Loss of the hepatic glycogen-binding subunit (G_L) of protein phosphatase 1 underlies deficient glycogen synthesis in insulin-dependent diabetic rats and in adrenalectomized starved rats

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Hepatic glycogen synthesis is impaired in insulin-dependent diabetic rats and in adrenalectomized starved rats, and although this is known to be due to defective activation of glycogen synthase by glycogen synthase phosphatase, the underlying molecular mechanism has not been delineated. Glycogen synthase phosphatase comprises the catalytic subunit of protein phosphatase 1 (PP1) complexed with the hepatic glycogen-binding subunit, termed G_L . In liver extracts of insulin-dependent diabetic and adrenalectomized starved rats, the level of G_L was shown by immunoblotting to be substantially reduced compared with that in control extracts, whereas the level of PP1 catalytic subunit was

not affected by these treatments. Insulin administration to diabetic rats restored the level of G_L and prolonged administration raised it above the control levels, whereas re-feeding partially restored the G_L level in adrenalectomized starved rats. The regulation of G_L protein levels by insulin and starvation/feeding was shown to correlate with changes in the level of the G_L mRNA, indicating that the long-term regulation of the hepatic glycogen-associated form of PP1 by insulin, and hence the activity of hepatic glycogen synthase, is predominantly mediated through changes in the level of the G_L mRNA.

INTRODUCTION

Protein phosphatase 1 (PP1) regulates many cellular processes, including glycogen metabolism, and its diversity of action resides in the ability of the PP1 catalytic subunit to form complexes with many different regulatory subunits, which may target it to different subcellular locations, modulate its substrate specificity and allow PP1 to be controlled by hormones, other extracellular signals and intracellular effectors [1–3]. The hepatic glycogen-binding subunit (G_L) targets PP1 to glycogen, where it may dephosphorylate and inhibit phosphorylase, in addition to dephosphorylating and activating glycogen synthase, thereby increasing hepatic glycogen synthesis. The interaction of G_L with PP1 enhances the glycogen synthase phosphatase, partially suppresses the phosphorylase phosphatase activity of the PP1 catalytic subunit and allows the glycogen synthase phosphatase activity to be regulated allosterically by phosphorylase *a*.

Phosphorylase *a* has been found to play a key role in the short-term regulation of hepatic glycogen synthase phosphatase [4–8], causing inhibition of the phosphatase activity by an allosteric effect [9]. This provides a mechanism for inhibiting glycogen synthesis as glycogenolysis is activated and vice versa. The level of hepatic phosphorylase *a* is increased *in vivo* by signals that elevate cAMP or calcium ions and is decreased by insulin or glucose. The potency of inhibition of synthase phosphatase by phosphorylase *a* is enhanced about 20-fold in the presence of glycogen, which explains why this control mechanism only operates in the fed and not the fasted state [4,10].

Purification of the phosphorylase *a* sensitive glycogen synthase phosphatase to near homogeneity from rat liver glycogen particles showed that it was composed of the PP1 catalytic subunit complexed to a 33 kDa protein (G_L), which was re-

sponsible for association with glycogen [11]. The G_L component interacted with phosphorylase *a* at nanomolar concentrations, suggesting that the binding of phosphorylase *a* to G_L underlies the inhibition of the glycogen synthase phosphatase activity. A cDNA encoding G_L was isolated from a rat liver library and sequenced [12]. The 284 residue, 32.6 kDa protein is 23 % identical with (39 % similar to) the N-terminal region of the glycogen-binding (G_M) subunit of PP1 G_M , the form of PP1 associated with glycogen in striated muscle. The bacterially expressed G_L bound phosphorylase *a* at nanomolar concentrations and interacted with the catalytic subunit of PP1, modulating its substrate specificity.

Diabetes induced in rats by destruction of the pancreatic β -cells with streptozotocin or alloxan provides an animal model for human Type I (insulin-dependent) diabetes mellitus, in which loss of insulin production results from autoimmune destruction of the pancreatic β -cells. One of the major effects noted in Type I diabetes is disturbance of hepatic glycogen metabolism, the activation of glycogen synthase in response to raised glucose levels being severely impaired [13–15]. The cause of the defective activation of glycogen synthase in diabetic rats was identified as a specific loss of the glycogen-bound glycogen synthase phosphatase activity [16,17], which was later shown to be a type 1 protein phosphatase [18,19]. Treatment of insulin-dependent diabetic rats with insulin restored the glycogen synthase phosphatase activity [16,20], but the mechanism by which insulin mediates this effect was not elucidated.

Glucocorticoids induce the synthesis of glycogen synthase and glycogen synthase phosphatase, initiating the gradual accumulation of hepatic glycogen in fetal liver [21,22]. Adrenalectomy in the adult, which abolishes the synthesis of glucocorticoids, reduces the glycogen synthase phosphatase activity in liver

Abbreviations used: G_L , hepatic glycogen-binding subunit; GST, glutathione S-transferase; MBP, maltose-binding protein; PP1, protein phosphatase 1.

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extracts by 30–40 % [23,24]. Starvation of rats for 48 h causes a similar reduction in synthase phosphatase activity, whereas a combination of adrenalectomy and starvation results in a 90 % reduction in glycogen synthase phosphatase activity [23–25]. The effects can be reversed by re-feeding and by administration of glucocorticoids. Re-feeding restores the levels of circulating insulin, which are very low in adrenalectomized starved rats, whereas glucocorticoids exert their effect without increasing insulin levels [26,27]. These data suggest that insulin and glucocorticoids act synergistically to maintain hepatic glycogen synthase phosphatase activity.

The findings described above indicate that the PP1- G_L complex is responsible for the dephosphorylation of glycogen synthase in hepatic glycogen particles and raise the question of whether the PP1- G_L complex is the source of the glycogen-associated synthase phosphatase activity that disappears in diabetic or adrenalectomized starved animals. We have therefore studied the effects of diabetes and adrenalectomy plus starvation on the G_L protein and PP1. The results demonstrate that the G_L protein levels are severely reduced by these treatments, which explains why glycogen synthase cannot be activated in severely diabetic animals.

MATERIALS AND METHODS

Treatment of animals and subcellular fractionation

All experiments were performed with male Wistar rats weighing about 250 g and fed *ad libitum*, unless otherwise stated. The animals were killed by decapitation without prior anaesthesia between 09:00 h and 11:00 h. Diabetes was induced by an intravenous injection of streptozotocin (55 mg/kg) and animals were killed 4 days later, or they received at that time one or four daily subcutaneous injections of insulin (5 units) and were killed 24 h or 96 h later respectively. Blood glucose levels, measured as in [16], ranged from 6.2 to 7.9 mg/ml in diabetic animals before insulin treatment. Bilateral adrenalectomy was performed under ether anaesthesia. The adrenalectomized rats were kept for 5 days under normal feeding conditions and with 0.15 M NaCl as drinking water. Then they were either fed *ad libitum* for an additional 48 h ('adrenalectomized'), or starved for 48 h ('adrenalectomized starved'), or first starved for 48 h and then re-fed for 48 h ('adrenalectomized starved and re-fed').

A part of the liver was immediately freeze-clamped for the preparation of RNA. The remainder of the liver was homogenized in a buffer containing 50 mM glycylglycine at pH 7.4, 3 mM EGTA, 5 % glycerol, 0.5 mM dithiothreitol, 5 mM 2-mercaptoethanol, 0.5 mM benzamidine and 0.3 mM PMSF. Liver extracts were prepared by centrifugation of the homogenates for 15 min at 10000 g. The glycogen fraction was prepared from the liver of glucagon-treated, overnight starved rats or from adrenalectomized starved rats by the addition of glycogen to the cytosolic fraction and re-isolation of the protein-glycogen complex, as described in [23].

Protein phosphatase assays

Glycogen synthase phosphatase activities were measured in liver extracts at a final dilution of 2 %, or in the glycogen fraction at a final dilution of 20 %, by the activation of purified glycogen synthase *b* from dog liver as described previously [23]. The phosphorylase phosphatase activity in the glycogen fraction was derived from the rate of dephosphorylation of ^{32}P -labelled muscle phosphorylase *a*. The phosphorylase phosphatase activities were assayed either as such ('spontaneous' activity) or after pre-incubation with trypsin (0.1 mg/ml) for 5 min at 30 °C ('total' activity), resulting in the release of free, C-terminally nicked

catalytic subunit. The action of trypsin was arrested by the addition of soybean trypsin inhibitor (1 mg/ml).

Immunoblotting

A glutathione S-transferase (GST)- G_L construct [12] was expressed in *Escherichia coli* BL21 and the insoluble GST- G_L was purified from the inclusion bodies and subjected to SDS/PAGE as described in [28]. The major band visible 2–5 min after immersion of the gel in 3 M KCl was excised, eluted by incubating the ground gel slice for 16 h at 37 °C in 0.137 M NaCl/2.68 mM KCl/1.76 mM KH_2PO_4 /10.1 mM Na_2HPO_4 (pH 7.4)/0.01 % SDS and injected into sheep at the Scottish Antibody Production Unit (Carluke, Lanarkshire, U.K.). Bacterially expressed maltose-binding protein (MBP)- G_L (expression vector from New England Biolabs, Inc., Beverly, MA, U.S.A.) was purified from inclusion bodies as described above and coupled to CNBr-Sepharose. The GST- G_L antibodies raised were affinity purified on MBP- G_L -Sepharose columns. Antibodies to bacterially expressed human PP1 γ [29] and to the N-terminal 55 amino acids of PP4 [28] fused to GST were also raised at the Scottish Antibody Production Unit and affinity purified.

Proteins in the liver extracts and glycogen particles were separated by SDS/PAGE, transferred to nitrocellulose membranes and probed with 0.1 $\mu\text{g/ml}$ affinity purified antibodies. Staining was detected using anti-sheep IgG antibodies conjugated to horseradish peroxidase (Pierce and Warriner, Chester, U.K.), followed by enhanced chemiluminescence (Amersham International, Amersham, Bucks., U.K.). Densitometric analysis of signal intensity was performed on an Astra 1200S flat bed scanner using an NIH Image analysis computer program. The levels of G_L were normalized to the levels of a protein phosphatase catalytic subunit (PP4 or PP1).

RNA analyses

Total RNA was prepared using the Rapid Total RNA Isolation Kit (5 Prime-3 Prime Inc., Boulder, CO, U.S.A.) according to the manufacturer's protocol. The RNA, denatured in 50 % formamide/6.5 % formaldehyde/0.1 M Mops (pH 7.0)/0.25 % ethidium bromide at 65 °C for 5 min, was examined by electrophoresis in a 1 % agarose gel in 0.1 M Mops (pH 7.0) containing 6.5 % formamide. The RNA was transferred to positively charged nylon membranes (Fluka, Gillingham, Dorset, U.K.) by overnight capillary transfer in 3 M NaCl/0.3 M sodium citrate (pH 7.0) and cross-linked to the membrane by heating for 2 h at 80 °C. RNA on the blot was examined by hybridization to an [α - ^{32}P]dATP-labelled cDNA corresponding to the coding region for G_L [12]. Blots were stripped by immersion in 1 % SDS at 100 °C, cooled to room temperature and rehybridized with a ^{32}P -labelled β -actin probe.

RESULTS

The level of hepatic G_L is substantially reduced in streptozotocin-diabetic rats and in adrenalectomized starved rats

The level of the PP1 G_L was examined in liver extracts of diabetic and adrenalectomized starved rats by immunoblotting. Figure 1(A) shows that the G_L protein levels are substantially reduced in streptozotocin-diabetic rats compared with fed control animals. In contrast, the level of PP1 catalytic subunit did not vary. An antibody against the unrelated protein phosphatase, PP4, was used as a control to show that the loading of the samples was constant. Treatment of the streptozotocin-diabetic rats with 5 units of insulin for 24 h led to a partial restoration of the G_L

Figure 1 Reduction of levels of the G_L protein in the livers of streptozotocin-diabetic rats

(A) Liver extracts from rats were subjected to gel electrophoresis, transferred to nitrocellulose membranes and probed with anti-G_L, anti-PP4 or anti-PP1 antibodies. Lanes 1, 2, 5 and 6, fed controls; lanes 3, 4, 7 and 8, streptozotocin-diabetic rats; lanes 9 and 10 streptozotocin-diabetic rats treated with 5 units of insulin for 24 h; lanes 11 and 12, streptozotocin-diabetic rats treated with 4 × 5 units of insulin for 96 h. (B) Comparison of the levels of G_L protein in liver extracts (black bar), the glycogen synthase phosphatase activity in liver extracts (hatched bar) and the blood glucose levels (grey bar) from fed control rats, diabetic rats and diabetic rats treated with insulin for 24 h or 96 h. The left panel shows the mean levels (± S.E.M.) from four rats used in the first experiment and corresponds to samples shown in the left panel of (A). The right panel shows the mean levels (± individual values) from two rats used in the second experiment and corresponds to samples shown in the right panel of (A).

protein level, whereas administration of 4 × 5 units of insulin for 96 h led to an increase in the level of the G_L protein slightly above that seen in control samples. In contrast, insulin treatment did not affect the level of the PP1 catalytic subunit. The changes in the levels of G_L protein correlate well with the activity of glycogen synthase phosphatase measured in these samples (Figure 1B). There was no positive correlation of G_L protein levels with blood glucose levels.

In adrenalectomized starved rats, the level of the G_L protein was drastically reduced compared with fed controls, whereas the levels of PP1 and PP4 did not vary significantly (Figure 2A). An examination of the level of G_L in glycogen particles confirmed that the reduction in this protein seen in adrenalectomized starved rats (Figure 2B) was even greater than that seen in streptozotocin-diabetic animals and correlated with the > 90 % reduction in synthase phosphatase activity measured in the livers of adrenalectomized starved animals compared with fed controls.

Figure 2 Reduction in levels of the G_L protein in the livers of adrenalectomized starved rats

Liver extracts (A) or glycogen particles (B) from rats were subjected to gel electrophoresis, transferred to nitrocellulose membranes and probed with anti-G_L, anti-PP4 or anti-PP1 antibodies. Lanes 1, 2, 7 and 8, starved rats; lanes 5 and 6, fed controls; lanes 9 and 10, adrenalectomized rats; lanes 3, 4, 11 and 12, adrenalectomized starved rats; lanes 13 and 14, adrenalectomized starved and re-fed rats. (C) Comparison of the level of the G_L protein (black bar) and glycogen synthase phosphatase activity (hatched bar) in liver extracts from fed control rats, starved rats, adrenalectomized rats, adrenalectomized starved rats and adrenalectomized starved and re-fed rats. The results represent the mean levels (± individual values) from two rats.

Starvation alone or adrenalectomy alone reduced the level of the G_L protein to approx. 70 % of the control level, whereas a combination of the two treatments resulted in G_L protein levels falling below the detection threshold in liver extracts. Re-feeding of adrenalectomized starved animals partially restored the level of the G_L protein (Figure 2A). These results correlate well with the changes in glycogen synthase phosphatase activities measured in liver extracts and glycogen particles (Figure 2C). In contrast, the virtual absence of G_L protein in adrenalectomized starved rats did not correlate well with the reduction of phosphorylase phosphatase activity measured in the liver glycogen fraction from these rats. Under these conditions, glycogen-bound phosphorylase phosphatase activity (spontaneous) declined by only 54 %, and a similar reduction of 47 % in the total glycogen-bound phosphorylase phosphatase activity (measured after mild trypsin treatment) was observed (Table 1).

The level of mRNA encoding the hepatic G_L subunit is markedly reduced in streptozotocin-diabetic and in adrenalectomized starved rats

The level of mRNA encoding G_L was examined by hybridization of total liver RNA to a G_L probe after Northern blotting. Figure

Table 1 Loss of the hepatic glycogen-associated protein phosphatase activity in adrenalectomized starved rats is substrate dependent

Glycogen synthase phosphatase and phosphorylase phosphatase activities were measured in the protein–glycogen complex isolated from the livers of overnight-fasted rats and of adrenalectomized starved rats, as detailed in the Experimental section. The results represent the means $\pm$ S.E.M. for four animals.

Treatment	Synthase phosphatase (m-units/g of liver)	Phosphorylase phosphatase (units/g of liver)	
		Spontaneous	Total
Control	269 $\pm$ 14	6.1 $\pm$ 0.4	38 $\pm$ 3
Adrenalectomized starved	27 $\pm$ 14	2.8 $\pm$ 0.3	20 $\pm$ 3

Figure 3 Effects of streptozotocin-induced diabetes and adrenalectomy/starvation on the levels of G_L mRNA

After electrophoresis and transfer to nylon membranes, the RNA was hybridized with G_L cDNA. The membranes were stripped and the RNA was rehybridized with a β -actin cDNA. Lanes 1 and 2, fed controls; lanes 3 and 4, streptozotocin-diabetic rats; lanes 5 and 6, streptozotocin-diabetic rats treated with 4×5 units of insulin for 96 h; lanes 7 and 8, adrenalectomized starved rats; and lanes 9 and 10, adrenalectomized starved and re-fed rats.

3 shows that the G_L mRNA levels were substantially reduced in streptozotocin-diabetic rats compared with fed controls and that the levels were restored to above the control levels by treatment with 4×5 units of insulin for 96 h. G_L mRNA levels were undetectable in adrenalectomized starved rats, whereas re-feeding restored the G_L mRNA levels.

DISCUSSION

The results presented here demonstrate that changes in the level of the G_L component of the PP1– G_L complex are responsible for the long-term regulation of glycogen synthase phosphatase, and consequently glycogen synthase activity, by insulin, starvation and feeding. Reduction in the level of the G_L protein correlates well with the 70–90% reduction in activity of hepatic glycogen synthase phosphatase observed in streptozotocin-diabetic rats, whereas insulin restores the level of the G_L protein and, when administered over a longer period, raises it above the control levels. The absence of a positive correlation of induction of the G_L protein after insulin administration with changes in blood glucose levels (Figure 1B) suggests that insulin rather than glycaemia regulates the level of the G_L protein. In addition, it has previously been shown that in diabetes, insulin and not glucose concentration controls the glycogen synthase phosphatase activity [17], and hence presumably also the concentration of the G_L subunit (present study). A control of the G_L subunit by

insulin would account for its decreased level during starvation and increased level on re-feeding (Figure 2). Regulation of G_L protein levels by insulin and starvation/feeding is shown to correlate with changes in the level of the G_L mRNA (Figure 3). In this respect, the long-term effect of insulin on G_L is similar to that observed on other hepatic enzymes reviewed in [30].

The levels of hepatic glycogen are regulated by the opposing activities of glycogen phosphorylase and glycogen synthase. However, it has been observed that although glycogen synthase phosphatase activity was reduced by 90% in glycogen particles from the livers of adrenalectomized starved rats, the phosphorylase phosphatase activity was only reduced by approx. 50% ([23] and Table 1). It is not clear why there is not a greater reduction in the phosphorylase phosphatase activity if it originates from the PP1– G_L complex. The > 95% loss of G_L protein demonstrated here would be expected to lead to the release of its associated PP1 catalytic subunit from the glycogen particles, and the residual phosphorylase phosphatase in the glycogen pellet is unlikely to be due to changed specificity of the free catalytic subunit, since the latter does not bind to glycogen. The simplest explanation is that the phosphorylase phosphatase activity arises from the presence of another form(s) of PP1, which has a much higher phosphorylase phosphatase/glycogen synthase phosphatase activity ratio than G_L . Two other PP1 glycogen-targeting subunits, termed PPP1R5 [31] and PPP1R6 [32], have recently been cloned. PPP1R5 (called PTG in the mouse [33]), which shows 42% identity with G_L , is present in a variety of tissues, but its mRNA is most abundant in liver and skeletal muscle. PPP1R6 shows 31% identity with G_L , and its mRNA is found at similar levels in a wide variety of tissues, including liver. A further glycogen-bound protein, which comprises PP1 complexed to a 160 kDa component, has also been purified [34,35]. These observations raise the possibility that dephosphorylation of glycogen synthase and phosphorylase may be catalysed by different PP1 complexes *in vivo*.

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REFERENCES

- Hubbard, M. J. and Cohen, P. (1993) Trends Biochem. Sci. **18**, 172–177
- Johnson, D. F., Moorhead, G., Caudwell, F. B., Cohen, P., Chen, Y. H., Chen, M. X. and Cohen, P. T. W. (1996) Eur. J. Biochem. **239**, 317–325
- Egloff, M.-P., Johnson, F., Moorhead, G., Cohen, P. T. W., Cohen, P. and Barford, D. (1997) EMBO J. **16**, 1876–1887
- Mvumbi, L. and Stalmans, W. (1987) Biochem. J. **246**, 367–374
- Mvumbi, L., Doperé, F. and Stalmans, W. (1983) Biochem. J. **212**, 407–416
- Hers, H. G. (1976) Annu. Rev. Biochem. **45**, 167–189
- Stalmans, W. (1976) Curr. Top. Cell. Regul. **11**, 51–97
- Hue, L., Bontemps, F. and Hers, H. G. (1975) Biochem. J. **152**, 105–114
- Aleman, S. and Cohen, P. (1986) FEBS Lett. **198**, 194–202
- Schelling, D. L., Leader, D. P., Zammit, V. A. and Cohen, P. (1988) Biochim. Biophys. Acta **927**, 221–231
- Moorhead, G., MacKintosh, C., Morrice, N. and Cohen, P. (1995) FEBS Lett. **362**, 101–105
- Doherty, M. J., Moorhead, G., Morrice, N., Cohen, P. and Cohen, P. T. W. (1995) FEBS Lett. **375**, 294–298
- Bollen, M. and Stalmans, W. (1992) Crit. Rev. Biochem. Mol. Biol. **27**, 227–281
- Miller, T. B., Hazen, R. and Larner, J. (1973) Biochem. Biophys. Res. Commun. **53**, 466–474
- Whitton, P. D. and Hems, D. A. (1975) Biochem. J. **150**, 153–165
- Bollen, M. and Stalmans, W. (1984) Biochem. J. **217**, 427–434
- Bollen, M., Keppens, S. and Stalmans, W. (1988) Diabetologia **31**, 711–713

- 18 Alemany, S., Pelech, S., Brierley, C. H. and Cohen, P. (1986) *Eur. J. Biochem.* **156**, 101–110
- 19 Bollen, M., Vandenheede, J. R., Goris, J. and Stalmans, W. (1988) *Biochim. Biophys. Acta* **969**, 66–77
- 20 Miller, T. B., Garnache, A. and Crux, J. (1984) *J. Biol. Chem.* **259**, 12470–12474
- 21 Vanstapel, F., Doperé, F. and Stalmans, W. (1980) *Biochem. J.* **192**, 607–612
- 22 Stalmans, W. and Laloux, M. (1979) in *Glucocorticoid Hormone Action* (Baxter, J. D. and Rousseau, G. G., eds.), pp. 517–533, Springer-Verlag, Berlin and New York
- 23 Bollen, M., Doperé, F., Goris, J., Merlevede, W. and Stalmans, W. (1984) *Eur. J. Biochem.* **144**, 57–63
- 24 Tan, A. W. H. and Nuttall, F. Q. (1976) *Biochim. Biophys. Acta* **445**, 118–130
- 25 Mersmann, H. and Segal, H. L. (1969) *J. Biol. Chem.* **244**, 1701–1704
- 26 Margolis, R. N. and Curnow, R. T. (1983) *Endocrinology*, **113**, 2113–2119
- 27 Vanstapel, F., Bollen, M., de Wulf, H. and Stalmans, W. (1982) *Mol. Cell. Endocrinol.* **27**, 107–114
- 28 Brewis, N. D., Street, A. J., Prescott, A. R. and Cohen, P. T. W. (1993) *EMBO J.* **12**, 987–996
- 29 Barker, H. M., Craig, S. P., Spurr, N. K. and Cohen, P. T. W. (1993) *Biochim. Biophys. Acta* **1178**, 228–233
- 30 O'Brien, R. M. and Granner, D. K. (1996) *Physiol. Rev.* **76**, 1109–1161
- 31 Doherty, M. J., Young, P. R. and Cohen, P. T. W. (1996) *FEBS Lett.* **399**, 339–343
- 32 Armstrong, C. G., Browne, G. J., Cohen, P. and Cohen, P. T. W. (1997) *FEBS Lett.* **418**, 210–214
- 33 Printen, J. A., Brady, M. J. and Saltiel, A. R. (1997) *Science* **275**, 1475–1478
- 34 Wera, S., Bollen, M. and Stalmans, W. (1991) *J. Biol. Chem.* **266**, 339–345
- 35 Zhao, S., Xia, W. and Lee, E. Y. (1996) *325*, 82–90

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