In this study, we examined the role of the glycogen-associated regulatory subunit of protein phosphatase-1 (PP-1G) in L6 rat skeletal muscle cell myogenesis. The level of PP-1G was depleted by transfection with an inducible antisense-oriented PP-1G gene. Western blot analysis of the PP-1G-depleted cell line revealed a >90% depletion of PP-1G protein and a 45% reduction in cellular PP-1 activity, and abolished the ability of L6 myoblasts to differentiate into multinucleated myotubes. PP-1G-depleted cells also exhibited a marked reduction in the expression of the differentiation marker myogenin as well as creatine kinase. After 7 days in culture, PP-1G-depleted cells sustained myoblast levels of inhibitor of differentiation-2, whereas control L6 cells had a severely lower inhibitor of differentiation-2 level and progressed into myotubes. Myoblasts were unable to exit the cell cycle, as measured by the impaired induction of p27 cyclin-dependent kinase inhibitor, a >2-fold increase in DNA synthesis, and elevated levels of phosphorylated retinoblastoma protein (pRb). Replacement of the PP-1G gene restored PP-1G protein expression, PP-1 enzymatic activity, and the ability to differentiate into myotubes. We conclude that PP-1G plays a definite role in L6 myogenesis via its regulation of PP-1 catalytic activity.

Progression of myoblasts into myotubes involves the regulation of several skeletal muscle-specific genes encoding a group of proteins known collectively as the basic helix-loop-helix family (1, 2). Members of this family of proteins, including MyoD, myogenin, myf-5, MRF-4, and the myocyte enhancer binding factor 2, share highly conserved domains that have the ability to both bind DNA and to form heterodimers (3). Binding of these basic helix-loop-helix proteins to E2a gene products promotes myogenic activity by increasing their affinity for specific DNA sequences located in control regions of muscle-specific genes (4). Id2, a member of the helix-loop-helix family present in proliferating cells, appears to preferentially bind E2a and thereby prevent myogenic activity (5).

Myogenic activity is also blocked by other agents such as high concentrations of mitogens, fetal bovine serum, basic fibroblast growth factor 2, transforming growth factor-β, and wortmannin, a PI3-kinase inhibitor (6–8). Furthermore, recent studies with constitutively active and dominant negative mutants of PI3-kinase, as well as Akt (protein kinase B), one of the downstream targets of PI3-kinase, suggest that PI3-kinase-directed myogenic signaling requires Akt (9–11). More importantly, inhibition of myogenesis with okadaic acid (12), as well as other studies using microinjection of affinity purified PP-1 catalytic subunit (PP-1C) antibodies (13), suggested an essential role for PP-1 in the regulation of mammalian cell division and mitotic exit. Moreover, PP-1C associates with retinoblastoma protein (Rb) and therefore has been implicated in the regulation of its activation status (14–16).

PP-1 holoenzyme exists in vivo as a complex with the catalytic subunit PP-1C, attached to regulatory subunits that target the enzyme to particular subcellular locations, modify its substrate specificity, and regulate enzymatic activity (17). Thus, PP-1C is found associated with glycogen particles (PP-1G1), sarcoplasmic reticulum (PP-1SR), myofibrils (PP-1M), the cytosolic inhibitor-2 protein (PP-1I), and the nucleus (PP-1N) through a set of nuclear inhibitor proteins (18). These studies indicate that the intracellular distribution of PP-1 activity, via its regulatory subunits, may be an important aspect of its regulation.

Although PP-1 has long been implicated in the regulation of cell growth and myogenesis in mammalian cells, the form of PP-1 that participates in these processes and the exact molecular mechanism remains unclear. Recent studies from this laboratory have shown that the PP-1C subunit is expressed in L6 cells upon fusion and is absent in proliferating L6 myoblasts (19). The stimulatory effect of insulin on PP-1 activity parallels the gradual appearance of the PP-1C subunit in cells at fusion. Modulation of PP-1C plays an important role in insulin-stimulated glycogen synthesis and glucose transport (20). Whether PP-1C subunit expression is essential for terminal differentiation of L6 cells is unknown. These results, together with the above studies indicating a potential role for PP-1C in cell cycle arrest and myogenesis, prompted us to further explore the role of PP-1C, a known regulator of PP-1 in myogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents, antibiotics/antimycotics, fetal bovine serum, LipofectAMINE™, Geneticin® (G418), phosphorylase b, and phosphorylase kinase were purchased from Life Technologies, Inc. PP-1C, PP-1 catalytic subunit; PP-1G1, glycogen-associated regulatory subunit of PP-1; Rb, retinoblastoma.
[\gamma^{32}P]ATP (specific activity, \(\approx3000\) Ci/mmol) and \([\text{H}]\text{thymidine were purchased from NEN Life Science Products. Electrophoresis and Bradford protein assay reagents were from Bio-Rad. Bicinchoninic acid protein assay reagent was purchased from Pierce. Okadaic acid was from Moana Bioproducts (Honolulu, HI). Ganciclovir was a kind gift from Dr. Amira Klip (The Hospital for Sick Children, Toronto, Ontario, Canada). Antibodies against Rb and phospho-Rb were purchased from New England Biolabs (Beverly, MA). Enhanced chemiluminescence reagent was from Amersham Pharmacia Biotech. The inductible mammalian expression vector system LacSwitch™ was purchased from Stratagene (La Jolla, CA). The mammalian expression vector pcDNA3 was purchased from Invitrogen (San Diego, CA). The vector for used for the construction of knockouts by positive-negative selection (pKO Scrambler™ series) was purchased from Lexicon Genetics Inc. (The Woodlands, TX). Monoclonal FLAG antibody, FLAG peptide, and the FLAG expression vector were purchased from Eastman Kodak IBI (New Haven, CT). All other reagents including those for creatine kinase activity were purchased from Sigma. Porcine insulin was a kind gift from the Eli Lilly Co. (Indianapolis, IN). Antibody against PP-1G was generated as described previously (19).

### In vitro Transfection, and Selection of Stable Cell L6 Lines—

The spontaneously fusing rat skeletal muscle cell line L6, a kind gift from Dr. Amira Klip (The Hospital for Sick Children, Toronto, Ontario, Canada), was used as the parent strain for all transfection experiments. The LacSwitch™ mammalian expression system vector p3SS was initially transfected into L6 myoblasts with LipofectAMIN™ and selected with 1.0 mg/ml hygromycin B to confer lac repressor expression in the parent strain used for the antisense construct (20). It is worth noting that a single clone expressing the highest level of repressor protein was used for subsequent construction to minimize clonal variability. PP-1G depletion was accomplished by inserting the PP-1G gene in the antisense orientation within the expression vector pPOi3 and selected with 2.0 mg/ml G418. All transfections were performed as described previously (20). Independent clones were picked up with cloning discs and screened for the absence of PP-1G protein expression by immunoblot analysis of cell extracts prepared from 10–14-day-old cells using PP-1G antibody as described under “Immunoblot Analysis,” below. Screenin with PP-1G antibody led to the identification of three clones out of 200 G418 resistant clones that lacked PP-1G protein upon induction with isopropl-β-D-thiogalactopyranoside (IPTG). Clone R14, which displayed a >95% depletion of PP-1G protein, was chosen for the experiments. Control cells (referred to herein as L6) were transfected with the empty expression vector. Transfection per se did not affect the extent of differentiation of L6 cells.

In order to further ensure that the observed effects were due to the depletion of PP-1G protein, the PP-1G gene was restored to PP-1G-depleted cells. Because our depletion strategy was based upon inducible antisense expression of PP-1G, restoration of PP-1G expression was possible. Therefore, we created a PP-1G knockout strain by homologous recombination with positive-negative selection and restored PP-1G into these cells by a method of transfection similar to that described above. Briefly, PP-1G deletions were constructed in L6 cells by inserting the gene for hygromycin resistance within the PP-1G gene and adding a thymidine kinase gene at the 3′ end of the PP-1G gene to yield plasmid pKO/PP-1G-HygRO-PP-1G-thymidine kinase. Clones resistant to 1 mg/ml hygromycin B and able to grow in 2 μg ganciclovir were screened for the lack of PP-1G protein expression by immunoblot analysis of cell extracts using PP-1G antibody. The PP-1G gene was restored in this knockout cell line by transfection with vector pcDNA3/FLAG/PP-1G, which contains the PP-1G gene in the sense orientation, and the screening for stable clones was based upon the appearance of the epitope-tagged FLAG/PP-1G fusion protein (21). The FLAG epitope was attached in-frame to the amino-terminal end of PP-1G gene to ensure novel PP-1G expression. All constructions were confirmed by a combination of restriction analysis, polymerase chain reaction, Southern analysis, and automated sequencing analysis using BigDye® in an ABI Prism 3100 automated sequencing apparatus.

### Cell Culture—Wild-type L6 cells were grown and maintained in α-MEM containing 2% fetal bovine serum (FBS) and 1% antibiotic/antimycotics in an atmosphere of 5% CO₂ at 37 °C. Transfected cells were maintained as above with the addition of 150 μg/ml hygromycin B and 400 μg/ml G418. Transfected cells carrying the PP-1G antisense construct were induced with 1.0 mM IPTG from day 3 of culture to induce the expression of PP-1G antisense mRNA. Unless otherwise stated, cells were serum-starved for 15 h in serum-free α-MEM for all stated and used after 3, 5, and 7 days in culture.

### Extraction and Assay of Cellular PP-1 Activity—Serum-starved cells were fed with serum-free medium containing 5 mM glucose and incubated at 37 °C for 1 h. Identical dishes in triplicate were incubated in the second medium and presence of insulin (10 ng/ml) for 24 h. At the end of the incubation period, the medium was removed, and the cells were rinsed three times with ice-cold phosphate-buffered saline, followed by extraction with PP-1 extraction buffer as detailed in our earlier publications (19).

PP-1 activity was determined using [\gamma^{32}P]labeled glycogen phosphorylase a in the presence of 2.0 mM okadaic acid. At this concentration, okadaic acid inhibits only PP-2A, and the remaining activity represents the contribution of PP-1 (19).

### Morphology—A Nikon inverted microscope fitted with a Sony DXC-9000 video camera at ×10 magnification was used to document all morphological studies. Cells were generally photographed at days 3, 5, and 7 and digitally imported into Adobe® Photoshop® 5.0.

### Creatine Kinase Activity—Creatine kinase activity was assayed spectrophotometrically using a diagnostic kit available from Sigma. Briefly, day 3, 5, and 7 cultures grown in 60-mm dishes were washed in ice-cold phosphate-buffered saline three times and extracted in 100 μl of muscle creatine kinase buffer containing 50 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 10 μg/ml each of leupeptin, aprotinin, antipain, soybean trypsin inhibitor, and pepstatin A. The supernatant was sonicated in 4 °C and centrifuged at 4000 × g for 4 min. Typically, 50 μl of extract was assayed as per the manufacturer’s instructions, and the activity was expressed as units/mg. A unit of creatine kinase activity was defined as (ΔA₄₀₅ units/min • 1000)/6.22 (extinction coefficient).

### Thymidine Incorporation—The incorporation of [\text{H}]thymidine into DNA was determined by liquid scintillation spectroscopy (22). Incorporation was expressed as dpmin of protein/h.

### Statistics—Results are expressed as means ± S.E., and the Student’s t test or analysis of variance was used to evaluate significance.

### RESULTS

In this study, we examined the effects of PP-1G depletion on the process of myogenesis using PP-1G antisense mRNA expression. PP-1G protein was depleted from the insulin-sensitive rat skeletal muscle cell line L6 by transfection with an IPTG inducible mammalian expression vector carrying the PP-1G gene in the antisense orientation as detailed under “Experimental Procedures.” As demonstrated in our earlier publications (19), PP-1G immunoblot analysis of whole cell extracts isolated from L6 cells revealed no expression of the 160-kDa PP-1G protein at day 3 (Fig. 1). At day 5, however, extracts isolated from L6 began to express PP-1G protein, with maximal expression observed at
day 7. PP-1G antisense cells (clone R14), however, had virtually no PP-1G expression in IPTG-induced cells after day 5 and only about 5% of the PP-1G found in L6 cells after day 7 (Fig. 1). Noninduced R14 cells also exhibited reduced PP-1G levels due to leaky expression from the LacSwitch™ system occasionally observed (20, 26). R14 was used as a representative clone throughout this study, but similar results were observed with two other PP-1G antisense clones.

In order to examine the impact of PP-1G depletion on cellular PP-1 enzymatic activity, extracts isolated from 7-day-old cells were assayed for PP-1 enzymatic activity. IPTG-induced R14 cells exhibited a 32% decrease in cellular PP-1 activity in the basal state when compared with wild-type L6 cells (Fig. 2). In addition, PP-1G-depleted cells exhibited a complete lack of insulin-stimulated PP-1 enzymatic activity when compared with wild-type cells (Fig. 2).

Wild-type L6 myoblasts grown in α-MEM containing 2% FBS formed multinucleated myotubes after 7 days in culture (Fig. 3). In contrast, PP-1G-depleted cells lined up but failed to differentiate into myotubes even after 7 days in culture (Fig. 3). This lack of differentiation was observed until the cells eventually fall apart after approximately 18 days.

Because PP-1G-depleted cells did not appear to be fusing into myotubes, the rate of cell growth and proliferation was compared between wild-type and PP-1G-depleted cells by measurement of [3H]thymidine incorporation into DNA. As seen in Fig. 4, R14 cells exhibit a 2-fold increase in DNA synthesis when compared with L6 cells over all seven days in the basal as well as serum-induced conditions. In wild-type L6 cells, differentiation was accompanied by a progressive decrease in the DNA synthesis rate in both basal and serum-induced cells from day 3 through day 7 of growth. However, the difference in growth rate persisted over the entire 7-day period studied in R14 cells even though these cells exhibited a considerable reduction in DNA synthesis in 7-day-old cells when compared with day 3 cells, but the rate of serum-induced DNA synthesis in 7-day-old cells was as high as 3-day-old proliferating L6 cells.

In conjunction with differences observed in the growth rate of R14 cells the activity of the myotube-specific marker, creatine kinase, was assayed and observed to be markedly reduced. As shown in Fig. 5, L6 cells exhibit an approximately 10-fold increase in creatine kinase activity during the transition from a fusing myoblasts at day 5 to fully differentiated myotubes at day 7. In contrast, R14 cells exhibit very little creatine kinase activity on day 5, with no increase in activity up to the 7 days of culture studied (Fig. 5).

We next examined the effect of PP-1G depletion on the expression of the muscle-specific differentiation markers myogenin, Id2, and p27Cdk inhibitor. Myogenin expression, although absent at day 3 of growth, was observed in wild-type L6 cells after 5 days of growth, with comparable levels observed after day 7 (Fig. 6A). Although myogenin also begins to appear after day 5 in R14 cells, the level is approximately 10-fold less than L6 levels. This decreased myogenin level is maintained in IPTG-induced PP-1G antisense R14 cells, even after 7 days in culture (Fig. 6A). The level of Id2, an inhibitor of differentiation, although similar in both cell types at day 3, remained expressed in PP-1G-depleted cells (IPTG-induced R14) even after day 7 and dropped to nearly 30% of day 3 levels in wild-type L6 cells (Fig. 6B). The levels of Id1 did not change during myogenesis in L6 cell line (data not shown). The p27Cdk inhibitor, which appears in L6 during myogenesis, was approximately 25% of wild-type in PP-1G-depleted cells studied at days 5 and 7 (Fig. 6C). The levels of p21 did not change during myogenesis in L6 cells (data not shown).

The retinoblastoma gene (Rb) encodes a nuclear phosphoprotein that functions as a critical negative regulator of mammalian cell cycle progression (27). The function of phospho-Rb appears to be controlled by the phosphorylation status of the
protein, and dephosphorylation via PP-1 appears to activate the protein causing cell cycle arrest (15–16). Therefore, we examined the level of Rb and phospho-Rb in both L6 and R14 cells. In proliferating L6 and antisense R14 cells at day 3 of growth, the level of phospho-Rb is comparable both in the basal state and after stimulation by FBS (Fig. 7). Whereas L6 cells show responsiveness to FBS in terms of Rb phosphorylation at day 3, they gradually lose the susceptibility to phosphorylation at days 5 and 7, despite an increase in the content of Rb protein. This is in contrast to R14 cells, which maintain elevated basal and serum-induced phospho-Rb levels at days 5 and 7. Induction of R14 cells with IPTG did result in a considerable increase in basal phospho-Rb levels at days 5 and 7 (Fig. 7, compare lane 8 versus lane 7 and lane 14 versus lane 13). It should be noted that L6 cells exhibited a gradual increase in Rb protein levels at day 5, with the highest expression observed at day 7. R14 cells exhibited an increase in Rb protein levels only at day 7. However, the content of Rb protein was lower than that of L6 cells (Fig. 7).

To further confirm that PP-1G indeed has a regulatory function in myogenesis, PP-1G expression was restored in a PP-1G knockout cell line created by homologous recombination (see under “Experimental Procedures”) by transfection with a recombinant PP-1G gene carrying a FLAG epitope attached to the N-terminus of the PP-1G gene in order to ensure novel PP-1G expression versus a return of endogenous PP-1G expression. The resulting strain, FLAG/PP-1G, had its cellular morphology and fusion patterns restored to normal, as shown in Fig. 8A and expressed PP-1G protein and myogenin at levels comparable to wild-type L6 myotubes at day 7, as well as diminished Id2 protein expression when compared with PP-1G knockout cells (Fig. 8B). This was accompanied by up-regulation of creatine kinase activity (Fig. 8C). Furthermore, up-regulation of endogenous PP-1G levels by induced stable PP-1G overexpression also resulted in 5-fold higher myogenin levels (Fig. 8D) and accelerated myotube formation (data not shown) when compared...
with control wild-type cells.

Earlier studies by Kim et al. (12) have shown that the activities of MyoD and myogenin are inhibited by phosphorylation. To examine whether PP-1G depletion is accompanied by excessive myogenin phosphorylation, we performed immunoprecipitation of lysates prepared from 32P-labeled 7-day-old cells using myogenin antibody. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane, followed by autoradiography. As shown in Fig. 9, both clone R14 and PP-1G knockout cells exhibit a marked increase in myogenin phosphorylation when compared with wild-type L6 and FLAG/PP-1G-expressing cells, which exhibit very little myogenin phosphorylation despite an increase in myogenin content.

DISCUSSION

In this study, we have demonstrated a unique role for PP-1G, one of the major regulatory subunits of PP-1, in the regulation of terminal differentiation in L6 cells, using a transfected cell line expressing PP-1G antisense mRNA, as well as a recombi-
FIG. 9. Myogenin is phosphorylated in PP-1G-depleted cells. Overexpression of PP-1G is accompanied by restoration of myogenin levels and reduction in its phosphorylation status. Equal amounts of 32P-labeled cell lysate proteins were immunoprecipitated with anti-myogenin antibody followed by separation of the immunoprecipitated proteins by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membrane followed by autoradiography. Top panel, a representative autoradiogram is shown. Bottom panel, the blots were probed with anti-myogenin antibody to quantitate the amount of myogenin immunoprecipitated from each cell lysate.

FIG. 10. Subcellular localization of PP-1G and PP-1C subunits in L6 and recombinant cell lines. Equal amounts of proteins (25 μg) from extracts (E), cytosolic fraction (C), and nuclear fraction (N) were separated by SDS-polyacrylamide gel electrophoresis followed by Western blot analyses with PP-1G and PP-1C antibodies. A representative autoradiogram is shown.

Determining factors for myogenic signaling for several reasons. First, PP-1G depletion severely impaired the induction of myogenin expression, the marker for L6 skeletal muscle cell differentiation, and completely inhibited terminal differentiation of myoblasts into myotubes. This defect is accompanied by (i) persistent levels of Id2, which is normally down-regulated as the cells start to form myotubes, and (ii) impaired induction of the p27Cdk inhibitor. Additionally, the somewhat elevated Id2 protein levels observed in PP-1G-depleted cells may cause further inactivation of the limited amount of myogenin by forming heterodimers and thereby silence its transcriptional activity. It is known that Id proteins bind E2a with greater affinity than myogenin, resulting in the inhibition of DNA binding and biological activities of E2a. In addition, PP-1G-depleted cells exhibit an increase in myogenin phosphorylation status, causing inactivation of myogenin. A second consequence of myogenin depletion and inactivation is that the expression of p27Cdk inhibitor is blocked. This may cause the activation of cyclin-dependent kinases and inactivation of Rb protein, due to increased phosphorylation, and therefore may impair cells from exiting mitosis. Finally, replacement of the FLAG-epitope-tagged PP-1G subunit into knockout cells restored basal PP-1 catalytic activity, its stimulation by insulin, expression of myogenin, down-regulation of Id2, expression of p27Cdk inhibitor, reduction in the levels of phospho-Rb, and the ability to form myotubes along with concomitant restoration of creatine kinase activity. These results confirm that the defects observed in terminal differentiation in PP-1G knockout cells were due to the lack of an active PP-1 holoenzyme and not to nonspecific events.

Previous studies by Fernandez et al. (13) have shown that PP-1 catalytic subunit gets localized to the nucleus as cells progress from G1 to G2/M phase in rat 1 fibroblasts. No evidence, however, exists on the processes regulating this increase in the nuclear localization of PP-1. The present study adds a new dimension to the role of PP-1 in muscle cell differentiation via its glycogen/SR associated regulatory subunit in L6 cells. It is known from several studies that withdrawal from the cell cycle during myogenesis is accompanied by a decrease in cyclin-dependent kinase activity. These results confirm that the defects observed in terminal differentiation in PP-1G knockout cells were due to the lack of an active PP-1 holoenzyme and not to nonspecific events.
tumor suppressor protein RB and PP-1 have been observed in cells from mid-mitosis to early G1 (14–16). We have observed elevated levels of phospho-Rb in cells lacking the PP-1G subunit. Given the knowledge that PP-1 dephosphorylates Rb protein, leading to its activation, it is plausible that impaired activation of PP-1, due to the absence of PP-1G, may result in increased phosphorylation of Rb protein, leading to its inactivation. However, Western blot analyses of nuclear fractions for the presence of PP-1CB, one of the isoforms of PP-1 C subunit, revealed no differences in PP-1CB contents between wild-type L6 and PP-1 G KO cells. This observation is not unexpected, given that PP-1 C binds to nuclear inhibitor proteins NIPP-1 and NIPP-2, leading to inactivation of PP-1 enzymatic activity. We observed a decrease in cellular PP-1 enzymatic activity in PP-1G-depleted cells. Clearly, further studies are needed to understand the exact role of PP-1 G subunit in the nuclear localization of PP-1 and its relationship to terminal differentiation of muscle cells.

In summary, the results of this study indicate that the glycogen/SR-associated regulatory subunit of protein phosphatase-1, PP-1G, may play a vital role in coupling initial events of myogenesis to terminal differentiation, thereby facilitating the exit of cells from mitosis via increased dephosphorylation of proteins involved in mitosis.

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Inhibition of Myogenesis by Depletion of the Glycogen-associated Regulatory Subunit of Protein Phosphatase-1 in Rat Skeletal Muscle Cells
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