Differential induction of mafF, mafG and mafK expression by electrophile-response-element activators

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The three small Maf proteins, MafF, MafG and MafK, have been implicated in a number of physiological processes, including development, differentiation, haematopoiesis and stress response. Here we report the constitutive expression of mafF, mafG and mafK in six human cell lines derived from various tissues (HepG2, IMR-32, K-562, HEK-293, RD and A549). The expression patterns of mafF, mafG and mafK varied widely among cell lines. Because small Maf proteins have been implicated in electrophile response element (EpRE)-mediated stress response, the ability of three EpRE activators [pyrrolidinedithiocarbamate (PDTC), phenylethyl isothiocyanate (PEITC) and t-butylhydroquinone (tBHQ)] to induce small Maf expression was examined in detail in HepG2 cells. Both PDTC and PEITC induced mafF, mafG and mafK expression, whereas tBHQ failed to markedly induce any of the three small Mafs. Where a response was observed, mafF was induced to the greatest extent compared with mafG and mafK, and this response was transcriptionally mediated. PDTC also induced small Maf expression in the other cell lines examined, with patterns of induction varying among cell lines. The differences in expression among the cell lines examined, coupled with the induction patterns observed, indicate that the three small maf genes are stress-responsive, but may be regulated via differing mechanisms. Furthermore, the fact that tBHQ, PDTC and PEITC induce EpRE activity, but that tBHQ fails to markedly induce any of the small Mafs, suggests that up-regulation of small Mafs is not an absolute requirement for EpRE-mediated gene expression.

Key words: PDTC, PEITC, stress response, tBHQ, tissue-specific.

INTRODUCTION

The Maf basic leucine zipper (bZip) transcription factors have recently garnered much attention as potential players in a variety of physiological processes, including embryogenesis, development, haematopoiesis and stress response (reviewed in [1]). The Maf family is divided into two subclasses, large Mafs (vMaf, cMaf, MafB and Nrl) [2] and small Mafs (MafF, MafK [3] and MafG [4]). Both subclasses contain leucine-zipper motifs, which allow homodimerization as well as heterodimerization with a variety of other bZip proteins, including alternative members of the Maf family [5,5a], activator protein (AP)-1 factors [5,5a,6] and Cap ‘N Collar (CNC) proteins (e.g. Nrf1, Nrf2, NF-E2 and p45) [7–10]. Recognition of and binding to specific DNA sequences by Maf-containing dimers is mediated, in large part, by a basic region with an adjacent extended homology region unique to Maf proteins [11]. The specific regulatory elements recognized by Maf-containing dimers vary somewhat, based on the identity of the dimerization partner present. Homodimers and interfamily heterodimers bind Maf-recognition elements (MAREs) [6], whereas CNC–small Maf heterodimers recognize the NF-E2 cis element, which resembles a MARE half site linked to a TPA (PMA) response element (TRE) half site [12]. Large Mafs also contain an acidic transactivation domain absent in the small Maf proteins. Although they possess no inherent trans-activation activity, small Maf proteins can act as positive regulators of transcription by targeting transcriptionally active dimerization partners to specific DNA regulatory elements. Conversely, small Mafs can act as negative regulators of transcription by recruiting transcriptional repressors or by forming homodimers that can then displace active dimers. Motohashi et al. [13] observed that the level of small Maf proteins affected both binding and transcriptional activity at a MARE (which also contained an NF-E2 site) in transgenic mice exhibiting varying degrees of MafK and/or MafG expression.

Recently, the involvement of Mafs in stress response has been the subject of much investigation. It has been suggested that CNC–Maf heterodimers mediate expression of stress-responsive genes, such as those involved in phase-II detoxification and GSH homeostasis by associating with the electrophile response element (EpRE; also known as the antioxidant response element or ARE) [14,15]. The EpRE consensus sequence [16–18] closely resembles the consensus sequence of the NF-E2 site, the recognition element for CNC–small Maf dimers. Much evidence exists in support of a role for CNC factors in EpRE-mediated transcription. Transgenic mice null for nrf2 (nrf2−/−) exhibited deficiencies in basal as well as butylated hydroxylanisole (BHA)-inducible expression of NAD(P)H quinone oxidoreductase 1 (NQO1) and glutathione S-transferase, as compared with their heterozygous littermates [14]. Impaired induction of oxidant-responsive genes and elevated sensitivity to oxidant toxicity has also been reported in vitro in peritoneal macrophages derived from nrf2−/− mice [15]. Recently, Chan and Kwong [19] reported reduced expression of both the catalytic and modifier subunits of glutamate-cysteine ligase (GCLC and GCLM), the enzyme which catalyses the rate-limiting reaction in GSH synthesis, in fibroblasts derived from nrf2-null mice. Several investigators have observed activation of EpRE-, NF-E2- and MARE-con-

Abbreviations used: PDTC, pyrrolidinedithiocarbamate; PEITC, phenylethyl isothiocyanate; tBHQ, t-butylhydroquinone; EpRE, electrophile response element; CNC, Cap ‘N Collar; MARE, Maf-recognition element; GCLM, glutamate-cysteine ligase modifier subunit; GCLC, glutamate-cysteine ligase catalytic subunit; GADPH, glyceraldehyde-3-phosphate dehydrogenase; RPA, RNase-protection analysis; AP, activator protein; ARE, antioxidant response element.

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containing promoter/reporter constructs by Nrf1 [10,20,21], Nrf2 [5,5a,10,20,22,23] and p45 [5,5a,7,20,24]. Because CNC proteins do not homodimerize and fail to bind DNA in the absence of a dimerization partner, it has been assumed that the involvement of CNC factors in EpRE-mediated transcription obviates the involvement of a dimerization partner as well. Some of the above studies [7,10,20,23,24] reported enhanced transcriptional activation when low amounts of small Maf proteins were co-transfected with the activating CNC factor. In all, however, increasing levels of small Maf inhibited both basal and CNC-protein-inducible promoter activity, a result attributed by several to a putative shift towards Maf–Maf homodimer formation. Although the ability of small Maf proteins to dimerize with CNC factors and bind to DNA-recognition elements has been demonstrated by numerous researchers in various experimental systems in vitro, the identification of small Maf proteins as the dimerization partners in situ remains somewhat controversial. This is due, in part, to the fact that several other transcription factors are also able to dimerize or interact with CNC factors [25–28].

Also worth noting with regard to small Maf involvement in stress response is the recent report by Hale et al. [29], which demonstrates an active MARE in the promoter of the p53 tumour-suppressor gene, the gene product of which is involved in the cellular response to DNA-damaging agents.

The response of the three small maf genes to oxidative stress and other insults is not well elucidated. Crawford et al. [30] reported increased expression of a mafG-like message (adapt66) in HA-1 hamster fibroblasts following exposure to H2O2. Consistent with this early report, Wild et al. [23] demonstrated increased mafG expression in HepG2 cells exposed to pyrrolidine-dithiocarbamate (PDTC) or β-naphthoflavone. More recently, Suzuki et al. [31] have proposed that small Mafs are involved in the cellular stress response, based on their observations that heavy metals induced maf expression in human HeLa cells and that heat shock induced adapt66 expression in HA-1 hamster fibroblasts.

In order to better understand the stress-responsive nature of each of the three small maf genes, we have examined constitutive and PDTC-induced levels of endogenous mafF, mafG and mafK expression in several human cell lines derived from various tissues. Detailed characterization of the kinetics of induction of mafF, mafG and mafK expression in HepG2 cells exposed to PDTC, phenylethyl isothiocyanate (PEITC) and t-butylhydroquinone (tBHQ) is also reported.

**MATERIALS AND METHODS**

**Reagents**

PDTC, PEITC and tBHQ were purchased from Sigma. [α-32P]dUTP was purchased from Amersham Pharmacia Biotech. Cell-culture materials were purchased from Gibco BRL unless otherwise noted. Acryl/bis, 19:1, solution was purchased from Sigma. [α-32P]dUTP was run for 3–5 h at 37 °C for 30 min to empirically determine the amount of probe corresponding to the mRNA of interest as used as templates for T7 polymerase in vitro-transcription run-off reactions. An antisense probe against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was generated from pTRI-GAPDH (Ambion) and used as an internal control. 36B4 was also used as an internal control where indicated. Transcription reactions containing [α-32P]dUTP were run for 3–5 h at 37 °C, with the exception of the reaction for 36B4, which was run at 15 °C. The three small maf probes were gel-purified (5 % polyacrylamide/8 M urea) excised and eluted overnight at 37 °C into probe-elution buffer provided in the RPA II kit (Ambion). The specific labelling of 36B4 and GAPDH was determined by trichloroacetic acid precipitation.

Expression levels of specific mRNAs were determined by RPA using the RPA III kit (Ambion) according to the manufacturer's procedure and as described previously [23]. Briefly, total RNA from treated cells was co-precipitated with an excess (determined empirically) of probe corresponding to the mRNA of interest as well as for an internal control (GAPDH or 36B4) and allowed to hybridize overnight at 60 °C. maf determinations were made using 100 μg of total RNA/hybridization. Hybridized samples were digested with a 1:33 dilution of RNase A/T1 provided in the RPA III kit. Protected fragments were resolved by denaturing PAGE (5 % polyacrylamide/8 M urea). Gels were exposed on PhosphorImager cassettes and visualized. PhosphorImager scans were analyzed by ImageQuant 1.2 (Molecular Dynamics).

**Statistics**

Data were corrected for loading, and fold inductions (treated/un-treated) were determined at each time point. Fold inductions were considered significant if the value obtained was greater than 1 and less than 0.5.
Basal and inducible expression of mafF, mafG and mafK were analysed by ANOVA and Fisher’s protected least significant difference (PLSD). Reported *P* values are for fold inductions at the indicated time points as compared with fold inductions at 0 h.

RESULTS

Cell-type-specific differences in basal expression of small mafs

An RNase-protection assay designed to distinguish between the mRNAs corresponding to each of the small mafs was used to determine mafF, mafG and mafK expression in A549, HEK-293, HepG2, IMR-32, K-562 and RD cells. The constitutive levels of mafF, mafG and mafK expression varied among the cell lines examined (Figure 1). Constitutive mafF expression was highest in HepG2, K-562 and RD cells, intermediate in A549 and HEK-293 cells and low in IMR-32 cells. In contrast, mafG was observed to be highest in K-562, at intermediate levels in A549, HEK-293 and HepG2 cells and lowest in IMR-32 and RD cells. mafK was highest in A549 cells, high in HEK-293 and K-562 cells, at intermediate levels in HepG2 and RD cells and present at very low levels in IMR-32 cells.

Small maf expression in HepG2 cells exposed to PDTC, PEITC and tBHQ

The induction of mafF, mafG and mafK expression by PDTC, PEITC and tBHQ was examined in detail in HepG2 cells. All three small maf messages were increased in HepG2 cells exposed to 100 µM PDTC. mafF, mafG and mafK were induced to a statistically significant extent as early as 2 h after PDTC treatment and returned to basal levels 8 h after treatment. Although the kinetics of induction did not appear to vary among the three small mafs, the magnitude of induction did (Figure 2); mafF was induced ≈ 5-fold, mafK ≈ 4-fold and mafG ≈ 2-fold. As was observed following PDTC treatment, all three small maf mRNAs were induced in HepG2 cells exposed to 10 µM PEITC (Figure 3). The relative magnitudes of induction (mafF > mafK > mafG) were also similar after PEITC treatment, although slight differences in the kinetics of induction were observed. mafF and mafG were elevated to a statistically significant extent by 3 h and had returned to basal levels 24 h after treatment. The induction of mafK was slightly delayed as compared with that of mafF or mafG, but had reached statistical significance 6 h after treatment with PEITC (Figure 2). None of the transcripts were markedly induced in HepG2 cells exposed to 50 µM tBHQ at any time point (Figure 4).

Cell-type-specific differences in PDTC-inducible expression of small mafs

Because basal expression of the small mafs varied among cell lines, we further investigated whether the PDTC responsiveness of mafF, mafG and mafK would also differ among cell lines. To examine cell-line-specific differences in small maf induction, A549, HEK-293, HepG2, IMR-32, K-562 and RD cells were treated for 0, 3, 6 and 12 h with 100 µM PDTC or a vehicle control. Preliminary analysis demonstrated that maximal induction for all the small mafs was seen at 3 h in HepG2 and RD cells.
cells and at 6 h in A549, HEK-293, IMR-32 and K-562 cells (results not shown). mafF was induced to the highest levels in all cell lines examined (Figure 5), although there were dramatic cell-type-specific differences in the magnitude of induction, which ranged from 25-fold in RD cells to 5-fold in IMR-32 and K-562 cells. mafG and mafK were induced to comparable levels in HEK-293, K-562 and RD cells (2–3-fold in all three cell lines). In A549 cells, mafK was induced 3-fold whereas mafG was not induced. In IMR 32 cells, mafG was induced 3.5-fold, and mafK was not induced.

PDTC-mediated increases in mafF expression were transcriptionally mediated

Induction of mafF expression following PDTC exposure was greater than mafG and mafK in all cell lines examined, except IMR-32. In order to determine whether this induction was transcriptionally mediated, HepG2 cells were pre-treated with 10 μg/ml actinomycin D for 1–1.5 h prior to PDTC exposure. Actinomycin D not only prevented PDTC-induced increases in mafF message, but also resulted in a slight decrease in basal levels (Figure 6).

DISCUSSION

Maf proteins are highly conserved across species. There is also remarkable amino acid sequence similarity among the three small Maf proteins (reviewed in [1]), which has led to speculation regarding functional redundancy. Indeed, in various in vitro assays MafF, MafG and MafK are functionally interchangeable [4,7], and overexpression of one small Maf can, in some cases, compensate for the loss of another in transgenic animals [13,32], mafF-null [33], mafK-null [32] and mafF::mafK-null [34] mice did not demonstrate any phenotype discernable from the wild type. By contrast, mafG-null mice were smaller than their heterozygous littermates and demonstrated clear haematological and neurological abnormalities. Elimination of mafF expression against a mafG-null background resulted in only a slight exacerbation of the mafG-null phenotype, whereas in mafK::mafG-null mice the exacerbation of the mafG-null phenotype was severe [34]. The above studies are consistent with partial redundancy among MafF, MafG and MafK, but also indicate a lack of complete functional redundancy among small Mafs in vivo. Additionally, the three small Mafs exhibit distinct spatial and temporal expression profiles in developing mouse embryos [32,33], as well as differing patterns of tissue-specific expression in adult mice [33,34]. These differences in small Maf expression profiles hint at varied mechanisms of regulation among the three genes. We observed differences in the degree to which each small Maf was expressed and induced within a given cell line. This observation is consistent with reported expression profiles in adult mice and provides further support for alternative regulatory mechanisms of gene expression and response among the three individual small maf genes. The cell-line-specific differences in expression of mafF, mafG and mafK may reflect the tissue type of origin, degree of cell differentiation, varied proliferation or a combination of these and other factors.
Basal and inducible expression of mafF, mafG and mafK

Figure 5 Cell-type-specific variations in induction of small maf expression by PDTC

(A) Representative RNase-protection assays showing induction of small mafs after treatment with 100 μM PDTC for 3 h (RD) or 6 h (A549, HEK-293, IMR-32 and K-562). (B) Fold inductions over untreated controls. Results are the means ± S.E.M. from three separate experiments.

PDTC, PEITC and tBHQ are compounds that have been associated with the induction of stress-response genes, probably via activation of EpRE/ARE [17,35–38]. Since small Mafs are thought to play a role in EpRE-mediated transcription, we examined the ability of these EpRE activators to alter small maf expression. Although both PDTC and PEITC induced mafF, mafG and mafK expression in HepG2 cells, tBHQ did not. Induction was fairly rapid and transient. Differences in the ability of tBHQ as compared with PDTC and PEITC to induce small maf expression may reflect differences in the nature of the redox or stress signal generated by these compounds. PDTC exhibits both antioxidant and pro-oxidant characteristics, depending on the experimental system. PDTC’s cytotoxicity in thymocytes has been attributed to its ability to chelate redox-active copper and shuttle it into the cell, where it can participate in the generation of reactive oxygen species [39]. Alternatively, PDTC itself can be oxidized to ultimately form the corresponding thuram disulphide [40]. Thuram disulphides are reactive compounds capable of oxidizing GSH [40] and other reduced thiols [41]. Increased expression of GCLC and GCLM in HepG2 cells exposed to PDTC was associated with elevated levels of GSSG, but did not appear to be mediated by increased intracellular reactive oxygen species [42]. The potency of various isothiocyanates to induce EpRE-mediated transcription has been correlated with their ability to form GSH conjugates, which accumulate intracellularly and can be further metabolized in vivo to generate thiocarbamates [43–45]. The fact that PEITC elicited a pattern of small maf induction similar to that observed following PDTC exposure may be partially attributable to its metabolism to a thiocarbamate. In contrast with what Wild and Mulcahy [42] observed in HepG2 cells treated with PDTC, Yamane et al. [46] reported increased intracellular reactive oxygen species in rat hepatoma cells treated with tBHQ, an observation also reported by Pinkus et al. [47] in HepG2 cells. Pinkus et al. [47] further

Figure 6 Actinomycin D inhibited PDTC-mediated induction of mafF

HepG2 cells were treated with 10 μg/ml actinomycin D (ActD) for 1–1.5 h prior to PDTC treatment. The expression of mafF 4–4.5 h after PDTC treatment was determined by RPA as described in the Materials and methods section. (A) The PhosphorImage is representative of three separate experiments. (B) Bands were quantified and indicate the ratio of mafF/36B4 for each lane. Data are presented as means ± S.E.M. from three separate experiments.
reported induced transcription from the mouse GSTYa promoter as well as binding in vitro to an AP-1 site following PDTC or tBHQ exposure, whereas only tBHQ and not PDTC induced binding to a nuclear factor x binding sequence. This difference supports the possibility that, while both PDTC and tBHQ are oxidants and effective inducers of EpRE activity, these two compounds may generate different signals with regard to oxidative stress response, and hence exert different effects on maf gene expression.

Of the three small mafs, mafF appeared to be the most responsive following PDTC exposure in all cell lines examined and in HepG2 cells exposed to either PDTC or PEITC. It is interesting to note that previous reports in the literature have focused on the inducibility of mafG [23,30,31], although Suzuki et al. [31] did report increases in maf-related messages that were not specifically identified. The ability of actinomycin D to eliminate PDTC-induced increases in mafF mRNA levels suggests that this increase was transcriptionally mediated. The actinomycin D-mediated reduction in basal levels is consistent with a labile steady state accomplished by rapid induction and rapid degradation of mafF, which may also be reflected in the sharp induction and rapid return to basal levels observed in HepG2 cells exposed to PDTC or PEITC.

We conclude that small maf expression varies among human cell lines, as does the magnitude of small maf response to PDTC treatment. All three small mafS appear to be responsive to PDTC and PEITC, but tBHQ failed to elicit a marked effect in expression of any of the three small mafS. Because tBHQ, PDTC and PEITC are all effective inducers of EpRE activity, we conclude that up-regulation of small mafS is not an absolute requirement for EpRE-mediated gene expression. Of the three small mafS, mafF was induced to the greatest extent in all cell lines examined and, in HepG2 cells at least, this induction was transcriptionally mediated. These results suggest that, even within a single cell line, expression of mafF, mafG and mafK genes may be regulated by distinct mechanisms.

This work was supported by National Institute of Environmental Health Sciences grant ES05749. J.A.M. and E.L.D. are supported in part by National Institutes of Health (NIH) grant T32 ES07015; J.A.M. is also supported in part by NIH grants F32 CA88466.

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