The Homodimeric Form of Glycine *N*-Methyltransferase Acts as a Polycyclic Aromatic Hydrocarbon-Binding Receptor†

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ABSTRACT: In the rat, cytochrome P4501A1 gene expression is thought to be regulated by several *trans*-acting factors including the 4S polycyclic aromatic hydrocarbon (PAH)-binding protein, which was recently identified as glycine *N*-methyltransferase [Raha, A., Wagner, C., MacDonald, R. G., and Bresnick, E. (1994) *J. Biol. Chem. 268*, 5750−5756]. Glycine *N*-methyltransferase (GNMT) is one of those unique proteins which exhibit diversity in function. Different subunit configurations are involved in its enzymatic role as a methyltransferase and as PAH-binding receptor. Here we report a systematic study of the oligomeric state of GNMT in the presence of benzo[a]pyrene (B[a]P) *in vivo* and *in vitro*. We have used chemical cross-linking and denaturing polyacrylamide gel electrophoresis to show that the B[a]P-binding unit of GNMT is a homodimer. We recently reported that phosphorylation is involved in the interaction of B[a]P with the 4S PAH-binding protein [Bhat, R., Weaver, J. A., Wagner, C., Bodwell, J. E., and Bresnick, E. (1996) *J. Biol. Chem. 271*, 32551−32556]. In the present study, this observation has been amplified by using bacterially expressed GNMT, which was post-translationally modified by a reticulocyte lysate and ATP-generating system. This modification was also accompanied by the formation of homodimers in the presence of B[a]P. These results indicate that post-translational modification is involved in determining the final configuration, i.e., dimeric form, of GNMT which then acts as a PAH-binding receptor.

One of the members of the cytochrome P450 family, CYP1A1,[1] which is induced severalfold by PAHs, such as B[a]P, is responsible for the bioactivation of these chemicals to metabolites that may play a role in environmental carcinogenesis (*1*). Evidence exists that such induction may be facilitated at least in part through a cytosolic receptor, the 4S PAH-binding protein (*2*−*8*). The 4S protein specifically binds certain PAHs with high affinity and in a saturable manner (*2*, *9*, *10*) and undergoes a nuclear translocation (*2*, *10*, *11*). The cytosolic 4S protein has recently been identified as glycine *N*-methyltransferase (GNMT) (*12*), an enzyme which catalyzes the synthesis of sarcosine from S-adenosylmethionine and glycine and whose activity is inhibited by 5-methyltetrahydrofolate polyglutamate (*31*). The cytosolic 4S PAH-binding protein has also been demonstrated to serve as a transcriptional activator of *CYP1A1* expression (*13*). Thus, the 4S protein belongs to a unique class of proteins that exhibit multiple functions, as an enzyme and as an activator of transcription.

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[1] Abbreviations: CYP1A1, cytochrome P4501A1; PAH, polycyclic aromatic hydrocarbon; B[a]P, benzo[a]pyrene; GNMT, glycine *N*-methyltransferase; *CYP1A1*, the CYP1A1 gene; SDS, sodium dodecyl sulfate; DSS, disuccinimidyl suberate; PMSF, phenylmethanesulfonyl fluoride.

GNMT, which was first found in an extract of guinea pig liver (*14*), was postulated to be involved in the oxidation of the methyl carbon of methionine. Purified rabbit liver GNMT was reported to consist of a minimum of three nonidentical subunits with M_r values between 27 000 and 33 000 (*15*). On the other hand, purified rat liver GNMT includes four identical subunits, each with a M_r of 31 500; the enzymatic homotetramer has a M_r of 132 000 (*16*).

A major role of GNMT in the oxidation of the methyl carbon of methionine is questionable as it accounts for only 20% of the total methionine methyl metabolism (*17*). However, GNMT may play a role in the regulation of the relative levels of S-adenosylmethionine and S-adenosylhomocysteine in the cell (*15*). In a later study by Cook and Wagner (*18*), GNMT was demonstrated to act as a folate-binding protein in rat liver cytosol. In our laboratory, we identified the 4S PAH-binding protein of rat liver as GNMT (*12*). As mentioned previously, rat liver GNMT, in its enzymatic role, occurs as a homotetramer (*16*); it exhibits independent binding sites for S-adenosylmethionine and folate (*18*−*20*), as well as for B[a]P (*12*). The enzymatic form of GNMT, i.e., the homotetramer, is inactive as a B[a]P-binding protein while nothing is known on the nature of the B[a]P-binding form. Therefore, we have investigated the structure/function relationship of GNMT as a PAH-binding protein in rat liver and these results are reported herein.

EXPERIMENTAL PROCEDURES

Materials. Rainbow protein markers for SDS−polyacrylamide gel electrophoresis were purchased from Pharmacia; the chemical cross-linker DSS from Pierce; reticulocyte

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lysate, ATP, phosphocreatine, and creatine phosphokinase were from Sigma; Immobilon P transfer membranes from Millipore; and BM^{v} Chemiluminesence western blotting kit from Boehringer-Mannheim, Inc. Affinity-purified antibodies to GNMT have been prepared, and their efficacy has been verified (*21*).

Purification of Bacterially Expressed GNMT. The plasmid containing the cDNA for rat liver GNMT was a generous gift of Dr. Hirofumi Ogawa. It was transferred into pRSET A using *Hin*dIII and *Nde*I, and expression was carried out in a methionine auxotrophic strain of BL21(DE3). Cells were grown in minimal media containing 40 mM MOPS buffer, pH 7.2, containing 20 mM glucose, 1.32 mM potassium phosphate, and micro nutrients (*22*). Methionine was added as a mixture of selenomethionine and sulfur methionine in a ratio of 4 to 1 at 4 mg/L. This provides GNMT that is enriched for selenomethionine. The selenomethionine-enriched enzyme has the same kinetic parameters as the native enzyme and purifies in the same way. Its crystal structure is the same as that of the enzyme containing only sulfur-methionine (unpublished data). Cells were induced with IPTG overnight at 30 °C. The cells were suspended in 50 mM Tris buffer, pH 7.5, containing 2 mM EDTA and 5 mM 2-mercaptoethanol (20 μL/g cells). Cells were lysed using lysozyme, freeze−thawing, and sonication and then centrifuged at 25 000*g* for 10 min to obtain a supernatant. The extract was applied to a DE-52 (Whatman) column equilibrated with 10 mM potassium phosphate buffer, pH 7.2, containing 50 mM NaCl. The flow-through fractions containing enzyme activity were concentrated using $(NH_4)_2SO_4$ by collecting the material that precipitated between 21 and 31 g/100 mL of solution. The pellet was dissolved in 10 mM potassium phosphate buffer, pH 7.2, containing 50 mM NaCl and chromatographed by gel filtration on a Sephacryl S-200 (Pharmacia) column equilibrated with the same buffer. Active fractions were again concentrated by $(NH_4)_2SO_4$ precipitation. The pellet was dissolved in 50 mM Tris buffer, pH 8.8, containing 5 mM DTT, 10 mM 2-mercaptoethanol, and 1 mM Na azide. Final purification was carried out using FPLC and a 15 μ Baker QUAT cation exchange column equilibrated with the same buffer and eluted with a NaCl gradient. A single isolated peak of protein was collected and was apparently homogenous by SDS−PAGE stained with Coomassie Blue. Enzyme activity was measured as described by Cook and Wagner (*18*).

Animals. Male Sprague−Dawley rats, 150 g in mass, were injected intraperitoneally (ip) with B[a]P (in corn oil) at 70 mg/kg body weight and were used 5 h later. Control rats were treated with corn oil alone. After treatment, the animals were sacrificed by cervical dislocation and were exsanguinated by decapitation. The livers were perfused *in vivo* with ice-cold buffer A (10 mM Hepes, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 10% glycerol), extirpated, and rinsed. The livers were subsequently minced and disrupted on ice in a Dounce glass homogenizer (Wheaton) in the same buffer at 2 mL/g of tissue. The homogenate was centrifuged at 10000*g* for 30 min at 4 °C and supernatant was collected as the *cytosolic fraction*. The resultant nuclear pellet was washed three times with buffer A and finally resuspended at 4 °C in the same buffer containing 0.5 M KCl. The suspension was mixed on a rotating wheel at 4 °C for 30 min, followed by homogenization in a Dounce apparatus to ensure complete lysis of the nuclei. This mixture was centrifuged at 10000*g* for 30 min, and the supernate was removed as the *nuclear fraction* and dialyzed twice for 1 h each time at 4 °C against 1 L of buffer A. Both the cytosolic and nuclear extracts were concentrated by Centricon 10 centrifugation, and the protein concentration was determined as described previously (*12*).

Chemical Cross-Linking and Western Blot Analysis. Two microliters of a DSS solution (final concentration, 1.5 mM) was added to 18 μL of cytosolic and nuclear extracts (∼50 μg of protein), and the reaction mixtures were incubated at room temperature for 30 min. The reaction was terminated by the addition of 6 μL of ethanolamine. Samples were resolved by SDS−PAGE on 10% polyacrylamide gels. After electrophoresis at 100 V for 90 min, the gels were transferred to Immobilon P membranes at 4 °C and western blotting was performed as described previously (*23*).

Reticulocyte Treatment and Assay for Specific Binding of Bacterially Expressed GNMT. Untreated rabbit reticulocyte lysate was added to the purified preparation of GNMT (1:9) with an ATP-generating system (this system consisted of 3 mM phosphocreatine, disodium salt, and 5 units/mL creatine phosphokinase) and the resultant mixture was incubated at 30 °C for 1 h. B[a]P-binding activity was determined as described previously (*21*).

RESULTS

Western Blot Analysis of Chemically Cross-Linked Cytosolic and Nuclear Extracts from Control and B[a]P-Treated Rats. In order to investigate the effect of B[a]P treatment on the oligomeric state of GNMT, the cytosolic and nuclear extracts were chemically cross-linked with DSS. When the 4S PAH-binding protein in these preparations was probed by western blot analysis, different subunit configurations were observed in the cytosolic and nuclear compartments of the livers from control and B[a]P-treated rats (Figure 1). In untreated rats, most of the liver GNMT is located in the cytosol (compare lanes a and b) and when chemically cross-linked, all of the GNMT is found in the tetrameric configuration (lanes c and d).

On the other hand, after B[a]P administration, a translocation of the GNMT from cytosol to nucleus is noted (compare lanes a and b to e and f). Upon chemical cross-linking, only the dimeric form of the GNMT appears in the nuclear compartment of the liver cells (lane h), whereas a mixture of tetrameric and dimeric forms (45:55 by densitometry) is found in the cytosolic compartment (lane g). The cytosolic and nuclear fractions obtained from the livers of B[a]P-treated rats were extensively dialyzed, and the interaction with labeled B[a]P was determined by sucrose density gradient analysis. The specifically bound B[a]P in the cytosolic and nuclear fractions was approximately 15 000 and 17 000 dpm, respectively. The specific binding was taken to be the difference between the radioactivities remaining in the cytosolic and nuclear preparations after incubation of [^{3}H]B[a]P with and without the 160-fold excess of unlabeled hydrocarbon (*24*). The fractions from the sucrose density gradients at the 4S position were also analyzed for dimer formation after chemical cross-linking followed by western blotting with affinity-purified antibodies to GNMT (Figure 1b). As is shown, both cytosolic and nuclear preparations were obtained in dimeric configuration

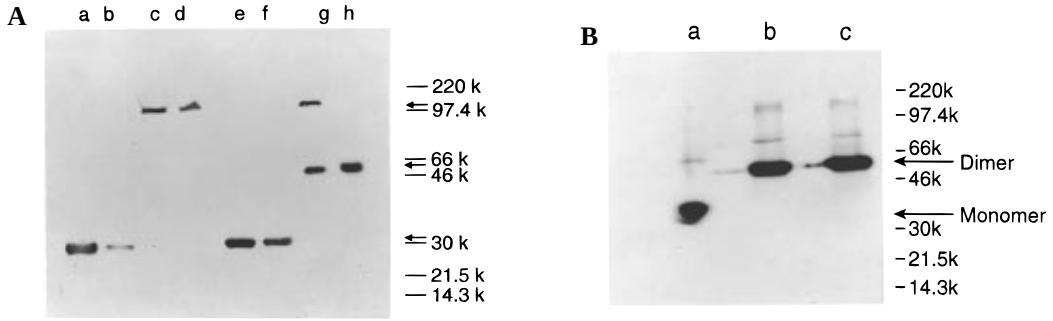

FIGURE 1: (A)Western analysis of chemically cross-linked cytosolic and nuclear fractions from control and B[a]P-treated rats. Rats were injected ip with B[a]P (70 mg/kg body weight) while control rats received the vehicle (corn oil). Cytosolic and nuclear extracts were prepared from the livers after 5 h and the proteins (50 μg) were chemically cross-linked with DSS (in dimethyl sulfoxide) as described in the Experimental Procedures. The proteins were analyzed on SDS−denaturing polyacrylamide gels, transferred to Immobilon P membranes, and probed with affinity-purified antibody to GNMT. Lanes a and b represent cytosolic and nuclear fractions from control rat liver; lanes c and d, the same fractions in the presence of DSS; lanes e and f, cytosolic and nuclear fractions from the livers of B[a]P-treated rats; lanes g and h, the same fractions in the presence of DSS. Molecular mass standards are given in the right lane; the positions of monomeric, dimeric, and tetrameric GNMT are indicated by arrows. (B) Western analysis of chemically cross-linked GNMT containing rat cytosolic and nuclear fractions isolated on sucrose density gradients. The cytosolic and nuclear fractions from control and B[a]P-treated rats were extensively dialyzed against buffer A, concentrated by centricon 10, and assayed for [^{3}H]B[a]P binding activity by sucrose density gradient analysis. The [^{3}H]B[a]P-containing fractions at the 4S position were again dialyzed, concentrated, and assayed for dimer formation by chemical cross-linking as above. Lane a, control GNMT in the absence of cross-linker; lanes b and c, cytosolic and nuclear preparations, respectively, treated with DSS. Molecular mass standards are given in the right lane; the positions of monomeric and dimeric GNMT are indicated by the arrows.

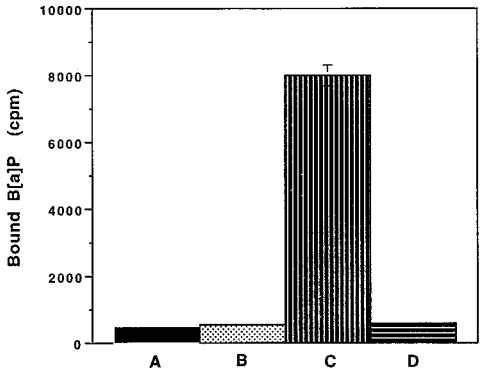

FIGURE 2: Sucrose density gradient analysis of B[a]P binding with reticulocyte lysate-treated bacterially expressed GNMT. Specific B[a]P-binding activity was assessed by sucrose gradient analysis. Briefly, 2−5 μg of bacterially expressed GNMT was treated with 9:1 reticulocyte lysate for 1 h at 30 °C, followed by incubation for 1 h at 4 °C with 10 nM [^{3}H]B[a]P. Fractions were layered onto linear 5−20% sucrose density gradients and centrifuged for 2 h at 372000*g* in a Beckman VTI-65 rotor. Ten drop fractions were collected and assayed for radioactivity in a liquid scintillation spectrometer. Column A, bacterially expressed GNMT; Column B, reticulocyte lysate + ATP-generating system; column C, bacterially expressed GNMT treated with reticulocyte lysate + ATP-generating system; column D, bacterially expressed GNMT treated with reticulocyte lysate + ATP-generating system + a 200-fold excess of nonradioactive B[a]P. The values represent the mean ± SE from three independent experiments.

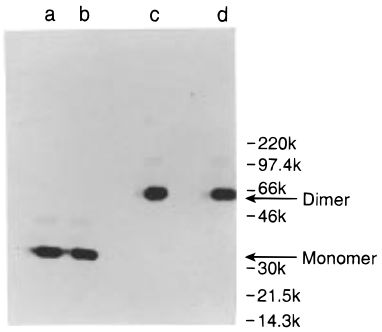

FIGURE 3: Western analysis of chemically cross-linked GNMT containing fractions isolated on sucrose density gradients. The fractions C and D from Figure 2 were extensively dialyzed against buffer A, concentrated by centricon 10 and assayed for dimer formation by chemical cross-linking. Lanes a and b are fractions C and D (from Figure 2) in the absence of DSS while lanes c and d represent the same fractions treated with DSS, respectively. Molecular mass standards are given in the right lane; the positions of monomeric and dimeric GNMT are indicated by the arrows.

(lanes b and c, respectively). In conjunction with our previous communication (*23*), the results suggest that the dimeric form of GNMT acts as the PAH-binding receptor and transcriptional activator of *CYP1A1*.

Effect of Reticulocyte Lysate Treatment on the B[a]P-Binding Activity and Oligomeric State of Bacterially Expressed GNMT. We have previously shown that phosphorylation plays an important role in the ligand-interacting activity of the 4S PAH-binding protein (*23*). The interaction of the bacterially expressed GNMT with B[a]P has been examined. Bacterially expressed GNMT is inactive as a PAH-binding protein (Figure 2), as it lacks the post-translational modification that is required for B[a]P interaction. A reticulocyte lysate has been used previously by other laboratories to post-translationally modify bacterially expressed proteins, e.g., the aldosterone receptor (*25*). We have investigated the effect of the reticulocyte lysate system on the ligand-binding activity of bacterially expressed GNMT (Figure 2). After treatment of the protein with the reticulocyte lysate in the presence of an ATP-generating system, [^{3}H]B[a]P-binding activity was recovered (Column C), which was completely inhibited when reactions were incubated in presence of 200-fold excess of unlabeled B[a]P (column D). In the absence of the ATP-generating system, little if any binding activity was observed (column A). The reticulocyte lysate itself was not able to specifically bind B[a]P (column B).

The protein fractions from preparations C and D (Figure 2) were extensively dialyzed against buffer A, concentrated by centricon 10, and analyzed for dimer formation by chemical cross-linking followed by western blotting (Figure 3). Both preparations incubated in the presence of [^{3}H]B[a]P (lane c) and [^{3}H]B[a]P + a 200-fold excess of unlabeled

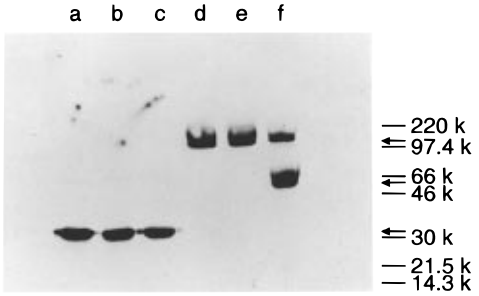

FIGURE 4: Effect of B[a]P treatment on the oligomeric state of bacterially expressed GNMT in the presence and absence of a reticulocyte lysate (and ATP-generating system). Bacterially expressed GNMT (0.5−1 μg) was treated with reticulocyte lysate followed by the addition of 4 μM B[a]P for 1 h at room temperature. Samples were chemically cross-linked with DSS and analyzed by western blotting with GNMT antibody as the probe. Lanes a−c represent bacterially expressed GNMT, GNMT + reticulocyte lysate (and ATP-generating system), and GNMT + reticulocyte lysate (and ATP-generating system) + B[a]P, respectively; lanes d−f are same samples in the presence of the chemical cross-linker, DSS. The positions of molecular mass standards are indicated on the right. The positions of the monomeric (33 kDa), dimeric (66 kDa), and tetrameric (132 kDa) GNMT are depicted by arrows (on the right).

B[a]P (lane d) generated homodimeric GNMT after chemical cross-linking while in the absence of treatment with the chemical cross-linker, only the monomeric form was observed (lanes a and b).

In order to investigate directly the effect of B[a]P treatment on the oligomeric state of the phosphorylated GNMT (treatment with the reticulocyte lysate and the ATP-generating system), reaction samples were chemically cross-linked with DSS, separated by denaturing polyacrylamide gel electrophoresis, and analyzed by western blotting (Figure 4). In the absence of chemical cross-linking, denaturing gel electrophoresis only shows the monomeric form of the bacterially expressed GNMT in all the reaction samples (lanes a−c). When chemically cross-linked, however, the tetrameric form of the protein is observed in the absence of B[a]P treatment (lanes d and e). In the presence of the phosphorylating system and after treatment with B[a]P, a mixture of tetrameric and dimeric configurations is observed (Lane f). These results are in confirmation of the dimeric form of GNMT acting as a PAH-binding protein.

DISCUSSION

Previous work from this laboratory (*12*) has identified the 4S PAH-binding protein as GNMT on the basis of several criteria. These included protein purification and protein sequencing of a 33 kDa band to yield GNMT, immunoprecipitation of PAH-binding activity from rat liver cytosol with polyclonal antibodies to GNMT, and copurification and colocalization of these two proteins in specific compartments of cell lines and tissues. In addition, upon introduction into hepatoma cells, both antisense to GNMT cDNA and antibody to GNMT significantly reduced the induction of *CYP1A1* in response to B[a]P but not to dioxins. These results established that GNMT, a protein that catalyzes sarcosine formation from glycine and S-adenosylmethionine, can also serve as a transcriptional activator of a totally unrelated system, CYP1A1.

Several other proteins, previously described as enzymes, have been demonstrated to function in nuclear regulation. These include the iron response element-binding protein, which regulates ferritin expression; it is identical to cytosolic aconitase (*26*). 4α-Carbinolaminedehydroxylase, a component of the phenylalanine hydroxylase system, is identical to a hepatic nuclear transcription factor (*27*).

There are also reports that proteins which exhibit multiple functions may exhibit different subunit configurations that are involved in these different functions. For instance, the 37 kDa subunit of glyceraldehyde 3-phosphate dehydrogenase acts as a DNA repair enzyme, nuclear uracil DNA glycosylase (*28*). Similarly, the different enzymatic and transcriptional activator properties of GNMT appear to be fulfilled by different subunit configurations. Methyltransferase activity requires the tetramerization of the protomeric 33 kDa subunit (*18*), whereas the role of transcriptional activator requires a dimeric configuration. Addition of B[a]P to rat cytosolic preparations did not exert any remarkable affect on methyltransferase activity. This is probably because GNMT is present in high concentration (1−2%) in rat liver cytosol and B[a]P is not able to saturate the interconversion of tetrameric and dimeric forms. As shown in the present study, the monomer or dimer of GNMT is not found in either the cytosol or the nucleus of control liver and dimeric conformation is produced only in response to B[a]P treatment of the animals or addition to cell-free systems. Consequently, the interconversion of the subunit configurations is important in determining the function of the resultant homooligomer which in turn is dependent on ligand treatment.

We have recently reported that phosphorylation is important in the interaction of B[a]P with GNMT and in its nuclear translocation (*23*). In addition, ligand binding stimulates nuclear translocation. A number of other transcription factors, such as NF-AT (*29*), NF IL-6 (*30*), and ISGF3 (*31*, *32*), are activated by stimuli which then regulate the translocation of either one or more of the subunits or the entire oligomeric factor into the nucleus.

It is evident from the present investigation that the dimeric form of GNMT translocates into nucleus in response to B[a]P binding. Phosphorylation (or other post-translational modification) is a prerequisite for the conversion of GNMT to the B[a]P-binding dimeric form. It is probable that phosphorylation alters the configuration of the protein in response to the availability of the ligand, which leads to a diversity of function. Many DNA-binding proteins function in dimeric form, which may require phosphorylation. This post-translational modification may result in an enhanced stability of the dimer, although examples are not widely reported. Since the GNMT in control rat liver (and in untreated rat hepatoma cells) occurs almost exclusively as the tetramer with little if any monomer noted, it is tempting to speculate that interaction of the protein with B[a]P occurs at the level of the tetramer, which then results in the rapid disaggregation to the dimeric state, i.e., the state for transcriptional activation. Hence, the rate-limiting factor in transcriptional regulation of *CYP1A1* expression may not be the amount of GNMT *per se* but the concentration of the dimer, which appears to be dependent on the concentration of B[a]P. In addition to the extent of dimerization and the subcellular localization, post-translational phosphorylation of the GNMT dimer may affect its stability or the extent of DNA binding, all of which could influence the overall property of transcriptional activation of this and other *trans*-acting factors (*33*).

In conclusion, we have demonstrated that the dimeric configuration of GNMT acts as a 4S PAH-binding protein and that the conversion from the predominant tetramer to the dimer is the result of complex interactions involving post-translational modifications, such as phosphorylation, and of the availability of the ligand.

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