Four Forms of Cytochrome P-450 in Human Fetal Liver: Purification and Their Capacity to Activate Promutagens

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Four forms of cytochrome P-450 were separated and purified to electrophoretic homogeneity from human fetal livers. These forms of cytochrome P-450, termed P-450HFLa, P-450HFLb, P-450HFLc and P-450HFLd, were distinguishable from each other in their molecular weights, spectral properties, immunochemical properties and mutagen-producing activities from promutagens. The molecular weights of P-450HFLa, b, c and d were estimated to be 51,500, 49,000, 51,500 and 50,000, respectively. Antibodies to P-450HFLa recognized P-450HFLc but not P-450HFLb or d, and antibodies to rat P-448-H (P-450IA2) cross-reacted with P-450HFLb but not with other forms of cytochrome P-450. The N-terminal amino acid sequence of P-450HFLc was highly homologous, but not identical, to that of P-450HFLa. Each form of cytochrome P-450 catalyzed mutagenic activation of aflatoxin B1 (AFB1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) at different rates. P-450 HFLa showed activities to produce mutagen(s) from AFB1, IQ and to a lesser extent from Glu-P-1. P-450 HFLb activated IQ at a faster rate than did the other forms. P-450 HFLc produced a mutagen from AFB1 and Glu-P-1 but not from IQ. P-450 HFLd did not activate these promutagens at significant rates.

Key words: Human fetal liver — Cytochrome P-450 — Purification — Promutagens

Human fetuses are capable of metabolizing structurally unrelated substrates including xenobiotics and endogenous compounds at an early gestational age in contrast to fetuses of other experimental animals.[1, 2] Since the majority of compounds readily cross the placenta,[3] human fetuses are exposed to a wide variety of environmental xenobiotics and drugs. Various environmental promutagens have been shown to be activated metabolically to mutagens in human fetal liver.[4, 5] It is, therefore, likely that human fetuses are at greater risk from exogenous compounds compared to fetuses of experimental animals. In a previous study, we purified one of the major forms of cytochrome P-450 (designated as P-450 HFLa) from human fetal liver; this form is responsible for the mutagenic activation of aflatoxin B1 (AFB1[4]).[5, 6] In regard to fetal carcinogenesis and teratogenesis, it seems very important to clarify the characteristics of human fetal cytochrome P-450. We show here that three of the four forms of cytochrome P-450 purified in this study are capable of activating various promutagens.

MATERIALS AND METHODS

Materials NADPH, NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Tokyo. Protein A-Sepharose and dilauroyl-L-3-phosphatidylcholine (DLPC) were from Pharmacia, Uppsala, and Serdary Research Laboratories, Ontario, respectively. *S. typhimurium* TA1535/ pSK 1002 and Emulgen 911 were kindly provided by Dr. Shimada of Osaka Prefectural Institute of Public Health and Kao-Atlas, Tokyo, respectively. Other chemicals were of the highest grade commercially available.

Purification of cytochrome P-450 from human fetal livers Livers from stillborn fetuses (around 20 w) were frozen at −70°C until use. Purification of the four forms of cytochrome P-450 from fetal liver homogenate was carried out as shown in Fig. 1. Liver homogenates were solubilized with sodium cholate (0.6% w/v) and then applied to an aminooctyl Sepharose 4B column which had been equilibrated with 100 mM potassium phosphate

[4] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; AFB1, aflatoxin B1; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; DLPC, dilauroyl-L-3-phosphatidylcholine.

In the present study, the apparent molecular weight of P-450 HFLa was estimated to be 51,500 rather than 51,000[6] when bovine serum albumin, catalase and aldolase were used as the standard proteins and when the mobility of P-450 HFLa was compared to that of P-450NF.[33] We therefore wish to revise the molecular weight of P-450 HFLa to 51,500.
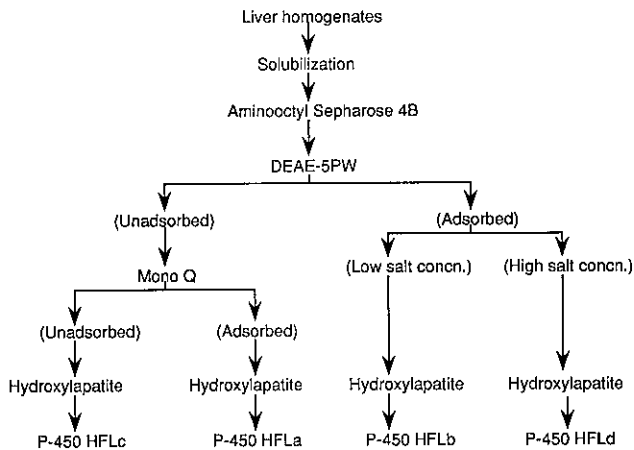
Fig. 1. Procedure for purification of four forms of cytochrome P-450 from human fetal livers.

buffer (pH 7.25) containing 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 0.6% sodium cholate. The column was washed with the equilibrating buffer, and cytochrome P-450 was eluted with 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.1% sodium cholate and 0.5% Emulgen 911. Fractions containing cytochrome P-450 were pooled and concentrated by ultrafiltration on a UK-50 membrane (Toyo, Tokyo). The concentrated fraction was diluted about 10-fold with 20 mM Tris-acetate buffer (pH 7.5) containing 20% glycerol and then injected into an FPLC system (Pharmacia) equipped with a preparative DEAE-5PW column (Toso, Tokyo) which had been equilibrated with 20 mM Tris-acetate buffer (pH 7.5) containing 20% glycerol and 0.4% Emulgen 911 (buffer A). Cytochrome P-450 was eluted with the equilibrating buffer, followed by a linear gradient of sodium acetate (0 to 300 mM) in the equilibrating buffer.

The cytochrome P-450 unadsorbed on the DEAE column was pooled and concentrated by ultrafiltration on a UK-50 membrane and then applied to a Mono Q column (Pharmacia) which had been equilibrated with buffer A. The fractions containing cytochrome P-450 eluted with buffer A and those eluted with a linear gradient of sodium acetate (0 to 500 mM) in buffer A were pooled separately. Further purification of the cytochrome P-450 was carried out by hydroxylapatite column (Kohen, Tokyo) chromatography.

The cytochrome P-450 adsorbed on the DEAE-5PW column was eluted in two peak fractions with a linear gradient of sodium acetate. The cytochromes P-450 were eluted from the column with the elution buffers containing around 150 mM and 210 mM sodium acetate, respec-

tively. Each fraction containing cytochrome P-450 was concentrated by ultrafiltration, and further purification was conducted by means of the FPLC system equipped with a hydroxylapatite column. Each pooled fraction from Mono Q or DEAE-5PW column chromatography was applied to a hydroxylapatite column which had been equilibrated with 10 mM sodium phosphate buffer (pH 7.5) containing 20% glycerol and 0.4% Emulgen 911. The cytochrome P-450 was eluted from the column by increasing the concentration of sodium phosphate in the equilibrating buffer.

Reconstitution of the monooxygenase system   The reconstituted system consisted of 50 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (0.5 mM NADP, 5 mM glucose 6-phosphate, 6 mM $MgCl_2$ and 0.5 unit of glucose 6-phosphate dehydrogenase), 25 μg of DLPC, 20 pmol of purified cytochrome P-450, 0.2 unit of NADPH-cytochrome P-450 reductase, 10 μM promutagen and bacteria in a final volume of 1 ml. If necessary, 20 pmol of cytochrome b_5 was added to the reconstituted system.

Assay for mutagen-producing activity   The induction of *umu* gene expression by metabolic activation of promutagens was measured using the tester strain, *S. typhimurium* TA 1535/pSK 1002 carrying the plasmid pSK 1002, which contains an *umu* C′-lac Z-fused gene that produces a hybrid protein with β-galactosidase.[7] The activity of β-galactosidase was measured spectrophotometrically using o-nitrophenyl-β-D-galactoside as the substrate.

Other methods   Cytochromes P-450 and b_5 were measured by the method of Omura and Sato.[8] Cytochrome P-448-H (P-450 IA2), cytochrome b_5 and NADPH-cytochrome P-450 reductase were purified from rat liver microsomes as described previously.[9-11] The activity of NADPH-cytochrome P-450 reductase was measured using cytochrome c as an electron acceptor.[12] One unit of the reductase was defined as the amount of enzyme which catalyzed the reduction of 1 μmol of cytochrome c per min. Antibodies to purified P-450 HFLa and P-448-H were raised in rabbits as described elsewhere.[13] SDS-PAGE and peroxidase staining were carried out according to the method described previously.[14, 15] The protein concentration was estimated by the method of Lowry *et al.*[16] using bovine serum albumin as a standard.

RESULTS

About 80% of cytochrome P-450 applied to an aminooctyl-Sepharose 4B column was recovered by washing the column with 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.1% sodium cholate and 0.5% Emulgen 911. Fig. 2 shows a typical elution profile of

cytochrome P-450 from a preparative DEAE-5PW column. As shown in the figure, cytochrome P-450 was eluted in three peak fractions with the equilibrating buffer containing a linear gradient of sodium acetate (0–300 mM). Each fraction was subjected to SDS-PAGE

before fractions were pooled. The fractions shown by oblique lines were pooled separately. Unadsorbed cytochrome P-450 eluted in the first peak fractions was further purified by means of Mono Q column chromatography as described in "Materials and Methods." As
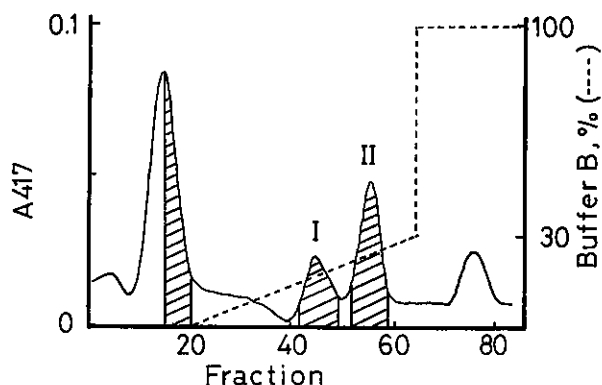

Fig. 2. Elution profile of cytochrome P-450 from a preparative DEAE-5PW column. Cytochrome P-450 was eluted by washing the column as described in "Materials and Methods." Buffer B consisted of 20 mM Tris-acetate (pH 7.5), 20% glycerol, 0.4% Emulgen 911 and 1 M sodium acetate. The eluate was collected to 3 ml fractions and monitored for cytochrome P-450 by measuring the absorbance at 417 nm.
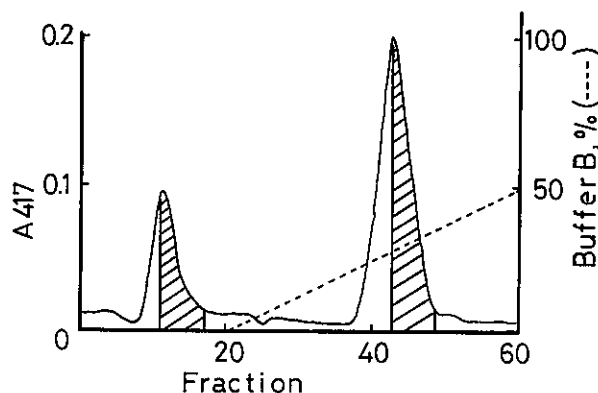
Fig. 3. Elution profile of cytochrome P-450 from a preparative Mono Q column. Cytochrome P-450 was eluted by washing the column as described in "Materials and Methods." Buffer B contained the same components as described in the legend to Fig. 2. The eluate was collected to 1.5 ml fractions and monitored for cytochrome P-450 by measuring the absorbance at 417 nm.

Table I. Summary of Purification of Cytochrome P-450 from Human Fetal Livers

Purification step	Total protein (mg)	Cytochrome P-450		Recovery (%)
		TC (nmol)	SC (nmol/mg)	
Solubilized supernatant	880	200	0.2	100
Aminooctyl Sepharose 4B	90	165	1.8	82
DEAE-5PW				
Unadsorbed	9.5	40	4.2	20
Mono Q				
Unadsorbed	1.0	7	7.0	3.5
Hydroxylapatite				
(P-450 HFLc)	0.2	2	10.0	1.0
Adsorbed	2.0	12	6.0	6.0
Hydroxylapatite				
(P-450 HFLa)	0.5	6	12.0	3.0
Adsorbed (low salt concn.)				
Hydroxylapatite	1.2	4	3.3	2.0
(P-450 HFLb)	0.2	1.5	7.5	0.8
Adsorbed (high salt concn.)				
Hydroxylapatite	0.9	3.8	4.2	1.9
(P-450 HFLd)	0.2	1.9	9.5	0.9

TC: total content. SC: specific content. Cytochrome P-450 was measured spectrophotometrically according to the method of Omura and Sato.[8]

shown in Fig. 3, cytochrome P-450 was eluted in two peak fractions from a Mono Q column. These fractions were separately pooled after each fraction had been subjected to SDS-PAGE. Cytochrome P-450 eluted in fractions indicated by oblique lines was applied to a hydroxylapatite column for further purification. The cytochromes P-450 eluted in the first and second peak fractions from a Mono Q column showed a single protein band in each case. These forms of cytochrome P-450 were referred to as P-450 HFLc and P-450 HFLa, re-

spectively. The forms of cytochrome P-450 eluted in the 2nd and 3rd peak fractions from a preparative DEAE-5PW column were purified by hydroxylapatite column chromatography. The cytochromes P-450 eluted in the 2nd and 3rd peak fractions from DEAE-5PW column were termed P-450 HFLb and P-450 HFLd, respectively.

The result of a typical purification of the four forms of cytochrome P-450, P-450 HFLa, b, c and d, from human fetal livers is summarized in Table I. Specific contents of purified P-450 HFLa, b, c and d were 12.0, 7.5, 10.0 and 9.5 nmol/mg of protein, respectively, and the recoveries of the cytochromes were 3.0, 0.8, 1.0 and 0.9% of the total cytochrome P-450 present in liver homogenates, respectively. As shown in Fig. 4, each of these final preparations gave a single protein band on SDS-PAGE. From the results of SDS-PAGE using bovine serum albumin, catalase and aldolase as standard protein markers, the apparent molecular weights of P-450 HFLa,
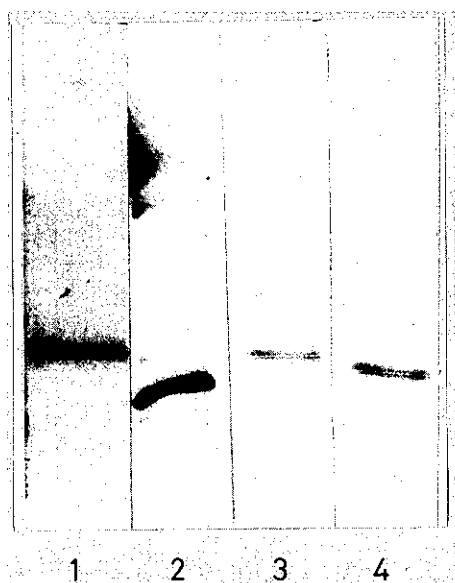

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified cytochrome P-450 from human fetal liver. Lanes 1 to 4 contain P-450 HFLa (0.75 pmol), P-450HFLb (1.5 pmol), P-450 HFLc (0.5 pmol) and P-450 HFLd (0.5 pmol), respectively.
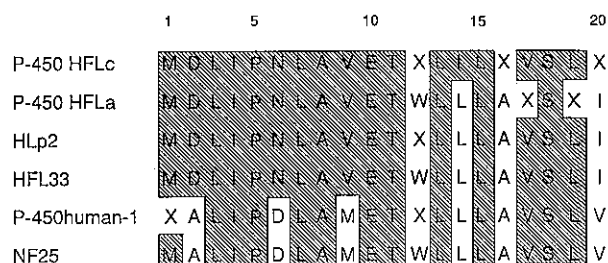

Fig. 5. Comparison of the N-terminal amino acid sequence of P-450HFLa with those of P-450HFLa and other forms of human cytochrome P-450 classified into the P-450III gene family. Amino acid residues marked X were unidentified. Amino acid sequences were obtained from the references given in parentheses; HFL33 (18), NF25 (19), P-450HLp2 (31) and P-450human-1 (32).

Table II. Metabolic Activation of Promutagens by Purified Cytochrome P-450 in a Reconstituted Monooxygenase System

Promutagen	System	Cytochrome P-450			
		P-450 HFLa	P-450 HFLb	P-450 HFLc	P-450 HFLd
		umu gene expression			
		(β-galactosidase unit/min/nmol P-450)			
AFB1	Complete	15.5	20.4	35.2	4.7
	plus cyt. b5	48.3	16.0	55.1	3.5
IQ	Complete	12.8	42.7	nd	4.5
	plus cyt. b5	26.4	20.8	nd	2.0
Glu-P-1	Complete	2.6	nd	24.0	nd
	plus cyt. b5	3.8	nd	36.1	nd

nd: < 15. cyt. b5: cytochrome b_5.

b, c and d were estimated to be 51,500, 49,000, 51,500 and 50,000, respectively. The oxidized forms of P-450 HFLa, b, c and d showed Soret peaks at 416.5, 416.5, 416.0 and 417 nm, respectively. The carbon monoxide-bound reduced forms of these cytochromes showed Soret peaks at 449.5, 448.5, 449.0 and 449.0 nm, respectively.

P-450 HFLc cross-reacted with antibodies to P-450 HFLa but not with antibodies to rat P-448-H (P-450IA2). P-450 HFLb, in contrast to P-450 HFLc, was cross-reactive with antibodies to the rat P-450IA2 but not with the antibodies to P-450 HFLa. Neither anti-P-450 HFLa antibodies nor anti-P-450IA2 antibodies recognized P-450 HFLd (data not shown). Although P-450 HFLa and P-450 HFLc were separately eluted from a Mono Q column, these cytochromes were indistinguishable in their immunochemical properties and molecular weights. Therefore, the N-terminal amino acid sequence of P-450 HFLc was compared with that of P-450 HFLa (Fig. 5). The N-terminal amino acid sequence of P-450 HFLc was highly homologous, but not identical, to those of P-450 HFLa and other forms of human cytochrome P-450 belonging to the P-450III gene family.

Table II shows the mutagen-producing activities of each form of cytochrome P-450 in a reconstituted system. AFB1 was activated to a genotoxic product by P-450 HFLc and P-450 HFLb as well as P-450 HFLa. P-450 HFLc activated AFB1 at a rate comparable with P-450 HFLa. P-450 HFLb as well as P-450 HFLa activated IQ efficiently, whereas P-450 HFLc was virtually ineffective for its activation. In contrast, P-450 HFLc was capable of activating Glu-P-1, while P-450 HFLb and P-450 HFLd were not. P-450 HFLa also catalyzed mutagen-production from Glu-P-1 but the activity of P-450 HFLa was much lower than that of P-450 HFLc.

DISCUSSION

It has been suggested that multiple forms of cytochrome P-450 exist in human fetal livers and catalyze biotransformation of a number of drugs.[17] In the present study, we purified four forms of cytochrome P-450 to electrophoretic homogeneity from human fetal livers. It is unclear at present why the specific contents of these forms of cytochrome P-450 are lower than those expected from their molecular weights. It is likely that low specific contents of the purified enzymes may be due to the presence of apoproteins. However, the possibility that impurities with the same mobility on SDS-PAGE as the cytochrome P-450 may be present in the final preparation cannot be excluded.

The purified cytochromes were distinct from each other in their molecular weights, immunochemical properties and mutagen-producing activities. We obtained a cDNA clone (λHFL33) containing the entire coding

region for a cytochrome P-450 related to P-450 HFLa.[18] The nucleotide and the deduced amino acid sequences of λHFL33 have been shown to be highly homologous to, but distinct from those of NF25[19] and HLp[20] cDNAs, which code for the forms of cytochrome P-450 classified into the P-450III gene family. P-450 HFLa and P-450 HFLc were very similar in their molecular weights and immunochemical properties but were different from each other in their chromatographic behavior and mutagen-producing activities. From the analyses of the total genomic DNA related to HLp, which is one of the forms of cytochrome P-450 classified into the P-450III family, it appears that there are three to five genes in the human P-450III family.[20] Since the N-terminal amino acid sequence of P-450 HFLc was highly related to but distinct from those of P-450 HFLa and other forms in the P-450-III family, it is likely that P-450 HFLc might be a new form of cytochrome P-450 belonging to the same gene family. In a previous study, we found that anti-P-450 HFLa antibodies almost completely inhibited the activity of 6β-hydroxylation of testosterone in human fetal liver.[21] Since, in the present study, anti-P-450 HFLa antibodies recognized not only P-450 HFLa but also P-450 HFLc, the possibility that P-450 HFLc is also responsible for testosterone 6β-hydroxylation cannot be excluded. Furthermore, the content of P-450 HFLa in human fetal liver appears to have been overestimated in a previous report[6] because the molecular weight of P-450 HFLc was indistinguishable from that of P-450 HFLa on SDS-PAGE, in addition to their immunochemical similarities.

Our recent study has shown that P-450 HFLa is expressed specifically in fetal liver but not in adult liver as evidenced by Northern blot analysis using oligonucleotide-specific regions of λHFL33 as probes.[22] It remains to be clarified whether or not P-450 HFLc is a form of cytochrome P-450 expressed only in fetal liver. We have recently shown that anti-P-450 HFLa antibodies inhibited more than 80% and 60% of the genotoxic product formation from AFB1 and IQ, respectively, in human fetal liver.[5, 23] Both P-450 HFLa and P-450 HFLc exhibited significant mutagen-producing activities from mycotoxin and protein pyrolysate products, indicating that these forms of cytochrome P-450 play toxicologically important roles in the human fetus. However, since anti-P-450 HFLa antibodies reacted with both P-450 HFLa and P-450 HFLc, the contribution of each form of cytochrome P-450 to the mutagenic activation of these promutagens is unknown at present.

Cytochrome P-450 related to rat P-450IA2 exists in adult human liver and functions in the metabolic activation of pyrolysates such as IQ and Glu-P-1.[24–27] We were able to purify a form of cytochrome P-450 immunochemically related to rat P-450IA2 from human fetal

liver. Although the purified cytochrome P-450 (P-450 HFLb) was apparently different from P-450PA (human P-450IA2)[28, 29] in molecular weight, P-450 HFLb also activated the pyrolysate products. Anti-rat P-450IA2 antibodies recognized a single protein with the same mobility on SDS-PAGE as purified P-450 HFLb in all liver samples from human fetuses (n=5) (data not shown), suggesting that P-450PA is not expressed in human fetal liver. It is, furthermore, likely that P-450 HFLb related to P-450IA2 may be one of the forms of cytochrome P-450 expressed constitutively in human fetal liver. Anti-rat P-450IA2 antibodies, which cross-reacted with P-450 HFLb, inhibited the mutagenic activation of IQ by about 40% but not that of AFB1 in human fetal liver,[23] suggesting that P-450 HFLb is one of the major forms of cytochrome P-450 responsible for the genotoxic product formation from IQ but not from AFB1. Recently, Omiecinski et al.[30] have suggested that cytochrome P-450 related to the P-450IA1 family is expressed in human fetal liver. However, at present, the relation between the presence of P-450 HFLb and the expression of P-450IA1 mRNA in human fetal liver remains to be elucidated.

It is well known that there are significant differences in the appearance of the forms of cytochrome P-450 in fetal livers of rats and humans. These differences between the rat and human in the expression of cytochrome P-450 in fetal liver suggest that the rat may not be an appropriate model animal for fetal toxicity studies.

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