keap1 as a Target for Chemopreventive Drugs

Modification of Cys residues in Keap1 by electrophiles

As chemopreventive agents that induce ARE-driven gene expression are capable of reacting with thiol groups (106, 297), it is reasonable to suppose that modification of Cys residues in Keap1 might be sufficient to block the ubiquitylation of Nrf2 by Cul3–Rbx1 and trigger induction of ARE-driven genes. Mouse and human Keap1 both comprise 624 amino acids, and contain 25 and 27 Cys residues, respectively; all the cysteines in mouse Keap1 are present in human Keap1, though the latter protein contains two additional Cys residues at positions 12 and 13. Ten of the 25 cysteines in mouse Keap1 (at positions 23, 38, 151, 226, 241, 273, 288, 297, 319, and 613), and ten of the 27 cysteines in human Keap1 (at positions 13, 38, 151, 226, 241, 273, 288, 297, 319, and 613), are situated adjacent to Arg, Lys, or His residues, and would therefore be expected to be more reactive towards electrophiles because the pK_a of their thiol side chains would be lowered through being situated in a basic environment (20, 271, 284). Based on these observations, it was anticipated that one or more of the reactive Cys residues in Keap1, half of which are located in the IVR domain, might serve as a 'sensor' for inducing agents.

The hypothesis that chemopreventive agents can form adducts with Keap1 has been explored using mass spectroscopy. Through a collaboration between the research groups of Paul Talalay and Masayuki Yamamoto, it was initially shown that cysteines in the IVR of mouse Keap1 could be covalently modified *in vitro* by dexamethasone mesylate (Dex-mes), an inducer of Nqo1 activity that irreversibly alkylates proteins (56) (Table 5). In particular, Cys-257, Cys-273, Cys-288, and Cys-297 were specifically modified by relatively low concentrations of Dex-mes, though at higher doses of the inducer other Cys residues were modified. Subsequently, Dan Liebler and his colleagues at Vanderbilt University used the electrophiles *N*-iodoacetyl-*N*-biotinylhexylenediamine (IAB) and 1-biotinamido-4-(4′-[maleimidoethyl-cyclohexane]-carboxamido)butane (BMCC) to probe human Keap1 in a series of *in vitro* experiments (121). Whilst they found that IAB modified Cys-257 and Cys-288 in human Keap1, it did not form adducts with either Cys-273 or Cys-297, but rather it also reacted with Cys-196, Cys-226, and Cys-241. Unexpectedly, BMCC failed to modify Cys-257, Cys-273, Cys-288, or Cys-297

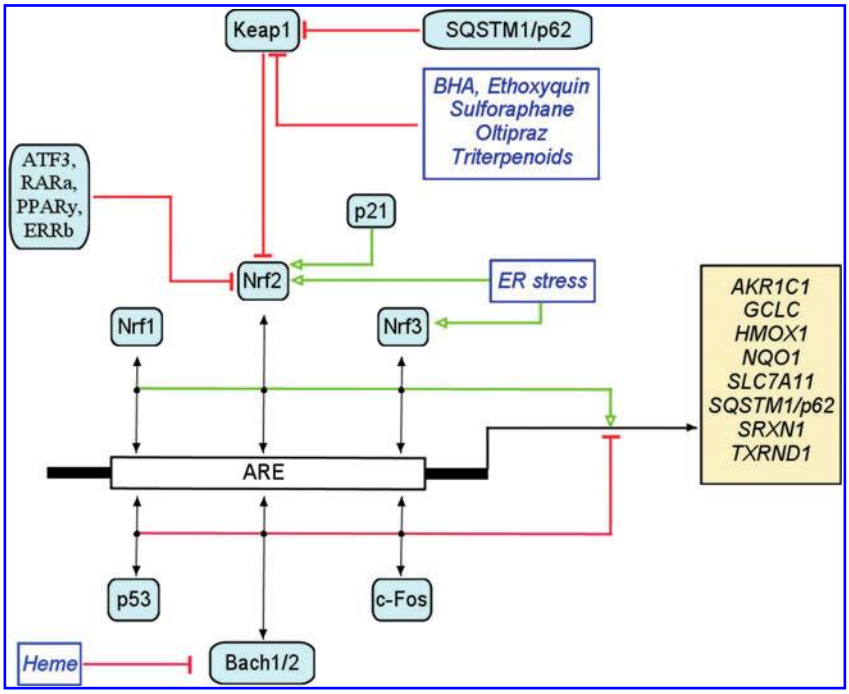

FIG. 10. Regulation of the human ARE-gene battery by Nrf2 and other transcription factors. Keap1 is the principal repressor of Nrf2 under normal homeostatic conditions. Inhibition of the substrate adaptor activity of Keap1 by chemopreventive agents such as BHA, ethoxyquin, sulforaphane, oltipraz, and triterpenoids (*blunt-ended arrow*) results in accumulation of Nrf2 protein and induction of ARE-driven gene expression (211, 235). The SQSTM1/p62 protein also inhibits Keap1 (139, 169). Nrf2 can be activated by p21 through an interaction that prevents Keap1-mediated ubiquitylation of the CNC–bZIP factor (34). Nrf2 and Nrf3 can also be activated by endoplasmic reticulum (ER) stress (44, 349). Besides repression by Keap1, Nrf2 is also subject to inhibition by ATF3, SFERRβ (ERRb), PPARγ, (PPARg), and RARα (RARa), and this can influence expression of the ARE-gene battery (8, 27, 133, 317, 353). The transcription factors Nrf1, Nrf3, p53, Bach1, Fra1, and c-Fos can also be recruited to the ARE and may attenuate Nrf2 activity (75, 308, 327, 349). In human HaCaT cells, genes that show maximal induction upon inhibition of Keap1 include *AKR1C1, GCLC, SLC7A11, SRXN1,* and *TXNRD1* (202), though *SQSTM1/p62* is also included because it can antagonize Keap1 (139, 169). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

in human Keap1, and instead modified Cys-77, Cys-196, Cys-249, Cys-368, and Cys-489. The Vanderbilt group also examined modification of human Keap1 by sulforaphane, and in this case discovered yet further variation in adduct formation with principally Cys-77, Cys-226, Cys-249, Cys-489, Cys-513, Cys-518, Cys-583, and Cys-624 being modified by the isothiocyanate, giving rise to the notion that different electrophiles produce distinct patterns of adduction (120, 121). A separate series of investigations by Mesecar and colleagues at the University of Illinois into modification of human Keap1 by IAB, sulforaphane, xanthohumol, isoliquiritigenin, and 10-shogaol, have indicated that most of the cysteines in Keap1 can form adducts with thiol-reactive compounds (65, 201). These workers have also revealed that methodology influences the recovery of adducted peptides, and have come to the conclusion that only a limited number of cysteines is important in the triggering of ARE-driven gene induction (66). Using an *in vivo* labeling procedure, Copple *et al.* (43) have shown that the electrophile produced from the anelgesic acetaminophen, *N*-acetyl-*p*-benzoquinoneimine, primarily modified Cys-288 in mouse Keap1, though Cys-226, Cys-273, and Cys-434 were adducted in certain, but not all, experiments. These workers similarly demonstrated that Cys-288 in Keap1 is the principal target of iodoacetamide, but that Cys-23, Cys-38, Cys-151, and Cys-273 were also occasionally modified in some experiments (43). It is important to recognize that a clear view has not emerged from the mass spectroscopy experiments about which Cys residue(s) serves as the 'sensor' of either chemopreventive agents or electrophiles. It will be important to link the modification of specific Cys residues in Keap1 with its inactivation as a substrate adaptor.

Figure 10 shows a molecular interaction map (168) depicting how Nrf1, Nrf2, and Nrf3 regulate the human ARE-gene battery, and in particular how chemopreventive agents such as BHA and sulforaphane induce gene expression. Inclusion of Bach1 as a negative regulator of ARE-driven gene expression only appears to occur in the case of *HMOX1* (202). Note that evidence endoplasmic reticulum stress activates Nrf2 and Nrf3 comes from data presented by Cullinan and Diehl (44) and Zhang *et al.* (350), respectively.

Identification of critical Cys residues in Keap1 for repressor activity

Site-directed mutagenesis has proved valuable in helping to identify Cys residues in Keap1 that are required for it to respond to chemopreventive agents. Amongst a number of excellent studies, that by Zhang and Hannink (343) in which Cys-151 was discovered to be essential for inactivation of the BTB-Kelch protein by tBHQ and sulforaphane has possibly been the most influential. Thus, these workers discovered that ectopic expression of a Keap1^{C151S} mutant in NIH 3T3 cells constitutively repressed Nrf2, as assessed by ARE-driven luciferase reporter activity, and could not be inactivated by tBHQ or sulforaphane. They also found that Cys-273 and Cys-288 were necessary for Keap1 to repress Nrf2 but did not find genetic evidence that further cysteines were involved in the process. At around the same time, other research groups also demonstrated that Cys-273 and Cys-288 in Keap1 are required for repression of Nrf2, through the finding that forced expression of Keap1^{C273S} and Keap1^{C288S} mutants in HEK-293 cells failed to repress Nrf2-mediated induction of a

GCLM-luciferase transgene (181) and by the discovery that knock-in of Keap1^{C273A} and Keap1^{C288A} mutants into *Keap1*$^{-/-}$ MEFs failed to reduce high basal ARE-luciferase activity (314). Subsequent examination of cysteines across the IVR domain has confirmed that Cys-273 and Cys-288 are necessary for Keap1-directed ubiquitylation of Nrf2 by Cul3-Rbx1, whereas Cys-226, Cys-241, Cys-249, Cys-257, and Cys-297 are dispensable (167). More recently it has been shown that Cys-23 in Keap1 is also required for its repressor activity (240) but this residue does not appear to be modified frequently by inducing agents (Table 5).

The molecular basis for the key function played by Cys-151 in Keap1 substrate adaptor activity is uncertain. It has been proposed from study of a Keap1^{C151W} mutant that modification of this cysteine by electrophiles might cause steric clashes that would lead to changes in its interaction with Cul3 (67). In a similar, but more radical vein, it has also been suggested that modification of Cys-151 in Keap1 would cause it to dissociate from Cul3 (259).

Currently, in the absence of a crystal structure for Keap1, it is not known why Cys-273 and Cys-288 are essential for its substrate adaptor function. It has been found that Keap1 is a zinc-containing metalloprotein (58), and it is possible that structural requirements dictate that Keap1 chelates the metal for its substrate adaptor function. As mutations in Cys-273 and Cys-288 greatly reduce the ability of Keap1 to bind zinc, it may be proposed that failure to chelate zinc leads to loss of its substrate adaptor activity.

Redox-dependent disulfide bridge formation in Keap1

As Keap1 contains many reactive cysteines, several groups have studied whether it can form disulfide bridges. Wakabayashi *et al.* (314) first reported that treatment with inducing agents, such as sulforaphane, 1,2-dithiole-3-thione, or bis(2-hydroxybenzylidene)acetone, caused the formation of intermolecular disulfide bridges between Cys-273 in one subunit of dimeric Keap1 with Cys-288 of another subunit, and that formation of the two bridges inhibited its ability to bind Nrf2. It has also been reported by Fourquet *et al.* (82) that the Cys-151 residue in Keap1 can form an intermolecular disulfide bridge between different subunits upon treatment with H_2O_2, nitric oxide, hypochlorous acid, or *S*-nitrosocysteine, and that this may impair recruitment of Cul3 to the substrate adaptor, thereby preventing ubiquitylation of Nrf2. These workers also suggested that Cys-226 is capable of forming an intramolecular disulfide bridge with Cys-613, an observation that is consistent with the proposal that the IVR is wrapped around the Kelch-repeat and CTR domains. Upon challenge with oxidants, the formation of intermolecular and intramolecular disulfide bridges in Keap1 is relatively transient, and is readily reversed in a glutathione- and thioredoxin-dependent manner (82).

In vivo *evidence for Cys residues in Keap1 as targets of chemoprotective agents*

As homozygous Keap1-null mice die at about 6 weeks of age (313), it has been possible to carry out complementation rescue experiments, by knocking-in Keap1^{C151S}, Keap1^{C273A}, Keap1^{C288A}, or Keap1^{C273A,C288A} mutants, to test the physiological importance of cysteines in repression of Nrf2 (333). These elegant experiments reported by the Yamamoto laboratory revealed that only transgenic expression of Keap1^{C151S} could rescue the *Keap1*$^{-/-}$ mouse; transgenic expression of Keap1^{C273A}, Keap1^{C288A}, and Keap1^{C273A,C288A} did not extend the life of the *Keap1*$^{-/-}$ mice. From the perspective of chemoprevention, it is interesting to note that MEFs prepared from transgenic mice expressing Keap1^{C151S} expressed lower basal Nqo1 mRNA levels than fibroblasts from either wild-type mice or *Keap1*$^{-/-}$ mice, but that the mRNA was still found to be inducible by treatment with 25 μM tBHQ. The finding that knock-in of Keap1^{C151S} represses basal Nqo1 expression but still allows induction by tBHQ is a most surprising result and suggests the existence of multiple sensors in Keap1. The rescue experiments support the hypothesis that Cys-151 in Keap1 serves as a 'sensor' for electrophiles. It also appears that replacement of either Cys-273 or Cys-288 in Keap1 with another amino acid abolishes its substrate adaptor activity.

Concluding Comments: Transcription Factor Nrf2 Influences Sensitivity to Genotoxic Chemicals

Although targeted disruption of Nrf2 yields mice that are viable and happily survive under normal laboratory conditions, they cannot cope with environmental stressors (157). This is to be expected from the observation that Nrf2 mutant mice display reduced basal expression of many ARE-driven genes. In the case of chemical carcinogens, *Nrf2*$^{-/-}$ mice show between 1.5- and 2-fold increases in susceptibility to forestomach tumorigenesis caused by benzo[*a*]pyrene (73, 260), urinary bladder cancer caused by *N*-nitrosobutyl(4-hydroxybutyl)amine (130), skin cancer caused by 7,12-dimethylbenz[*a*]anthracene/TPA (331), and colorectal cancer caused by azoxymethane/dextran sodium sulfate (161); Nrf2 protects against bladder cancer caused by *N*-nitrosobutyl(4-hydroxybutyl)amine in a cooperative fashion with p53 (131). Furthermore, *Nrf2*$^{-/-}$ mice cannot be protected against tumorigenesis caused by these carcinogens through pretreatment with oltipraz or sulforaphane (73, 260, 261, 331). In the case of challenge with benzo[*a*]pyrene, *Nrf2*$^{-/-}$ mice formed 2.5-fold more DNA adducts in the lung than *Nrf2*$^{+/+}$ mice (261). When exposed to AFB_1, *Nrf2*$^{-/-}$ mice formed 2.5-fold more DNA adducts in the liver than did wild-type mice (175). These deleterious effects are not restricted to model carcinogens as both cigarette smoke and diesel exhaust fumes are more harmful to *Nrf2*$^{-/-}$ mice than their wild-type counterparts (9, 10, 36, 186, 281, 295).

Using primary MEFs from *Nrf2*$^{-/-}$ and *Nrf2*$^{+/+}$ animals as an experimental model, it was found by Higgins *et al.* (115) that the CNC–bZIP factor is responsible for intrinsic resistance against a wide range of xenobiotics, including isothiocyanates, α,β-unsaturated carbonyls, aryl halides, epoxides, peroxides, free radical-generating compounds, heavy metals, mutagens, and anticancer drugs. Typically, the mutant MEFs were only able to tolerate between 30% and 70% of the dose that the wild-type MEFs could withstand. This difference in the intrinsic resistance of *Nrf2*$^{-/-}$ and *Nrf2*$^{+/+}$ MEFs was attributed, at least in part, to the diminished levels of glutathione in the mutant fibroblasts. Pretreatment (*i.e.*, priming) of the MEFs with a nontoxic dose of sulforaphane conferred significant resistance on the wild-type cells, but not

on the Nrf2 null cells, against many stressors. In particular, priming $Nrf2^{+/+}$ fibroblasts with sulforaphane conferred 2.8-fold resistance against acrolein, 2.7-fold resistance against chlorambucil, 3.2-fold resistance against cumene hydroperoxide, and 2.5-fold resistance against menadione. Much of the protection against acrolein, chlorambucil, and cumene hydoperoxide appeared to be due to increased production of GSH in the $Nrf2^{+/+}$ MEFs, because treatment with buthionine sulfoximine blocked the sulforaphane-induced resistance (115). However, sulforaphane-induced resistance against menadione could not be prevented by treatment with buthionine sulfoximine, and it was speculated that resistance in this case was due to upregulation of Nqo1. Although this work was conducted in MEFs, they are relevant to cancer chemoprevention because both acrolein and chlorambucil are genotoxic chemicals. Interestingly, knockdown of Keap1 in human HaCaT keratinocytes increased glutathione levels ~1.7-fold (50) and conferred between 1.4- and 1.6-fold resistance against acrolein, chlorambucil, cumene hydroperoxide, and menadione (202), indicating the cytoprotective mechanisms are conserved between mice and men. As anticipated, knockdown of Nrf2 in HaCaT cells decreased their resistance to acrolein, chlorambucil, cumene hydroperoxide, and menadione to between 0.65 and 0.90 of that of mock-transfected keratinocytes (202). Knockdown of Nrf2 in human A549 and H460 cells has similarly been found to increase their sensitivity to carboplatin and etoposide (280).

It is clear that enormous advances have been made over the past 10 years in our understanding of the mechanisms of action of chemopreventive blocking agents. Much of the work has been undertaken in mice, and there is now an urgent need to translate this knowledge into the human. Many of the principles by which Nrf2 provides protection against carcinogenesis are also relevant to other degenerative diseases. Recent reviews by Nguyen *et al.* (236) and by Sykiotis and Bohmann (296) have discussed the potential value of the Keap1-Nrf2 pathway as a therapeutic target in chronic obstructive pulmonary disease, Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis.

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Abbreviations Used

AFB$_1$ = aflatoxin B$_1$
AFM$_1$ = aflatoxin M$_1$
AKR = aldo-keto reductase
ALDH = aldehyde dehydrogenase
AP1 = activator protein-1
ARE = antioxidant response element
ATF3 = activating transcription factor 3
Bach1 = bric-à-brac, tramtrack and broad complex and CNC homology 1
BHA = butylated hydroxyanisole
BHT = butylated hydroxytoluene
BMCC = 1-biotinamido-4-(4′-[maleimidoethyl-cyclohexane]-carboxamido) butane
β-NF = β-naphthoflavone
BP = benzo[*a*]pyrene
BRG1 = Brahma-related gene 1
BTB = bric-à-brac, tramtrack and broad complex
bZIP = basic-region leucine zipper
CBP = cAMP response element-binding protein (CREB) binding protein
CBR = carbonyl reductase
CDDO = 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid
CDDO-Im = 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazolide
C/EBP = CCAAT enhancer binding protein
CNC = cap'n'collar
CTR = C-terminal region
CYP = cytochrome P450
Dex-mes = dexamethasone mesylate
DMBA = 7,12-dimethylbenz[*a*]anthracene
ECH = erythroid-derived protein with CNC homology
EPH1 = microsomal epoxide hydrolase
EpRE = electrophile response element
EQ = ethoxyquin
FTH = ferritin heavy
FTL = Ferritin light
G6PD = glucose-6-phosphate dehydrogenase
GADD45 = growth arrest and DNA-damage-inducible 45
GCLC = glutamate-cysteine ligase catalytic
GCLM = glutamate-cysteine ligase modifier
GPEI = glutathione transferase P enhancer I
GPx = glutathione peroxidase
GSH = reduced glutathione
GSR = glutathione reductase
GSSG = oxidised glutathione
GST = glutathione *S*-transferase
HMOX1 = heme oxygenase 1
IAB = *N*-iodoacetyl-*N*-biotinylhexylenediamine
IVR = intervening region
Keap1 = Kelch-like ECH-associated protein 1
Maf = musculo-aponeurotic fibrosarcoma
MAPEG = membrane-associated proteins in eicosanoid and glutathione metabolism
MARE = Maf recognition element
ME1 = malic enzyme
MEF = mouse embryonic fibroblast
MGST = microsomal glutathione *S*-transferase
MRP2 = multidrug resistance-associated protein 2
MT = metallothionein
Neh = Nrf2-ECH homology
NF-E2 = nuclear factor-erythroid 2
NF-κB = nuclear factor-κB
NQO1 = NAD(P)H:quinone oxidoreductase 1
Nrf = nuclear factor-erythroid 2 p45-related factor
PGAM5 = phosphoglycerate mutase family member 5
PGD = 6-phosphogluconate dehydrogenase
PPARγ = peroxisome proliferator-activated receptor-γ
PRDX = peroxiredoxin
PTGR = prostaglandin reductase
RARα = retinoic acid receptor α
ROS = reactive oxygen species
SFERRβ = short form estrogen-related receptor β
SLC7A11 = solute carrier family 7, member 11
SQSTM1 = sequestosome 1
SRXN1 = sulfiredoxin
StRE = stress response element
Sulforaphane = 4-methylsulfinylbutyl isothiocyanate
SULT = sulfotransferase
tBHQ = *tert*-butyl-1,4-hydroquinone
TPA = 12-*O*-tetradecanoylphorbol 13-acetate
TRX = thioredoxin
TXNRD1 = thioredoxin reductase
UGT = UDP-glucuronosyl transferase

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