Regulatory Heme and Trichloroethylene Intoxication: A Possible Explanation of the Case of “A Civil Action”

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Abstract

In 1998, a movie entitled “A Civil Action” was released. The movie described the Woburn case, begun in 1982 and concluded in 1990, one of the most famous cases of trichloroethylene pollution. In a small town near Boston, twelve children died of leukemia, which seemed attributable to trichloroethylene contamination of the drinking water. The victims, however, could not win the case, since evidence that the identified chemicals could cause leukemia and other human illnesses was rather sketchy. There have been many cases of trichloroethylene pollution in industrial nations including Japan, therefore, we reconsidered the missing link. Our conclusion is that the disease occurred not by a direct effect of the chemical hazard on biological macromolecules but by an indirect effect through the physiological system such as signal transduction and transcriptional regulation. In 1984, we reported a marked reduction in the regulatory heme pool by trichloroethylene exposure, however, the biological significance was not well understood. Recently, we found that the DNA binding activity of Bach1, a negative regulator of genes, is controlled by heme, the regulation of which seems to explain how leukemia develops. The heterodimer of Bach1 with MafK recognizes Maf recognition elements (MAREs) competing with the erythroid type positive regulator, a complex of NF-E2 with MafK. Bach1/MafK occupies MAREs under lower heme conditions, whereas MAREs are open to NF-E2/MafK along with increasing heme concentration. Since the NF-E2/MafK function is closely related to normal erythroid differentiation, of which disorders such as sideroblastic anemia are often related to neoplasia; i.e., a clonal disorder that can progress to leukemia. Thus, a marked decline in regulatory heme by trichloroethylene intoxication could be one of the pathways to leukemia.

Key words: δ -aminolevulinate synthase, δ -aminolevulinate dehydratase, heme, NF-E2, Bach1, trichloroethylene

Introduction

In the last two decades, many cases of ground water pollution have been discovered not only in the United States but also in Japan, due to modern industries related to electronics, IT, and so on (1). One of the most frequent pollutants was trichloroethylene, a widely used industrial solvent (2), and forty percent of all Superfund sites are contaminated with this chemical (3). Among trichloroethylene contaminations of the environment, the Woburn case became famous, since the legal case, begun in May 1982 and concluded in March 1990, was published in 1995 as a nonfiction novel entitled “A Civil Action” (4). The novel was also dramatized as a movie under the same title in 1998. In May 1979, state investigators discovered that two municipal wells in Woburn were contaminated with trichloroethylene (5). Biostatistics suggested that habitants who received most of their water from the two wells

suffered from more than the normal number of leukemia cases and immune-system disorders (6). Thus one of the most important causes of these diseases was suspected to be trichloroethylene intoxication via drinking water. The accusers, however, lost the case, since the evidence that the chemical could cause leukemia and other human illnesses was rather sketchy (7–10). Other researchers examined whether toxic metals, such as arsenic and chromium, had elevated the incidence of childhood leukemia in Woburn (11), but in vain (12).

In Japan, a similar pollution case with as much as 500,000 tons of illegally scrapped industrial waste, including trichloroethylene and heavy metals, was found on a tiny island named Teshima. We, therefore, started to reconsider what was missing and what should be taken into account in trichloroethylene intoxication (13).

Something was missing

Most previous toxicology studies have focused on the direct effects of chemical hazards and/or their metabolite(s) on macromolecules, such as enzymes, receptors, and DNA (13, 14). For example, trichloroethylene is bioactivated by cytochrome P450,

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primarily cytochrome P450 2E1 (15, 16). In spite of the induction of cytochrome P450 2E1 by trichloroethylene exposure (17, 18), the isozyme activity reduced (17, 19) due to adduct formation with reactive intermediate(s) of trichloroethylene (19–21). A couple of studies demonstrated that the reactive intermediate(s) is covalently bound not only to cytochrome P450 2E1, but also to other proteins (20–25) and to nucleotides (25–27). One of the possible explanations of carcinogenicity is induced oxidative DNA damage by reactive oxygen compounds from trichloroethylene (25). The oxidative DNA damage was, however, observed under only a high dose of trichloroethylene intoxication (25). As discussed in the early days of the Woburn case, the contaminated trichloroethylene was at a very low level (7), and it seemed unlikely to have caused the elevated leukemia rate (8).

What is missing? One possible answer is that the mechanism of carcinogenesis is not due to the direct effect of the chemical and its metabolites, but through regulatory machinery controlling normal cell function, such as differentiation and proliferation.

Once upon a time

In the 1970s, it was already accepted that trichloroethylene is oxidized by the hepatic cytochrome P450 to form reactive intermediate(s), which irreversibly bind(s) to proteins and DNA, and affect cytochrome P450 activity through accelerated degradation (28–34). Since hepatic cytochrome P450 is generally considered to be one of the indicators of the free (or regulatory) heme pool (35), the decline in cytochrome P450 under trichloroethylene intoxication might affect some cell functions through the regulatory heme.

We therefore started to examine the effects of trichloroethylene on heme metabolism (Fig. 1). δ -Aminolevulinatase, catalyzing the second step of heme biosynthesis (36), was selected as the item to be elucidated, since the enzyme contains as many as 64 moles of the Cys/molecule, including 8 essential SH residues (37–39). When rats were exposed to various concentrations of trichloroethylene vapor (50 to 800 ppm), the activity of hepatic δ -aminolevulinatase decreased in a dose-dependent manner (40). Since the enzyme concentration in tested rats were essentially similar to that in the controls, the activity loss seems to be a result of the covalent binding of trichloroethylene or its metabolites. In vitro experiments revealed that the presence of trichloroethylene as well as the mixed function oxidase system in the assay mixture was essential to inhibit δ -aminolevulinatase, suggesting a covalent binding of reactive intermediate(s) with the enzyme (Fig. 1a).

Then, we examined the effects of δ -aminolevulinatase inhibition by trichloroethylene on heme metabolism in the liver (Fig. 1b). It is well known that lead also inhibits δ -aminolevulinatase to cause typical chemical porphyria with elevated excretion of the substrate in urine (37, 38, 41, 42). Similar to the lead intoxication, trichloroethylene-treated animals showed significantly elevated urinary δ -aminolevulinic acid. The elevated substrate level suggested a reduction in heme biosynthesis, therefore, two indicators of free heme in the liver were examined. One was the heme saturation of tryptophan pyrrolase, taken as a sensitive indicator of free heme (43). The heme saturation was decreased to half the level of the control in rats, which received 50 ppm trichloroethylene vapor, suggesting that the free heme level is already affected by even a partial δ -aminolevulinatase

(A)

(B)

Fig. 1 Effects of trichloroethylene exposure on heme biosynthesis.

(A) Inhibition of δ -aminolevulinatase by trichloroethylene in vitro.

Purified δ -aminolevulinatase (6 μ g/ml=1.7 μ M of subunit, which contains 13.7 μ M of SH residues) in 0.5 ml of 100 mM Tris-acetate buffer, pH 7.1, was incubated with trichloroethylene (60 μ M) either in the presence or absence of the mixed function oxidase system for 60 minutes, followed by δ -aminolevulinatase activity determination in the presence of dithiothreitol and zinc. δ -Aminolevulinatase is inhibited after the bioactivation of trichloroethylene by cytochrome P-450. The activity without trichloroethylene and mixed function oxidase system is taken as 100%.

(B) Effect of trichloroethylene exposure on heme biosynthesis in rat liver.

Male Wistar rats (8 rats for each group) were exposed to various concentration of trichloroethylene vapor for 10 days. The liver was removed after perfusion with isotonic KCl to minimize blood contamination. Effects of trichloroethylene on δ -aminolevulinatase synthase activity (black squares), δ -aminolevulinatase activity (black circles), cytochrome P-450 concentration (white diamonds), heme saturation of tryptophan pyrrolase (black diamonds), urinary δ -aminolevulinic acid concentration (white squares), and urinary coproporphyrin concentration (white circles) were estimated.

dehydratase inhibition. The effect on cytochrome P450 concentration, the other indicator, by trichloroethylene was more severe than that by succinylacetone, one of the most potent inhibitors of δ -aminolevulinatase, with a comparable degree of

Fig. 2 A scheme of hepatic heme biosynthesis under trichloroethylene exposure.

Abbreviation: ALA, δ -aminolevulinic acid; PBG, porphobilinogen; URO III, uroporphyrinogen III; COPRO III, coproporphyrinogen III; PROTO IX, protoporphyrinogen IX; PROTO IX, protoporphyrin IX; ALAS, δ -aminolevulinic synthase; ALAD, δ -aminolevulinic dehydratase; PBGD, porphobilinogen deaminase; UROS, uroporphyrinogen cosynthase; UROD, uroporphyrinogen decarboxylase; CPO, coproporphyrinogen oxidase; PPO, protoporphyrinogen oxidase; FeC, ferrochelatase; CYP, cytochrome P-450, TrpP, tryptophan pyrrolase.

enzyme inhibition (44–46). It is suggested that the decrease in cytochrome P450 is due not only to δ -aminolevulinic dehydratase inhibition but also to the accelerated degradation of cytochrome P450 by trichloroethylene (33, 34).

Free heme is considered to be a gene regulator; for example, hepatic δ -aminolevulinic synthase, the rate-limiting step of heme biosynthesis, is controlled by heme in a negative feedback manner (35, 43). Our study demonstrates a significant induction of δ -aminolevulinic synthase in the liver of trichloroethylene-treated rats, forming a reciprocal mirror image of the inhibition of δ -aminolevulinic dehydratase. This is evidence, which shows a disturbance of gene regulation through heme in trichloroethylene-exposed mammals (Fig. 2).

Regulatory heme pool

There is a cluster of genes which are regulated by heme. The above study demonstrated that hepatic δ -aminolevulinic synthase is one of these. An immunochemical study of δ -aminolevulinic synthase indicated that an antibody against rat hepatic δ -aminolevulinic synthase could not recognize the purified enzyme from the erythrocytes of anemic rats (47), suggesting the presence of an erythroid-type isozyme. It was not clear if the erythroid and hepatic isozymes were regulated by heme distinctively or not, therefore, the expressions of both isozymes were examined in mouse erythroleukemia (MEL) cells (48) (Fig. 3). The results indicated that the hepatic isozyme of δ -aminolevulinic synthase

Fig. 3 Effects of heme on isozymes of δ -aminolevulinic synthase.

To elucidate the effects of heme on δ -aminolevulinic synthase, MEL cells (DS-19 clone) were treated with 0.5 mM succinylacetone (SA) or 0.1 mM hemin, respectively. Cells were harvested after 48 hours of treatment, and total RNA from cells was subjected to Northern blot analysis. Succinylacetone is a potent inhibitor of δ -aminolevulinic dehydratase, and the enzyme activity is essentially nil under the treatment.

Abbreviation: ALAS-E, erythroid isozyme of δ -aminolevulinic synthase; ALAS-N, nonspecific isozyme of δ -aminolevulinic synthase.

was expressed even in erythroid cells, controlled by heme under a negative feedback regulation. In contrast, the erythroid isozyme of δ -aminolevulinic synthase was expressed only in the cells of erythroid lineage, and was positively regulated by heme. Thus, heme was shown to control two δ -aminolevulinic synthase isozymes in a distinctive manner (48, 49).

Another enzyme well known to be activated by heme is heme oxygenase, the key enzyme of the heme catabolic pathway (50, 51). The induction of heme oxygenase by heme is physiologically important, since the step catalyzes the toxic pro-oxidant, heme, to form anti-oxidants, biliverdin and bilirubin. Heme oxygenase, nowadays termed heme oxygenase-1 as an inducible isozyme, is induced not only by heme but also by biological stress, such as heat shock, acute phase, UV, and heavy metals (52). The physiological significance of stress-induced heme oxygenase-1 by stress was not clear, since it was not known whether induced enzyme produced more biliverdin or not. Recently, Nakayama et al. (53) demonstrated that the induction of heme oxygenase-1 resulted in an accumulation of bilirubin, indicating the biological importance of the marked increase in anti-oxidants.

When heme in erythroid cells undergoing differentiation is synthesized, almost all of the chemical is utilized to form hemoglobin, one of the most studied hemoproteins. The transcription and/or translation of globin should be, therefore, co-regulated with heme synthesis. The differentiation-resistant clone of MEL cells, DR-1 cells, was analyzed to show that the cells could not undergo erythroid differentiation due to the absence of erythroid-type heme biosynthesis (48). Although DR-1 cells failed to activate globin genes under differentiation treatment, exogenous hemin can accumulate globin mRNA (54) (Fig. 4). Furthermore, we observed a marked reduction in globin synthesis in both MEL cells reducing mRNA encoding erythroid isozyme of δ -aminolevulinic synthase by antisense method (55) and in ES cells carrying a disrupted isozyme (56). Taken together, globin genes are also

Fig. 4 Effect of dimethylsulfoxide and hemin treatment on β -globin mRNA.

Normal MEL cells (DS-19) or DR-1 cells were treated with 1.5% (v/v) dimethylsulfoxide (DMSO) alone or with a combination of DMSO and 0.1 mM hemin. Cells were harvested after 48 hours treatment, followed by Northern blot analysis to detect β -globin mRNA. MEL cells (DS-19) undergo erythroid differentiation in the presence of 1.5% (v/v) DMSO, while DR-1 cells cannot.

under the control of heme.

Studies indicate that heme has a regulatory effect on genes as described above, however, the mechanism of controlling genes has not yet been elucidated.

Heme deficiency and gene regulation

NF-E2 is one of the erythroid-type transcription factors, originally found in the erythroid promoter of the human porphobilinogen deaminase gene (57). Igarashi et al. proved that the heterodimer formation of NF-E2 p45 with small Maf protein (MafK) is essential to regulate genes (58). Today, heterodimers of the Maf-related oncoprotein family with transcription factor NF-E2 and its related factors Nrf1, -2 and -3 (59–62) are known to bind to Maf recognition elements (MAREs) (58, 63–65). MAREs are present in the regulatory region of various genes involved in heme metabolism such as oxidative stress-responsive genes (66), globin genes (67–70), the heme oxygenase-1 gene (71, 72), and the gene encoding erythroid isozyme of δ -aminolevulinate synthase (73).

In 1995, we found that the suppression of heme supply, by means of the antisense RNA expression of the erythroid isozyme of δ -aminolevulinate synthase, inhibited the induction of NF-E2 mRNA with no influence on MafK expression (55). A similar phenomenon was also observed in DR-1 cells (74), suggesting that the deficient heme supply seems to affect gene regulation via NF-E2 p45. Furthermore, the expression of NF-E2 p45 protein, its binding activity to DNA, and its enhancer activity were markedly lower in heme-deficient DR-1 cells than those in normal MEL cells undergoing erythroid differentiation (Fig. 5) (74).

Since NF-E2 p45 has been shown to be phosphorylated in vitro (75), the effects of protein kinase inhibitors on the NF-E2 DNA binding activity and cis-regulatory activity of the MARE site were examined to further understand the mechanism. Treatment with 2-aminopurine, a broad specificity serine/threonine kinase inhibitor, suppressed the DNA binding activity in differentiating

Fig. 5 Effect of dimethylsulfoxide on the enhancer activity of NF-E2. A luciferase reporter plasmid with three NF-E2 binding sites and pSV- β -galactosidase was cotransfected into DS-19 and DR-1 cells as described previously (74). Cells were incubated with 1.5% dimethylsulfoxide (DMSO) for 72 hours. Luciferase activity was normalized to the β -galactosidase value and expressed as the mean of three separate transfection experiments.

Fig. 6 Effect of the inhibition of serine/threonine phosphorylation on erythroid differentiation. mRNA for β -globin (black slash bars) and mRNA for erythroid isozyme of δ -aminolevulinate synthase (gray bars). Lane 1, 1.5% DMSO treatment for 48 hours; lane 2, 1.5% DMSO and 10 mM 2-aminopurine treatment for 48 hours; lane 3, 1.5% DMSO treatment for 54 hours; lane 4, 1.5% DMSO treatment for 48 hours, then 10 mM 2-aminopurine was added and incubated for another 6 hours.

MEL cells, while treatment with genistein, a tyrosine kinase inhibitor, had no effect (74). In addition, 2-aminopurine treatment suppressed MARE site-dependent promoter activity in cells undergoing erythroid differentiation. The suppression of serine/threonine phosphorylation also reduced the accumulation of mRNAs for β -globin and erythroid isozyme of δ -aminolevulinate synthase in differentiating MEL cells (Fig. 6). Serine/threonine phosphorylation, therefore, is suggested to be involved in both the induction of binding activity and the cis-regulatory activity of NF-E2, resulting in the upregulation of the erythroid genes such as β -globin and erythroid isozyme of δ -aminolevulinate synthase.

The Ras-Raf-MAPKK cascade ultimately phosphorylates serine/threonine residues of effector molecules including transcription factors (76), and involvement of the cascade in the NF-E2 function was examined. The coexpression of H-ras (H-Ras^{V12}) with NF-E2 p45 and MafK in NIH3T3 cells markedly increased the reporter gene activity (approx. 25-times) when compared with cells without H-Ras^{V12}. A greater increase in the reporter gene activity (approx. 50-times) was observed in cells coexpressing the active form of MAPKK with NF-E2 p45 and MafK. Both H-Ras^{V12} and MAPKK coexpression with NF-E2 p45 and MafK also increase the DNA binding activity of NF-E2 heterodimer

Fig. 7 Hypothetical scheme of the regulation of NF-E2 function.

(Fig. 7). Further analysis revealed that the N-terminal 83-amino acid region of NF-E2 p45 is required for the Ras-Raf-MAPKK cascade dependent regulation of the NF-E2 function, however, no obvious contribution of free heme or hemoprotein was identified.

Although the NF-E2 system seems to have some relationships with regulatory heme as described above, the precise mechanism has been the missing link.

Bach1, a heme controlled transcription factor

Like the NF-E2 family proteins described above, the transcription factors Bach1 and Bach2, basic leucine zipper (bZip) proteins, form heterodimers with the Maf proteins to bind MAREs, functioning primarily as repressors (69, 70, 59, 77, 78). Thus, the gene regulation operating through MAREs might be based on the balance between activation and repression. Purified Bach1 protein has a brown color, therefore, we thought that Bach1 was a hemoprotein. To examine this, we expressed various portions of Bach1 as fusion proteins with glutathione S-transferase (GST) and carried out binding assays (79). Bach1 has at least two functional domains, i.e., a BTB/POZ domain involving protein interaction at the N-terminal region and a DNA binding bZip domain at the C-terminal region (Fig. 8). When a portion of Bach1 without the BTB/POZ domain (amino acids 174–739) was incubated with hemin, a Soret band with a peak at 421 nm was observed which indicated Bach1 as a heme-binding protein. Scatchard analysis revealed that 1.3 mol of hemin bound to 1 mol of the portion of Bach1 (amino acids 174–739) with a K_d of 140 nM.

Using several GST fusions of Bach1 derivatives (portions of amino acids 174–739, 174–415, 417–739, 417–645, and 558–739), the heme-binding region was explored (Fig. 9). Though the N-terminal portion (amino acids 174–415) showed no specific binding to heme, the C-terminal portion (amino acids 417–739) showed a high affinity for heme that was as high as a portion of Bach1 without the BTB/POZ domain (amino acids 174–739). Two subfragments (417–645 and 558–739) still showed comparable heme-binding activities, almost half of that of the C-terminal

Fig. 8 Scheme of Bach1. CP motif (gray oval). Number indicates amino acid from the N-terminal.

Fig. 9 Binding of hemin to Bach1 subfragments. GST-Bach1 fusions (1 nmol) were immobilized onto Sepharose beads and incubated with 1 μ M hemin. The amounts of bound hemin were determined using spectrofluorometry. Each data bar represents the average and SEM of several independent experiments. The number indicates amino acid from the N-terminal of Bach1 protein.

portion (amino acids 417–739). In the C-terminal portion (amino acids 417–739), there are 4 Cys and Pro dipeptide (CP) motifs, which have been found in heme regulatory motifs (HRMs) to play an important role in heme binding (80–85). When we examined GST fusion proteins with single amino acid substitutions of each CP motif by changing Cys to Ala, no decline in heme binding activity was observed. In contrast, replacing 3 clustered CPs in the upstream of bZip domain or all 4 CPs reduced the heme-binding activity to 1/3 or to 1/10 of the control, respectively. Thus, the heme binding of Bach1 is mainly regulated by 4 CP motifs in the C-terminal portion (amino acids 417–739).

The specific binding of heme by CP motifs suggests that heme in Bach1 molecule has certain functions, therefore, the effects of heme on MARE binding activity were analyzed (Fig. 10). The addition of hemin to the DNA binding reactions showed a dose-dependent inhibition of binding activity by Bach1-MafK heterodimer. Even 0.03 μ M heme caused reproducible inhibition, and almost complete inhibition was observed in the presence of 0.01 μ M heme. Cys to Ala substitution of 4 CP motifs in Bach1 abolished the heme-mediated inhibition of DNA binding. It is, therefore, suggested that 4 CP motifs play an essential role in the regulatory function of heme via Bach1 binding. There are two possible mechanisms to reduce DNA binding by heme; one is the direct inhibition of Bach1-MafK heterodimer binding to MARE, and the other is through reduced heterodimer formation. The latter possibility was immunochemically examined. The addition of

Fig. 10 Involvement of the CP motifs of Bach1 in regulating its DNA-binding activity by heme.

BA1G417-739 (a Bach1 fragment of amino acid from 417 to 739) or BA1G417-739CP3-6AP (a mutated Bach1 fragment with CP to AP substitutions) and MafK were incubated in the absence or presence of various concentrations of hemin. Binding to the C β E probe was examined by EMSA. Heme concentrations were 0.1 μ M (lanes 4 and 9), 0.3 μ M (lanes 5 and 10), 0.5 μ M (lanes 6 and 11) and 1.0 μ M (lanes 7 and 12).

Fig. 11 Hypothetical scheme of the regulation of Bach1 by heme.

1 μ M hemin had no effect on the leucine zipper-mediated formation of a Bach1-MafK, suggesting that heme inhibits DNA binding activity of the heterodimer.

Functional effects of the interaction between heme and Bach1 in cells were examined using a MARE-dependent reporter

plasmid. Without hemin treatment, Bach1 repressed expression of the reporter gene, while 10 μ M of hemin in the medium abolished the repression by Bach1. When all CPs were altered to APs, Bach1 repressed the gene even in the presence of 10 μ M of hemin. It is, therefore, proved that one of the mechanisms of heme-controlled

transcription is regulation of the repression activity of Bach1 interacting with CP motifs.

Regulatory heme, gene regulation, and cell differentiation

When we consider the mechanism of gene regulation through Bach1 (Fig. 11), the Bach1 heterodimer represses genes with MAREs in their regulatory regions in cells with a lower heme concentration. If cellular heme increases, heme reduces the DNA-binding activity of Bach1 heterodimer, resulting in dissociation of Bach1 factor from the enhancers. A higher level of heme, therefore, opens MAREs for competing activators such as Nrf2 (72, 66) and NF-E2 p45 (69). We have reported that NF-E2 p45-MafK heterodimer binding activity is markedly induced in erythroid differentiating MEL cells as described in the preceding section (74). There is a remarkable induction in heme biosynthesis during erythroid differentiation (86), and the cellular heme level might decrease DNA-binding activity of Bach1 to increase the NF-E2-DNA complex. No increase in the NF-E2-DNA complex in DR cells (74) may support this hypothesis. The physiological functions of the Bach1/heme system seem to be biological modulation of the Ras-Raf-MAPKK signaling cascade through the function of NF-E2, which plays a significant role in erythroid lineage cell differentiation. One type of disorder in erythroid lineage cells is sideroblastic anemia, a heterogeneous disorder of erythroid differentiation, whose etiology is insufficient heme biosynthesis,

especially relating to dysfunction of the erythroid isozyme of δ -aminolevulinate synthase (87, 88). It is generally accepted that a part of sideroblastic anemia may be related to neoplasia; i.e., a clonal disorder that can progress to leukemia (87). Thus, heme-controlled Bach1, suggesting one of the essential factors for normal cell differentiation, might play a key role in chemical induced leukemia.

Further studies are, of course, obviously necessary to examine this possibility. For example, many environmental hazards decrease cellular heme concentration either by the inhibition of heme biosynthesis or by induction in heme degradation (13); among these chemicals, it has not yet been elucidated why some cause leukemia and the others do not. Nevertheless, our studies and the others described here demonstrated an important possibility that not the direct effect of environmental hazards but the affected cellular heme, a biological modulator, by environmental pollutants is the mechanism of leukemia through disturbances of normal cellular functions, including differentiation. In the field of toxicology, the study of such types on chemical hazards is necessary in the near future.

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