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Differential expression and activation of a family of murine peroxisome proliferator-activated receptors

(nuclear hormone receptors/fatty acid/β-oxidation)

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ABSTRACT **To gain insight into the function of peroxisome proliferator-activated receptor (PPAR) isoforms in mammals, we have cloned and characterized two PPARα-related cDNAs (designated PPARγ and -δ, respectively) from mouse. The three PPAR isoforms display widely divergent patterns of expression during embryogenesis and in the adult. Surprisingly, PPARγ and -δ are not activated by pirinixic acid (Wy 14,643), a potent peroxisome proliferator and activator of PPARα. However, PPARγ and -δ are activated by the structurally distinct peroxisome proliferator LY-171883 and linoleic acid, respectively, indicating that each of the isoforms can act as a regulated activator of transcription. These data suggest that tissue-specific responsiveness to peroxisome proliferators, including certain fatty acids, is in part a consequence of differential expression of multiple, pharmacologically distinct PPAR isoforms.**

Peroxisome proliferators are a structurally diverse group of compounds which, when administered to rodents, elicit dramatic increases in the size and number of hepatic and renal peroxisomes as well as concomitant increases in the capacity of peroxisomes to metabolize fatty acids via increased expression of the enzymes required for the β-oxidation cycle (for review, see refs. 1–3). Chemicals included in this group are the fibrate class of hypolipidemic drugs, herbicides, and phthalate plasticizers (4). Peroxisome proliferation can also be elicited by dietary or physiological factors such as a high-fat diet and cold acclimatization.

Insight into the mechanism whereby peroxisome proliferators exert their pleiotropic effects was provided by the identification of a member of the nuclear hormone receptor superfamily activated by these chemicals (5). This receptor, termed peroxisome proliferator-activated receptor α (PPARα), was subsequently shown to be activated by a variety of medium- and long-chain fatty acids and to stimulate expression of the genes encoding rat acyl-CoA oxidase and hydratase–dehydrogenase, enzymes required for peroxisomal β-oxidation, as well as rabbit cytochrome $P450_{4A6}$, a fatty acid ω-hydroxylase (6–10). These data support a physiological role for PPARα in the regulation of lipid metabolism. PPARα activates transcription by binding to DNA sequence elements, termed peroxisome proliferator response elements (PPREs), as a heterodimer with the retinoid X receptor (RXR), itself a receptor for 9-*cis*-retinoic acid (11–15). As the PPARα–RXR complex can be activated by peroxisome proliferators and/or 9-*cis*-retinoic acid, the retinoid and fatty acid signaling pathways converge in modulating lipid metabolism.

Several PPARα-related proteins have been described in *Xenopus* and human; unlike PPARα, these additional isoforms are only weakly activated by peroxisome proliferators, raising the question of their physiologic function (16, 17). In this report, we describe the identification and characterization of two PPARα-related gene products present in mouse.§

MATERIALS AND METHODS

Screening of cDNA Libraries. PPARγ was isolated by screening an adult mouse liver λZAP cDNA library (Stratagene) with a synthetic oligonucleotide, 5′-GGNTTYCAY-TAYGGNGTNCAYGC-3′, under conditions previously described (18). This oligonucleotide is a mixture of all possible DNA sequences encoding the amino acid sequence GF-HYGVHA, a sequence present in the loop of the first zinc finger in the *Xenopus* PPARα, -β, and -γ isoforms. PPARδ was isolated by screening a 6.5-day embryonic mouse λZAP-II cDNA library (a gift of D. E. Weng and J. D. Gerhart, The Johns Hopkins University) under low stringency with a cDNA fragment encoding the human retinoic acid receptor α DNA-binding domain (19). In both screens, positive clones were converted to plasmids by the automatic excision process.

Cotransfection Assay. The mammalian expression vectors pCMX-PPARα, pCMX-PPARγ, and pCMX-PPARδ were constructed by inserting the cDNA inserts of PPARα, PPARγ, and PPARδ into pCMX (20). Construction of the reporter $PPRE_3$-TK-LUC (luciferase gene under control of the herpes simplex virus thymidine kinase promoter and three PPREs) has been described (11). Cotransfection assays in CV-1 monkey kidney cells were done in 48-well plates by using *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) according to the manufacturer's instructions (Boehringer Mannheim). Transfection mixtures contained 10 ng of receptor expression plasmid vector, 20 ng of the reporter $PPRE_3$-TK-LUC, 60 ng of pCMX-βGAL (β-galactosidase) as an internal control, and 210 ng of carrier plasmid pGEM. Cells were incubated in the presence of DOTAP for 8 hr, washed, and incubated in the presence of peroxisome proliferators or fatty acids for 36 hr. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity as described (20). All experimental points were done in triplicate.

Northern Analysis. Preparation of poly(A)$^+$ RNA from rat tissues and Northern analysis were performed as described (21).

DNA Binding Assays. Gel mobility-shift assays were performed as described (11). PPARα, -γ, and -δ and RXRα, -β,

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Abbreviations: PPAR, peroxisome proliferator-activated receptor; mPPAR, mouse PPAR; xPPAR, *Xenopus* PPAR, PPRE, peroxisome proliferator response element; RXR, retinoid X receptor.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U10374 and U10375).

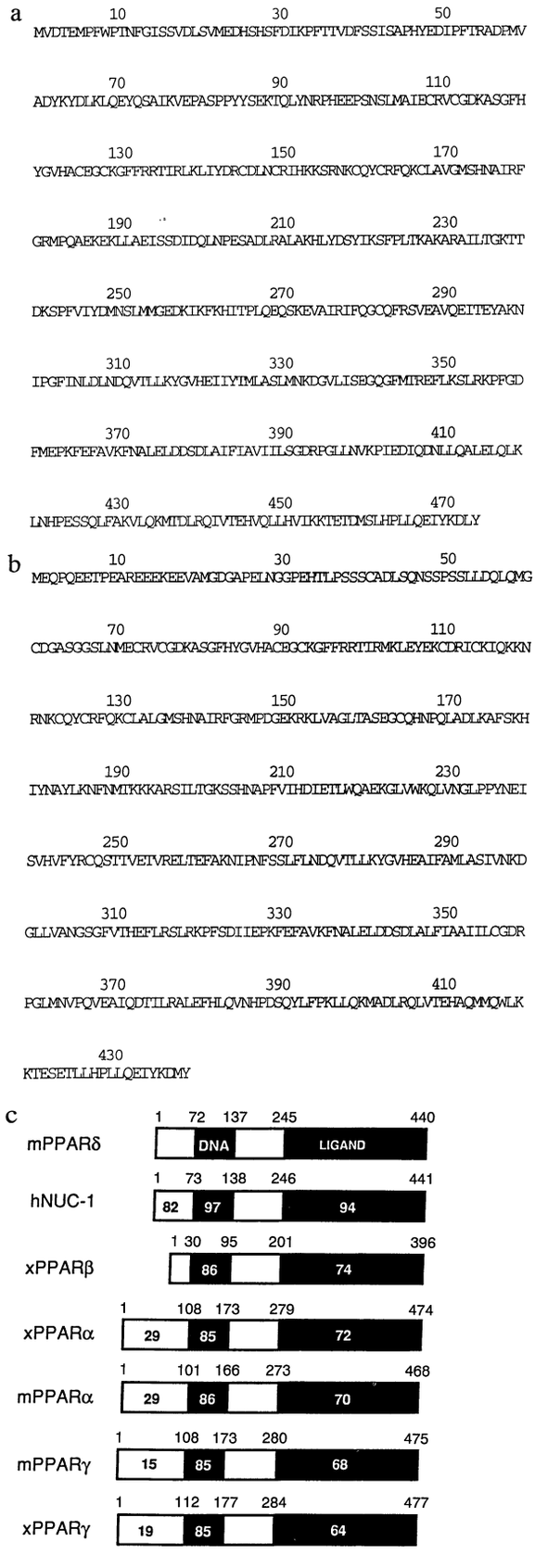

FIG. 1. Three mPPAR isoforms. (*a*) Sequence of the mPPARγ protein. (*b*) Sequence of the mPPARδ protein. (*c*). Schematic comparison of the members of the PPAR gene family using PPARδ as a reference. Comparisons among the different domains of the proteins are expressed as percent amino acid identity.

and -γ were synthesized *in vitro* by using the TNT coupled transcription/translation system (Promega) according to the manufacturer's instructions.

RESULTS

Isolation of Three Murine PPAR Isoforms. The function of peroxisome proliferators has been most extensively studied in rodents, where treatment with these compounds results in marked increases in peroxisome size and number and concomitant increases in the expression of the genes encoding the enzymes of the peroxisomal β-oxidation pathway. To gain insight into the function of PPAR isoforms in rodents, we screened mouse embryonic and adult liver libraries for PPARα-related gene products. In addition to PPARα, two types of PPARα-related clones were isolated. The first can encode a 475-amino acid protein that is 56% identical to mouse (m) PPARα and 76% identical to *Xenopus* (x) PPARγ, as this clone is 97% and 84% identical to the DNA-binding and ligand-binding domains of xPPARγ, respectively, we designate it mPPARγ1 (Fig. 1*A*). The second clone can encode a 440-amino acid protein that is closely related to NUC-1 (Fig. 1 *B* and *C*), a PPARα-related receptor isolated from a human osteosarcoma library (17). As this second clone is not highly homologous to mPPARα or the xPPARα, -β, and -γ isoforms (Fig. 1*C*), we believe it represents a novel receptor and designate it mPPARδ. Of the ≈50 positive clones characterized during the course of screening, no mouse homolog of xPPARβ was identified.

PPARα, -γ, and -δ Are Differentially Expressed in the Adult and Embryo. The expression patterns of the murine PPAR isoforms were examined in the embryo and adult. Northern analysis of poly(A)$^+$ RNA isolated from adult male rat tissues revealed differential yet overlapping patterns of expression of the three isoforms. Both PPARα and -δ were widely expressed, with PPARα message levels highest in the liver, kidney, heart, and adrenal, and PPARδ message highest in the heart, adrenal, and intestine (Fig. 2*A*). In contrast, PPARγ displayed a more restricted distribution pattern, with abundant expression in only the adrenal and spleen, although message was also detected in the heart, kidney, and intestine (Fig. 2*A*).

The developmental expression of the PPAR isoforms was also examined through Northern analysis of whole mouse embryo RNA. PPARα and -γ displayed similar expression patterns during mouse embryogenesis, with message first appearing at day 13.5 postconception and increasing until birth (Fig. 2*B*). In contrast, PPARδ message was abundant at all the embryonic time points tested, suggesting a broad role for this isoform during development (Fig. 2*B*). Thus, the PPAR isoforms are differentially expressed in both the embryo and the adult.

Evidence for Pharmacological Differences Between PPARα, -γ, and -δ. The relatively high degree of conservation within their ligand-binding domains suggested that the PPAR isoforms might respond to the same activators. Accordingly, each of the PPAR isoforms was first tested for responsiveness to Wy 14,643, a peroxisome proliferator and potent activator of PPARα (5). Cotransfection of PPARα expression plasmid resulted in a >100-fold increase in activation of a reporter construct containing three copies of the acyl-CoA oxidase PPRE upstream of the thymidine kinase promoter driving luciferase expression ($PPRE_3$-TK-LUC) in response to Wy 14,643 (Fig. 3). In contrast, no activation of reporter expression was seen in the presence of Wy 14,643 upon cotransfection of PPARγ or -δ expression plasmids (Fig. 3). This lack of activation is unlikely to reflect differences in binding-site specificity, as each of the PPAR isoforms bound efficiently to the acyl-CoA oxidase PPRE as a heterodimer with RXR (Fig. 4). Additional experiments revealed that overexpression of

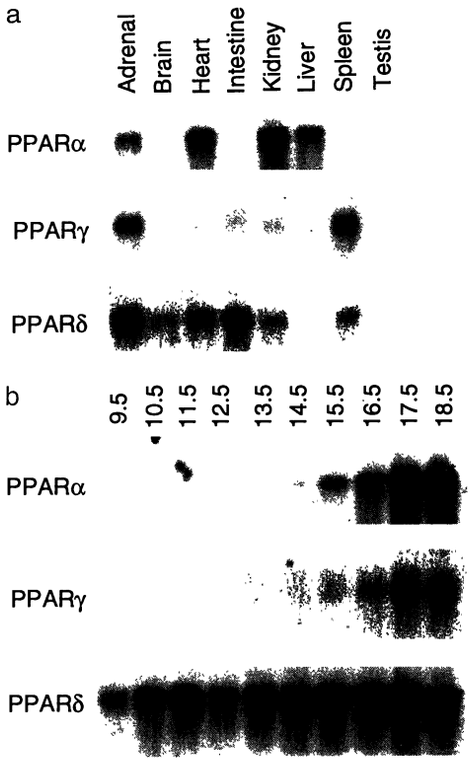

FIG. 2. Northern blot analysis of PPAR mRNA in adult and embryonic tissue. Adult male rat tissues (*a*) and mouse embryos from gestation days 9.5 to 18.5 (*b*). The exposure time for each of the blots was 48 hr. Sizes of the transcripts based on RNA size markers were 8.5 kb (PPARα), 1.9 kb (PPARγ), and 3.5 kb (PPARδ).

PPARγ and -δ interfered with the ability of PPARα to activate through the acyl-CoA oxidase PPRE (Fig. 5). Thus, both PPARγ and -δ are expressed and can function as dominant repressors of PPARα-mediated responsiveness to Wy 14,643.

As we failed to detect activation of PPARγ and -δ with Wy 14,643, other potential activators were tested, including a broad spectrum of peroxisome proliferators and fatty acids. Significant activation of PPARγ was obtained upon treatment with LY-171883 (Fig. 6), a leukotriene antagonist and peroxisome proliferator which lacks the carboxyl group typically found in this class of compounds (22). No activation of PPARγ was seen in the presence of linoleic acid (Fig. 6). In contrast, PPARδ was activated in the presence of linoleic acid but not upon treatment with LY-171883. Both LY-171883 and linoleic acid are strong activators of PPARα (Fig. 6). Interestingly, each of the three PPAR isoforms was activated with a distinct rank order of efficacy by these compounds (PPARα, Wy 14,643 > LY-171883 > linoleic acid; PPARγ-LY 171883 > linoleic acid > Wy 14,643; PPARδ

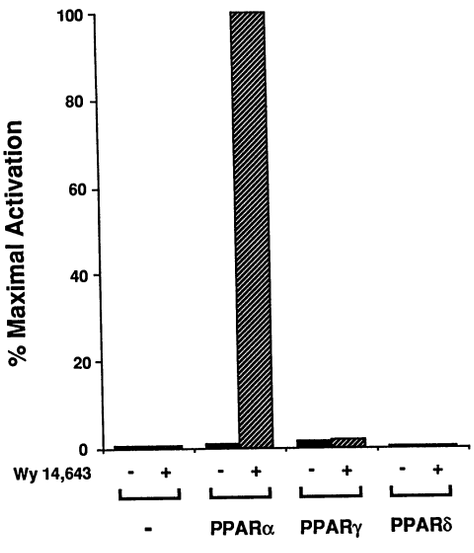

FIG. 3. PPARγ and -δ fail to respond to peroxisome proliferator Wy 14,643. CV-1 cells were cotransfected with reporter plasmid $PPRE_3$-TK-LUC and either no receptor expression plasmid (−), CMX-PPARα, CMX-PPARγ, or CMX-PPARδ and then incubated in either the absence (−) or presence (+) of 5 μM Wy 14,643. Luciferase activities are expressed as percentages of the activity obtained with PPARα in the presence of 5 μM Wy 14,643.

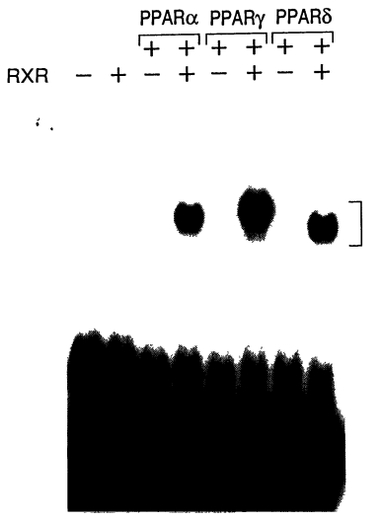

FIG. 4. PPARα, -γ, and -δ bind to the acyl-CoA oxidase PPRE as heterodimers with the RXR. Gel mobility-shift assays were done using *in vitro* synthesized PPARα, -γ, and -δ, with or without RXRγ as indicated, and ^{32}P-labeled acyl-CoA oxidase PPRE oligonucleotide. The positions of the PPAR–RXR–oligonucleotide complexes are indicated by the bracket. Similar results were obtained when either RXRα or -β was substituted for RXRγ (data not shown).

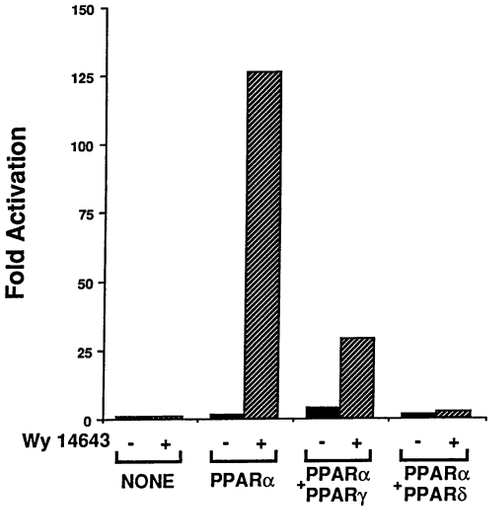

FIG. 5. PPARγ and -δ can repress PPARα-mediated responsiveness to Wy 14,643. CV-1 cells were cotransfected with reporter plasmid $PPRE_3$-TK-LUC and either no receptor expression plasmid (NONE) or CMX-PPARα (10 ng) in either the absence or presence of CMX-PPARγ (100 ng) or CMX-PPARδ (100 ng). Cells were then incubated in either the absence (−) or presence (+) of 5 μM Wy 14,643. Luciferase activities are presented as fold activation relative to cells which were not transfected with receptor expression plasmid and were not treated with Wy 14,643.

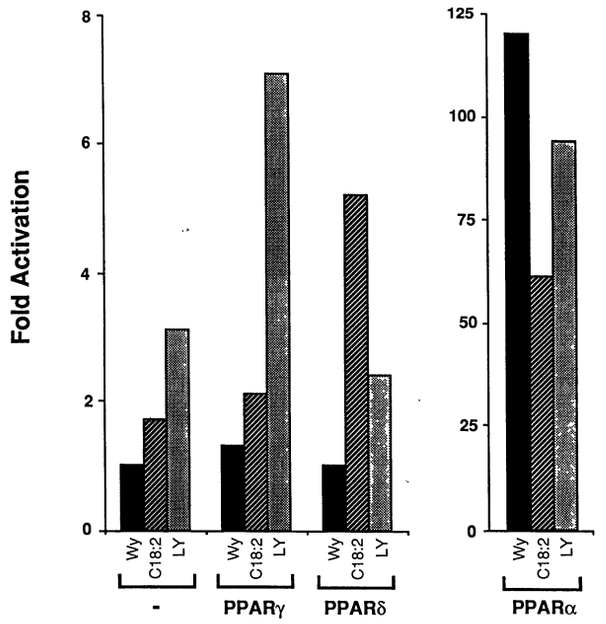

FIG. 6. PPAR isoforms are pharmacologically distinct. CV-1 cells were cotransfected with reporter plasmid $PPRE_3$-TK-LUC and either no receptor expression plasmid (−), CMX-PPARα, CMX-PPARγ, or CMX-PPARδ in either the absence or presence of 5 μM Wy 14,643 (WY), 30 μM linoleic acid (C18:2), or 30 μM LY-171883 (LY). Luciferase activities are presented as the fold activation achieved in compound-treated versus mock-treated cells. Similar results were obtained in triplicate in three independent experiments.

linoleic acid > LY-171883 and Wy 14,643) (Fig. 6). These data provide evidence that PPARγ and -δ can function as regulated activators of gene expression and that the three PPAR isoforms are pharmacologically distinct.

DISCUSSION

To further understand the basis for the effects of peroxisome proliferators in rodents, we have cloned and characterized two PPARα-related receptors in mouse. The three PPAR isoforms display marked differences in their responsiveness to peroxisome proliferators and fatty acids as well as differences in their temporal and spatial patterns of expression. These observations suggest a broad role for the PPAR family during development and in adult physiology.

What is the significance of multiple PPAR isoforms with distinct expression patterns? One possibility is that the three isoforms have different ligand specificities. Indeed, we have shown that the PPAR isoforms are pharmacologically distinct. PPARα, -γ, and -δ are most efficiently activated by Wy 14,643, LY-171883, and linoleic acid, respectively. Remarkably, Wy 14,643, which results in an ≈100-fold induction in reporter expression in the presence of PPARα, fails to activate either PPARγ or PPARδ. With regard to this differential responsiveness to activators, the relationship among the PPAR isoforms may be analogous to that between the glucocorticoid and mineralocorticoid receptors (GR and MR, respectively). While both receptors can bind to the same response elements and respond to mineralocorticoids and corticosteroids, the MR and GR display differential-sensitivities to aldosterone and specific glucocorticoids such as dexamethasone, respectively (23). Thus, the ratio of these receptors and their ligands provide a means of determining tissue-specific expression of target genes. Similarly, the existence of multiple PPAR isoforms with overlapping ligand specificities may provide the means for tissue-specific regulation of gene programs regulated by peroxisome proliferators and fatty acids, such as the peroxisomal β-oxidation cycle.

In addition to their differential responsiveness to peroxisome proliferators, the three PPAR isoforms also display distinct yet overlapping expression patterns. As previously shown, PPARα mRNA is abundant in liver and kidney (5, 24), tissues in which peroxisomes proliferators result in dramatic increases in the numbers of peroxisomes and concomitant increases in peroxisomal β-oxidation (3). In contrast, the message levels of PPARγ and -δ, which can act as dominant repressors of PPARα-mediated responsiveness to Wy 14,643, are low in these tissues. Thus, a pattern emerges in which tissues that are most responsive to peroxisome proliferators such as Wy 14,643 express high amounts of PPARα RNA and relatively low amounts of PPARγ and -δ (Fig. 2*A*). These data suggest that the ratio of the PPAR isoforms is likely to play a critical role in establishing the degree of responsiveness of tissues to specific peroxisome proliferators.

The widespread expression of PPARδ both in the embryo and in adult tissues suggests that this isoform may play a general "housekeeping" role. In contrast, PPARγ is selectively expressed in the adrenal and spleen. The expression of all three PPAR isoforms in the adrenal is particularly intriguing, as diseases which result in peroxisome dysfunction (e.g., adrenoleukodystrophy and Zellweger syndrome) cause gross morphological changes in adrenal cells and, eventually, adrenal deficiency, suggesting a critical role for peroxisomes in this tissue (reviewed in ref. 2). Interestingly, peroxisomes can be induced to proliferate in hamster adrenals in response to treatment with adrenocorticotropic hormone and corticosteroids (25), indicating the presence of adrenal-specific signaling pathway(s) for peroxisome proliferation. The differential expression of PPARγ in the adrenal suggests that this isoform may respond to an adrenal-enriched ligand.

We and others have recently shown that PPARα binds to its cognate response elements as a heterodimer with the RXR (11–13); the PPARα–RXR complex can respond to both peroxisome proliferators and 9-*cis*-retinoic acid (11). Likewise, PPARγ and -δ cooperate in binding to DNA as heterodimers with RXR. Thus, the identification of multiple PPAR isoforms clearly further augments the complexity of retinoid signaling. Ultimately, the regulation of peroxisome physiology is likely to be a consequence of a complex interplay among the multiple PPAR and RXR isoforms and the ligands for these receptors.

Finally, we note that while this manuscript was in preparation, two additional groups reported the cloning of mPPARγ (26, 27).

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