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Regulation of Two Soluble Forms of Brain Glutamate Dehydrogenase Isoproteins by Protein Kinases

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We isolated two soluble forms of glutamate dehydrogenase isoproteins, GDH I and GDH II, from bovine brain. The regulation of GDH I and GDH II by phosphorylation and dephosphorylation has been examined in various conditions. There were dose- and time- dependent activation of the GDH isoproteins when phosphorylated by cAMP-dependent protein kinase. The phosphorylated GDH had 1.1 mol of covalently bound phosphate/mol of subunit and a 2-fold increased specific activity. The phosphorylated amino acid was identified as serine. When treated with alkaline phosphatase, the activities of the phosphorylated GDH isoproteins were reduced in dose and time dependent manner and returned to those of unphosphorylated enzymes. There were no significant differences between GDH I and GDH II in their sensitivities to the action of phosphorylation and dephosphorylation, demonstrating that the microenvironmental structures of the phosphorylation site in GDH isoproteins are similar to each other. These results suggest that the inter-conversion between less active form of brain GDH isoproteins and more active form is regulated by phosphorylation through cAMP-dependent protein kinases.

Due to its neurotoxic potentials, glutamate may be involved in the pathogenesis of human degenerative disorders (McGeer and McGeer, 1976; Plaitakis et al., 1982). One enzyme central to the metabolism of glutamate is glutamate dehydrogenase (GDH). The importance of the pathophysiological nature of the GDH-deficient neurological disorders has attracted considerable interest. Hussain et al. (1989) detected four different forms of GDH isoproteins from human cerebellum of normal subjects and patients with neurodegenerative disorders. The enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed marked reduction of one of the GDH isoproteins (Hussain et al., 1989; Plaitakis et al., 1993). The origin of the GDH polymorphism is not known. The presence of four differently sized mRNAs and multiple gene copies for GDH in the human brain have been reported (Mavrothalassitis et al., 1988).

The mystery of GDH isoproteins and their role in neurobiology have just begun to be unraveled. As many proteins have functions distinct from those for which they were originally identified, it will also be of interest to assess other roles of GDH isoproteins. It has been reported that GDH from pig liver and brain possesses an ATP-dependent high-affinity microtubule-binding activity (Rajas and Rouset, 1993; Rajas et al., 1996). McDaniel (1995) has identified the complete primary structure of nuclear GDH from bovine liver and suggested the possibility that the most important role of nuclear GDH may be regulation of transcription. Preiss et al. (1993, 1995, 1997) have reported that GDH contains a binding site for heteropolymeric RNA with the highest affinity for an as yet undefined nucleotide consensus sequence or structure. It also has been reported that mitochondrial GDH from *Leishmania tarentolae* is a guide RNA-binding protein (Bringaud et al., 1997). Recent studies have shown that a novel nerve tissue-specific human GDH shows a different thermostability and ADP regulation to that of previously reported human GDH (Shashidharan et al., 1994). Cavallaro et al. (1997) have identified GDH as one of late memory-related genes in the hippocampus and Frattini et al. (1997) have identified GDH as a new member of the ring finger gene family in Xq24-25.

We isolated two soluble forms of glutamate dehydrogenase isoproteins, designated GDH I and GDH II, from bovine brain (Cho et al., 1995). They are hexameric enzymes with a subunit molecular mass of 56,500 Da. We identified GTP binding site within the GDH isoproteins using photoaffinity labeling (Cho et al., 1996). The results from our previous studies (Cho et al., 1995; Cho et al., 1996; Cho and Lee, 1996;

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Kim et al., 1997) demonstrated that the GDH isoproteins from bovine brain are different gene products rather than they are the products of posttranslational modifications. Although the three-dimensional structure of GDH from microorganisms is available (Baker et al., 1992), little is known about the structure of mammalian GDH, especially brain enzymes. Comparison of the detailed structure of active sites and regulatory sites of any GDH isoproteins has been rarely reported. It is, therefore, essential to have a detailed infomation on structure and function of the various types of brain GDH to elucidate the pathophysiological nature of the GDH-deficient neurological disorders.

It is well known that activities of some enzymes are regulated by a phosphorylation-dephosphorylation mechanism and that the reactions involved in the phosphorylation and dephosphorylation of proteins are catalyzed by protein kinases and phosphatases (Krebs and Beabo, 1979). In the present work, we show for the first time that two types of GDH isoproteins from bovine brain are activated through phosphorylation by cAMP-dependent protein kinases and reversibly inactivated by dephosphorylation.

Materials and Methods

Materials

NADH, 2-oxoglutarate, ADP, cAMP, ATP, cAMP-dependent protein kinases, and alkaline phosphatase were purchased from Sigma Chemical Co. The GDH isoproteins were purified from bovine brain by the method developed in our laboratory (Cho et al., 1995) and were homogeneous as judged by Coomassie-stained gradient SDS-polyacrylamide gel electrophoresis. All other chemicals and solvents were reagent grade or better.

Enzyme assay

GDH activity was measured spectrophotometrically in the direction of reductive amination of 2-oxoglutarate by following the decrease in absorbance at 340 nm as described before (Cho et al., 1995). All assays were performed in duplicate, and initial velocity data were correlated with a standard assay mixture containing 50 mM triethanolamine, pH 8.0, 100 mM ammonium acetate, 0.1 mM NADH, and 2.6 mM EDTA at 25℃. Under these conditions, the purified GDH isoproteins contained no detectable amount of phosphate. GDH concentrations were adjusted to give a measured rate of less than 0.04 absorbance units per min. The reaction was started by the addition of 2-oxoglutarate to 10 mM final concentration. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min at 25℃.

Protein phosphorylation

The phosphorylation of GDH isoproteins was carried out as follows. The reaction mixture contained 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 100 μM cAMP, and 50 μM ATP. The reaction was initiated by the addition of various amount of cAMP-dependent protein kinase and carried out at 30℃ with shaking. At various time interval, aliquots of the reaction mixture were withdrawn and the reaction was terminated by the addition of the stop solution containing 30 mM Tris/HCl (pH 7.5), 3 mM EDTA, 9% glycerol, and 1 M β-mercaptoethanol. The terminated samples were assayed for GDH activity by addition of GDH isoproteins and standard GDH assay mixture as described above.

Protein dephosphorylation

The phosphorylated GDH isoproteins were diluted with alkaline phosphatase buffer containing 100 mM Tris/HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$, and 5 mM $MnCl_2$ and treated with various amount of alkaline phosphatase at 30℃. At various time interval, aliquots of the reaction mixture were withdrawn, diluted with GDH assay buffer, and assayed for GDH activity by addition of GDH isoproteins and standard GDH assay mixture as described above.

Identification of phosphorylation site

^{32}P-Labeled GDH isoproteins were hydrolyzed in 0.5 ml of 6 N HCl for 5 h at 100℃. The hydrolysate was lyophilized, dissolved in 10 mM HCl with 0.5 mg of pure phosphoserine and phosphothreonine, and applied to a Dowex AG 50W-12 column (1 x 35 cm) equilibrated with 10 mM HCl. The flow rate was 15 ml/h and 1-ml fractions were analyzed for radioactivity and for ninhydrin positive material.

Results and Discussion

The functional significance of GDH in nerve tissue remains uncertain. The observations that one of the GDH isoproteins is reduced in patients with multisystemic neurological disorders have been obtained by many (Konagaya et al., 1986; Abe et al., 1992) but not all (Aubby et al., 1988; Duvoisin et al., 1988) investigators. Molecular biological studies revealed that multiple GDH-specific genes are present in the human and at least two of these genes are functional (Mavrothalassitis et al., 1988; Shashidharan et al., 1994). Similar studies showed that the presence of two GDH activities in rat brain differing in their relative resistance to thermal inactivation, solubility, and allosteric regulation characteristics (Colon et al., 1986). Studies in our laboratory have shown that two different GDH isoproteins (GDH I and GDH II) are also present in bovine brain (Cho et al., 1995). Unlike most previous reports, which present a soluble and a particulate form of GDH (Plaitakis et al., 1993; Rajas and Rouset, 1993), both GDH I and GDH II were readily solublized and no detergents were required for the initial extrac-

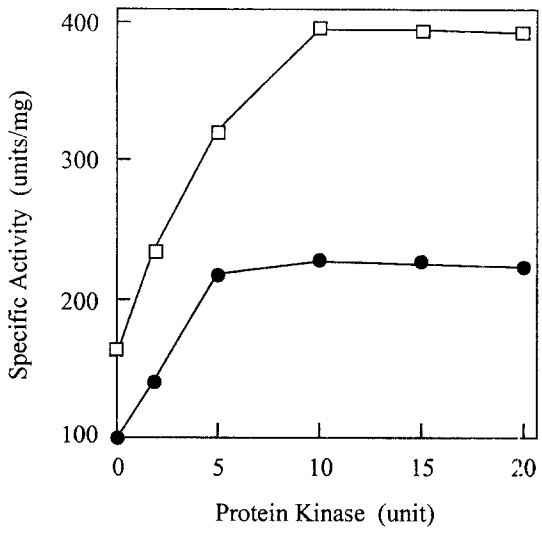

Fig. 1. Dose-dependent activation of GDH I (□) and GDH II (●) by cAMP-dependent protein kinase.

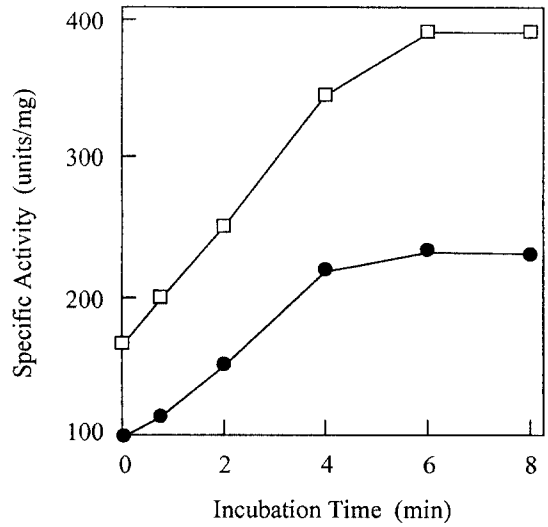

Fig. 2. Time-dependent activation of GDH I (□) and GDH II (●) by cAMP-dependent protein kinase. The phosphorylation of GDH isoproteins was carried out with 15 units of cAMP-dependent protein kinase.

tion step (Cho et al., 1995). The purified GDH I and GDH II showed different characteristics in their heat stability, sensitivities to the action of ADP, and remaining activities after limited proteolysis with trypsin.

Although the origin of the GDH polymorphism is not understood, biochemical studies in our laboratory have suggested that the two GDH isoproteins are produced from different genes based on their differences in amino acid sequences of N-termius and GTP-binding site (Cho et al., 1995; Cho et al., 1996). This possibility is supported by previous studies suggesting that human GDHs are encoded by a multigene family (Michaelidis et al., 1993) as well as the demonstration of two loci for human GDH genes on chromosomes 10 and X, respectively (Shashidharan et al., 1994). However, little is known about the structural and functional differences between the GDH isoproteins due to the lack of detailed information for the three-dimensional structure of mammalian GDHs, although very recent study has reported the crystallization of bovine liver GDH for the first time from the mammalian sources (Peterson et al., 1997).

In the present work, the effects of phosphorylation on GDH activities have been studied at various conditions for both GDH I and GDH II. Our results show that two types of GDH isoproteins from bovine brain are activated through phosphorylation by cAMP-dependent protein kinases and is reversibly inactivated by dephosphorylation. When effects of phosphorylation by cAMP-dependent protein kinase on GDH activities were studied in the direction of reductive amination of 2-oxoglutarate, a marked activation was observed for both isoproteins at kinase concentrations up to 20 units as shown in Fig. 1. These results indicate that the activation of brain GDH isoproteins by protein kinase was proportional to the extent of phosphorylation of the enzymes. The results in Fig. 2 show a time dependent activation of the GDH isoproteins up to a 2-fold in their specific activities when phosphorylated by cAMP-dependent protein kinase. There were no significant difference between GDH I and GDH II in their sensitivities to the action of phosphorylation.

When the phosphorylated GDH isoproteins were treated with alkaline phosphatase, the activities of the phosphorylated GDH isoproteins were reduced and returned to those of the unphosphorylated enzymes as shown in Fig. 3. The extents of decrease of the phosphorylated GDH activities were proportional to the concentration of alkaline phosphatase and incubation time (Figs. 3 and 4). Once again, there were no

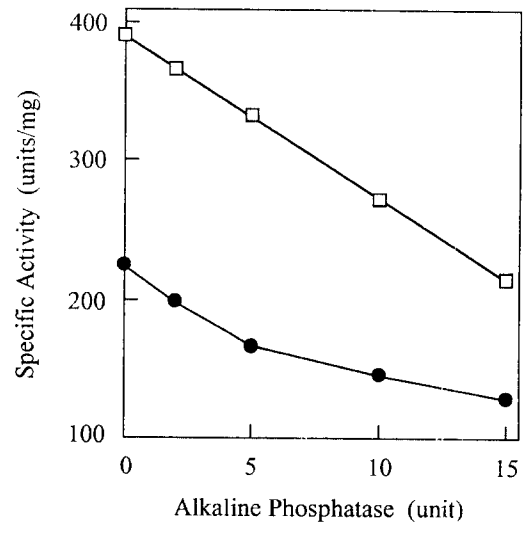

Fig. 3. Dose-dependent inactivation of the phosphorylated GDH I (□) and GDH II (●) by alkaline phosphatase.

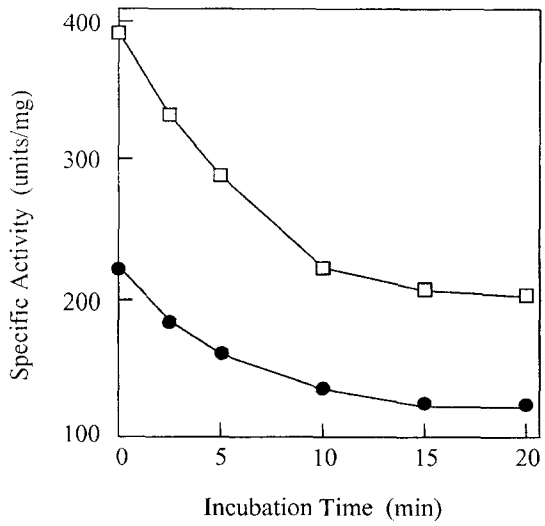

Fig. 4. Time-dependent inactivation of the phosphorylated GDH I (□) and GDH II (●) by alkaline phosphatase. The phosphorylated GDH isoproteins were treated with 15 units of alkaline phosphatase.

significant difference between GDH I and GDH II in their sensitivities to dephosphorylation, demonstrating that microenvironmental structures of the phosphorylation site in GDH isoproteins are similar to each other. These results strongly suggest that the inter-conversion between less active forms of brain GDH isoproteins and more active forms is regulated by phosphorylation and dephosphorylation. Previously, similar results were reported that the phosphorylation of NAD-dependent GDH from *Candida utilis* was promoted by glutamate starvation and was completely reversible (Hemmings, 1978). It was also reported that the interconversion of NAD-dependent GDH between active and inactive forms in *Saccharomyces cerevisiae* is regulated *in vitro* by the phosphorylation-dephosphorylation mechanism as indicated in *Candida utilis* (Hemmings, 1980). These results suggest that GDH is one of common substrates of cAMP-dependent protein kinases, although it remains to be studied whether the regulation of GDH activity by the phosphorylation-dephosphorylation mechanism occurs at the same manner *in vivo*.

A number of samples of phosphorylated GDH and dephosphorylated GDH were further analyzed for content of phosphate bound covalently to GDH isoproteins and compared with those of unphosphorylated GDH isoproteins. The results in Table 1 show that phosphorylated GDH isoproteins contained 1.1 mol of phosphate/mol of subunit, whereas dephosphorylated contained a small amount of phosphate, about 0.1 mol/mol of subunit. The amount of phosphate in the intact GDH isoproteins, on the other hand, was undetectable. The difference in specific activities between the phosphorylated enzymes and the dephosphorylated or intact enzymes was about 2-fold. These results suggest a linear relationship between extent of phosphorylation and activities of GDH isoproteins. To identify the

Table 1. Contents of phosphate covalently bound to phosphoGDH, dephosphoGDH, and intact GDH isoproteins

Enzyme	$[PO_4]$/[subunit][a]
PhosphoGDH I	1.11
PhosphoGDH II	1.09
DephosphoGDH I	0.08
DephosphoGDH II	0.09
Intact GDH I	ND[b]
Intact GDH II	ND[b]

[a]Mol of phosphate/mol of enzyme subunit. Phosphate determinations were carried out in duplicate according to the procedure described by Nimmo et al. (1976). With this method, 10 nmol gives A_{820} of 0.25. Mole of subunit was determined based on the molecular mass of 56,500 Da
[b]Not detectable.

phosphorylated amino acid, acid hydrolysis was carried out. Chromatography of acid-hydrolyzed ^{32}P-labeled enzymes on Dowex AG 50W-X12 yielded two peaks of radioactivity which were identified as a free phosphate and a phosphoserine (data not shown). This result indicates that the phosphorylated amino acid is serine, although identification of the exact phosphorylation residue in the overall sequence is necessary to confirm.

In conclusion, our present studies indicate that phosphorylation and dephosphorylation by protein kinase may play a role as GDH regulating enzymes, and therefore the subsequent physiological regulation of glutamate, one of the major excitatory neurotransmitters. It remains to be studied whether the distinct properties of the brain GDH isoproteins are essential for the regulation of in vivo glutamate metabolism.

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References

Abe T, Ishiguro SI, Saito H, Kiyosawa M, and Tamai M (1992) Partial deficient glutamate dehydrogenase activity and attenuated oscillatory potentials in patients with spinocerebellar degeneration. *Invest Ophthalmol & Visual Sci* 33: 447-452.

Aubby D, Saggu HK, Jenner P, Quinn NP, Harding AE, and Marsden CD (1988) Leukocyte glutamate dehydrogenase activity in patients with degererative neurological disorders. *J Neurol Neurosurg Psych* 51: 893-902.

Baker PJ, Britton KL, Rice DW, Rob A, and Stillman TJ (1992) Structural consequences of sequence patterns in the fingerprint region of the nucleotide binding fold: implications for nucleotide specificity. *J Mol Biol* 228: 662-671.

Bringaud F, Stripecke R, French GC, Freedland S, Turck C, Byrne EM, and Simpson L (1997) Mitochondrial glutamate dehydrogenase from *Leishmania tarentolae* is a guide RNA-binding protein. *Mol Cell Biol* 17: 3915-3923.

Cavallaro S, Meiri N, Yi CL, Musco S, Ma W, Goldberg J, and Alkon DL (1997) Late memory-related genes in the hippocampus revealed by RNA fingerprinting. *Proc Natl Acad Sci USA* 94: 9669-9673.

Cho S-W, Ahn J-Y, Lee J, and Choi SY (1996) Identification of a peptide of the guanosine triphosphate binding site within brain glutamate dehydrogenase isoproteins using 8-azidoguanosine triphosphate. *Biochemistry* 35: 13907-13913.

Cho S-W and Lee JE (1996) Modification of brain glutamate dehydrogenase isoproteins with pyridoxal 5'-phosphate. *Biochimie* 78: 817-821.

Cho S-W, Lee J, and Choi SY (1995) Two doluble forms of glutamate dehydrogenase isoproteins from bovine brain. *Eur J Biochem* 233: 340-346.

Colon A, Plaitakis A, Perakis A, Berl S, and Clarke DD (1986) Purification and characterization of a soluble and a particulate glutamate dehydrogenase from a rat brain. *J Neurochem* 46: 1811-1819.

Duvoisin RC, Nicklas WJ, Ritchie V, Sage J, and Chokroverty S (1988) Low leukocyte glutamate dehydrogenase activity does not correlate with a particular type of multiple system atrophy. *J Neurol Neurosurg Psych* 33: 1322-1326.

Frattini A, Faranda S, Bagnasco L, Patrosso C, Nulli P, Zucchi I, and Vezzoni P (1997) Identification of a new member (ZNF183) of the ring finger gene family in Xq24-25. *Gene* 192: 291-298.

Hemmings BA (1978) Phosphorylation of NAD-dependent glutamate dehydrogenase from yeast. *J Biol Chem* 253: 5255-5258.

Hemmings BA (1980) Purification and properties of the phospho and dephospho forms of yeast NAD-dependent glutamate dehydrogenase. *J Biol Chem* 255: 7925-7932.

Hussain MH, Zannis VI, and Plaitakis A (1989) Characterization of glutamate dehydrogenase isoproteins purified from the cerebellum of normal subjects and patients with degenerative neurological disorders, and from human neoplastic cell lines. *J Biol Chem* 264: 20730-20735.

Kim SW, Lee J, Song MS, Choi SY, and Cho SW (1997) Essential active-site lysine of brain glutamate dehydrogenase isoproteins. *J Neurochem* 69: 418-422.

Konagaya Y, Konagaya M, and Takayanagi T (1986) Glutamate dehydrogenase and its isoenzyme activity in olivopontocerebellar atrophy. *J Neurol Sci* 74: 231-236.

Krebs EG and Beabo JA (1979) Phosphorylation-dephosphorylation of enzymes. *Annu Rev Biochem* 48: 923-959.

Mavrothalassitis G, Tzimagiorgis G, Mitsialis A, Zannis VI, Plaitakis A, Papamatheakis J, and Moschonas NK (1988) Isolation and characterization of cDNA clones encoding human liver glutamate dehydrogenase: evidence for a small gene family. *Proc Natl Acad Sci USA* 85: 3494-3498.

McDaniel HG (1995) Comparison of the primary structure of nuclear and mitochondrial glutamate dehydrogenase from bovine liver. *Arch Biochem Biophys* 319: 316-321.

McGeer EG and McGeer PL (1976) Duplication of biochemical changes of Huntington's chorea by intrastriatal injection of glutamic and kainic acid. *Nature* 263: 517-519.

Michaelidis TM, Tzimagiorgis G, Moschonas N, and Papamatheakis J (1993) The human glutamate dehydrogenase gene family: gene organization and structural characterization. *Genomics* 16: 150-160.

Nimmo HG, Proud CG, and Cohen P (1976) The purification and properties of rabbit skeletal muscle glycogen synthase. *Eur J Biochem* 68: 21-30.

Peterson PE, Pierce J, and Smith TJ (1997) Crystallization and characterization of bovine liver glutamate dehydrogenase. *J Struct Biol* 120: 73-77.

Plaitakis A, Berl S, and Yahr MD (1982) Abnormal glutamate dehydrogenase deficiency in three patients with spinocerebellar syndrome. *Science* 216: 193-196.

Plaitakis A, Flessas P, Natsiou AB, and Shashidharan P (1993) Glutamate dehydrogenase deficiency in cerebellar degenerations: clinical, biochemical and molecular genetic aspects. *Can J Neurol Sci Suppl* 3: S109-S116.

Preiss T, Hall AG, and Lightowlers RN (1993) Identification of bovine glutamate dehydrogenase as an RNA-binding protein. *J Biol Chem* 268: 24523-24526.

Preiss T, Sang A, Chrzanowska-Lightowlers ZMA, and Lightowlers RN (1995) The mRNA-binding protein COLBP is glutamate dehydrogenase. *FEBS Lett* 367: 291-296.

Preiss T, Chrzanowska-Lightowlers ZM, and Lightowlers RN (1997) Glutamate dehydrogenase: an organelle-specific mRNA-binding protein. *Trends Biochem Sci* 22: 290.

Rajas F and Rouset B (1993) A membrane-bound form of glutamate dehydrogenase possesses an ATP-dependent high-affinity microtubule-binding activity. *Biochem J* 295: 447-455.

Rajas F, Gire V, and Rouset B (1996) Involvement of a membrane-bound form of glutamate dehydrogenase in the association of lysosomes to microtubles. *J Biol Chem* 271: 29882-29890.

Shashidharan P, Michaelidis TM, Robakis NK, Kresovali A, Papamatheakis J, and Plaitakis A (1994) Novel human glutamate dehydrogenase expressed in neural and testicular tissues and encoded by an X-linked intronless gene. *J Biol Chem* 269: 16971-16976.

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