Liver Glycogen Synthase but Not the Muscle Isoform Differentiates between Glucose 6-Phosphate Produced by Glucokinase or Hexokinase*

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Using adenovirus-mediated gene transfer into FTO-2B cells, a rat hepatoma cell line, we have overexpressed hexokinase I (HK I), glucokinase (GK), liver glycogen synthase (LGS), muscle glycogen synthase (MGS), and combinations of each of the two glucose-phosphorylating enzymes with each one of the GS isoforms. FTO-2B cells do not synthesize glycogen even when incubated with high doses of glucose. Adenovirus-induced overexpression of HK I and/or LGS, two enzymes endogenously expressed by these cells, did not produce a significant increase in the levels of active GS and the total glycogen content. In contrast, GK overexpression led to the glucose-dependent activation of endogenous or overexpressed LGS and to the accumulation of glycogen. Similarly overexpressed MGS was readily activated by the glucose-6-phosphate (Glc-6-P) produced by either endogenous or overexpressed HK I and by overexpressed GK. These results indicate the existence of at least two pools of Glc-6-P in the cell, one of them is accessible to both isoforms of GS and is replenished by the action of GK, whereas LGS is excluded from the cellular compartment where the Glc-6-P produced by HK I is directed. These findings are interpreted in terms of the metabolic role that the two pairs of enzymes, HK I-MGS in the muscle and GK-LGS in the hepatocyte, perform in their respective tissues.

Glycogen synthase (GS) catalyzes the incorporation of glucose residues to the non-reducing end of a growing glycogen molecule via α-1,4-glycosidic bonds using UDP-glucose as substrate. GS can be inactivated by phosphorylation at multiple sites, but even highly phosphorylated forms of the enzyme become active “in vitro” in the presence of high concentrations of glucose-6-phosphate (Glc-6-P), which acts as an allosteric activator (1). “In vivo,” Glc-6-P binding to GS also converts the enzyme into a better substrate for phosphatases and induces its dephosphorylation and activation (1).

The two main isoforms of mammalian GS are designated as muscle and liver. Most tissues express the former, whereas the latter appears to be tissue-specific (2). Although the two forms have 70% identical amino acid sequence, the N and C termini, which contain the phosphorylation sites that regulate the activity of the enzyme, show a lower degree of homology (3). Moreover the two isoforms have dissimilar intracellular distribution both in the absence and in the presence of glucose (4, 5), suggesting that there are significant differences in the molecular mechanisms involved in the control of glycogen synthesis in muscle and in liver.

Using adenovirus-mediated gene transfer in cultured hepatocytes, we have previously shown that overexpression of glucokinase (GK, hexokinase IV or hexokinase D), the isoenzyme of the mammalian hexokinase group characteristic of hepatocytes and pancreatic β cells, enhances glycogen synthesis, whereas overexpression of hexokinase I (HK I) has no effect (6). This is attributed to a different capacity of Glc-6-P produced by GK or HK I to induce the activation of liver glycogen synthase (LGS) (7). Further studies in cultured hepatocytes have shown that LGS shares the control of glycogen synthesis with GK (8). In contrast, the control of glycogen deposition from glucose in muscle is shared between glucose transport and muscle glycogen synthase (MGS) (9).

The FTO-2B rat hepatoma cell line has a highly differentiated hepatic phenotype (10, 11) and expresses genes characteristic of the liver, such as albumin and insulin-like growth factor II receptor. However, FTO-2B cells do not express GK and instead express high levels of HK I (12). In this hepatoma cell line, anaerobic glycolysis is impaired, and there is no glycogen deposition even in the presence of high concentrations of glucose (12). The retrovirus-mediated expression of GK in FTO-2B cells stimulates glucose uptake and utilization and restores the ability of these cells to synthesize glycogen. It has been suggested that the increase in the levels of Glc-6-P in the GK-expressing cells is responsible for the glycogen deposition (12).

In this study, we have taken advantage of the characteristics of the FTO-2B hepatoma cell line to show that LGS, but not the muscle isoform, is dependent on GK as the source of Glc-6-P for activation. The latter isoform, MGS, is activated and able to synthesize glycogen in response to glucose independently of the type of hexokinase present. This observation underlines the differences between the liver and muscle glycogen metabolism.

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The abbreviations used are: GS, glycogen synthase; LGS, liver glycogen synthase; MGS, muscle glycogen synthase; Glc-6-P, glucose 6-phosphate; GK, glucokinase; HK I, hexokinase I; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein.
Preparation of Recombinant Adenovirus—AdCMV-GK (7), AdCMV-HK I (7), and AdCMV-LGS (8) were described previously. AdCMV-GFP and AdCMV-MGS were constructed following the procedure described by Becker et al. (13) using the CDNAs of the green fluorescent protein (GFP) and that of the human muscle glycogen synthase (14), respectively. In the metabolic impact studies, FTO-2B cells infected with AdCMV-GFP were used as control cells.

**FTO-2B Culture Conditions and Treatment with Recombinant Adenovirus**—FTO-2B rat hepatoma cells were cultured in 60-mm plates. Cells were kept in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 mM glucose and 5% fetal bovine serum. Cells were treated for 2 h with adenovirus at a multiplicity of infection of 5 except for AdCMV-HK I, which was used at a multiplicity of infection of 10. Media were replaced with DMEM containing 10 mM glucose, and cells were incubated for 24 h at 37 °C. Media were then replaced by DMEM, and another incubation of 12–14 h was carried out. Cells were then incubated in DMEM at several concentrations of glucose as detailed in the text and figure legends. At the end of each manipulation, cell monolayers were washed in phosphate-buffered saline and frozen in liquid N2 until analysis.

**Metabolite Determinations**—For the measurement of glycogen content, cell monolayers were scraped into 30% KOH, and the extract was then boiled for 15 min and centrifuged at 5,000 × g for 15 min. Glycogen was measured in the cleared supernatants as described previously (15). The intracellular concentration of Glc-6-P was measured by a spectrophotometric assay (16).

**Enzyme Activity Assays**—Frozen cell monolayers from 60-mm-diameter plates were scraped using 100 μl of homogenization buffer, which consisted of 10 mM Tris-HCl (pH 7.0), 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Thawing plus sonication caused cell bursting. Protein concentration was measured following Bradford (17) using a Bio-Rad assay reagent. Glucose phosphorylating activity was measured spectrophotometrically in the supernatant fraction of FTO-2B cell extracts centrifuged at 10,000 × g for 15 min using 1 or 100 mM glucose at 30 °C as described previously (18). GS activity was measured in homogenates in the absence or presence of 6.6 mM Glc-6-P as described previously (19). The activity measured in the absence of Glc-6-P represents the active form of the enzyme (1 or a form), whereas that measured in the presence of 6.6 mM Glc-6-P represents total GS activity.

**Electrophoresis and Immunoblotting**—Hall and Vettor (20) described the procedure for preparation of the adenovirus-treated FTO-2B cells. A, cells were treated with AdCMV-GFP (i), AdCMV-HK I (ii), AdCMV-LGS/HK I (iii), or AdCMV-MGS/HK I (iv). B, cells were treated with AdCMV-GFP (i), AdCMV-GK (ii), AdCMV-LGS/GK (iii), or AdCMV-MGS/GK (iv). C, cells were treated with AdCMV-GFP (i), AdCMV-LGS (ii), AdCMV-LGS/GK (iii), or AdCMV-MGS/HK I (iv). D, cells were treated with AdCMV-GFP (i), AdCMV-MGS (ii), AdCMV-MGS/GK (iii), or AdCMV-MGS/HK I (iv) as described under "Materials and Methods." The multiplicity of infection was 5 for all the viruses except for AdCMV-HKI, which was used at a multiplicity of infection of 10. After infection, cells were incubated for 24 h with DMEM containing 10 mM glucose, the medium was then replaced by DMEM without glucose, and incubation was continued for 12–14 h. Finally, cells were incubated for 2 h in DMEM with 25 mM glucose and collected, and homogenates were analyzed by Western blotting using antibodies against HK I (A), GK (B), LGS (C), or MGS (D). The bands corresponding to each one of the proteins analyzed ran at their appropriate respective molecular weight.

**TABLE I**

| Protein | HK I | GK | MGS | Total GS activity |
|---------|-----|----|-----|-------------------|
| Cultured hepatocytes | 4.5 ± 0.6 | 12.0 ± 1.8 | 3.1 ± 0.2 |
| AdCMV-GFP | 8.2 ± 1.6 | 0.3 ± 0.1 | 9.9 ± 1.8 |
| AdCMV-HK I | 44.7 ± 3.2 | 0.8 ± 0.2 | 10.4 ± 1.8 |
| AdCMV-GK | 9.2 ± 1.8 | 12.2 ± 2.2 | 11.5 ± 1.6 |
| AdCMV-LGS | 9.4 ± 1.8 | 1.0 ± 0.4 | 33.8 ± 3.5 |
| AdCMV-LGS/HK I | 49.4 ± 5.7 | 1.2 ± 0.3 | 22.4 ± 2.4 |
| AdCMV-LGS/GK | 7.2 ± 0.9 | 13.6 ± 3.3 | 26.7 ± 2.8 |
| AdCMV-MGS | 8.2 ± 1.6 | 0.6 ± 0.3 | 24.3 ± 3.6 |
| AdCMV-MGS/HK I | 55.6 ± 0.9 | 1.2 ± 0.2 | 23.4 ± 2.5 |
| AdCMV-MGS/GK | 6.5 ± 0.9 | 9.6 ± 2.3 | 30.1 ± 5.5 |

**RESULTS**

**Characterization of FTO-2B Cells**—The FTO-2B cells show the most consistent hepatocyte-like phenotype among the hepatoma cell lines (10). However, these cells expressed undetectable levels of GK, both measured as protein by Western blot (Fig. 1B, lane i) and by enzymatic activity (Table I), and instead expressed high levels of HK I (Fig. 1A, lane i, and Table I). These two observations are in agreement with previous results obtained with this cell line (11, 12). Unique among hepatic tumor cell lines, FTO-2B expressed the liver isofrom of GS (Fig. 1C, lane i) even at higher levels than cultured hepatocytes (Table I) and did not express detectable amounts of MGS (Fig. 1D, lane i).

**Adenovirus-mediated Overexpression of HK I, GK, LGS, or MGS**—Protein overexpression in FTO-2B cells was achieved very efficiently using recombinant adenoviruses. When AdCMV-GFP was used as control at a multiplicity of infection of 5, over 95% of the adenovirus-treated cells expressed GFP as detected by fluorescence microscopy (not shown).

Infection of FTO-2B cells with AdCMV-HK I produced a 5-fold increase in HK I activity and protein content (Fig. 1A,
lungs (and ii, and Table I). When the AdCMV-GK was used, GK reached levels similar to those found in cultured hepatocytes, measured both as protein by densitometric analysis (Fig. 1B, lane ii) or activity (Table I). AdCMV-LGS-infected cells showed a 3-fold increase in total GS activity (Table I). This increase was consistent with protein content analyzed by Western blot (Fig. 1C, lanes i and ii) with an antibody specific for rat LGS. Similarly MGS was overexpressed by infection of FTO-2B cells with AdCMV-MGS and also resulted in a 3-fold increase in total GS activity (Table I). The presence of the muscle isoform was also detected by Western blot using antibodies specific for MGS (Fig. 1D, lane ii). The multiplicity of infection used was 5 in all cases except with the AdCMV-HK I adenovirus, which was used at a multiplicity of infection of 10 to obtain intracellular levels of Glc-6-P similar to those obtained with the AdCMV-GK infection (see below).

In addition, FTO-2B cells were simultaneously co-infected with AdCMV-LGS plus AdCMV-HK I or AdCMV-GK and with AdCMV-MGS plus AdCMV-HK I or AdCMV-GK. In the double infection experiments, HK I, GK, and total GS activities were comparable to those obtained when the viruses were used alone (Table I). Likewise the levels of protein measured by Western blot were also similar to those attained in the single infections (Fig. 1, lanes iii and iv).

Metabolic Impact of the Overexpression of HK I or GK—Despite containing 3-fold more total LGS than cultured hepatocytes (Table I), FTO-2B cells synthesized negligible amounts of glycogen in response to increasing doses of glucose (Fig. 2A). Intracellular Glc-6-P levels increased sharply when the cells were incubated with 5 mM glucose but remained constant at ~2 nmol/mg of protein when higher concentrations of the sugar were used (Fig. 3A). Nevertheless this accumulation of Glc-6-P induced neither the activation of LGS (Fig. 4) nor the deposition of glycogen.

Adenovirus-mediated overexpression of HK I in FTO-2B cells led to a marked increase in Glc-6-P levels, which at 25 mM glucose were about ~3-fold higher than in control cells (Fig. 3A). Nevertheless HK I overexpression did not produce a significant increase in glycogen deposition (Fig. 2A), and the amount of active LGS was not significantly different from that found in control cells infected with the AdCMV-GFP adenovirus (Fig. 4A). On the contrary, GK overexpression had a dramatic effect on the capacity of FTO-2B cells to synthesize glycogen. At high glucose concentrations, AdCMV-GK-infected cells produced over 2-fold more glycogen than control cells or cells overexpressing HK I (Fig. 2A). In contrast to control cells, in GK-expressing cells, endogenous GS was activated by Glc-6-P in a concentration-dependent manner (Fig. 4A). Interestingly the large increase in the amount of active LGS in the AdCMV-GK-infected cells was attained at similar or even lower levels of Glc-6-P than those found in HK I-overexpressing cells (Fig. 4A). However, as indicated above, no GS activation was observed in HK I-overexpressing cells.

Metabolic Impact of the Overexpression of Liver and Muscle Glycogen Synthase—Overexpression of LGS up to 3-fold did not increase glycogen deposition even when infected FTO-2B cells were incubated with high doses of glucose (Fig. 2B). Overexpressed LGS behaved like the endogenous enzyme in the sense that it was not activated in response to an increase in the intracellular concentration of Glc-6-P (Fig. 4B). In contrast, when MGS was expressed to attain the same total GS activity as with LGS, there was a marked increase in glycogen deposition (Fig. 2C) in response to increasing doses of glucose. Although LGS or MGS overexpression did not alter Glc-6-P concentrations (Fig. 3, B and C), the amount of active GS was much larger in AdCMV-MGS-infected cells and increased linearly with Glc-6-P concentration (Fig. 4B).

Differential Metabolic Impact of Overexpression of LGS or MGS Concomitantly with HK I or GK—To compare the effect on glycogen synthesis of the overexpression of LGS or MGS concomitantly with each of the two hexokinases, we overexpressed LGS plus HK I or GK, and MGS plus HK I or GK.

In FTO-2B cells overexpressing LGS plus GK, glycogen synthesis was strongly enhanced (Fig. 2B) compared with control cells or cells overexpressing LGS alone. In contrast, when LGS was overexpressed concomitantly with HK I glycolgen deposition in response to increasing doses of glucose remained negligible (Fig. 2B). Furthermore LGS was very efficiently activated by Glc-6-P in a dose-dependent manner when GK was present but only experienced a minor activation when the source of Glc-6-P was overexpressed HK I (Fig. 4C).

When MGS was co-expressed with GK or HK I, glycogen synthesis was strongly enhanced compared with the cells overexpressing MGS alone. In both cases, glycogen accumulation increased in response to glucose (Fig. 2C), and the levels of active GS (Fig. 4D) increased with Glc-6-P in a dose-dependent manner independently of the hexokinase activity present in the cells. In contrast to what occurs with LGS, both hexokinases, HK I and GK, appeared to be equally effective in mediating the activation of MGS in response to glucose. The differences observed in the amount of glycogen and in the levels of active GS between the cells overexpressing MGS and HK I and those expressing MGS and GK can be attributed to the simultaneous activation of the endogenous LGS in the latter case.

DISCUSSION

In liver and muscle, GS activation occurs through the dephosphorylation of the enzyme, which is probably produced by protein phosphatases of type I (2, 23) and is enhanced by Glc-6-P. It is generally accepted that this metabolite binds to GS and triggers a conformational change that renders the enzyme a better substrate for protein phosphatases (1). Previous studies by our group, using cultured hepatocytes, have shown that Glc-6-P arising from the catalytic action of GK is much more effective in mediating the activation of LGS than the same metabolite produced by HK I (6, 7). In the present work we have taken advantage of the biochemical features of the FTO-2B cells to show that LGS but not MGS differentiates between Glc-6-P produced by GK or by HK I.

Using specific antibodies, here we show that FTO-2B cells express high levels of LGS and do not express measurable levels of MGS. However, these cells do not express GK, the main glucose-phosphorylating enzyme in hepatocytes, and instead express HK I, a ubiquitous hexokinase. Because of these characteristics, this system is ideally suited to study the dependence of GS activation on the source of Glc-6-P.

It has already been reported that FTO-2B cells do not accumulate glycogen when incubated with glucose but that the overexpression of GK restores this ability (12). It was hypothesized that the large increment in the levels of Glc-6-P derived from the expression of GK was solely responsible for this. However, here we show that the source of this metabolite is also crucial. In the first set of experiments, the adenovirus-mediated overexpression of GK led to the activation of the endogenous LGS present in FTO-2B cells and to the consequent accumulation of glycogen. In contrast, the overexpression of HK I, such that similar levels of Glc-6-P were attained, had no effect on the amount of active GS and glycogen content.

The observation that endogenous LGS was activated in response to increasing concentrations of glucose when GK was present but not when the source of Glc-6-P was HK I indicates the existence of at least two pools of Glc-6-P inside the cell. One
of these pools is replenished by the action of GK and is accessible to LGS, while the pool of Glc-6-P produced by HK I is localized to a cellular compartment from which LGS is excluded.

The second set of experiments, in which we overexpressed LGS and MGS, corroborated this conclusion. The overexpression of LGS did not have any effect on the levels of glycogen. Glc-6-P produced by the endogenous HK I of the FTO-2B cells did not facilitate the dephosphorylation and activation of overexpressed LGS. However, exogenous MGS was effectively activated in response to glucose, and MGS-overexpressing cells synthesized a large amount of glycogen. This finding indicates that, in contrast to LGS, MGS has access to the compartment where the Glc-6-P produced by HK I is directed.

The third set of experiments, consisting of double infections with each of the two GS isoforms combined with each one of the
two glucose-phosphorylating enzymes, further confirms the previous conclusions and allows us to make one additional conclusion. LGS was only activated by glucose when GK was present, but Glc-6-P produced by GK or HK I was equally effective in inducing the activation of MGS. Simultaneous overexpression of LGS and HK I had no net effect on glycogen deposition, while the co-expression of LGS and GK led to a much larger accumulation of glycogen than in FTO-2B cells singly infected with the AdCMV-GS or AdCMV-GK viruses. In contrast, glycogen accumulation was enhanced in FTO-2B cells overexpressing MGS when the glucose phosphorylating capacity of the cells was also increased regardless of whether the source of Glc-6-P was HK I or GK.

On the basis of the different effectiveness in inducing the activation of LGS by the Glc-6-P produced by GK or by HK I, the channeling of this sugar phosphate from GK to GS was hypothesized. However, in the light of the new data presented here, an alternative hypothesis emerges: compartmentation of Glc-6-P. GK delivers its product into a cellular compartment, which is accessible by several enzymes that use Glc-6-P, while the compartment where the Glc-6-P produced by HK I is directed has a more restricted access. In this study we show that overexpression of GK in FTO-2B cells enhances glycogen deposition irrespectively of the GS isoform present. Glycolysis, which is an alternative metabolic fate of Glc-6-P, is also greatly stimulated in GK-overexpressing FTO-2B cells (12). In cultured hepatocytes (6, 7, 24) and in cultured human muscle cells (25), GK overexpression also stimulates both processes, glycogen deposition and glycolysis. On the contrary, Glc-6-P produced by HK I is not sensed by LGS and therefore is not used for the synthesis of glycogen even when the intracellular levels of this metabolite are substantially increased.

Figure 3. Effects of HK I, GK, LGS, and MGS overexpression on Glc-6-P levels in FTO-2B cells. A, cells were treated with AdCMV-GFP (open squares), AdCMV-HK I (open triangles), or AdCMV-GK (filled triangles). B, cells were treated with AdCMV-GFP (open squares), AdCMV-LGS (filled squares), AdCMV-LGS plus AdCMV-HK I (filled triangles), or AdCMV-LGS plus AdCMV-GK (filled circles). C, cells were treated with AdCMV-GFP (open squares), AdCMV-MGS (filled triangles), AdCMV-MGS plus AdCMV-HK I (filled circles), or AdCMV-MGS plus AdCMV-GK (filled squares) and incubated as described in the legend of Fig. 2. Cells were then collected, and intracellular Glc-6-P concentrations were measured. Data represent the mean ± S.E. for six to eight independent experiments. Prot, protein.
The four enzymes overexpressed in this study have been shown to change their intracellular distribution in response to glucose. In the absence of the sugar, GK is localized to the nucleus of the hepatocyte but moves into the cytosol when the levels of glucose increase (21, 26). MGS is also concentrated in the nucleus at low glucose and translocates to the cytosol, where it adopts a particulate pattern, at high glucose concentrations (5, 14). In contrast, LGS presents a cytosolic distribution in the absence of glucose and concentrates at the periphery of the hepatocyte when the concentration of the hexose increases (27). Finally HK I has been shown to reversibly bind to the outer mitochondrial membrane through a hydrophobic N-terminal sequence (28). This association, which is at least in part controlled by the intracellular levels of Glc-6-P, plays a role in the regulation of HK I activity in vivo. Low levels of Glc-6-P favor the association with mitochondria and stimulate HK I activity, while high levels of this metabolite have the opposite effect (29).

HK I, which accounts for more than 75% of total hexokinase activity present in skeletal muscle (30), and MGS are, respectively, at the beginning and at the end of the glycogen synthetic pathway in muscle. Therefore, the ability of HK I to mediate the glucose-induced activation of MGS has a clear physiological meaning. The high affinity of HK I for glucose implies that this sugar is readily converted into Glc-6-P upon entering the cell, and thus the pair HK I-MGS is sensitive to the low concentrations of glucose present in the muscle cell. This explains why the control of glycogen deposition in muscle does not reside in the glucose phosphorylating capacity of the cell but rather in the insulin-stimulated import of the sugar by the GLUT-4...
transporter and in the MGS (9).

However, the situation in the liver is rather different. In the hepatocyte, extracellular glucose and intracellular glucose are in equilibrium partly due to the high capacity of the hepatic glucose transporter GLUT-2 (31). Although GK represents the main glucose phosphorylating activity of the hepatocyte, HK I is also present at considerable levels (32). When blood glucose levels are low (below ~5 mM), there is no significant flux through GK because of its high $K_m$ for glucose (32) and to the action of its regulatory protein, which further decreases the apparent affinity of GK for glucose in the hepatocyte (22).

Therefore, under these conditions only HK I can phosphorylate glucose, but as shown in this study, the Glc-6-P thus produced cannot be diverted toward the synthesis of glycogen since this metabolite does not activate LGS. Only when blood sugar concentration increases above a threshold level does GK translocate to the cytosol and start to produce Glc-6-P, thus giving the signal that triggers the synthesis of hepatic glycogen. In this case, the control of glycogen synthesis is not exerted by glucose transport but rather by HK and GS (8). It appears that the inability of the Glc-6-P produced by HK I to stimulate the uptake but rather by GK and GS (8). It appears that the inability of the Glc-6-P produced by HK I to stimulate the activation of LGS is one way for the hepatocyte to ensure that hepatic glycogen synthesis is only engaged when needed, that is, when blood glucose levels are high.

We conclude that the characteristics of the two pairs of isoenzymes LGS/GK and MGS/HK I and the relationships that they establish are tailored to suit specific metabolic roles of the tissues in which they are expressed.

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