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Maf Genes Are Involved in Multiple Stress Response in Human

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The *Maf* protein family consists of eight transcription factors containing a basic-leucine zipper (bZIP) domain. We have previously reported that the mRNA to one of these members, *mafG/adapt66*, is induced by oxidative stress in hamster HA-1 cells. It has subsequently been reported that *mafG* is induced bystress that activates the expression of genes under the control of the antioxidant/electrophile response element (ARE/EpRE), and that small Maf proteins are present in ARE/EpRE-protein complexes. Here we extend these studies to assess the effects of various types of stress on *maf* mRNA induction in human cells. The oxidative stressor cadmium, and the heavy metals cadmium, zinc, and arsenite induced *mafG* RNA levels within two hours, and maximally at five hours for cadmium and zinc. This induction was observed for multiple transcripts including two not normally associated with *mafG*, suggesting that these stress agents induced the expression of other related *maf* family RNAs. Modest induction of *mafG* mRNA was also observed with heat shock but not calcium elevation. These results suggest that *mafG* is a human stress-response gene induced by multiple stress, and that several *maf* (proto-)oncogene members play an important role in cellular stress response.

Key Words: **stress; oxidative; heavy metals; heat shock; calcium; bZIP; gene expression; HeLa cells.**

The ability to maintain cell viability following stress is an important component of normal cellular and organismal homeostasis. Cells have evolved a plethora of protective mechanisms to successfully cope with exposure to toxic stimuli, and many of these mechanisms have a strong genetic component. These include the well-characterized stress response gene families such as heat shock and glucose regulated protein (*grp*) genes as well as a growing list of newly identified genes (1). In addition to valuable insight regarding basic cellular response to stress, these genes also represent potential clinical diagnostic and therapeutic targets.

We have identified several genes, designated "*adapts*", that are induced by hydrogen peroxide in hamster HA-1 cells, and subsequently characterized their response in other cells including human (2–5). The expression of all the *adapts* characterized to date (*adapt15, 33, 66, 73,* and *78*) have been found to be induced by multiple stress (i.e., not just oxidative) including methyl methanesulphonate, *cis*(II)-platinum, hydrogen peroxide, 2-deoxyglucose, heavy metals, heat, and calcium elevation by calcium ionophore A23187 and thapsigargin (3–7).

One of the *adapt* genes that we found that was induced by peroxide in HA-1 cells, *adapt66*, was cloned and sequenced and subsequently identified as *mafG*, a member of the *maf* (proto-)oncogene family (5). *maf* genes code for basic-leucine zipper (bZIP) transcription factors and are derivatives of the avian transforming retroviral oncogene *v-maf*. They include c-*maf*, *mafK*, *mafF*, *mafA*, *mafB*, *mafG*, L-*maf*, and *nrl* (8–11). *MafB* and *nrl* are closest in homology to *v-maf*, containing amino-terminal trans-activating and carboxyl-terminal DNA-binding domains. The protein products of *mafF*, *mafG*, and *mafK* are referred to as the small Maf family proteins, lack the amino-terminal two-thirds of the v-Maf molecule. Evidence to date indicates that the small Maf proteins can either dimerize with themselves or other small Mafs; and/or heterodimerize with several other transcription factors including Fos, NF-EF p45, and Ech; to regulate transcription, both negatively and positively (8, 9). Our identification of *mafG*/*adapt66* as a peroxide inducible mRNA was the first report

Abbreviations used: MEM, Eagle minimal essential medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, sodium chloride/sodium citrate; ARE/EpRE, antioxidant/electrophile response element.

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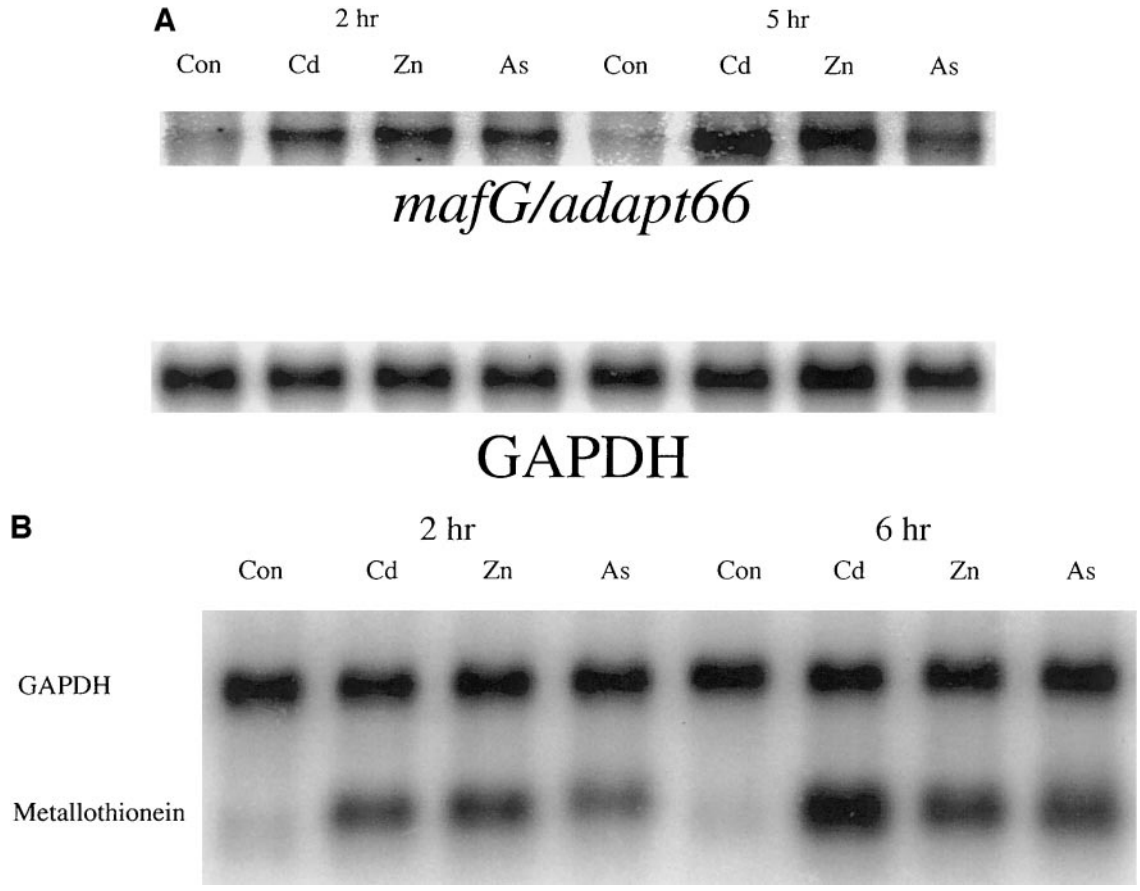

FIG. 1. Induction of *mafG/adapt66* mRNA in human HeLa cells by stress. (A) Human HeLa cells were treated for 2 and 5 h with 30 μM cadmium chloride, 240 μM zinc chloride, and 80 μM sodium arsenite. After 5 h, the cultures were washed with PBS, RNA extracted, polyA+-selected, electrophoresed, blotted, and the nylon blots hybridized with cDNA probes to human *mafG/adapt66* followed by human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This figure is representative of three independent analyses. (B) Same as above except that the HeLa cells were treated for 2 and 6 h, and extracted and blotted RNA hybridized to probes to human metallothionein-1A followed by GAPDH.

that any member of this important transcription factor family is induced by stress (5, 9). It has subsequently been reported that *mafG* is induced by stress that activates the expression of genes under the control of the antioxidant/electrophile response elements (ARE/EpRE), and that small Maf proteins have been found to be present in ARE/EpRE-protein complexes (12, 13).

Here, we extend these previous studies to assess the effects of several other types of stress agents on *mafG*, and other *maf*, mRNA expression. For these studies, we chose cadmium as our main stress agent. Cadmium is an important human stress agent for several reasons as it produces an intracellular oxidative stress, has toxic effects due to its heavy metal nature, and is a human health toxin (14, 15). The effect of other heavy metals, heat shock, and elevated calcium were also assessed toward gaining a better understanding of the types and extent of stress that affect *maf* expression in human cells.

MATERIALS AND METHODS

Cell culture. Epithelial-like human HeLa cells were grown in monolayer culture in media containing MEM supplemented with 7% HA-1 fibroblasts, a Chinese hamster ovary subline, were maintained in Eagle minimal essential medium (MEM) supplemented with 15% heat inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were grown in a humidified incubator atmosphere of 95% air and 5% CO_2 at 37°C.

Heavy metal treatments. HeLa cell cultures were exposed to heavy metals when they had grown to approximately 70% confluency. At the time of treatment, the cultures were taken from the incubator and the heavy metal added directly to the media to the desired final concentration. The cells were then returned to the incubator for the appropriate times until RNA extraction. Treatments included 15 μM cadmium chloride, 240 μM zinc chloride, and 80 μM sodium arsenite.

Heat shock treatments. HeLa cells grown to 70% confluency were divided into two groups. The first group was maintained at 37°C. The second group was elevated to 42°C. Six hours later, RNA was extracted from both groups.

RNA isolation and analysis. Following incubation, the cultures were washed with PBS and total RNA extracted using RNA extractor according to the manufacturer (Genosys, The Woodlands, Texas). PolyA+ mRNA was then selected using oligo-dT cellulose as described (16). The final RNA pellet was resuspended in diethylpyrocarbonate-treated distilled, deionized water and analyzed by Northern blot hybridization as previously described (16). The hybridization solution contained random primed (Oligolabeling kit, Pharmacia, Piscataway, New Jersey) radiolabeled probe to the cDNAs described below. After overnight hybridization at 65°C, the blots were washed 2 times for 15 min each at 65°C with 2× SSC plus 0.2% SDS, followed by 2 more washes for 30 min each at 65°C with 0.3× SSC plus 0.2% SDS. The blots were then exposed to X-ray film or phosphoimaged using a Storm 860 phosphoimager with Pathways software (Research Genetics, Huntsville, AL).

Probes. We obtained a human cDNA clone to both *mafG* and metallothionein-1A from the I.M.A.G.E. consortium through Research Genetics. These clones were grown and purified using a Qiagen plasmid purification kit as described by the manufacturer (Qiagen Inc., Valencia, CA). The stress-inducible human *hsp70B* DNA probe was obtained from StressGen Biotechnologies (Victoria BC, Canada). Whole inserts to near full-length cDNA clones for all three clones were used as probes.

RESULTS

Heavy metals induce the expression of mafG mRNA in human HeLa cells. HeLa cells in monolayer culture were exposed to 15 μM cadmium chloride, 240 μM zinc chloride, and 80 μM sodium arsenite and extracted, polyA+-selected mRNA analyzed as described under Materials and Methods. Both cadmium and zinc exposure led to a significant induction of the 5.8-kb *mafG* mRNA after 5 h as shown in Fig. 1A. While arsenite induction is observed for this experiment at the earlier 2 h time point, weak induction, if any, was observed at 5 h comparing three independent analyses (1.4 ± 0.5 mean ± SEM). The fold inductions for 5 h cadmium and zinc for these three experiments were nearly identical; respectively, 5.1 ± 0.7 and 5.0 ± 0.5. Thus, heavy metals induce the expression of *mafG* mRNA in human HeLa cells.

Figure 1B shows the induction of metallothionein, a positive control for our studies and an indication of a bona fide heavy metal effect on these cells at the level of mRNA.

Heat shock induces the expression of maf mRNA in human HeLa cells. A number of stress response mRNAs are induced by multiple stress including heat shock (1). Exposure of HeLa cells to mild heat shock (42°C) for 6 h led to a moderate induction of *mafG* mRNA. A 2.1-fold induction was observed in duplicate experiments, and a representative result shown in Fig. 2. As a positive control, heat-shock inducible *hsp70B* mRNA was also included.

Multiple maf-related mRNAs are induced by heavy metals. Under our hybridization and wash conditions, we observed several other *mafG/adapt66*-hybridizable bands on Northern blots containing samples of HeLa cells treated with heavy metals (Fig. 3).

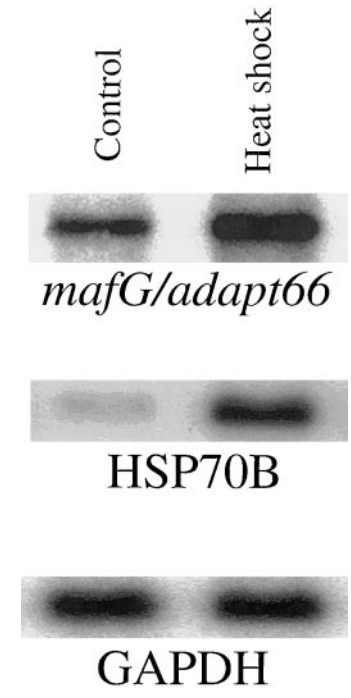

FIG. 2. The effects of mild heat shock on *mafG/adapt66* expression. Northern blots containing RNA from HA-1 cells containing control and mild heat shocked HeLa cells were extracted, blotted, and hybridized as above using *hsp70B* probe followed by GAPDH. This figure is representative of two independent analyses.

The distribution and sizes of these homologous bands is shown in Fig. 3. Since these bands were detected using a relatively high stringency wash (final 0.3× SSC, 0.2% SDS at 65°C), they are presumably *maf* gene family members. The other *maf* mRNA species that were significantly induced by heavy metals were 2.0 and 2.5 kb in size. The inductions of these two smaller *maf* homologs did not appear to be coordinate with that for *mafG* (5.8 kb) since arsenite induction of *mafG* at this time point was weak, if at all.

As indicated in the introduction, the *maf* gene family includes *mafK*, *mafF*, *mafA*, *mafB*, *mafG*, c-*maf*, and the retinal-specific *nrl*. *mafG* is identifiable by its characteristic size and induction (5;17) as shown in Fig. 3. At this time, we do not know the identity of the other bands. Two other *maf*-homologous bands also appeared that were even larger than *mafG*. A 7.8-kb mRNA was not modulated by any treatment (3 experiments). The largest band, 9.6 kb, also did not show any clear modulation (two comparisons) with the possible exception of a modest induction by arsenite at 5 h. It is possible that one or both of these bands represent *maf* RNA precursors.

DISCUSSION

Our results indicate that *mafG* is a human stress-response gene induced by oxidative stress, heavy met-

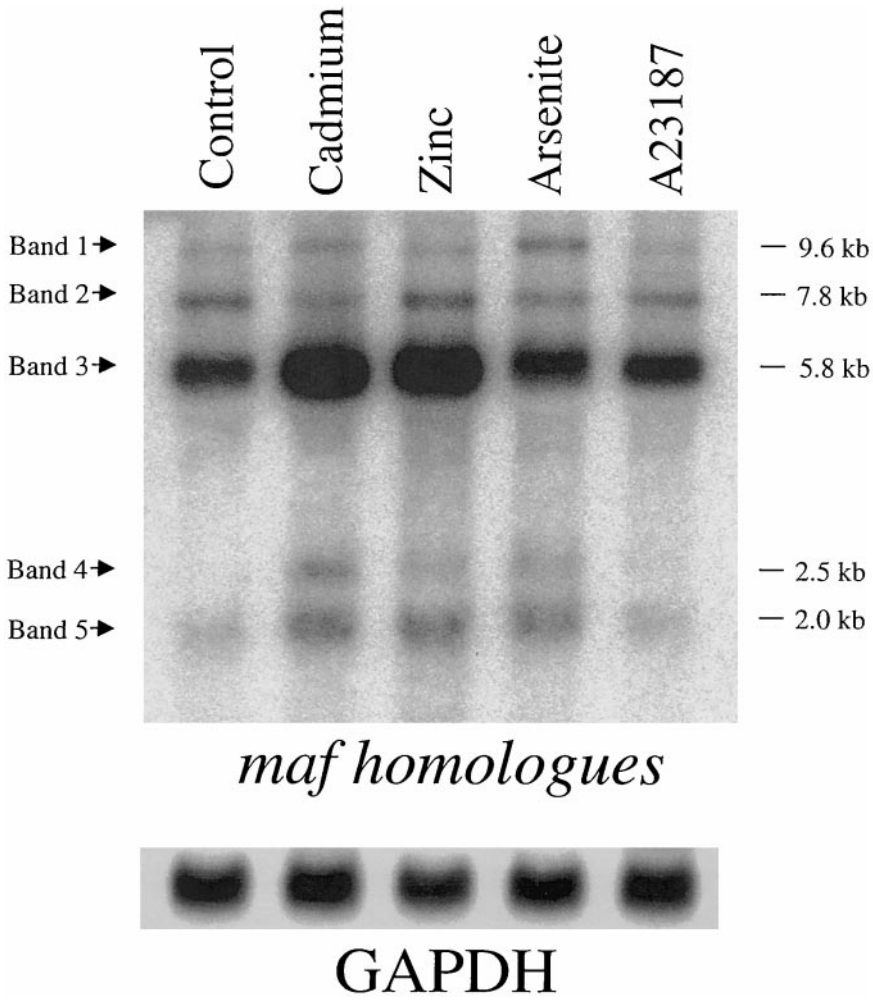

FIG. 3. Heavy metals induce multiple *maf*-related mRNAs. HeLa cells were treated for 5 h with cadmium, zinc, and arsenite and processed as described in Fig. 1 and the expression of all *maf*-homologous mRNAs assessed. Calcium ionophore (1 μg/ml) was also included in these studies. Five *maf*-homologous mRNAs were clearly distinguishable and sized as indicated.

als and heat shock, and demonstrate induction of other members of the *maf* family of genes as well by these stress inducers. Previous studies by our laboratory (5), Wild *et al.* (12), and Shimokawa *et al.* (18) indicate that *mafG* is induced by hydrogen peroxide, ARE/EpRE-inducers, and hypercapnic (high CO_2) stimulation. Combined, these studies suggest an important role for some or all of these induced *maf* homologues in cellular stress response, similar or complementary to other known stress response transcription factors including c-*fos*, c-*jun*, ATF, and NFKB.

Although we have now observed *mafG* mRNA induction using several different stress agents, it may be that at least some of these agents act through a common pathway; most likely, oxidative stress. For one, we originally detected the induction of *adapt66*/*mafG* in hamster cells using hydrogen peroxide, a prototype oxidant (5). Second, cadmium and arsenite are known to generate a cellular prooxidant state (14, 19). And third, Wild *et al.* found that pyrrolidine dithiocarbamate and β-napthoflavone induce *mafG* (12). Both compounds are known stimulators of the antioxidant/electrophile promoter response elements. There is less precedence for a prooxidant effect from zinc, heat shock or hypercapnia. There have been reports that zinc can act as a prooxidant but this depends upon the specific conditions, as it is also a reported antioxidant (19, 20).

The expression of all of the *adapt* genes studied to date, including hamster *mafG*/*adapt66*, is inducible by calcium (2–7). Curiously, no induction of *maf* mRNA by calcium ionophore A23187 was observed in HeLa cells (Fig. 3). This was not due to the lack of an effect of A23187 on the HeLa cells, as we did observe inductions of other *adapt* mRNAs known to be induced by calcium (data not shown). Thus, human *mafG*/*adapt66* inducibility by calcium appears to be cell- and/or calcium source-dependent.

In summary, these results indicate that *mafG* is a human stress-response gene induced by multiple stress, and suggest that several members of the *maf* family of genes may also play an important role in cellular stress response. We hope that this represents valuable insight into basic cellular stress response, and that these *maf*-inducible species have potential utility as clinical targets in the diagnosis and therapy of stress related diseases and disorders.

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