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# Glycogen phosphorylase is activated in response to glucose deprivation but is not responsible for enhanced glucose transport activity in 3T3-L1 adipocytes

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**Abstract**

We have previously shown that glucose deprivation activates glucose transport in a time- and protein synthesis-dependent fashion in 3T3-L1 adipocytes, a mouse cell line. Coincident with this is loss of glycogen. Because glycogen phosphorylase (GP) is responsible for glycogen degradation, we have examined its regulation to determine the relationship between transport activation and glycogen turnover. We first cloned the adipose GP cDNA and found sequence similarity to rat and human liver GP. Because the mouse liver GP cDNA sequence was unavailable, we cloned this cDNA as well and showed 100% identity between mouse adipose and liver sequences. A 3.1 kb transcript was readily observed in total RNA isolated from mouse liver or adipose by Northern blot analysis but, surprisingly, not in either total or poly(A) selected RNA from 3T3-L1 adipocytes. To evaluate regulation in 3T3-L1 adipocytes, we amplified GP mRNA from total RNA using multiplex, semi-quantitative PCR but found that expression did not change in response to deprivation. GP protein levels did not change either. However, endogenous GP activity from glucose-deprived cells was significantly elevated relative to controls, due to an increase in the phosphorylated form of GP (GP*a*). Finally, we overexpressed GP to determine its direct influence on the glucose transport system. These results were negative, which suggests that the nutrient control of glucose transport and GP occurs independently despite kinetic similarities in transport activation and glycogen turnover. 

*Keywords:* Glycogen phosphorylase; Glucose transport; 3T3-L1 adipocyte; Gene expression; Nutrient control

## 1. Introduction

Glucose availability controls a number of homeostatic processes in adipocytes at the transcriptional level. Among these are the genes for fatty acid synthase [1,2], S14 [3,4], and mitochondrial glycerophosphate acyl transferase [5,6], each of which play a role in triglyceride biosynthesis. Facilitating these studies was the development of cultured adipocyte model systems, such as the 3T3-L1 cell line, which allows the independent manipulation of nutrients from hormones. Using this cell line, we and others have shown that glucose deprivation increases glucose transport activity by at least 10-fold by a process which requires both new transcription and translation [7–9]. This process can be blocked by non-metabolizable glucose analogues, which suggests that glucose itself and not one of its metabolites regulates the process. This feature is further emphasized by the fact that hexoses which do not serve as substrates for the transporters, like fructose, do not block or reverse the activation. We now know that the two major transporters in 3T3-L1 adipocytes are GLUT1 and GLUT4 [10–13]. In our hands, there is little change in GLUT1 mRNA, protein, or compartmentation [9,14,15], although GLUT1 is the major transporter at the cell surface. GLUT4 mRNA and protein decrease in response to glucose deprivation [16,9]. This has led to the hypothesis that GLUT1 is activated by a novel protein whose synthesis is controlled by glucose. Such an activator has not yet been identified.

Glucose deprivation also causes a time-dependent loss

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of glycogen in 3T3-L1 adipocytes [17], indicating mobilization of this internal glucose pool. The kinetics of this loss are similar to the kinetics of transport activation. In addition, fructose, which is unable to prevent transport activation, is likewise unable to prevent glycogen loss [17]. Yet fructose is utilized by other metabolic pathways, such as the oligosaccharide biosynthetic path which prevents aberrant protein glycosylation [9]. Finally, glycogen phosphorylase (GP), the enzyme responsible for glycogen degradation, is one of the few proteins beside the transporter which binds free glucose [18]. Indeed, glucose is an allosteric inhibitor of GP*a*, the phosphorylated and more active form of the enzyme (for review, see [19]). Together, these common features provided the basis for the hypothesis that changes in GP expression and or activity might directly influence GLUT1 activity to increase glucose flux.

In this work, we thus examine the regulation of adipocyte GP in relation to glucose availability. Mouse adipose GP was unavailable in the databases so we initially cloned GP from an adipose library. This revealed similarity to known liver-specific sequences. To confirm this identity, we cloned the mouse liver cDNA as well to show 100% identity between mouse adipose and liver sequences. Despite our successful cloning efforts, we were unable to show either transcriptional or translational changes in response to glucose availability in 3T3-L1 adipocytes. Nevertheless, GP activity increased significantly at early time points in cells which were deprived of glucose. We show that an increase in GP*a*, relative to GP*b*, is responsible for the increase in activity. We finally assessed the direct influence of GP on glucose transport activity by overproduction of the endogenous isozyme in 3T3-L1 adipocytes. Although GP protein and activity increased significantly, glucose transport activity was unaltered. This argues that GP does not influence GLUT1 activity and that nutrient control of each system occurs independently.

## 2. Materials and methods

Dulbecco's modified Eagle's medium (DMEM, high glucose) and glucose-free DMEM were obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA, USA) and calf serum was purchased from Intergen (Purchase, NY, USA). FBS was dialyzed to remove glucose against three changes of phosphate buffered saline (pH 7.2) over 3 days using Snakeskin dialysis tubing (Pierce, Rockford, IL, USA) with a 10 000 molecular weight cutoff. Enhanced chemiluminescence reagents were purchased from Amersham. The Glucose (HK) 10 Kit (cat. No. 16-10) was purchased from Sigma. The inbred mouse strain used in this project was BTBR (black and tan, brachyury, Jackson Laboratory, Bar Harbor, ME, USA).

### 2.1. Cell culture

3T3-L1 fibroblasts were plated in high-glucose DMEM containing 10% calf serum. Two days after reaching confluence, cells were differentiated using 0.5 mM methyl isobutyl xanthine, 0.25 mM dexamethasone, and 172 nM insulin. Two days later the cells were re-fed with DMEM containing 10% FBS and 172 nM insulin. The cells were then fed every other day with DMEM and 10% FBS. The cells were used within days 8–12 post differentiation.

### 2.2. Initial cloning and isoform identification

Initial PCR primers were chosen from the most conserved regions of a generalized mouse GP consensus sequence created by aligning all existing mouse EST sequences homologous to the known human and rat cDNAs (muscle-, brain-, and liver-specific). The forward primer (located at nucleotide 1508 in this consensus sequence) was 5′-TTGGAGAGGATTATGTGAAAGA-3′ and the reverse primer (at nucleotide 2233) was 5′-CTGGCTGATTGGGAGAAAAGAA-3′. PCR amplification of a mouse adipose cDNA library (Clontech, Palo Alto, CA, USA) resulted in an approx. 750 bp fragment which was cloned and sequenced. The fragment had 91% identity to the rat liver-specific GP isoform. A second alignment of mouse EST sequences which were specifically homologous to the rat liver isoform of GP was performed and PCR primers were designed to amplify the regions that were missing or of poor quality in the EST liver consensus sequence.

### 2.3. Cloning and isolation of full length cDNAs

Total RNA was isolated from either 3T3-L1 cell cultures or mouse liver or adipose mRNA (RNeasy Total RNA, Qiagen, Santa Clara, CA, USA). For adipocytes, it was necessary to remove the abundant lipid layer after homogenization and before applying to the RNeasy column. Total RNA was reverse transcribed (Superscript Preamplification System, Gibco) using an oligo(dT) primer to generate cDNA for PCR. PCR using several liver isoform-specific primers yielded fragments from both 3T3-L1 and mouse liver cDNA preparations. These multiple overlapping fragments were cloned and sequenced, yielding the complete mouse adipocyte and liver GP coding sequence plus additional sequences from the 5′ and 3′ UTRs. A complete cDNA clone was then amplified from mouse liver cDNA using primers to the extreme 5′ and 3′ ends of the coding sequence. Sequencing was performed on three separate clones for verification.

### 2.4. Construction of recombinant pBMN-mlGP and 3T3-L1 fibroblast infection

The liver-specific GP isozyme was cloned into the TA cloning sites of pCR 2.1-TOPO (Invitrogen, Carlsbad, CA, USA). A 2.65 kb fragment, including the entire coding region, was subcloned into the *Bam*HI and *Hin*dIII sites of a pBMN retroviral vector [20], generating pBMN-mlGP. This construct and the control pBMN-GFP (green fluorescent protein) were transiently transfected into the ecotropic retroviral producer cell line Phoenix E. After a 30 h incubation at 32°C, retroviral supernatants were collected, filtered through a 0.45 μm membrane and added undiluted to cells in the presence of polybrene (5 μg/ml). Following incubation at 32°C for 1 day, the cells were refed with normal media and placed at 37°C. The efficiency of retroviral transduction was estimated to be ~95% based on the percentage of cells that were GFP positive by fluorescent microscopy (data not shown).

### 2.5. Isolation of poly(A) mRNA

3T3-L1 fibroblasts and adipocytes were grown to confluence and differentiated in 10 cm plates, as appropriate. Confluent fibroblasts were exposed to complete medium (25 mM glucose) supplemented with 10% calf serum for 12 h prior to RNA isolation. 3T3-L1 adipocytes were exposed to complete medium (+10% FBS) or glucose-free medium (+10% FBS), supplemented with 25 mM fructose for 12 h prior to RNA isolation. Cells were then washed with PBS and lysed with RLT lysis buffer (RNeasy Total RNA). As before, the top fat layer was removed. Total RNA from five 10 cm plates of 3T3-L1 adipocytes (~430 μg) was applied to an aliquot (12.5 mg, 150 μl) of oligo$(dT)_{25}$ cellulose bead slurry in 500 μl of loading buffer (0.5 M NaCl, 20 mM Tris–Cl (pH 7.5), 1.0 mM EDTA). After washing five times with loading buffer and once with 500 μl low salt buffer (0.1 M NaCl, 20 mM Tris–HCl (pH 7.5), 1.0 mM EDTA), poly(A) containing mRNA was eluted with RNase free water. Recovery was 26 μg of RNA (~6%).

### 2.6. Northern analysis

Samples of total RNA (15 μg) or poly(A) selected RNA (5 μg) were resuspended in RNA loading buffer (0.02 M MOPS, pH 7.0, 0.5 mM EDTA, pH 8.0, 5 mM NaOAc, pH 7.4, 6.475% formaldehyde, 50% formamide), heated to 65°C for 15 min, cooled on ice and loaded onto a 1% agarose gel (running buffer: 0.04 M MOPS (pH 7.0), 1 mM EDTA (pH 8.0), 10 M NaOAc (pH 7.4) and 6.6% formaldehyde). The RNA was blotted overnight onto a Duralon-UV nylon membrane (Stratagene, Cedar Creek, TX, USA) and the RNA cross-linked to the membrane with UV light. The membrane was prehybridized (1% BSA in 0.5 M NaPO4 (pH 7.2), 7% SDS, 1 M EDTA (pH 8.0), 30 min at 61°C) and then 200 ng of the complete 2023 bp fragment of liver GP cDNA labeled with [$^{32}$P]dCTP using a Random Primers DNA Labeling System (Gibco) were added and incubated overnight at 42°C. The membrane was washed four times with 0.04 M NaPO4 (pH 7.2), 1 mM EDTA (pH 8.0), 1% SDS at 68°C. Glyceraldehyde phosphate dehydrogenase was probed at the same time as GP using a radiolabeled human cDNA fragment.

### 2.7. Semi-quantitative multiplex RT-PCR

To measure the level of low abundance messages, we have followed the protocol described by Pernas-Alonso et al. [21]. RNA was collected from 3T3-L1 adipocytes treated with either complete medium (25 mM glucose), glucose-free medium containing 10% dialyzed FBS and 25 mM fructose, or glucose-free medium containing 10% dialyzed FBS for 12 h. Total RNA (3 μg) from 3T3-L1 adipocytes was reverse transcribed; cDNA was resuspended in 42 μl of RNase-free water. Duplicate samples (12 μl) of cDNA were used as the template in separate 100 μl PCR reactions. For GP, primers (see Table 1) amplified a 635 bp fragment. After cycles 16, 18, 20, 22, 24, 26, 28, 30, and 32 an aliquot (10 μl) of each reaction was removed. All 16 aliquots were loaded onto a 1% agarose gel and electrophoresed. A similar procedure was used to amplify a 421 bp fragment of GLUT1 and an 877 bp fragment of GRP78, an ER molecular chaperone that is transcriptionally induced by glucose deprivation [22]. Each gel was then blotted onto Duralon-UV nylon membrane and DNA cross-linked using UV light. The relative quantities of amplified product at the various cycles were determined by Southern analysis. Probes were generated using 200 ng of each DNA template (mouse liver GP cDNA, this work; human GLUT1 cDNA [23] and hamster

Table 1
Primers used for semi-quantitative multiplex PCR

| Primer | Sequence (5′ to 3′) | Nucleotide position |
|---|---|---|
| GP forward | TCA TTC CAG CCA CAG ACC TAT C | 2104 |
| GP reverse | AGC TCT AAA ACA CTC AAG TTC C | 2655 |
| GLUT1 forward | CAG TAT GTG GAG CAA CTG TGC G | 1456 |
| GLUT1 reverse | TAG ATC TGA GCA GCA GTC TTG C | 1877 |
| GRP78 forward | GTG GAG ATC ATA GCC AAC GAT CA | 162 |
| GRP78 reverse | ACA GGC TTC ATG GTA GAG CGG | 1039 |

GRP78 cDNA [24]) using 60 ng of a gene-specific primer, 50 μCi [$^{32}$P]dCTP, and the Klenow fragment of DNA polymerase I. Hybridization and washing was as described except the temperature was 68°C. Densitometric analysis was performed on the exposed film to determine the linear phase of PCR amplification for each of the three target DNAs. Multiplex PCR was then performed to concurrently amplify each mRNA in individual samples.

### 2.8. GP activity assay

Cells from five 10 cm plates were scraped into 1 ml of TES (20 mM Tris–HCl, pH 7.4; 1 mM EDTA, and 255 mM sucrose) containing 2.5 mM DTT, 1 μl of protease inhibitor cocktail (Sigma, cat. No. P8340), and 20 μg of phenylmethylsulfonyl fluoride. The cells were lysed in a Potter–Elvehjem homogenizer and the cellular extract was centrifuged at $12\,000\times g$ for 30 min in a Sorvall SS-34 rotor. The supernatant fraction was collected and protein concentration was determined using a modification of the Lowry assay [25]. One hundred micrograms of total crude protein were assayed for GP activity. The assay was performed in a 1 ml volume containing: 50 mM potassium phosphate (pH 7.5), 10 mM $MgCl_2$, 5 mM EDTA (pH 8.0), 0.5 mM NADP, 1.0 unit of phosphoglucomutase (Sigma G-7877), and 1.5 units of glucose-6-phosphate dehydrogenase (Sigma P-3379). Absorbance at 340 nm was measured every minute for a total of 60 min: 100 μg of rabbit liver glycogen was added after 10 min, 0.5 mM caffeine was added after 25 min, and 0.1 mM AMP was added after 40 min. The rate of GP activity was determined from the slope of the read-out using the molar extinction coefficient of NADH ($6.22\times10^3$).

### 2.9. Western blot analysis

One hundred micrograms of protein (prepared as described above) were separated on a 7.5% polyacrylamide gel and then transferred to nitrocellulose for Western blot analysis [9]. GP protein levels were determined using rabbit antisera generated against purified recombinant human liver GP (a gift from Dr. Judith Treadway, Pfizer Global Research and Development). This antibody recognizes all forms of GP.

## 3. Results

### 3.1. Cloning of mouse liver glycogen phosphorylase

We have shown previously that glucose deprivation in either the presence or absence of fructose leads to enhanced glucose transport activity [9,14] and loss of glycogen [17]. To determine the possibility that GP plays a regulatory role in transport activity, we pursued the regulation of GP.

Table 2
Percent identity of mouse liver GP to other isozymes[a]

| | Protein | mRNA |
|---|---|---|
| Mouse muscle GP | 78.8 | 74.6 |
| Rat liver GP | 98.5 | 94.1 |
| Rat muscle GP | 77.3 | 74.1 |
| Rat brain GP | 79.8 | 74.6 |
| Human liver GP | 94.2 | 88.4 |
| Human muscle GP | 78.7 | 74.5 |
| Human brain GP | 80.5 | 75.3 |

[a]Analysis was performed using GeneStream align of coding sequence, only. Sequence information was copied from the GenBank database.

Three isozymes of rat and human GP have been identified: brain-, muscle-, and liver-specific [19]. An initial search of the mouse EST database retrieved three groups of incomplete cDNA sequences with homology to the three known rat GP isozymes. As described in Section 2, oligonucleotides matching a region common to all three isozymes were used to amplify a conserved region of the GP coding sequence from a mouse adipose cDNA library. Sequencing of the resulting fragment showed similarity to the rat (and human) GP liver isozyme. This suggested that mouse adipose contains the liver-specific isozyme. Additional PCR amplification studies showed that this putative liver-specific fragment could also be detected in reverse-transcribed mRNA from differentiated 3T3-L1 adipocytes, as well as from mouse liver. Sequencing of the fragments from 3T3-L1 adipocytes and mouse liver showed 100% identity. While a complete and overlapping set of GP cDNA fragments could be amplified from reverse-transcribed 3T3-L1 mRNA, only reverse-transcribed mouse liver mRNA allowed amplification of an intact, full length clone, probably because of greater message abundance (see below). Fig. 1 shows the complete nucleotide and deduced protein sequence for the liver-specific isoform of GP, to which the 3T3-L1 adipocyte GP sequence has 100% identity. The full length sequence has two base pair differences from that of the incomplete consensus sequence compiled from the mouse EST database (at nucleotides 1743 and 2067). Both of these are silent, i.e. the sequences encode the same amino acid, presumably representing strain polymorphisms. The initial 747 bp fragment amplified from the mouse adipose cDNA library is underlined. As expected, the mouse liver-specific GP sequence is highly similar to that of the rat and human liver isozymes, and shares less homology with the muscle and brain isoforms (Table 2).

### 3.2. Transcriptional regulation

Attempts to examine the effect of glucose deprivation on GP mRNA levels by Northern blot analysis were inconclusive. GP mRNA could not be detected in 3T3-L1 cells, even after poly(A) selection (Fig. 2). This suggested a low level of expression which is consistent with our experience in cloning GP from these cells. By comparison, the mRNA was readily detected in mouse adipose and liver tissue,

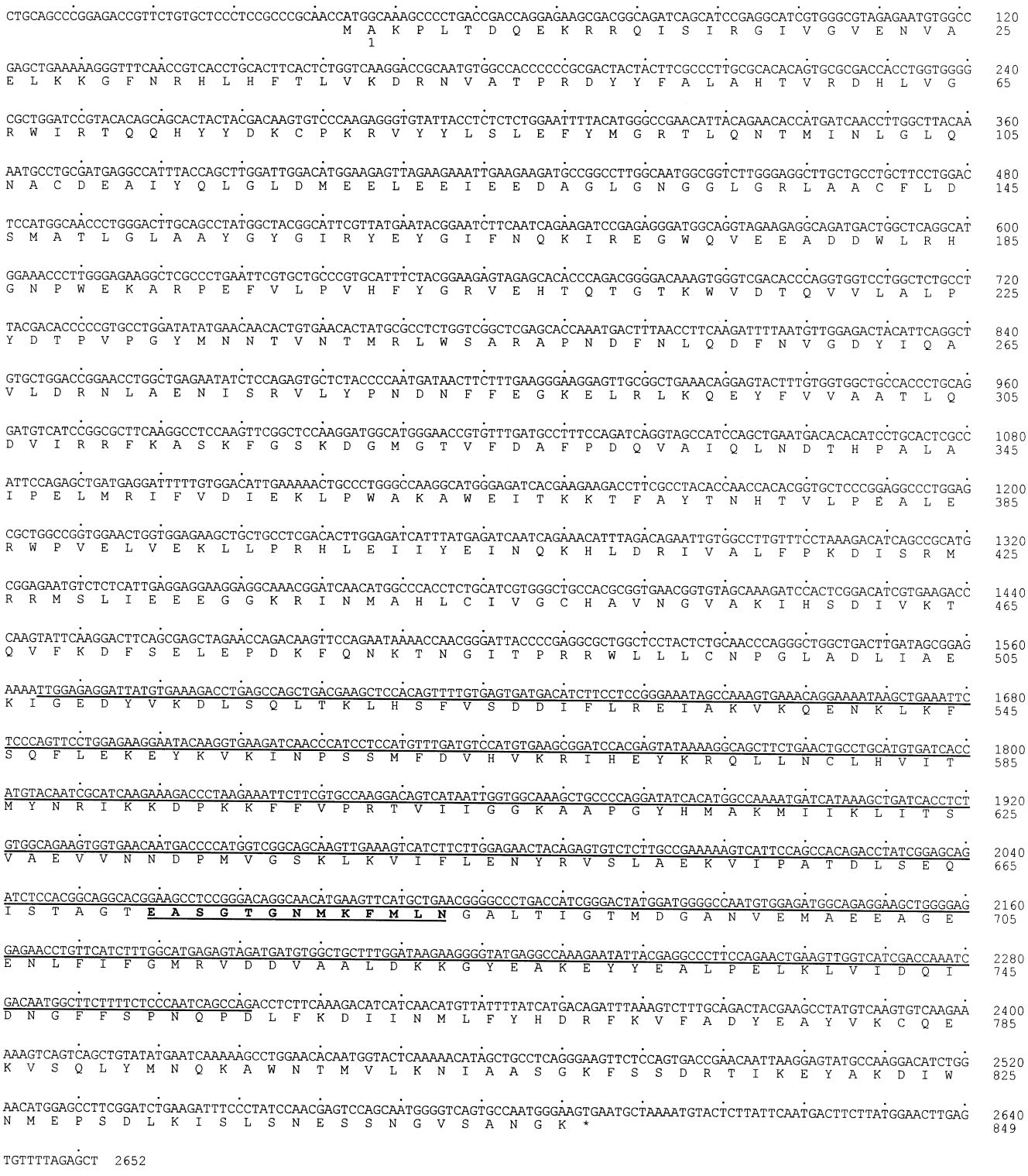


Fig. 1. Liver-specific phosphorylase cDNA. The sequence of adipocyte phosphorylase was determined to be identical to the liver-specific isoform. The underlined DNA sequence represents the original 750 bp fragment amplified from the adipose cDNA library. The protein sequence was deduced from the cDNA. By convention, the amino acids are numbered based on alignment with rabbit muscle glycogen phosphorylase [19]. The consensus sequence which defines the pyridoxyl phosphate attachment site is underlined and in bold print. The serine residue at position 14 is the conserved phosphorylation site. The residues that comprise the AMP binding site (Asp-42, Asn-44, Val-45, Gln-71, Gln-72 and Tyr-75) [40] and the moveable gate (Tyr-280, Pro-281, Asn-282, Asp-283, Asn-284, Phe-285) which protects the active site [41] are also highly conserved among species. The GenBank accession number for the cDNA sequence is AF288783.

migrating as a single species at 3.1 kb. We have not evaluated the reason(s) for these differences.

Due to the insensitivity of Northern analysis, we utilized semi-quantitative multiplex RT-PCR to examine transcriptional control in 3T3-L1 adipocytes. This methodology amplifies the initial amount of template message to within detectable levels. Adequate internal controls are necessary to correct for variability in individual reverse transcription and PCR reactions, and to insure detection of changes in mRNA. In our hands, GLUT1 mRNA changes little in response to glucose deprivation and if any change is noted it is a decrease in expression [9,26]. Thus GLUT1 served as our 'negative' control. GRP78, an ER-specific chaperone protein, is upregulated by complete hexose deprivation in 3T3-L1 adipocytes [26,27] and other cell types [10,17,21] and served as a 'positive' control. Initial experiments to determine conditions for amplification of the GP, GLUT1, and GRP78 mRNAs revealed that 22, 20, and 19 cycles, respectively, yielded linear amplification rates (data not shown). In the experiment shown in Fig. 3, RNA was isolated from cells exposed to three treatment paradigms. One set of cells was provided glucose for 12 h. A second set was deprived of glucose, but supplemented with fructose to prevent metabolite deprivation. As mentioned earlier, this allows for both the loss of glycogen and activation of glucose transport. A third set was deprived of glucose, with no additional supplementation. Amplification of each RT product was then accomplished in a single tube by initiating GP amplification first. The primers for GLUT1 were added after two cycles, and the primers for GRP78 were added after one additional cycle. PCR products were analyzed by Southern blotting (Fig. 3A). Densitometric comparison between samples showed that neither GP, GLUT1, nor GRP78 mRNA changed in cells treated in the absence of glucose but the presence of fructose in comparison to controls (Fig. 3B). In cells exposed to complete hexose deprivation, GLUT1 mRNA dropped slightly while GRP78 mRNA was significantly elevated relative to controls. However, GP mRNA did not change. Together, these data indicate that 3T3-L1 adipocyte GP mRNA is not under nutritional control.

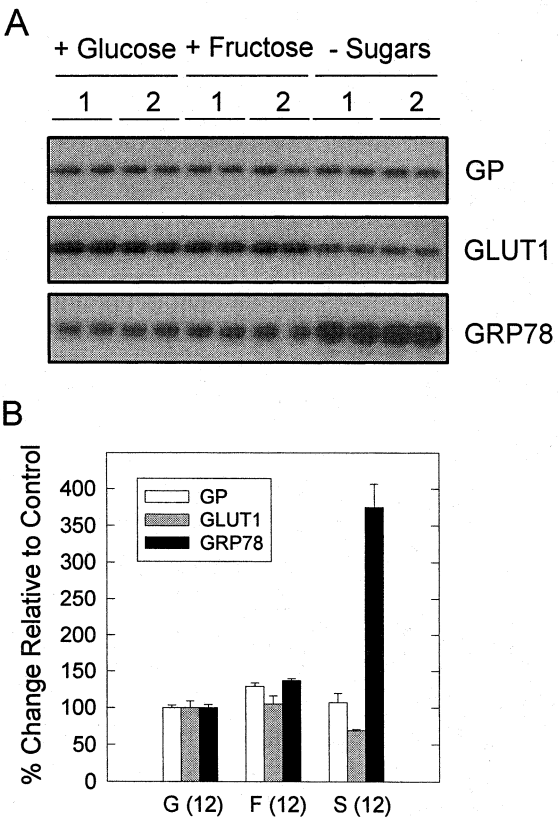


Fig. 3. Southern analysis of semi-quantitative multiplex RT-PCR. (A) Cells were incubated for 12 h in the presence of complete medium (+Glucose), glucose-free medium supplemented with fructose (+Fructose), or glucose-free medium with no supplementation (−Sugars). Duplicate RT reactions (1, 2) were performed on each sample to control for the amount of cDNA synthesized. Duplicate PCR reactions were performed on each RT reaction as a control for product amplification. PCR products were separated by electrophoresis and transferred to nylon membranes. Radiolabeled probes were hybridized to the blots to reveal GP, GLUT1, and GRP78 amplification products. (B) Bands in panel A were quantified by video densitometry and expressed as % of the control (+glucose) values. The averages ± S.E. of three independent experiments are shown. G(12), +glucose for 12 h; F(12), +fructose for 12 h; S(12), −sugars for 12 h, i.e. complete hexose deprivation.

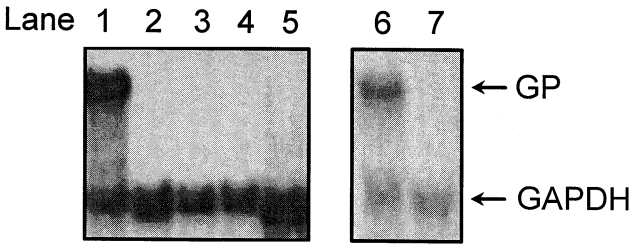


Fig. 2. Northern analysis of glycogen phosphorylase. Total RNA was isolated from mouse liver (lane 1), 3T3-L1 fibroblasts (lane 2), and 3T3-L1 adipocytes incubated in complete medium (lane 3) or glucose-free medium supplemented with 25 mM fructose (lane 4) for 12 h. Lane 5 contains poly(A) mRNA from adipocytes incubated as in lane 3. Lane 6 contains RNA isolated from mouse adipose. Lane 7 contains a separate sample of 3T3-L1 adipocyte RNA. Fifteen micrograms of total RNA or 5 μg of poly(A) selected RNA were separated by electrophoresis on a formaldehyde denaturing gel. GP mRNA was detected using a radiolabeled probe generated from mouse liver GP. Detection of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA is shown as a loading control.

### 3.3. Translational regulation

Levels of GP protein were examined by Western blotting. Cells were incubated in either complete medium or glucose-free medium supplemented with fructose. Time points were chosen based on the kinetics of glycogen turnover [17]. Detection of GP protein was accomplished using rabbit polyclonal antisera, generated against purified human liver GP. In our hands, this antibody detected a single protein migrating at 97 kDa, consistent with the calculated molecular mass of GP. The data show that over the

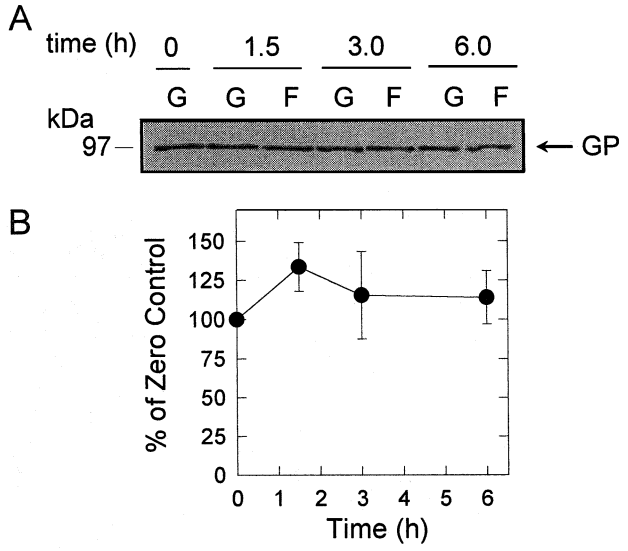


Fig. 4. Effect of glucose deprivation on glycogen phosphorylase expression. Cells were incubated in complete medium (G) or glucose-free medium plus fructose (F) for the indicated times. Cells were harvested and homogenized as described. A crude extract was prepared by centrifugation which contained 95% of total GP activity. (A) One hundred micrograms of protein were loaded onto a 7.5% SDS–polyacrylamide gel for protein separation. After transfer to nitrocellulose membrane, immunoblot detection was performed using anti-GP antibody. (B) Densitometric analysis of three independent experiments was performed. GP levels in glucose-deprived samples (F) are represented as the percentage of the glucose-fed samples (G) at each time point, ± S.E.

first 6 h of glucose deprivation, during which time glycogen is rapidly degraded, the amount of 3T3-L1 GP protein does not change relative to control cells (Fig. 4). This argues against translational control.

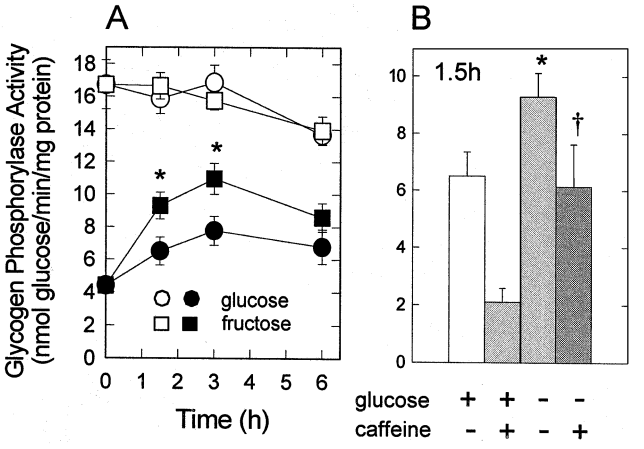


Fig. 5. Effect of glucose deprivation on GP activity. (A) GP activity in 100 μg of crude extract (prepared as in Fig. 4) from cells incubated in complete medium or glucose-free medium supplemented with fructose was measured in the presence of substrate (100 μg glycogen) (closed symbols) or with subsequent AMP addition (0.1 mM) (open symbols). The data represent the average ± S.E. of five independent experiments. $^{*}P < 0.05$. (B) At the 1.5 h time point, GP activity was measured in the presence of glycogen in the absence or presence of 0.5 mM caffeine. The data represent the average ± S.E. of five independent experiments. $^{*}P < 0.05$ for controls versus glucose-deprived; $^{\dagger}P < 0.02$ for controls+caffeine versus glucose-deprived+caffeine.

### 3.4. Regulation of phosphorylase activity

GP is a classic example of an enzyme which is regulated by allosteric control (see [28]). Therefore, GP enzyme activity was examined to determine if the loss of glycogen in the glucose-deprived state was due to changes in activity. Activity was measured at specific time points during the first 6 h of glucose deprivation. First, we determined the total level of GP activity as described by Stalmans and Hers [29]. This assay is performed in the presence of the activator, AMP, which provides a measure of the potential activity in the cell. In essence, this is a reflection of total GP protein. These data show that total 3T3-L1 adipocyte GP activity does not change in response to glucose deprivation (Fig. 5), which is consistent with the Western blotting data. Next, we measured endogenous activity. Endogenous activity is a fraction of the total. This reflects the actual activity at the time that GP is extracted from cells. In the presence of glycogen, the extract from glucose-deprived cells (Fig. 5A, black squares) exhibited significantly higher activity at 1.5 and 3 h of glucose deprivation when compared to that of the control cells (Fig. 5A, black circles). This suggests that GP is indeed activated. The increase in activity can be attributed to either enhanced phosphorylation of GP*b* to generate the more active GP*a*

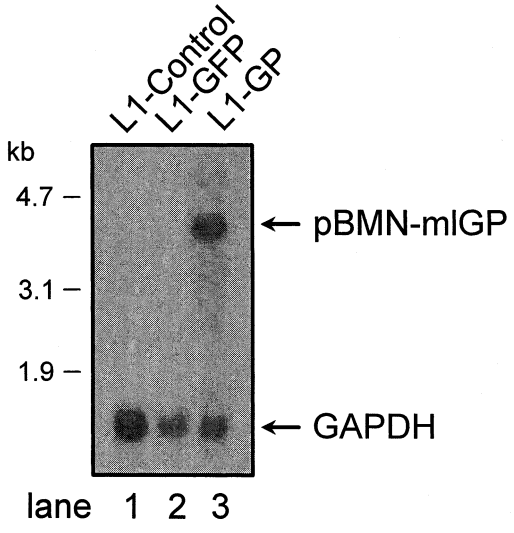


Fig. 6. Virally induced overexpression of GP increases GP mRNA in 3T3-L1 adipocytes. A cDNA fragment of GP, representing nucleotides 6–2652 in the mlGP sequence, was subcloned into the *Bam*HI and *Hin*dIII sites of a pBMN retroviral vector [20], generating pBMN-mlGP. This construct was transfected into ecotrophic Phoenix producer cells, and the supernatant was used to infect 3T3-L1 fibroblasts. Cells were differentiated to the adipocyte phenotype (L1-GP). In parallel, fibroblasts were infected with virus containing the green fluorescent protein and differentiated (L1-GFP). Differentiation was only 70% efficient in cells infected with virus compared with normal 3T3-L1 adipocytes (L1-Control). Total RNA was isolated and Northern blot analysis performed on equal amounts of RNA. pBMN-mlGP, viral vector containing the liver-specific GP sequence; GAPDH, glyceraldehyde phosphate dehydrogenase. The decrease in GAPDH in both virally infected cells likely represents the reduced efficiency of differentiation.

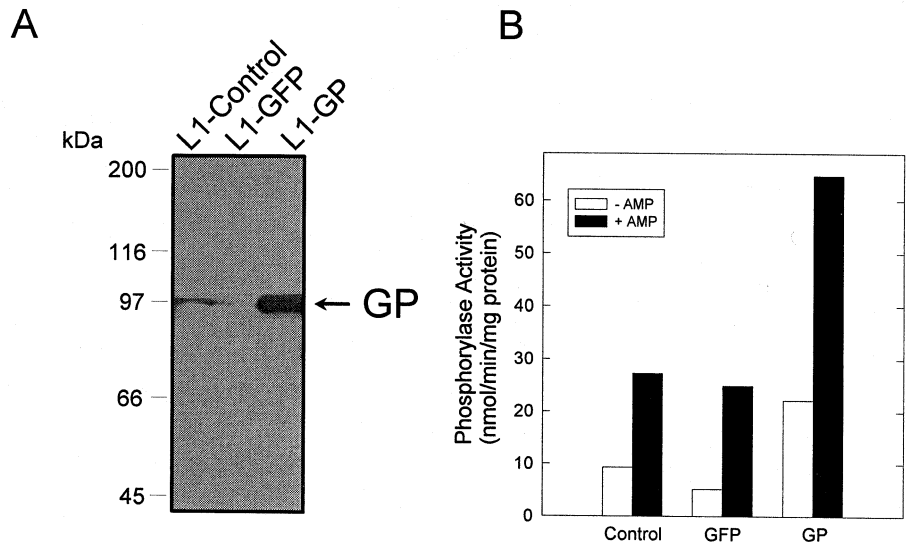


Fig. 7. Overexpression of GP leads to elevated protein and activity. (A) A crude cytoplasmic fraction was collected from normal 3T3-L1 adipocytes (L1-Control), cells overexpressing GFP (L1-GFP), or overexpressing GP (L1-GP). Fifty micrograms were analyzed by Western blot analysis. (B) Phosphorylase activity was analyzed in the same samples as depicted in panel A. Activity (in 100 μg) was analyzed for endogenous activity (−AMP) and total activity (+AMP) as described in Section 2. This was repeated on two additional sets of samples with similar results.

or allosteric activation of GP*b* by AMP. The latter is unlikely in that allosteric interaction would not be maintained through the isolation procedure. Further, there is little evidence that AMP increases in response to glucose deprivation in 3T3-L1 adipocytes [7]. To determine the role of phosphorylation, we examined the sensitivity of GP activity to caffeine, which selectively inhibits liver GP*b* [29]. At 1.5 h, GP activity in fed cells was inhibited by 68% which contrasts to only 34% inhibition in glucose-deprived cells (Fig. 5B). Similar inhibition was noted in the 3 h extracts (data not shown). In total, our data suggest that glucose deprivation exerts its effect post-translationally by stimulating the conversion of GP*b* to the GP*a* form, which enhances endogenous activity.

### 3.5. Overexpression of glycogen phosphorylase

Baque et al. have recently shown that overexpression, alone, of muscle GP in cultured human muscle cells increases glucose transport activity [30,31]. We thus tested the hypothesis that overexpression, and thus enhanced activity, of homologous GP in 3T3-L1 adipocytes would influence glucose transport activity. We used a transfection system based on the murine leukemia virus (see Section 2). To determine that GP mRNA was indeed overexpressed in 3T3-L1 adipocytes, we performed Northern blot analysis on normal cells, those infected with the vector containing our cloned GP sequence (pBMN-mlGP), or with vector containing GFP (pBMN-GFP). Fig. 6 shows the results. As before, mRNA could not be detected in normal 3T3-L1 adipocytes, which was also true for pBMN-GFP infected cells. However, a transcript was detected in adipocytes infected with pBMN-mlGP which migrated as a higher molecular mass species than that of authentic GP mRNA (3.1 kb) due to the transcription of both GP and viral sequence. Translation of this message species resulted in a protein which migrated identically to that of endogenous GP (Fig. 7A). Overexpression varied from 5- to 7-fold over control 3T3-L1 adipocytes and adipocytes infected with

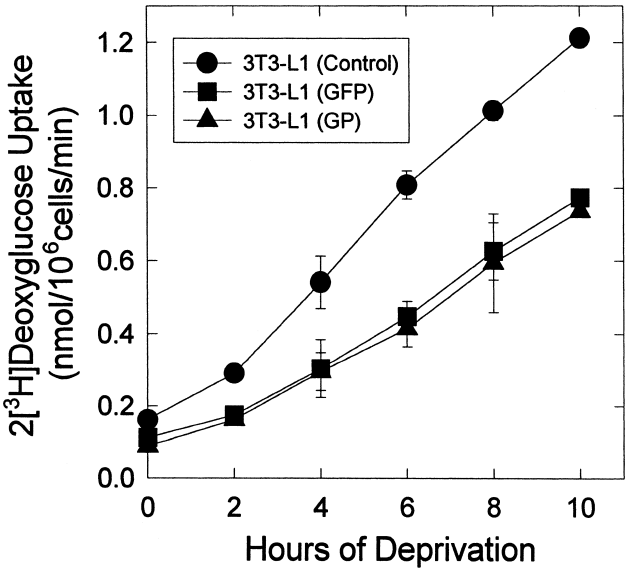


Fig. 8. Effect of GP overexpression on glucose transport activity. Cells were grown and differentiated on 35 mm plates for glucose transport analysis as described earlier [9]. Glucose uptake was analyzed in control cells (●), those overexpressing GFP (■) or GP (▲) at the indicated time points of glucose deprivation over a 10 min interval with [2-$^3$H]deoxyglucose (0.2 mM, 0.2 μCi). Uptake was stopped with ice-cold PBS washes. Cells were extracted in 0.1% SDS and counted for radioactivity. Quadruplicates in a single experiment are shown (±S.D.). The rates of activity change over time (d*r*/d*t*) were calculated using linear regression analysis from the 2 to the 10 h time points. The experiment was repeated three times with similar results.

pBMN-GFP ($n=5$). Glycogen phosphorylase activity was also elevated in pBMN-mlGP infected cells (Fig. 7B). Both endogenous activity and AMP-activated activity were increased by 3–4-fold in pBMN-mlGP cells compared to control cells or those harboring pBMN-GFP.

With the increase in protein and activity confirmed in the infected 3T3-L1 adipocytes, we then measured transport activity in response to glucose deprivation (Fig. 8). As expected, glucose deprivation caused a time-dependent increase in the rate of deoxyglucose uptake in control adipocytes ($dr/dt = 0.109$ nmol/$10^6$ cells/min). With infected cells, there was no difference in the basal transport activity (compare black squares and triangles at zero time). While transport activity in both pBMN-mlGP and pBMN-GFP infected cells did increase in response to glucose deprivation, there was no difference in the rate of increase ($dr/dt = 0.067$ nmol/$10^6$ cells/min in pBMN-mlGP vs. $dr/dt = 0.063$ nmol/$10^6$ cells/min in pBMN-GFP). Note that the rates are only about 60% of that of the control 3T3-L1 adipocytes. This directly reflects the less efficient differentiation of the infected cells (see Fig. 6), the cause of which is unknown. Despite this, we must conclude that glycogen phosphorylase does not influence glucose transport activity in 3T3-L1 adipocytes.

## 4. Discussion

In the present study, we have shown for the first time that 3T3-L1 adipocytes, as well as mouse adipose, express the liver-specific isoform of GP by cloning GP from each tissue. However, message was not detected in 3T3-L1 adipocytes by conventional methodology, suggesting a low steady-state level of mRNA. In contrast, mouse liver and adipose contained abundant mRNA that migrated as a single transcript of approx. 3.1 kb. A similar abundance and size were found in rat liver and adipose [32]. However, the minor transcript ($\sim$3.4 kb) identified in rat liver and attributed to a second polyadenylation signal was not detected in mouse. While the cause of the down-regulation of mRNA in cultured adipocytes is not known, it may be due in part to insufficient insulin, whose action has been reported to enhance transcription (see below).

In whole animals, the feeding/fasting paradigm has been used by many to examine gene regulation. Giffin et al. have shown that the expression of the phosphorylase gene in rat liver does not change under these conditions, leading to the conclusion that phosphorylase is not under nutritional regulation [33]. Of course, these results are potentially confounded by the complexity of whole animal studies where hormonal changes can also influence expression. However, our results essentially confirm this conclusion and extend it to adipocyte GP, as semi-quantitative PCR revealed no regulation by glucose or its metabolites. Further, GP protein expression did not change in response to glucose deprivation, as measured by Western blotting and determination of total phosphorylase activity.

While our data show that phosphorylase is under neither glucose-dependent transcriptional nor translational control, there is in vivo evidence that liver GP is regulated at this level. For example, the increase in GP activity during development correlates with an increase in the level of mRNA [34]. Regulation of GP is also seen in diabetes. In streptozotocin-treated rats (a model of human type I diabetics), a 45% decrease in hepatic GP mRNA has been reported [35]. This decrease is directly related to a decrease in enzyme activity. Both message abundance and activity are restored upon insulin therapy. In contrast, an increase in GP activity is observed in rodent models of type II diabetes which correlates with an increase in GP protein [36] and/or mRNA [37]. The latter is specifically related to an increase in mRNA stability. Thus, these data are consistent with some form of transcription regulation dependent on insulin action.

Post-translational (allosteric and covalent) modification is a well known mode of GP regulation. It appears that this type of regulation predominates in response to glucose availability in 3T3-L1 adipocytes. Thus, our data show that the increase in GP activity is due to an increase in the GP*a* pool. This may be due to an enhanced rate of phosphorylation, or the loss of glucose as an in vivo allosteric inhibitor (which might stabilize the GP*a* pool). In liver, the role of GP is to provide free glucose for extrahepatic tissue [19]. It has been argued that allosteric inhibition of liver-specific GP*a* by glucose is physiologically relevant because of the rapid equilibration of glucose across the plasma membrane. At any given time, the intracellular glucose ranges from 5 mM in fasted animals to 20 mM after oral glucose administration [38]. In vitro, muscle GP is as sensitive to glucose as is the liver isozyme, but because the in vivo intracellular concentration of free glucose is low in muscle, regulation by this effector is thought to be minimal. In that regard, it would seem that muscle and adipose should be more similar in that these tissues express the same low $K_m$ transporters. Yet adipose expresses the liver-specific isoform of phosphorylase. Whitesell et al. have revisited the issue of intracellular glucose concentration in 3T3-L1 adipocytes and showed that the steady-state intracellular glucose concentration ranged from 75 to 90% of extracellular glucose at 5 mM to 50% of the extracellular glucose at 24 mM [39]. This suggests that glucose may indeed play a role in the turnover of glycogen because the liver-specific GP isozyme has a $K_i$ of 8 mM for glucose. Thus, our data are consistent with the hypothesis that the increase in GP activity in glucose-deprived 3T3-L1 adipocytes is due, in part, to loss of allosteric inhibition by glucose.

In glucose-deprived adipocytes, 20% of the GLUT1 pool resides in the plasma membrane in contrast to only 4% of the GLUT4 pool, the insulin-sensitive glucose transporter [14]. This argues that the predominant transport

activity is mediated by GLUT1 in either fed or glucose-deprived cells. While both GP and GLUT1 appear to be regulated by glucose availability, our data suggest that GP activity does not influence that of GLUT1. Even overexpression of GP, despite the increase in activity, had no influence on glucose transport activity. This contrasts to recent studies from Baque et al., who showed that overexpression of the muscle GP isozyme increased the rate of transport in cultured human myocytes [30,31]. Interestingly, they showed that enhanced expression of GLUT4 was responsible for the elevated transport activity in cells overexpressing GP. In addition, insulin-stimulated transport was significantly elevated. We saw neither of these effects (data not shown). While both studies used viral vectors to mediate uptake, the increase in GP activity in the infected myocytes was about 10-fold [31], compared to only 4-fold in 3T3-L1 adipocytes. Assuming that the effect on GLUT4 could be titrated based on the total activity of GP, we would have expected at least a 2-fold increase in basal transport activity, which would be readily detected in our system. As this was not the case, we conclude that myocytes respond differently than do adipocytes to increased GP activity.

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## References

[1] F. Foufelle, B. Gouhot, J.-P. Pegorier, D. Perdereau, J. Girard, P. Ferre, J. Biol. Chem. 267 (1992) 20543–20546.
[2] C. Prip-Buus, D. Perdereau, F. Foufelle, J. Maury, P. Ferre, J. Girard, Eur. J. Biochem. 230 (2000) 309–315.
[3] F. Mourrieras, F. Foufelle, M. Foretz, J. Morin, S. Bouche, P. Ferre, Biochem. J. 326 (1997) 345–349.
[4] Y. Ota, A. Mariash, J.L. Wagner, C.N. Mariash, Mol. Cell. Endocrinol. 126 (1997) 75–81.
[5] E.D. Saggerson, C.A. Carpenter, Biochem. J. 243 (2000) 289–292.
[6] S.F. Yet, S. Lee, H.S. Sul, Biochemistry 32 (2000) 9486–9491.
[7] J.P.M. VanPutten, H.M.J. Krans, J. Biol. Chem. 260 (1985) 7996–8001.
[8] B.C. Reed, D. Shade, F. Alperovich, M. Vang, Arch. Biochem. Biophys. 279 (1990) 261–274.
[9] H.H. Kitzman Jr., R.J. McMahon, M.G. Williams, S.C. Frost, J. Biol. Chem. 268 (1993) 1320–1325.
[10] D.E. James, M. Strube, M. Mueckler, Nature 338 (1989) 83–87.
[11] K.H. Kaestner, R.J. Christy, J.C. McLenithan, L.T. Braiterman, P. Cornelius, P.H. Pekala, M.D. Lane, Proc. Natl. Acad. Sci. USA 86 (1989) 3150–3154.
[12] A.G. de Herreros, M.J. Birnbaum, J. Biol. Chem. 264 (1989) 19994–19999.
[13] D.M. Calderhead, K. Kitagawa, L.I. Tanner, G.D. Holman, G.E. Lienhard, J. Biol. Chem. 265 (1990) 13800–13808.
[14] M.D. Fisher, S.C. Frost, J. Biol. Chem. 271 (1996) 11806–11809.
[15] R.J. McMahon, J.B. Hwang, S.C. Frost, Biochem. Biophys. Res. Commun. 273 (2000) 859–864.
[16] K.M. Tordjman, K.A. Leingang, M. Mueckler, Biochem. J. 271 (1990) 201–207.
[17] R.J. McMahon, S.C. Frost, Am. J. Physiol. 1996 (1996) E640–E645.
[18] A. Carabaza, C.J. Ciudad, S. Baque, J.J. Guinovart, FEBS Lett. 296 (1992) 211–214.
[19] C.B. Newgard, P.K. Hwang, R.J. Fletterick, Crit. Rev. Biochem. Mol. Biol. 24 (1989) 69–99.
[20] S. Kinoshita, L. Su, M. Amano, L.A. Timmerman, H. Kaneshima, G.P. Nolan, Immunity 6 (1997) 235–244.
[21] R. Pernas-Alonso, F. Morelli, U. di-Porzio, C. Perrone-Capano, Brain Res. Protoc. 4 (1999) 395–406.
[22] A.S. Lee, A.M. Delegeane, V. Baker, P.C. Chow, J. Biol. Chem. 258 (1983) 597–603.
[23] M. Mueckler, C. Caruso, S.A. Baldwin, M. Panico, I. Blench, H.R. Morris, W.J. Allard, G.E. Lienhard, H. Lodish, Science 229 (1985) 941–945.
[24] J. Ting, S.K. Wooden, R. Kriz, K. Kelleher, R.J. Kaufman, A.S. Lee, Gene 55 (1987) 147–152.
[25] M.A.K. Markwell, S.M. Haas, L.L. Lieber, N.E. Tolbert, Anal. Biochem. 87 (1978) 206–210.
[26] H.H. Kitzman Jr., R.J. McMahon, A.M. Aslanian, P.M. Fadia, S.C. Frost, Mol. Cell. Biochem. 162 (1996) 51–58.
[27] J.B. Hwang, S.C. Frost, J. Biol. Chem. 274 (1999) 22813–22820.
[28] L.N. Johnson, FASEB J. 6 (1992) 2274–2282.
[29] W. Stalmans, H.-G. Hers, Eur. J. Biochem. 54 (1975) 341–350.
[30] S. Baque, J.J. Guinovart, A.M. Gomez-Foix, J. Biol. Chem. 271 (1996) 2594–2598.
[31] S. Baque, E. Montell, M. Camps, J.J. Guinovart, A. Zorzano, A.M. Gomez-Foix, Diabetes 47 (1998) 1185–1192.
[32] B.E. Froman, R.C. Tait, F.A. Gorin, B.A. Horwitz, J.S. Stern, Gene 109 (1991) 269–274.
[33] B.F. Giffin, R.L. Drake, R.E. Morris, R.R. Cardell, J. Histochem. Cytochem. 41 (1993) 1849–1862.
[34] C.A. Bloch, M.A. Ozbun, S.A. Khan, Biol. Neonate 63 (1993) 113–119.
[35] P.V. Rao, S. Pugazhenthi, R.L. Khandelwal, J. Biol. Chem. 270 (1995) 24955–24960.
[36] W.J. Roesler, R.L. Khandelwal, Arch. Biochem. Biophys. 244 (1986) 397–407.
[37] S. Aiston, M. Peak, L. Agius, Diabetologia 43 (2000) 589–596.
[38] C.B. Niewoehner, F.Q. Nuttall, Diabetes 37 (1988) 1559–1566.
[39] R.R. Whitesell, D.M. Regen, D. Pelletier, N.A. Abumrad, Diabetes 39 (1990) 1228–1234.
[40] J.W. Hudson, K.L. Hefferon, M.M. Crerar, Biochim. Biophys. Acta 1164 (1993) 197–208.
[41] V.L. Rath, M. Ammirati, P.K. LeMotte, K.F. Fennell, M.N. Mansour, D.E. Danley, T.R. Hynes, G.K. Schulte, L.C. Wasilko, J. Pandit, Mol. Cell 6 (2000) 139–148.