Regulation of glycogen accumulation in L6 myotubes cultured under optimized differentiation conditions

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Abstract: Regulation of glycogen accumulation in L6 myotubes cultured under optimized differentiation conditions. The present study is concerned with the differentiation of L6 myotubes cultured under optimized differentiation conditions. The regulation of glycogen accumulation in L6 myotubes cultured under optimized differentiation conditions was studied. Only a few studies have dealt with the effect of combinations of differentiation factors (55), and thus the possibility of more complete differentiation of muscle-specific characteristics of the cell line has not been explored. For example, the ability of L6 myotubes to accumulate glycogen is poorly investigated. Only one study on the effect of medium glucose concentration on glycogen accumulation (54) and two studies concerned with the effect of insulin on glycogen accumulation (26, 42) have been reported. However, none of these involved addition of differentiation factors.

The aims of the present investigation, therefore, were 1) to enhance the differentiation level of L6 cells by combinations of differentiation factors, including factors that have been reported to increase the expression of GLUT-4, 2) to identify conditions that stimulate glycogen accumulation, and 3) to study the regulation of glycogen metabolism under these conditions of optimized differentiation.

METHODS

Materials

L6 cells (L6.G8.C5) were obtained from the European Collection of Animal Cell Cultures and were routinely used after three passages. Fetal calf serum (FCS) was from Biological Industries. Penicillin was from Løvens Kemiske Fabrik. Insulin-like growth factor I (IGF-I) was a gift from Pharmacia. Dexamethasone (Dex) was from Merck, Sharp & Dohme. Insulin (porcine) was from Novo Nordisk. Culture dishes were from Nunc. 3-O-methyl-D-[1-3H]glucose, [U-14C]glucose 1-phosphate, and UDP-[U-14C]glucose were from Amersham Radiochemical Center. SeaPlaque agarose was from FMC Bioproducts. UDP-glucose, glucose 1-phosphate, glucose 6-phosphate (G-6-P), and glycogen were from Boehringer Mannheim. Trypsin was from GIBCO-BRL. DMEM, gentamicin, creatine (Cr), retinoic acid (Ret.a), triiodothyronine (T3), cytosine-1-p-o-arabinofuranoside (AraC), and all other chemicals were from Sigma Chemical.

Cell Culture

L6 myoblasts (56) were plated in 35-mm-diameter (glucose-transport experiments) or 60-mm-diameter (all other experiments) uncoated petri dishes at a density of ~4,500 cells/cm² and incubated at 37°C in 5% CO₂-95% air in a medium composed of 10% (vol/vol) FCS and DMEM with 5 mM glucose until confluence occurred (7 days). Penicillin and gentamicin were added to DMEM to a concentration of 100 IU/ml and 50 µg/ml, respectively. The medium was changed every 48 h. From day 7 to day 10, the medium was changed to DMEM with 2% (vol/vol) FCS, different combinations of differentiation effectors, and 10 µM AraC to prevent further proliferation of nonfused mononucleated myoblasts (53). From day 10 to the day of the experiment, the medium remained the same, except that AraC was excluded.

The present study is concerned with the differentiation and glycogen metabolism of the myogenic cell line L6 developed by Yaffe in 1968 (56). The L6 myotubes exhibit several myogenic characteristics, i.e., contractility (50), capacity to form myoneural junctions (51), resting membrane potentials of about −70 mV (23), acetylcholine receptors (41), synthesis of actinomyosin (30), insulin-sensitive glucose transport by the GLUT-4 transporter (33), active lactate transport (6), and expression of creatine kinase, albeit at low levels (44). However, the L6 myotubes also lack certain skeletal muscle characteristics, such as a high ratio of phosphocreatine to ATP (PCr/ATP) (32), high glycogen levels (36), and a low intracellular glucose concentration (17). Several differentiation factors have been reported to enhance myogenic differentiation of L6 cells (1, 7, 11, 15, 49, 55), but typically the effect of individual factors has been studied. Only a few studies have dealt with the effect of combinations of differentiation factors (55), and thus the possibility of more complete differentiation of muscle-specific characteristics of the cell line has not been explored. For example, the ability of L6 myotubes to accumulate glycogen is poorly investigated. Only one study on the effect of medium glucose concentration on glycogen accumulation (54) and two studies concerned with the effect of insulin on glycogen accumulation (26, 42) have been reported. However, none of these involved addition of differentiation factors.

The aims of the present investigation, therefore, were 1) to enhance the differentiation level of L6 cells by combinations of differentiation factors, including factors that have been reported to increase the expression of GLUT-4, 2) to identify conditions that stimulate glycogen accumulation, and 3) to study the regulation of glycogen metabolism under these conditions of optimized differentiation.
Analytic Procedures

Preparation of homogenates for measurement of enzyme activities and protein and DNA content. The medium was removed by suction from the dishes, and the cells were washed three times at 1–2°C with Hanks’ balanced salt solution (HBSS). Glycyl-glycine buffer (1,200 µl) (14) was added to each dish, and the cells were scraped loose with a rubber policeman and homogenized with the dish on ice by ultrasonication (Branson Sonifier B-12) at 50 W for 5 s. The homogenates were kept frozen at –80°C until analyzed.

Preparation of extracts for measurement of ATP and PCr. One dish at a time was placed on ice, the medium was quickly removed by suction, and 1,000 µl of ice-cold 0.7 M perchloric acid (PCA) were added immediately. The cells were scraped loose with a rubber policeman, and aliquots of the extract were kept frozen at –80°C until analyzed.

Preparation of extracts for measurement of G-6-P and intracellular glucose. One dish at a time was placed on ice, the medium was quickly removed by suction from the dishes, the cells were washed three times at 1–2°C with 2 ml HBSS containing 0.4 mM phloretin (from freshly made stock solution of 100 mM phloretin in 100% DMSO), and immediately after this 500 µl of ice-cold 1.2 M PCA were added. The cells were scraped loose with a rubber policeman, and aliquots of the extract were kept frozen at –80°C until analyzed. It was demonstrated that this washing procedure removed >99.8% of the medium glucose.

Preparation of extracts for measurement of glycogen. The medium was removed from the dishes by suction, and 1,000 µl of 0.4 N KOH were added to each dish. After 5 min at room temperature, aliquots of the homogenates were stored frozen at –20°C until analyzed.

Measurements

Enzymes. Creatine kinase (CK) activity was measured spectrophotometrically as described by Passonneau and Lowry (40). Glycogen synthase (GS) activity was measured radiochemically, as described by Golden, Wals, and Katz (13), at 37°C with an incubation time of 60 min. Glycogen phosphorylase (GPh) was measured radiochemically at 37°C in the direction of glycogen synthesis, as described by Gilboe et al. (12), except that Dowex columns replaced filter papers. The specifications of these columns and the elution procedure were as in the GS assay (13). Incubation time was 60 min.

Glucose transport. 3-O-Methyl-d-[1-3H]glucose uptake was measured at 25°C in 35-mm culture dishes. Before the uptake measurement, the dishes were prewashed three times at 25°C with 2 ml of buffer composed of 122 mM NaCl, 4.9 mM KCl, 2.5 mM MgSO₄, 36 mM Tris·HCl, pH 7.4, and 1 mM CaCl₂ (25). 3-O-Methyl-d-[1-3H]glucose uptake was measured over a 30-s interval in the same buffer + 10 µM 3-O-methyl-d-[1-3H]glucose (3 µCi/ml), 100 nM Ret.a, 5 nM Dex, and 10 mM Cr, with or without 5 nM IGF-I and with or without 100 nM insulin. Non-specific uptake was determined in the presence of 10 µM cytochalasin B, and the value was subtracted from the total uptake. After the uptake period, the dishes were washed three times at 1–2°C with 2 ml of the prewashing buffer described above + 1 mM HgCl₂, the cells were disrupted in 1,000 µl of 0.4 N KOH, and cellular radioactivity was counted by liquid scintillation.

Protein. Protein content was measured by the method of Lowry et al. (28) with bovine serum albumin as standard.

DNA. DNA content was measured by the method of Kissane and Robins (24).

PCr and ATP. PCr and ATP were measured spectrophotometrically as described by Passonneau and Lowry (38).

G-6-P and glucose, G-6-P and glucose were measured fluorometrically as described by Passonneau and Lowry (39).

Glycogen. Glycogen was determined as glucose after treatment with amyloglucosidase, as described by Katz et al. (21).

Light microscopy. The cells were examined each day by a Leitz Diavert phase-contrast microscope to evaluate the degree of differentiation.

Test of contractility. Contractility was evoked by a Digitimer D330 Multistim electrostimulator connected to the electrodes. Electrodes were constructed as described by Rössler et al. (43), with an electrode distance of 45 mm. The 4% agar in HBSS used by Rössler was replaced by 2% SeaPlaque agarose in DMEM. The cultures were stimulated for 10 s with 4 V/cm electrode distance in pulse trains of 500 ms at 100 Hz with a 2-ms pulse width. The interval between pulse trains was 500 ms.

Statistics

Data were analyzed by ANOVA and Tukey’s procedure. Significance was accepted at P < 0.05.

RESULTS

Differentiation

The L6 myoblasts reached confluence at day 6. At day 7 the cells aligned, and scattered small myotubes were observed (Fig. 1A). When medium was changed to fusion medium with FCS content of 2%, the fusion process accelerated until day 10 or 11, when most of the myoblasts were fused into multinucleated myotubes (Fig. 1, B and C). The nuclei remained in clusters and did not display any lateral displacement in the myotube. The subclone and passage used for the present experiments showed spontaneous contractions in the interval between days 10 and 15. Visible (light microscopy) cross-striation appeared one day after the first spontaneous contractions could be observed (Fig. 1C). From day 14 to day 15, cells began to degenerate. This in particular was the case for myotubes with added Dex.

Effect of differentiation factors. The specific activity of CK was used as a marker of myogenic differentiation. Effectors like T₃, Dex, IGF-I, insulin, Ret.a, and Cr, which have previously been ascribed potential as myogenic differentiators (1, 7, 11, 15, 16, 31, 49, 55), were tested in screening experiments either individually or in combinations from the day of onset of fusion of the myoblasts (day 7) to obtain the most optimal culture conditions for later experiments.

The largest effect on differentiation of a single factor was observed with 5 nM IGF-I or 100 nM insulin (results not shown). IGF-I showed a positive effect (~25%) on CK from day 7 to day 14. The effect of insulin was similar to the effect of IGF-I, but its presence during a longer period (>4 days) caused more cell debris than IGF-I (results not shown). When 100 nM retinoid acid was added in combination with 5 nM IGF-I (Table 1), CK was at the same level as with IGF-I alone. However, the myotubes appeared morphologically more uniform with respect to thickness and length. Addition of 5 nM T₃ showed no effect on CK activity alone or in combination with other effectors (results not shown).
In 10-day-old cultures, 5 nM Dex inhibited differentiation (Table 1), and when added to the combination of Ret.a and IGF-I, it abolished their effect on CK activity, which decreased to control level (Table 1). In 14-day-old cultures, Dex had no effect on differentiation (Table 1). However, the myotubes appeared morphologically more degenerated as judged by light microscopy.

Effect of CR. Addition of 10 mM Cr from day 7 caused an increase in the differentiation level. The largest effect was observed with the low CK levels, i.e., with 5 nM Dex, when Cr increased CK by 64 and 38% at days 10 and 14, respectively (Table 1). The lowest effect was seen when the CK activity was already high, i.e., 5 nM IGF-I + 100 nM Ret.a, when Cr gave rise to an increase of 22 and 18% at days 10 and 14, respectively (not significant at day 14; Table 1). Myotubes cultured under this condition showed the highest degree of differentiation [7.8 and 9.8 U of CK/mg protein at days 10 and 14, respectively (Table 1)]. PCr/ATP in L6 myotubes is reported to be rather low compared with that in the intact muscle (7.8 and 9.8 U of CK/mg protein at days 10 and 14, respectively (Table 1)]. PCr/ATP in L6 myotubes is reported to be rather low compared with that in the intact muscle (32). This was confirmed in the present study (Table 2). Addition of 10 mM Cr to the medium, however, resulted in a >10-fold increase of PCr/ATP from 0.24–0.28 to 2.8–4.1 (Table 2). Cr concen-

### Table 1. Specific activity of CK in L6 cells during differentiation

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>Differentiation Effectors</th>
<th>CK Activity, U/mg protein</th>
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<tbody>
<tr>
<td></td>
<td>Dex, 5 nM</td>
<td>Ret.a, 100 nM</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
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<tr>
<td>10</td>
<td>+</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data were divided into two groups, 10- and 14-day cultures. CK, creatine kinase; Dex, dexamethasone; Ret.a, retinoic acid; IGF-I, insulin-like growth factor I; Cr, creatine; -, without; +, with; ±, with or without. Differentiation conditions for medium days 0–7: DMEM, 5.05 mM glucose + 10% FCS. For cultures analyzed from day 7 to day 10, medium days 7–10: DMEM, 5.05 mM glucose + 2% FCS + 10 µM cytosine 1-β-D-arabinofuranoside (AraC) + differentiation effectors as indicated ± 10 mM Cr. For cultures analyzed at day 14: medium days 7–10: DMEM, 5.05 mM glucose + 2% FCS + 10 µM AraC + differentiation effectors as indicated (except Dex) ± 10 mM Cr. Medium days 10–11: DMEM, 5.05 mM glucose + 2% FCS + differentiation effectors as indicated (except Dex) ± 10 mM Cr. Medium days 11–14: DMEM, 5.05 mM glucose + 2% FCS + differentiation effectors as indicated ± 10 mM Cr. Statistical ANOVA for repeated measurement was performed for each group. Significant differences between values are marked with identical letters (with or without differentiation effectors) or numbers (with or without Cr):<sup>b</sup>, e, g, h, j, k, m, 3, 5: P < 0.05; <sup>4</sup>: P < 0.01; <sup>a</sup>, c, d, f, i, 1, 2: P < 0.001.

In 10-day-old cultures, 5 nM Dex inhibited differentiation (Table 1), and when added to the combination of Ret.a and IGF-I, it abolished their effect on CK activity, which decreased to control level (Table 1). In 14-day-old cultures, Dex had no effect on differentiation (Table 1). However, the myotubes appeared morphologically more degenerated as judged by light microscopy.

**Fig. 1.** L6 myotubes during differentiation. A: 7-day-old myoblasts fusing into small myotubes just before fusing process was accelerated. B: myotubes at day 11 cultured without differentiation factors + 10 mM creatine (Table 1). C: cross-striated myotubes at day 11 differentiated with 100 nM retinoic acid, 5 nM insulin-like growth factor I (IGF-I), and 10 mM creatine (Table 1). Scale bars, 100 µm.
glycogen content increased 3.5 times from Dex. The combination of 25 mM glucose and 100 nM insulin on 10- and 14-day-old myotubes in the absence of IGF-I, Ret.a, and 10 mM Cr had only a small effect on glycogen accumulation for the first 6 h, 25 and 17% increases in the absence and presence of Dex, respectively. From 6 to 24 h, the glycogen level declined to 55 and 62% of the 6-h values in the absence and presence of Dex, respectively.

In the 14-day-old myotubes (Fig. 2B), the basal level of glycogen was 48 ± 6 nmol glucosyl U/mg protein. Glycogen accumulation in these cells reached a maximum of 459–555 nmol glucosyl U/mg protein, unaffected by Dex and Cr addition after the first 6 h. In the

Table 2. Effect of medium Cr addition on PCr/ATP ratio in L6 cells

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>Dex, 5 nM</th>
<th>Ret.a, 100 nM</th>
<th>IGF-I, 5 nM</th>
<th>PCr/ATP</th>
<th>–Cr</th>
<th>+10 mM Cr</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.17 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.24 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>3.26 ± 0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>2.56 ± 0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2.76 ± 0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.28 ± 0.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4. Culture conditions were identical to conditions in Table 1. ATP content at days 7, 10, and 14 was 22, 20–27, and 24 nmol/mg protein, respectively. PCr/ATP, ratio of phosphocreatine to ATP.

Contractions of 0.1 or 1.0 mM were without significant effect on PCr/ATP.

Contractility. The subclone and passage used for the present experiments showed spontaneous contractions from days 10–11 to days 14–15. However, contractions were not coordinated in time or across the dish. When exposed to electrical stimulation, one-third to one-half of the myotubes were able to contract in concert. The earlier the appearance of spontaneous contractions was observed, the higher was the differentiation level, i.e., IGF-I + Ret.a + Cr. From day 14 to day 15, the tubes became too degenerated to contract either spontaneously or by electric stimulation.

As a consequence of the above results, further experiments were carried out with 5 nM IGF-I, 100 nM Ret.a, and 10 mM Cr from day 7.

Glycogen Accumulation and Glucose Transport

L6 myotubes cultured at control conditions are able to accumulate only low levels of glycogen (18, 37, 54). In screening experiments, combinations of different medium glucose concentrations (5, 10, and 25 mM) and insulin concentrations (0, 10, and 100 nM) were tested for effects on glycogen accumulation over 24 h on 14-day-old myotubes in the absence of IGF-I, Ret.a, and Dex. The combination of 25 mM glucose and 100 nM insulin increased the glycogen content 3.5 times from 50 ± 4 to 179 ± 17 nmol glucosyl U/dish (n = 2). The glycogen content increased to 107 ± 10 nmol glucosyl U/dish (n = 2) in the presence of 25 mM glucose and 10 nM insulin. No other combination of glucose and insulin affected the glycogen accumulation measured at 24 h.

Subsequent experiments on glycogen metabolism were carried out with 5 nM IGF-I, 100 nM Ret.a, and 100 nM insulin on 10- and 14-day-old myotubes. Under these conditions the glycogen content in 10-day-old myotubes increased from basal level at 51 ± 4 at time 0 to 369 ± 36 nmol glucosyl U/mg protein at 6 h (Fig. 2A). Addition of 5 nM Dex increased glycogen accumulation to 606 ± 46 nmol glucosyl U/mg protein at 6 h, corresponding to a 67% increase. This level of glycogen accumulation remained constant until 24 h in the absence as well as in the presence of Dex. Addition of 10

Fig. 2. Glycogen accumulation in 10-day-old (A) and 14-day-old (B) myotubes. Values are means ± SE; n = 4. Cr, creatine; Dex, dexamethasone; AraC, cytosine 1-β-d-arabinofuranoside; Ret.a, retinoic acid. Significant differences between values (by ANOVA and Tukey’s procedure) are marked with identical superscript letters. A: a, P < 0.05; b, P < 0.01; c, P < 0.001; d, P < 0.0001; e, P < 0.00001. Differentiation and culture conditions were as follows for cultures analyzed at day 10 and day 14. Days 0–7, DMEM, 5.05 mM glucose + 10% FCS. For cultures analyzed at day 10: days 7–10, DMEM and 5.05 mM glucose + 2% FCS + 10 µM AraC + 100 nM Ret.a + 5 mM IGF-I + 5 mM Dex + 10 mM Cr. For cultures analyzed at day 14: days 7–10, DMEM and 5.05 mM glucose + 2% FCS + 10 µM AraC + 100 nM Ret.a + 10 nM IGF-I + 10 mM Cr; days 10–11, identical to days 7–10 but without AraC; days 11–14, DMEM and 5.05 mM glucose + 2% FCS + 100 nM Ret.a + 5 mM IGF-I + 5 mM Dex + 10 mM Cr. Glycogen accumulation conditions, for both day 10 and day 14, were DMEM and 25 mM glucose + 2% FCS + 100 nM Ret.a + 5 mM IGF-I + 5 mM Dex + 10 mM Cr + 100 nM insulin. □, with both Cr and Dex; ■, with Cr but without Dex; ○, without Cr but with Dex; ●, with both Cr + Dex. The indicated Cr and Dex concentrations were present during both differentiation and glycogen accumulation periods.
presence of Dex, independent of Cr addition, the glycogen levels were almost constant from 6 to 24 h (95% of the 6-h values). When Dex was absent, the glycogen level declined to 60% of the 6-h values.

Glucose transport was measured as 3-O-methylglucose uptake in 11-day-old cultures. On day 10, the medium was changed to DMEM without IGF-I but with 5 nM Dex, 100 nM Ret.a, and 10 mM Cr added as we have described (Fig. 2). On day 11, the medium was changed to DMEM, 25 mM glucose + 2% FCS + 100 nM Ret.a + 10 mM Cr with or without 5 nM IGF-I and with or without 100 nM insulin. At 2 h after this medium change, 3-O-methylglucose uptake was (in μmol/mg protein⁻¹·min⁻¹): 45.2 ± 8.5 in the absence of both insulin and IGF-I, 64.1 ± 4.9 in the presence of 5 nM IGF-I, 70.6 ± 5.8 in the presence of 100 nM insulin, and 78.3 ± 2.8 in the presence of both insulin and IGF-I (n = 4). Thus glucose uptake was significantly stimulated by insulin (P < 0.05) and by the combination of insulin and IGF-I (P < 0.01) after induction of glycogen accumulation.

GS and GPh Activity, G-6-P, and Glucose During Glycogen Accumulation

Glycogen accumulation was studied in detail in 10-day-old myotubes differentiated with 5 nM IGF-I + 100 nM Ret.a + 5 nM Dex + 10 mM Cr. Glycogen accumulation was initiated with a medium including the same differentiation factors and 25 mM glucose + 100 nM insulin. This combination was chosen because it represented the best compromise with respect to PCR/ATP, specific activity of CK, and level of glycogen accumulated.

Glycogen accumulated at a rather constant rate of 315–349 nmol glucosyl U·mg protein⁻¹·h⁻¹ for the first 2 h (Table 3 and Fig. 3). From 2 to 4 h, the accumulation rate decreased gradually to zero (−22 ± 27 nmol glucosyl U·mg protein⁻¹·h⁻¹). The maximum level of glycogen, 982 ± 26 nmol glucosyl U/mg, was reached at 4 h, and at 6 h the value was 798 ± 35 nmol glucosyl U/mg (Table 3).

GS and GPh activity. The total activity (a + b form) of GS and GPh was constant during the accumulation period. The a form of GS was constant from 0 to 0.5 h (30.1 ± 0.9 and 34.7 ± 2.5% of total activity, respectively), but from 0.5 h the activity declined in an exponential manner to 2.8 ± 0.1% at 6 h. This progressive decline in GS activity was not observed under conditions in which the medium glucose concentration was 0 mM, and no glycogen accumulation was observed (results not shown). The a form of GPh declined from 87 to 75% of the total activity from 0 to 0.5 h (P < 0.05). From 0.5 h, the a form increased to 98% of total activity at 6 h. Although the ratio of GS to GPh (GSA/GPh) decreased 6.2-fold during the time from 0.5 to 2 h, the glycogen accumulation rate was constant (Fig. 4). Between 2 and 4 h, the glycogen accumulation rate decreased rapidly to zero and GSA/GPh increased 6.2-fold. From 4 to 6 h the glycogen accumulation rate became negative, whereas GSA/GPh decreased only 1.1-fold.

G-6-P and glucose. At the onset of the glycogen accumulation period, the cell content of G-6-P and glucose was low at 0.5 ± 0.0 and 5 ± 2 nmol/mg protein, respectively (Table 3). The change from conditioned medium with low glucose concentration (5.05 mM at the start of the 72-h period) to medium with 25 mM glucose + 5 nM Dex + 100 nM insulin caused a 9- to 11-fold increase in glucose and a 5- to 6-fold increase

Table 3. Glycogen synthase and phosphorylase activities, G-6-P, and intracellular glucose content during glycogen accumulation in 10-day-old L6 myotubes

<table>
<thead>
<tr>
<th>Incubation Time, h</th>
<th>Glycogen Synthase, nmol/mg protein</th>
<th>Phosphorylase, nmol/mg protein</th>
<th>Glycogen, nmol/mg protein</th>
<th>ΔGlycogen/At, nmol·mg protein⁻¹·h⁻¹</th>
<th>G-6-P, nmol/mg protein</th>
<th>Glucose, nmol/mg protein</th>
<th>G-6-P/glucose</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>4.5 ± 0.1</td>
<td>0.1</td>
<td>38.8 ± 1.3</td>
<td>43.1 ± 0.6</td>
<td>24 ± 1</td>
<td>333 ± 15</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>0.5</td>
<td>4.2 ± 0.4</td>
<td>0.1</td>
<td>12.4 ± 1.0</td>
<td>12.4 ± 1.0</td>
<td>43 ± 1</td>
<td>125 ± 6</td>
<td>5.0 ± 0.0</td>
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<tr>
<td>1</td>
<td>2.0 ± 0.1</td>
<td>0.1</td>
<td>13.4 ± 0.4</td>
<td>32.7 ± 0.9</td>
<td>349 ± 9</td>
<td>349 ± 9</td>
<td>3.2 ± 1.1</td>
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<tr>
<td>2</td>
<td>0.8 ± 0.0</td>
<td>0.1</td>
<td>13.4 ± 0.4</td>
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<td>328 ± 30</td>
<td>7.7 ± 1.7</td>
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<td>4</td>
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<td>0.1</td>
<td>12.3 ± 0.2</td>
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<td>982 ± 26</td>
<td>982 ± 26</td>
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<tr>
<td>6</td>
<td>0.3 ± 0.0</td>
<td>0.1</td>
<td>12.1 ± 0.1</td>
<td>40.6 ± 1.4</td>
<td>798 ± 35</td>
<td>798 ± 35</td>
<td>8.5 ± 2.7</td>
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</table>

Values are means ± SE; n = 4. ΔGlycogen/At is the slope of cubic spline fit, which describes the glycogen accumulation curve (see Fig. 3). Differences and culture conditions were as follows. For days 0–7, DMEM, 5.05 mM glucose + 10% FCS; for days 7–10, DMEM and 5.05 mM glucose + 2% FCS + 10 μM AraC + 100 nM Ret.a + 5 nM IGF-I + 5 nM Dex + 10 mM Cr. Glycogen accumulation conditions were DMEM, 25 mM glucose + 2% FCS + 100 nM Ret.a + 5 nM IGF-I + 5 nM Dex + 10 mM Cr + 100 mM insulin.
The maximal CK activity was judged by the CK activity (Table 1) and the morphology of the cells.

PCr/ATP. Cr was added to the medium to increase the PCr content and thereby PCr/ATP. The presence of Cr increased the ratio to the levels of skeletal muscles in vivo and did in fact also promote the differentiation level, as reflected in the CK activity. The effect of Cr on PCr/ATP was observed only at a very high concentration of Cr (10 mM), which could indicate that the ability to concentrate Cr, normally displayed by muscle cells (32), is lost in L6 cells. This would seem to contradict the findings of Loike et al. (27), who concluded that Cr transport in L6 cells is an active process in which the Cr transporter, however, is gradually downregulated at a Cr concentration > 1 µM.

**Glycogen Metabolism**

Glycogen accumulation. Reported glycogen levels in rat skeletal muscle are maximally ~850 nmol/mg protein (36). In our experiments, ~50 nmol/mg protein accumulated at basal conditions, and ~1,000 nmol/mg protein accumulated with 25 mM glucose, insulin, and Dex (Table 3). Reported levels of glycogen in L6 cells (in nmol/mg protein with the glucose concentration of medium shown in parentheses) are 22.5 (7 mM) by Pardridge et al. (37), 62 (9 mM) by Huang and Tao (18), and 250 (25 mM) by Wahrmann et al. (54). The low levels of glycogen accumulation in L6 myotubes demonstrated by Pardridge et al. and Huang and Tao may be explained by low intracellular G-6-P levels in response to a low initial extracellular glucose concentration (7 and 9 mM) without insulin or IGF-I addition. This condition will cause low glucose transport, even though the authors demonstrate a high ratio of intracellular glucose to G-6-P. The relatively high glycogen level reported by Wahrmann et al. of 250 nmol/mg protein is at approximately the same level as that observed in the present study, when no differentiation effectors were added. Wahrmann et al. also used a glucose concentration of 25 mM, but no insulin or IGF-I. This suggests that, in their subclone, glucose transport by the GLUT-1 transporter was sufficient to cause glycogen accumulation.

The high glycogen level reached in our experiments is probably a result of increased GS activity (2, 5) and GLUT-4 expression (15, 30, 48) due to the addition of IGF-I, insulin, Ret.a, and Dex combined with increased glucose transport in the presence of a high glucose concentration (25 mM) (25, 30, 33).

The expression of CK and the ability to accumulate glycogen were not correlated when differentiation conditions were improved. Thus the highest CK activities were measured in 14-day-old myotubes, when IGF-I, retinoic acid, and creatine were present, whereas the highest level of glycogen accumulation was measured in 10-day-old myotubes in the presence of IGF-I, Ret.a, Dex, and Cr.

Ten-day-old and 14-day-old cultures exhibited some unexplained differences with respect to the effect of Dex and Cr on glycogen accumulation, suggesting that the regulation of glycogen metabolism was changing with...
the age of the myotubes. Thus Dex enhanced the accumulation of glycogen in 10-day-old myotubes but appeared to be without effect in 14-day-old myotubes. In 10-day-old myotubes, the glycogen level was constant from 6 to 24 h whether Dex was present or not, whereas Dex had to be present to avoid a fall in the glycogen level after 6 h in 14-day-old myotubes (Fig. 2, A and B). Cr caused a declining glycogen level from 6 to 24 h in 10-day-old myotubes but was without effect in 14-day-old myotubes (Fig. 2, A and B).

GS and GPh. The total activity of GS and GPh was ~15 and 40 mU/mg protein, respectively (Table 3) compared with ~20 and 100–500 mU/mg protein in rat skeletal muscle [GPh measured in the direction of glycogen synthesis and depending on the fiber type composition of the muscle (34, 35)]. The total GS activity was correlated to the CK activity, which was the sole regulatory factors for glycogen accumulation in 10-day-old myotubes but did not depend on the differentiation level, in contrast to the results reported by Wahrmann et al.

In our experiments, the a form constituted 30.1% of the total GS and 86.4% of the total GPh at the onset of the glycogen accumulation period. The GS activity did not increase further when glycogen accumulation was induced by insulin, as described by Lazar et al. (26) and Ragolia and Begum (42), presumably because the GS was already activated by inhibition of glycogen synthase kinase-3 by IGF-I, mediated by protein kinase B (2, 5). The level of enzyme phosphorylation in the present study is in agreement with values observed by Wahrmann et al. (54) in L6 myotubes cultured for 9 days with 25 mM glucose (25% for GS and 85% for GPh). These authors also demonstrated that the phosphorylation state changed from 85 to 40% and 25 to 60% for GPh and GS, respectively, by a change in the glucose concentration from 25 to 2.5 mM, thereby decreasing the glycogen level from 125 to 42 nmol/mg protein in 24 h. Huang and Tao (18) found 0.5% GSa and ~80% GPh in 9-day-old myotubes cultured with 9 mM glucose. They observed no change in the phosphorylation state of GS and GPh during 48 h of glycogen accumulation from zero to 62 nmol/mg protein, and they showed that the phosphoprotein phosphatase activity was continuously suppressed by inhibitors. GSa activity decreased with time concomitant with accumulation of glycogen (Fig. 3) but was constant with time if the rate of glycogen accumulation was minimized by using 0 instead of 25 mM glucose (results not shown), suggesting that the phosphorylation state of GS is subject to feedback regulation by glycogen.

Regulation of the glycogen content. In textbooks of biochemistry, it is often stated that the processes of glycogen synthesis and degradation are mutually exclusive because of concomitant phosphorylation/dephosphorylation of the GS and GPh enzymes (52). However, phosphorylation and dephosphorylation are clearly not the sole regulatory factors for glycogen accumulation in intact skeletal muscles, as pointed out by Chasiotis (4) and Shulman and co-workers (45, 46), and in L6 cells, as shown by the following calculations. At the start of the glycogen accumulation period, the GSa and the GPha activities corresponded to 270 and 1,000 nmol glucosyl U·mg protein⁻¹·h⁻¹, respectively [when a 2.5-fold lower GPh activity is assumed when assayed in the direction of glycogen degradation compared with the direction of glycogen synthesis (22)]. These figures would seem to exclude any glycogen accumulation at all. Furthermore, the GS activity decreased, and GPha activity increased during the first 2 h of glycogen accumulation (Fig. 3) despite the fact that the rate of accumulation was constant (Fig. 4). Finally, the fall in GSa with increasing glycogen levels (Fig. 3) indicates lack of correlation between glycogen accumulation and the dephosphorylated form of GS. Consequently, it would seem that regulatory mechanisms other than phosphorylation/dephosphorylation of GS and GPh are playing a major role in L6 cells. These factors might be G-6-P and glucose. The concentration of G-6-P in the cells was 0.5–1.6 mM, when a protein content of 0.11 mg/mg wet weight and a water content of 60% are assumed (18) (Table 3). Those concentrations would cause a 16–39% activation of GSb and partially phosphorylated forms of GS (calculated from Ref. 18) and could explain the high glycogen accumulation rate. The glucose concentration was 8–22 mM during the glycogen accumulation period (Table 3 and the same assumptions as for the calculations of G-6-P concentration). Thus GPha could be inhibited by these high concentrations of glucose, corresponding to 5–13 times the inhibitory constant (20). That GPha is in fact inhibited in L6 cells is corroborated by the observation that the initial rate of glycogen degradation in the presence of epinephrine and in the absence of glucose is ~10% of the GPha activity (results not shown). The much lower rate of glycogen degradation than the actual GPha activity might, of course, also reflect compartmentation of the enzyme and/or its substrate glycogen and/or rate-limiting activity of the debranching enzyme.

The factor limiting the maximal amount of glycogen accumulated in the incubation period could be the number of available glycogenin/proglycogen molecules, which will limit the number of β-glycogen molecules that can be synthesized (3, 45). The shape of the curve in Fig. 4 indicates that this could in fact be the case.

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