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Distinct Hormone Stimulation and Counteraction by Insulin of the Expression of the Two Components of Glucose 6-Phosphatase in HepG2 Cells

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We found recently (*J. Biol. Chem*. 274, 33866–33869, 1999) that the expression of the catalytic subunit (p36) and putative glucose 6-phosphate translocase (p46) of the liver glucose 6-phosphatase system was stimulated by cyclic AMP and glucose and repressed by insulin. We now further show in HepG2 cells that whereas insulin (0.01–10 nM) suppressed p36 mRNA, it only reduced p46 mRNA by half at 1 μM. Cyclic AMP (0.01–100 μM) caused a 2.7-fold increase in p36 mRNA but barely increased p46 mRNA. In contrast, dexamethasone (0.1–100 nM) increased both p36 and p46 mRNA by more than 3-fold. The effects of cyclic AMP and dexamethasone were counteracted by 1 μM insulin. The endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (1–100 nM) increased p36 mRNA by 2-fold but not p46 mRNA. It thus appears that the hormonal changes which affect p36 alone concur with known modifications in glucose production; those that affect both p36 and p46 are rather consistent with glucose storage.

Key Words: **glucose 6-phosphatase; catalytic subunit; putative glucose 6-phosphate translocase; gene expression; insulin; glucagon; dexamethasone; cyclic AMP; calcium.**

Liver glucose production is ultimately realized by glucose 6-phosphatase (G6Pase) (EC 3.1.3.9) (reviewed in Refs. 1 and 2). Insulin deficiency, the presence of unopposed counterregulatory hormones, and hyperglycemia may each contribute in the streptozotocin-treated diabetic rat to enhanced hepatic glucose formation, through an up-regulation of G6Pase that affects both the 36-kDa catalytic subunit (p36) (3, 4) and the putative glucose 6-phosphate (G6P) translocase, a 46-kDa protein (p46) (5). Glucose and cyclic AMP (cAMP) indeed increased both p36 and p46 mRNA but the p46 protein level was unaffected by cAMP (5). Insulin severely represses the expression of p36 (6) and reduces that of p46 (5). Taken together, these properties indicate that both p36 and p46 are regulated in parallel; however, the comparative sensitivity of p36 and p46 to hormones remains to be defined. In this study we evaluated the differential response of p36 and p46 mRNA to physiological concentrations of insulin and cAMP, but also to dexamethasone and to changes in cytosolic free calcium ($[Ca^{2+}]_i$) induced by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (7) in HepG2 cells. The results indicate that p36 is sensitive to cAMP and thapsigargin stimulation and insulin inhibition whereas p46 is virtually not, but that dexamethasone has similar effects on both p36 and p46 gene expression. The possible role of p36 and p46 in the G6Pase system is discussed in the light of this differential expression of the two genes.

Abbreviations used: G6Pase, glucose 6-phosphatase; G6P, glucose 6-phosphate; p36, catalytic subunit of G6Pase; p46, putative G6P translocase; cAMP, cyclic AMP; MIX, methyl isobutylxanthine; $[Ca^{2+}]_i$, cytosolic free calcium.

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MATERIALS AND METHODS

Materials. Bovine serum albumin and fetal bovine serum and Dulbecco's modified Eagle medium (DMEM), as well as insulin (lyophilized), were purchased from Life Technologies Inc. (Birlington, ON, Canada). Glucagon was from Eli Lilly Inc. (Toronto, ON, Canada). Adenosine 3′:5′-cycle monophosphate (cAMP), methyl isobutylxanthine (MIX), dexamethasone and thapsigargin were from Sigma (Oakville, ON, Canada). Insulin, glucagon, cAMP and MIX were dissolved in 0.9% NaCl, while dexamethasone and thapsigargin were dissolved in dimethyl sulfoxide (DMSO).

Cell culture. HepG2 cells were maintained in DMEM containing 10% bovine fetal serum. Before treatment, cells were adapted in serum-free DMEM (but containing 0.1% BSA) overnight. Insulin, glucagon, cAMP (in the presence of 50 μM MIX to inhibit cAMP phosphodiesterases), dexamethasone or the Ca^{2+}-ATPase inhibitor thapsigargin was added to 80–90% confluent HepG2 cells in serum-free DMEM at the indicated concentrations and times, in the pres-

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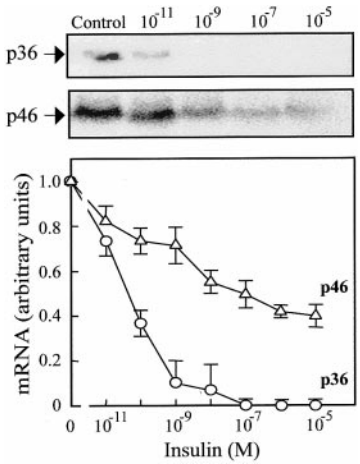

FIG. 1. Dose response of p36 and p46 mRNA to insulin in HepG2 cells. Northern analysis of p36 (○) and p46 (△) on total RNAs extracted from HepG2 cells incubated for 24 h with the indicated concentrations of insulin, as described under Materials and Methods. Densitometric scanning values are presented as means ± S.D. (n = 3). Representative blots are shown on top, in which all controls represent samples without any treatment and are considered as one arbitrary unit for quantitative analysis.

ence of 5 mM glucose. Control cells were treated with the same final concentration (0.01%) of DMSO in the cases of dexamethasone and thapsigargin. Cells were harvested by scraping after different times of treatment and kept at −80°C for subsequent analysis. With thapsigargin, cells were exposed for 10 min with the inhibitor and further incubated without it for the indicated time.

Northern blot analysis. Total RNA was isolated from HepG2 cells with Trizol LS reagent (Life Technologies Inc.) following the protocol of the manufacturer. Fifteen μg of total RNA was electrophoresed on a 1% formaldehyde-denatured agarose gel in 1× MOPS running buffer. RNA was transferred to a nylon membrane (Bio-Rad, Mississauga, ON, Canada) by capillary action overnight. The membrane was then hybridized with ^{32}P-labelled p36 (8) or p46 (9) probes overnight at 68°C in ExpressHyb Hybridization solution (CLONTECH, Palo Alto, CA). After hybridization, membranes were washed five times with different proportions of SSC/SDS buffer and exposed to a X-ray film at −80°C. Both p36 and p46 probes were full length human cDNA fragments labelled by a random primer method with Ready-To-Go DNA labelling beads (Amersham Pharmacia Biotech, Baie d' Urfe, QC, Canada). Probes were then purified with MicroSpin G-50 columns (Amersham Pharmacia Biotech, Baie d' Urfe, QC, Canada). Quantification was performed with a scanning densitometer (Dual light Transilluminator) and equal amount of loading was verified by the 18S and 28S ribosomal RNA signal obtained by ethidium bromide staining.

Statistic analysis. The results were presented as the mean ± S.D. of three or four independent experiments.

RESULTS

Dose response of p36 and p46 mRNA to insulin. Figure 1 shows that after 24 h insulin caused a dose-dependent decrease in the G6Pase catalytic subunit p36 mRNA in HepG2 cells that was half maximal at about 0.03 nM. At 10 nM insulin, p36 mRNA was not detectable anymore. Similar results were reported in FAO cells (6, 10). In contrast to p36, p46 mRNA was not reduced more than by 60% at the highest (0.1–10 μM) insulin concentrations investigated. Changes in p46 protein that occur at 1 μM insulin were found before to be parallel to those of the corresponding mRNA (5).

Dose response of p36 and p46 mRNA to cAMP and counteraction by insulin. The effect of increasing concentrations of cAMP (in the presence of 50 μM MIX) on p36 and p46 transcripts was examined in HepG2 cells after 6 h (Fig. 2), because longer incubation times reversed this effect in FAO cells (6). It can be seen that cAMP produced a 2.7-fold increase in the p36 mRNA level with a maximal effect at 1 μM, whereas the p46 mRNA was only slightly (1.25-fold) affected. This latter result is in agreement with our previous observations that cAMP did not increase the p46 protein (5). Consistently, glucagon (1 μM) increased p36 and p46 to similar extends as with an optimal concentration of cAMP (Fig. 2). Insulin (1 μM) counteracted the cAMP-induced increase in p36 mRNA but did not decrease the p46 mRNA more than when added alone (see Fig. 1).

Dose response of p36 and p46 mRNA to dexamethasone and counteraction by insulin. Dexamethasone (0.1–100 nM) increased in 24 h the level of both p36

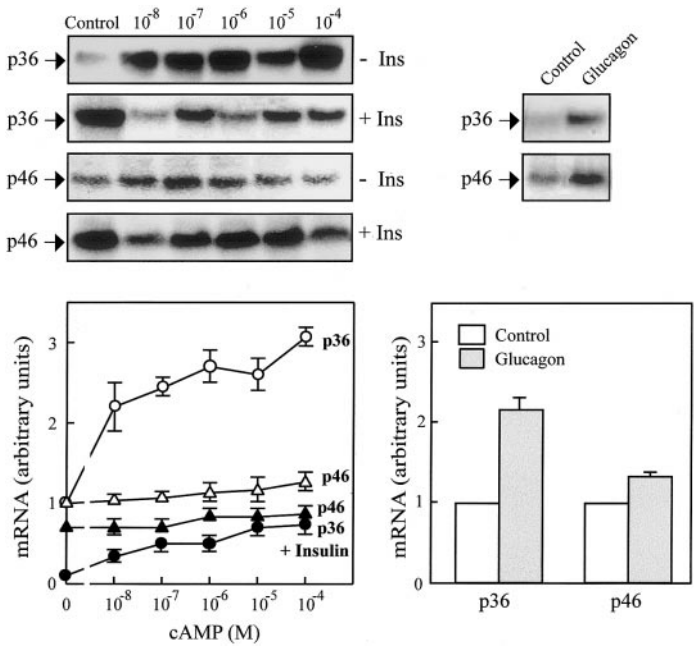

FIG. 2. Dose response of p36 and p46 mRNA to cAMP and to 1 μM glucagon and antagonism by insulin in HepG2 cells. (Left) Northern analysis of p36 (○) and p46 (△) on total RNAs extracted from HepG2 cells incubated for 6 h with the indicated concentrations of cAMP and 50 μM MIX with (●, ▲) or without (○, △) 1 μM insulin. (Right) Northern analysis of p36 and p46 on total RNAs extracted from HepG2 cells incubated for 6 h with 1 μM glucagon. Densitometric scanning values are presented as means ± S.D. (n = 3). Representative blots are shown on top, in which all controls represent samples without any treatment and are considered as one arbitrary unit for quantitative analysis.

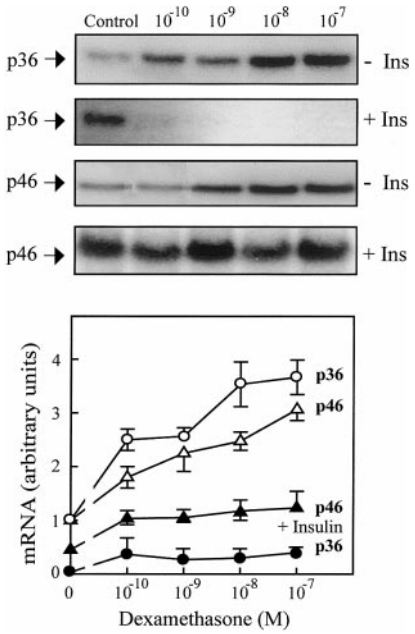

FIG. 3. Dose response of p36 and p46 mRNA to dexamethasone and antagonism by insulin in HepG2 cells. Northern analysis of p36 (○) and p46 (△) on total RNAs extracted from HepG2 cells incubated for 24 h with the indicated concentrations of dexamethasone with (●, ▲) or without (○, △) 1 μM insulin. Densitometric scanning values are presented as means ± S.D. (n = 3 or 4). Representative blots are shown on top, in which all controls represent samples without any treatment and are considered as one arbitrary unit for quantitative analysis.

and p46 message by a factor between 2- and 3-fold with similar sensitivity (Fig. 3). This stimulatory effect was inhibited by 1 μM insulin on p36 as well as on p46 mRNA (Fig. 3), in agreement with the property of insulin to inhibit glucocorticoid receptor activity (11).

Dose response of p36 and p46 mRNA to thapsigargin in HepG2 cells. In order to evaluate the effect of increased $[Ca^{2+}]_i$ on the p36 and p46 message, independent of inositol trisphosphate, we used an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (7). Because prolonged application of this drug resulted in cell death, we exposed the HepG2 cultures for 10 min to increasing concentrations of thapsigargin and further cultured them up to 6 h before harvesting and mRNA analysis. It has been emphasized before that a $[Ca^{2+}]_i$ perturbation of a few minutes is sufficient for full induction of transcripts (12). Figure 4 shows that p36 mRNA was increased up to 2-fold by increasing thapsigargin concentrations (1–100 nM) whereas p46 mRNA was unchanged under the same conditions.

DISCUSSION

Insulin, cAMP and calcium affect differently p36 and p46 mRNA. The negative effect of insulin on p36 mRNA, which is partly attributable to a decreased transcription rate (6), was found to be within its physiological concentration range (0.05–0.1 nM), indicating that the expression of G6Pase must be very sensitive to down-regulation by insulin. This is not the case for p46, the mRNA of which was only reduced by 20–30% by normal insulin concentrations. Suppression of hepatic glucose production after a meal can thus appropriately be achieved by increased insulin secretion that occurs in that situation followed by decreased G6Pase activity. Conversely, glucagon and cAMP, which stimulate glycogenolysis and gluconeogenesis, increase p36 message and enhance glucose release by the liver without significantly affecting p46. Therefore both inhibition and stimulation of G6Pase seem to be principally the consequence of changes in the catalytic subunit of G6Pase. A similar conclusion can be drawn from the effect of thapsigargin. The elevation in $[Ca^{2+}]_i$ expected to occur following inhibition of the endoplasmic reticulum Ca^{2+}-ATPase in HepG2 cells by thapsigargin increased by up to twofold the p36 mRNA without affecting p46 mRNA. Although cAMP and calcium increase p36 mRNA to the same extent, calcium however is a less potent signal than cAMP in inducing glucose production because the former increases fructose-2,6-bisphosphate and flux through glycolysis whereas the latter lowers fructose-2,6-bisphosphate and inhibits glycolysis, thereby directing G6P towards glucose formation.

Although the regulation of p36 and p46 mRNA tends to be affected in parallel, the dose responses of the respective messages to insulin, cAMP and thapsigargin are quite different for these two components of the

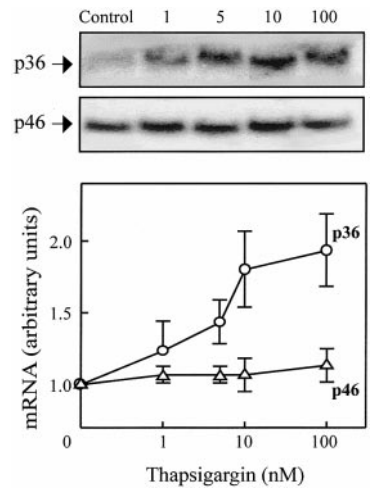

FIG. 4. Dose response of p36 and p46 mRNA to thapsigargin in HepG2 cells. Northern analysis of p36 (○) and p46 (△) on total RNAs extracted from HepG2 cells incubated with the indicated concentrations of thapsigargin for 10 min and further cultured for 6 h. Densitometric scanning values are presented as means ± S.D. (n = 3). Representative blots are shown on top, in which all controls represent samples without any treatment and are considered as one arbitrary unit for quantitative analysis.

G6Pase system. Hormonal changes compatible with increased hepatic glucose production essentially modify the expression of the catalytic subunit of G6Pase, p36, without significant effect on that of p46.

Dexamethasone affects similarly p36 and p46. Effectors such as glucose (5) and the synthetic glucocorticoid dexamethasone (this work), which enhance glycogen synthesis, increase both p36 and p46 mRNA. Glucocorticoids stimulate gluconeogenesis as well as glycogen synthesis and have only a marginal effect on glucose production (13, 14). Taken together, these properties suggest that p46 favors carbon flux through G6P towards glycogen, even if G6Pase activity is concomitantly increased. Such mechanism would be compatible with the proposition of Aiston *et al.* (15) that G6Pase acts as a buffer for G6P. G6P hydrolysis might serve either for glucose export by the liver (increased p36 alone) or to feed a glucose/G6P substrate cycle to keep intracellular G6P available for glycogen synthesis (increased p46 and p36). This cycle is indeed increased in diabetes (16) and could be a metabolic adaptation whereby hyperglycemia appropriately induces both components of the liver G6Pase system (5). Similarly, hyperglycemia partially compensates the down-regulation of synthase phosphatase induced by insulin deficiency in diabetes (17).

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REFERENCES

1. Foster, J. D., Pederson, B. A., and Nordlie, R. C. (1997) Glucose-6-phosphatase structure, regulation, and function: An update. *Proc. Soc. Exp. Biol. Med.* **215,** 314–332.
2. van de Werve, G., Lange, A., Newgard, C., Méchin, M.-C., Li, Y., and Berteloot, A. (2000) New lessons in the regulation of glucose metabolism taught by the glucose 6-phosphatase system. *Eur. J. Biochem.* **267,** 1533–1549.
3. Liu, Z. Q., Barrett, E. J., Dalkin, A. C., Zwart, A. D., and Chou, J. Y. (1994) Effect of acute diabetes on rat hepatic glucose-6-phosphatase activity and its messenger RNA level. *Biochem. Biophys. Res. Commun.* **205,** 680–686, doi:10.1006/bbrc.1994.2719.
4. Massillon, D., Barzilai, N., Chen, W., Hu, M., and Rossetti, L. (1996) Glucose regulates in vivo glucose-6-phosphatase gene expression in the liver of diabetic rats. *J. Biol. Chem.* **271,** 9871–9874.
5. Li, Y. Z., Méchin, M.-C., and van de Werve, G. (1999) Diabetes affects similarly the catalytic subunit and putative glucose-6-phosphate translocase of glucose-6-phosphatase. *J. Biol. Chem.* **274,** 33866–33869.
6. Arguad, D., Zhang, Q., Pan, W., Maitra, S., Pilkis, S. J., and Lange, A. J. (1996) Regulation of rat liver glucose-6-phosphatase gene expression in different nutritional and hormonal states: Gene structure and 5′-flanking sequence. *Diabetes* **45,** 1563–1571.
7. Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P. (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. *Proc. Natl. Acad. Sci. USA* **87,** 2466–2470.
8. Shelly, L. L., Lei, K.-J., Pan, C.-J., Sakata, S. F., Ruppert, S., Schutz, G., and Chou, J. Y. (1993) Isolation of the gene for murine glucose-6-phosphatase, the enzyme deficient in glycogen storage disease type 1A. *J. Biol. Chem.* **268,** 21482–21485.
9. Gerin, I., Veiga-da-Cunha, M., Achouri, Y., Collet, J. F., and Van Schaftingen, E. (1997) Sequence of a putative glucose 6-phosphate translocase, mutated in glycogen storage disease type Ib. *FEBS Lett.* **419,** 235–238.
10. Lange, A. J., Argaud, D., El-Maghrabi, M. R., Pan, W., Maitra, S. R., and Pilkis, S. J. (1994) Isolation of a cDNA for the catalytic subunit of rat liver glucose-6-phosphatase: Regulation of gene expression in FAO hepatoma cells by insulin, dexamethasone and cAMP. *Biochem. Biophys. Res. Commun.* **201,** 302–309, doi: 10.1006/bbrc.1994.1702.
11. O'Brien, R. M., and Granner, D. K. (1996) Regulation of gene expression by insulin. *Physiol. Rev.* **76,** 1109–1161.
12. Werlen, G., Belin, D., Conne, B., Roche, E., Lew, D. P., and Prentki, M. (1993) Intracellular Ca2+ and the regulation of early response gene expression in HL-60 myeloid leukemia cells. *J. Biol. Chem.* **268,** 16596–16601.
13. Kobayashi, T., Iwai, H., Uchimoto, R., Ohta, M., Shiota, M., and Sugano, T. (1989) Gluconeogenesis in perfused livers from dexamethasone-treated chickens. *Am. J. Physiol.* **256,** R907–R914.
14. Voice, M. W., Webster, A. P., and Burchell, A. (1997) The in vivo regulation of liver and kidney glucose-6-phosphatase by dexamethasone. *Horm. Metab. Res.* **29,** 97–100.
15. Aiston, S., Trinh, K. Y., Lange, A. J., Newgard, C. B., and Agius, L. J. (1999) Glucose-6-phosphatase overexpression lowers glucose 6-phosphate and inhibits glycogen synthesis and glycolysis in hepatocytes without affecting glucokinase translocation. Evidence against feedback inhibition of glucokinase. *J. Biol. Chem.* **274,** 24559–24566.
16. Fisher, S. J., Lekas, M. C., McCall, R. H., Shi, Z. Q., Giacca, A., and Vranic, M. (1996) Determinants of glucose turnover in the pathophysiology of diabetes: An in vivo analysis in diabetic dogs. *Diabetes Metab.* **22,** 111–121.
17. Lavoie, L., Dimitrakoudis, D., Marette, A., Annabi, B., Klip, A., Vranic, M., and van de Werve, G. (1993) Opposite effects of hyperglycemia and insulin deficiency on liver glycogen synthase phosphatase activity in the diabetic rat. *Diabetes* **42,** 363–366.