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Phosphatidylinositol 3-Kinase Inhibitors Block Differentiation of Skeletal Muscle Cells*

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Skeletal muscle differentiation involves myoblast alignment, elongation, and fusion into multinucleate myotubes, together with the induction of regulatory and structural muscle-specific genes. Here we show that two phosphatidylinositol 3-kinase inhibitors, LY294002 and wortmannin, blocked an essential step in the differentiation of two skeletal muscle cell models. Both inhibitors abolished the capacity of L6E9 myoblasts to form myotubes, without affecting myoblast proliferation, elongation, or alignment. Myogenic events like the induction of myogenin and of glucose carrier GLUT4 were also blocked and myoblasts could not exit the cell cycle, as measured by the lack of mRNA induction of p21 cyclin-dependent kinase inhibitor. Overexpresssion of MyoD in 10T1/2 cells was not sufficient to bypass the myogenic differentiation blockade by LY294002. Upon serum withdrawal, 10T1/2-MyoD cells formed myotubes and showed increased levels of myogenin and p21. In contrast, LY294002-treated cells exhibited none of these myogenic characteristics and maintained high levels of Id, a negative regulator of myogenesis. These data indicate that whereas phosphatidylinositol 3-kinase is not indispensable for cell proliferation or in the initial events of myoblast differentiation, *i.e.* elongation and alignment, it appears to be essential for terminal differentiation of muscle cells.

The development of skeletal muscle is a multistep process that involves the determination of pluripotential mesodermal cells to give rise to myoblasts, withdrawal of the myoblasts from the cell cycle and differentiation into muscle cells, and finally growth and maturation of skeletal muscle fibers (1).

At the molecular level, myogenic commitment and muscle-specific gene expression involve the skeletal muscle-specific helix-loop-helix (bHLH)[1] MyoD family of proteins, which includes MyoD, myogenin, myf-5, and MRF4, and the myocyte enhancer-binding factor 2 (MEF2). Each of the MyoD-family proteins and also MEF2A indiscriminately direct non-muscle cells into the myogenic lineage by auto- and cross-activating bHLH genes (2–4). The myogenic activity of the MyoD family proteins requires heterodimerization with ubiquitously expressed E2a gene products to form functional transcription complexes (5, 6). The DNA binding activity of MyoD family proteins is attenuated by Id, which forms complexes with E2a gene products in proliferating cells and is down-regulated when they are induced to differentiate (7, 8).

The decision to differentiate into myotubes is influenced negatively by several factors. Treatment of myoblasts with fetal bovine serum, basic fibroblast growth factor 2, or transforming growth factor β1 is known to inhibit differentiation of myoblasts (9, 10). Myogenesis is also regulated negatively by oncogenes such as c-*myc*, c-*jun*, c-*fos*, H-*ras*, and E1a (11–16).

There is very little information regarding the signaling that is triggered in the myoblast upon serum withdrawal which leads to the induction of the MyoD family gene expression and to muscle differentiation. Myogenic differentiation seems to depend on the activation of integrins present on the plasma membrane of myoblasts (17, 18), suggesting the operation of an "outside-in" biochemical pathway in which integrin is the upstream molecular species. Interactions of insulin-like growth factor (IGF)-I and -II with their receptors are also positive regulators of skeletal muscle differentiation (19). However, the intracellular events occurring between receptor activation and terminal differentiation remain obscure.

Phosphatidylinositol 3-kinases (PI 3-kinases) are involved in receptor signal transduction via tyrosine kinase receptors and in protein trafficking in eukaryotic cells. Multiple forms of PI 3-kinases have been reported. A well characterized group of PI 3-kinases are heterodimers composed of a regulatory p85 subunit and a catalytic p110 subunit (20, 21). In contrast, the recently cloned PI 3-kinase γ (22) does not couple to p85 and is activated by G$\beta\gamma$ subunits (23, 24). A third group of PI 3-kinases includes the VPS34 gene product from *Saccharomyces cerevisiae* (25, 26) and its human homolog (27). The Vps34p only phosphorylates the 3′ position of PI and is required for vacuolar protein sorting (25). This last group of PI 3-kinases associate with a 150–160-kDa protein, which in *S. cerevisiae* is a serine/threonine kinase essential for Vps34p activity (28). All PI 3-kinase isoforms so far described are potently inhibited in the nanomolar or low micromolar range by two structurally unrelated membrane permeant reagents: wortmannin (for review, see Ref. 29) and LY294002 (30).

While much information has been accumulated on the role of PI 3-kinase activities in tyrosine kinase receptor signal transduction or vesicle trafficking, little is known about the possible role of PI 3-kinases in cell differentiation. In this regard, it has been observed that PI 3-kinase might be involved in neurite outgrowth of PC12 cells (31). In a previous report, we showed that PI 3-kinase activity and insulin-stimulated glucose transport in L6E9 muscle cells were inhibited in a dose-response

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[1] The abbreviations used are: bHLH, basic helix-loop-helix; IGF, insulin-like growth factor; PI 3-kinase, phosphatidylinositol 3-kinase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

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manner by a 1-h treatment with wortmannin (32). Here we show that chronic treatment with LY294002 or wortmannin blocked morphological and biochemical changes associated with L6E9 myoblast terminal differentiation and that this inhibition is not bypassed by the overexpression of MyoD in 10T1/2 cells. Data presented here implicate PI 3-kinase as an essential positive regulator of terminal differentiation of skeletal muscle cells.

EXPERIMENTAL PROCEDURES

*Materials—*Wortmannin was kindly given by Dr. Trevor Payne (Sandoz, Basel) and LY294002 was from BioMol Research Laboratories (Plymouth Meeting, PA). L6E9 rat skeletal muscle cell line was kindly provided by Dr. B. Nadal-Ginard (Harvard University). 10T1/2 cells transfected with MyoD were obtained from Dr. Vicente Andrés (St. Elizabeth's Medical Center, Boston, MA).

The polyclonal antibody OSCRX was raised against the C terminus of GLUT4 (33). The polyclonal antibodies against rat p85 PI 3-kinase were from Upstate Biotechnology Inc. (Lake Placid, NY). A rabbit polyclonal antibody against β_1-integrin was kindly given by Dr. Carles Enrich (University of Barcelona) (34). cDNAs encoding for myogenin and p21 were kindly given by Dr. Eric Olson (University of Texas, Houston, TX) and David Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), respectively. cDNA for Id was obtained from Dr. Harold Weintraub (Fred Hutchinson Cancer Research Center, Seattle, WA).

*Cell Culture—*Rat skeletal muscle L6E9 myoblasts were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (10,000 units/ml penicillin G and 10 mg/ml streptomycin). Confluent myoblasts were differentiated by lowering FBS to a final concentration of 2% (v/v). 10T1/2 fibroblasts transfected with MyoD were grown as L6E9 cells in the presence of 0.5 mg/ml geneticin, and differentiated in Dulbecco's modified Eagle's medium containing 5% (v/v) horse serum with antibiotics and geneticin.

To analyze the effect of PI 3-kinase inhibitors on cell differentiation, confluent cells were washed twice in phosphate-buffered saline solution (PBS) and low serum medium was added without or with LY294002 (10, 20, or 50 μM) or wortmannin (1 μM). Both inhibitors were initially dissolved in dimethyl sulfoxide and in all experimental series, control cells were treated with the corresponding volumes of dimethyl sulfoxide. Due to the instability of wortmannin in aqueous solutions (31), after washing twice in PBS, medium from cells treated with wortmannin and untreated controls was replaced every 5 h during the differentiation period (2–4 days).

*RNA Isolation and Northern Blot Analysis—*Total RNA from cells was extracted using the phenol/chloroform method (35). All samples had a 260/280 absorbance ratio above 1.7.

After quantification, total RNA (30 μg) was denatured at 65 °C in the presence of formamide, formaldehyde, and ethidium bromide (36). RNA was separated on a 1.8% agarose/formaldehyde gel, blotted on Hybond N filters, and processed as previously reported (33). The blots were then hybridized to the corresponding probes overnight at 42 °C in 50% formamide, 5 × Denhardt's, 0.1% SDS, 5 × SSPE, 10% dextran sulfate, and 200 μg/ml denatured salmon sperm DNA. The mouse cDNA probe for myogenin was a 1,100-base pair *Eco*RI fragment, the mouse cDNA probe for Id was a 900-bp *Sma*I fragment, and the human cDNA probe for p21 was a 600-bp *Not*I fragment. The cDNA probes were labeled with [α-^{32}P]dCTP by random oligonucleotide priming. The probes were included at 1–2 × 10^6 cpm/ml.

*Preparation of Membrane Fractions from Muscle Cells—*Cells were homogenized by 20 strokes with a Dounce A homogenizer in 10 volumes of ice-cold buffer containing 20 mM Hepes, 250 mM sucrose, 2 mM EGTA, 5 mM NaN_3, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin, pH 7.4. Homogenates were centrifuged at 760 × *g* for 10 min at 4 °C. The supernatants were then centrifuged at 200,000 × *g* for 90 min at 4 °C to obtain the membrane fractions. The membrane pellets were resuspended in homogenization buffer and repeatedly passed through a 25-gauge needle before storage at −20 °C. Proteins were measured by the method of Bradford (37) using γ-globulin as a standard and 30 μg of total membranes from each experimental series were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

*Immunoprecipitation of p85 Subunit of PI 3-Kinase—*Immunoprecipitation from solubilized cells was performed as described previously (32). The immunopellets were resuspended in SDS-PAGE sample buffer under reduction conditions and electrophoresed.

*Electrophoresis and Immunoblotting of Membranes—*SDS-PAGE was performed following Laemmli (38). Proteins were transferred to Immobilon in buffer consisting of 20% methanol, 200 mM glycine, 25 mM Tris, pH 8.3. Following transfer, the filters were blocked with 5% nonfat dry milk and 0.02% NaN_3 in PBS for 1 h at 37 °C and then incubated overnight at 4 °C with antibodies against GLUT4 (1:400), β_1-integrin (1:1000), and p85 (1:1000) in PBS containing 1% nonfat dry milk and 0.02% sodium azide. β_1-Integrin was detected using ^{125}I-protein A for 3 h at room temperature. GLUT4 and p85 were detected by ECL chemiluminiscence system.

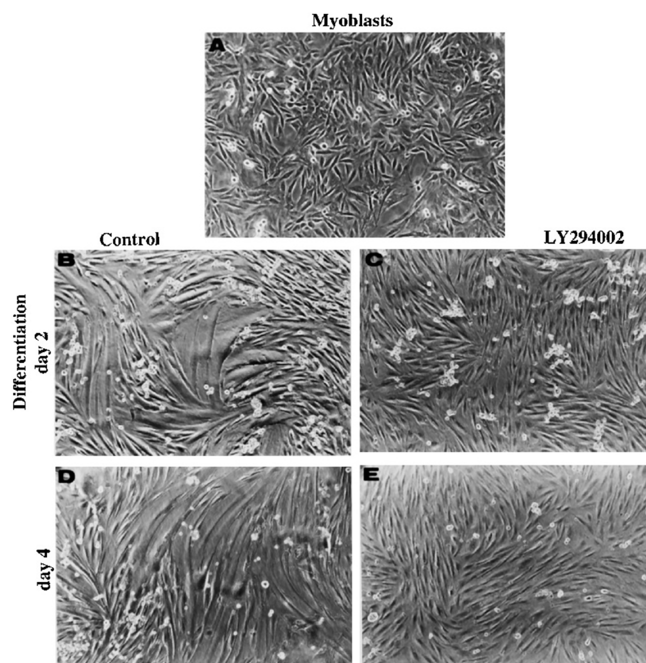

FIG. 1. **LY294002 inhibits myotube formation in L6E9 muscle cells.** L6E9 myoblasts were grown to confluence in a 10% FBS-containing medium (*A*) and then allowed to differentiate by serum deprivation (2% FBS-containing medium) in the absence (*B* and *D*) or presence (*C* and *E*) of 10 μM LY294002. Cells were photographed after 2 days (*B* and *C*) or 4 days (*D* and *E*) in low serum medium. Magnification: 125 ×.

RESULTS

*LY29004 and Wortmannin Inhibit the Fusion of L6E9 Myoblasts into Myotubes—*L6E9 skeletal muscle myoblasts were grown in a proliferating medium containing 10% FBS (Fig. 1*A*) and when confluent, myoblasts were incubated in a 2% FBS-containing medium (differentiation medium). Under these conditions, myoblasts elongated and aligned with each other during the first 24 h and multinucleate myotube formation was observed during the following 72 h (Fig. 1, *B* and *D*, day 2 and 4, respectively). When the differentiation medium was added together with the PI 3-kinase inhibitor LY294002 (10, 20, or 50 μM), L6E9 myoblasts aligned as did the untreated control myoblasts. However, no fusion into myotubes was observed during the 4 days required for full differentiation. Fig. 1 shows myoblasts treated with 10 μM LY294002 in differentiation medium for 2 and 4 days (*C* and *E*, respectively). Identical effects were observed at 20 or 50 μM LY294002 (data not shown).

Myoblasts fusion was also blocked when confluent myoblasts were allowed to differentiate in a medium containing 1 μM wortmannin, another inhibitor of PI 3-kinases. It has been shown that wortmannin is highly unstable in aqueous solutions (29). For this, our dose-response studies were limited to LY294002; in experiments using 1 μM wortmannin, after washing twice in PBS, culture medium from control and treated cells was replaced by fresh medium with dimethyl sulfoxide or with wortmannin, respectively, every 5 h throughout the differentiation period (2–4 days). Under these conditions, control myoblasts aligned with each other during the first 24 h in differ-

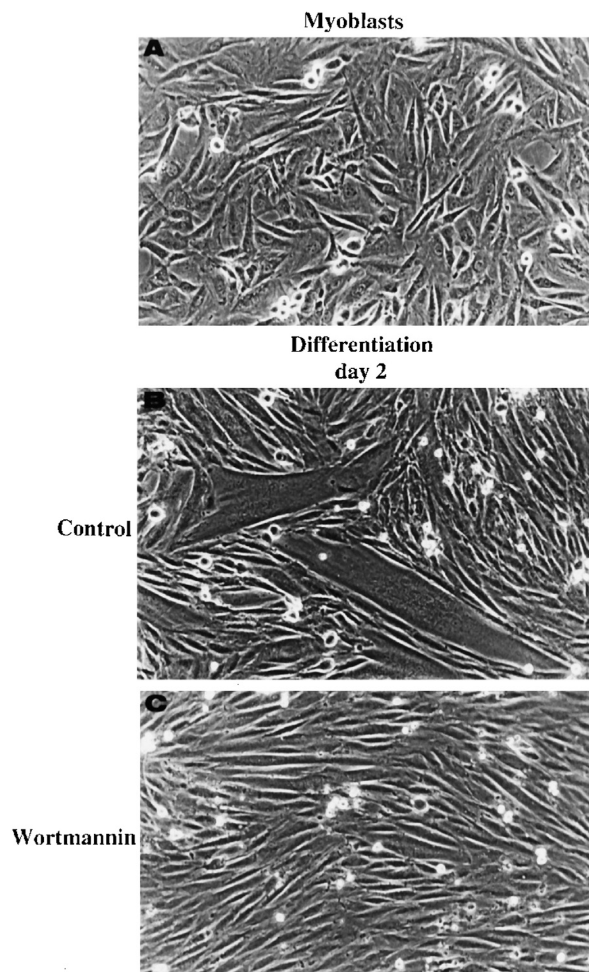

FIG. 2. **Wortmannin inhibits myotube formation in L6E9 muscle cells.** L6E9 myoblasts were grown to confluence in a 10% FBS-containing medium (*A*) and then allowed to differentiate by serum deprivation (2% FBS) during 2 days in the absence (*B*) or presence (*C*) of 1 μM wortmannin. Magnification: 320 ×.

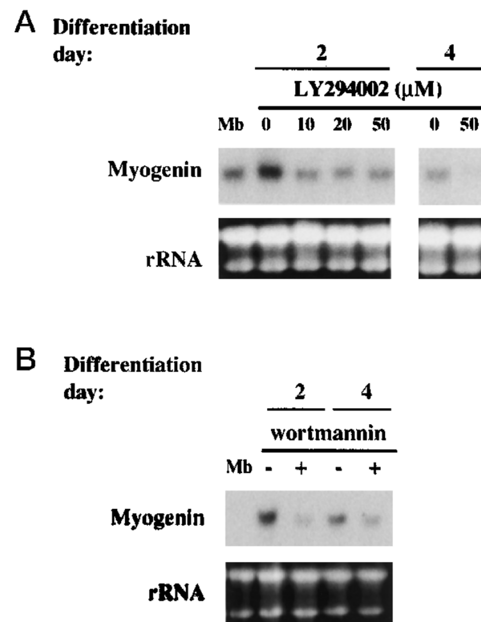

FIG. 3. **LY294002 and wortmannin inhibit the induction of myogenin mRNA in L6E9 muscle cells.** Confluent L6E9 myoblasts (*Mb*) were allowed to differentiate in low serum medium for 2 or 4 days in the absence or presence of LY294002 (*A*: 10, 20 or 50 μM) or 1 μM wortmannin (*B*). Total RNA was obtained from the different experimental groups and 30 μg of RNA was laid on gels. After blotting, myogenin mRNA was detected by hybridization with a 1,100-base pair *Eco*RI fragment as a cDNA probe. Representative autoradiograms after 2 days of exposure, from three to five experiments, are shown. The integrity and relative amounts of RNA in each sample were checked by ethidium bromide staining (rRNA).

entiation medium (not shown) and formed multinucleate myotubes after 2 days (Fig. 2*B*). In contrast, myoblasts in differentiation medium with 1 μM wortmannin aligned with each other but did not fuse into myotubes (Fig. 2*C*). Identical effects were observed after a 4-day treatment with wortmannin (data not shown).

LY29004 and Wortmannin Inhibit Myogenin mRNA Induction Associated with Differentiation—It has been described that the lack of the bHLH muscle regulatory factor myogenin reduces myotube formation *in vivo* (39, 40), indicating that myogenin has a unique function in the transition from a determined myoblast to a fully differentiated myotube. Based on this, we next analyzed the effect of PI 3-kinase inhibitors on myogenin mRNA levels during the differentiation period. As shown in Fig. 3*A*, LY294002 blocked the induction of myogenin mRNA which is normally observed on differentiation days 2 and 4. The maximal effect of LY294002 was observed at a concentration of 10 μM. Similar results were obtained when 1 μM wortmannin was added to the differentiation medium and myogenin mRNA levels were determined at days 2 and 4 (Fig. 3*B*).

LY29004 and Wortmannin Inhibit mRNA Induction of p21^{cdk} Inhibitor Associated with Differentiation—An early event during the muscle differentiation process is the exit of myoblasts from the cell cycle. A marker of this event is the induction of the mRNA expression of p21^{cdk} inhibitor. Indeed, it is known that ectopic expression of p21 in growing myoblasts is sufficient to inhibit cell cycle progression (41). As expected, 2 days after serum deprivation, L6E9 myotubes showed increased mRNA levels of p21 compared to proliferating cells (Fig. 4). In contrast, the absence of p21 mRNA induction in LY294002-treated cells (10 μM or 20 μM), strongly suggests that PI 3-kinase activity may be essential to signal the cell cycle exit in differentiating muscle cells.

LY29004 and Wortmannin Inhibit the Muscle-specific Induction of the Glucose Transporter GLUT4 in L6E9 Cells—To determine whether the blockade of multinucleate myotube formation by LY294002 and wortmannin was associated with the inhibition of differentiation at the biochemical level, we analyzed the expression of GLUT4, a skeletal muscle marker, in cells allowed to differentiate in the absence or presence of LY294002 or wortmannin.

After 4 days in differentiation medium, the total membrane content of GLUT4 was increased by 2.42 ± 0.46-fold ($n = 3$) in myotubes compared to myoblasts (Fig. 5, *Mt versus Mb*). When 50 μM LY294002 or 1 μM wortmannin were present during the 4 days of differentiation, little or no induction in GLUT4 expression was observed (Fig. 5, *LY* or *W versus Mb*). In contrast, PI 3-kinase inhibitors did not affect the expression of two non-muscle-specific proteins: the structural plasma membrane component β_1-integrin and the p85 regulatory subunit of PI 3-kinase. As shown in Fig. 5, the expression level of these proteins was unaffected after 4 days in differentiation medium either in the absence or presence of PI 3-kinase inhibitors.

LY294002 Inhibits Myotube Formation in 10T1/2 Cells Stably Transfected with MyoD—Multinucleate myotube formation together with muscle-specific gene onset can be induced by overexpression of MyoD or the other members of myogenic bHLH transcription factors (myogenin, MyoD, myf5, and MRF4); each of these four myogenic factors indiscriminately

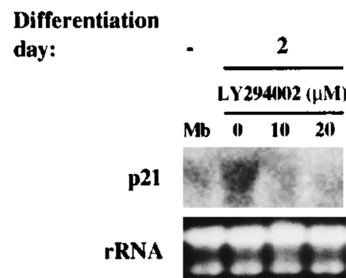

FIG. 4. **LY294002 inhibits the induction of p21^{cdk} inhibitor mRNA levels in L6E9 muscle cells.** Confluent L6E9 myoblasts (*Mb*) were allowed to differentiate in low serum medium for 2 days in the absence or presence of LY294002 (10 or 20 μM). Total RNA was obtained from the different experimental groups and 30 μg of RNA was laid on gels. After blotting, p21 mRNA was detected by hybridization with a 600-base pair *Not*I fragment as a cDNA probe. A representative autoradiogram after 2 weeks of exposure, from three separate experiments, is shown. The integrity and relative amounts of RNA in each sample were checked by ethidium bromide staining (rRNA).

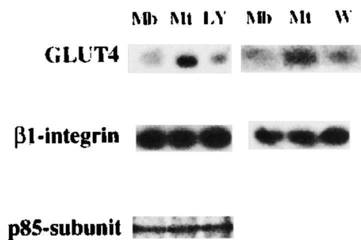

FIG. 5. **LY294002 and wortmannin inhibit the induction of the skeletal muscle marker GLUT4 protein content in L6E9 muscle cells.** Confluent L6E9 myoblasts were allowed to differentiate for 4 days in the absence or presence of 50 μM LY294002 or 1 μM wortmannin. Total membrane proteins from confluent myoblasts (*Mb*) and from cells in differentiation medium without (*Mt*) or with PI 3-kinase inhibitors (*LY*, 50 μM LY294002; *W*, 1 μM wortmannin) were obtained. GLUT4 glucose transporter and β_1-integrin content was analyzed by immunoblotting 30 μg of total membrane proteins from the different experimental groups. PI 3-kinase p85-subunit was analyzed by immunoprecipitating 2.5 mg of solubilized proteins from the different experimental groups with anti-p85 polyclonal antibody and then immunoblotting with the same antibody. Representative autoradiograms from three separate experiments of each group are shown.

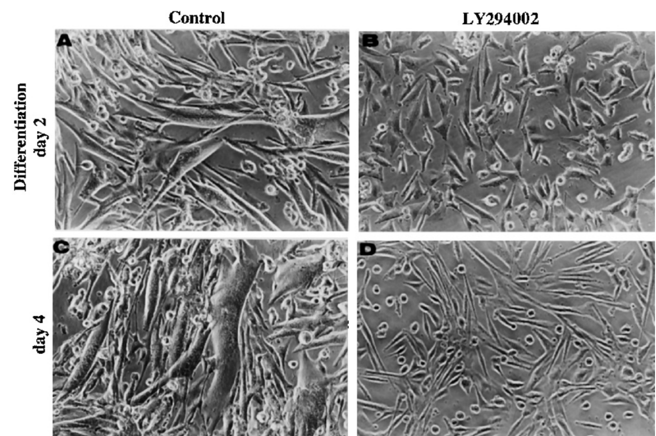

FIG. 6. **LY294002 inhibits myotube formation in 10T1/2 cells transformed with MyoD.** 10T1/2-MyoD cells were grown to confluence in a 10% FBS-containing medium and then allowed to differentiate following serum deprivation (5% horse serum containing medium) in the absence (*A* and *C*) or presence (*B* and *D*) of 50 μM LY294002. Cells were photographed after 2 days (*A* and *B*) or 4 days (*C* and *D*) in low serum medium. Magnification: 215 ×.

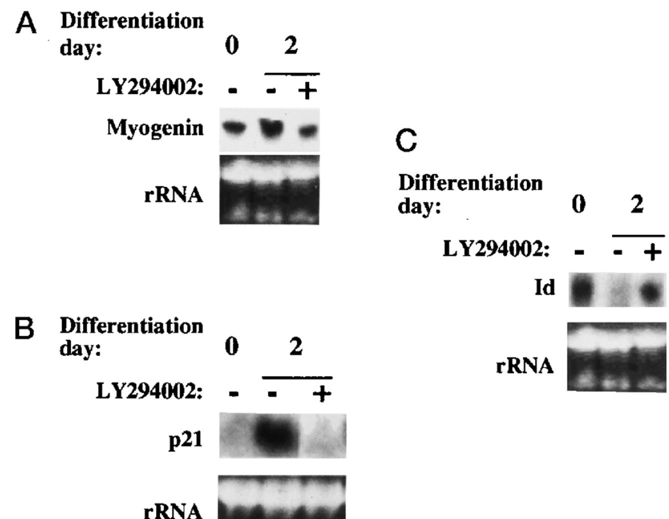

FIG. 7. **LY294002 inhibits the induction of myogenin and p21 mRNA and the down-regulation of Id mRNA in serum-deprived 10T1/2-MyoD cells.** Confluent 10T1/2-MyoD cells (day 0) were allowed to differentiate in low serum medium for 2 days in the absence or presence of 50 μM LY294002. Total RNA was obtained from the different experimental groups and 30 μg of RNA were laid on gels. After blotting, myogenin was detected by a 1,100-bp *Eco*RI fragment as a cDNA probe (*A*); p21 mRNA was detected by hybridization with a 600-bp *Not*I fragment (*B*) and Id was detected by a 900-bp *Sma*I fragment (*C*). Representative autoradiograms after 1–2 days of exposure, from three separate experiments, are shown. The integrity and relative amounts of RNA in each sample were checked by ethidium bromide staining (rRNA).

directs non-muscle cells into the myogenic lineage by auto- and cross-activating bHLH genes (2, 3). Consistent with these observations, 10T1/2 cells stably transfected with MyoD (10T1/2-MyoD) were able to fuse to each other upon serum withdrawal. Large multinucleate myotubes were observed from differentiation day 2 up to day 4 (Fig. 6, *A* and *C*). In contrast, when confluent 10T1/2-MyoD cells were serum-deprived in the presence of 50 μM LY294002, cells progressively adopted an elongated shape but were unable to form myotubes (Fig. 6, *B* and *D*), indicating that the overexpression of MyoD could not bypass the blockade of skeletal muscle differentiation by the PI 3-kinase inhibitor LY294002.

LY294002 Blocks Biochemical Differentiation in 10T1/2-MyoD Cells—We next analyzed whether LY294002 inhibition of 10T1/2-MyoD myotube formation was accompanied by a blockade of biochemical differentiation, as we had previously observed in L6E9 cells. To this end, we first determined the mRNA levels of myogenin in undifferentiated cells and in cells allowed to differentiate in the absence or presence of 50 μM LY294002 for 2 days. As seen in Fig. 7*A*, myogenin mRNA levels were induced after serum withdrawal in 10T1/2-MyoD cells and this induction was blocked in the presence of the PI 3-kinase inhibitor LY294002.

As observed for L6E9 cells and consistent with the observation that MyoD induces p21^{cdk} inhibitor expression *in vitro* (41, 42), serum-deprived 10T1/2-MyoD cells showed increased mRNA levels of p21 compared to proliferating cells (Fig. 7*B*). However, as observed in L6E9 cells, p21 mRNA induction was blocked by LY294002, indicating that the PI 3-kinase inhibitor impaired the cell cycle exit.

We next determined the effect of LY294002 on the mRNA levels of Id. It is known that undifferentiated 10T1/2-MyoD cells express high levels of this dominant negative bHLH protein, which inactivates MyoD by heterodimerization with E2a gene products and which is down-regulated during myogenesis (7, 8). As expected, Id mRNA level in 10T1/2-MyoD cells was decreased after 2 days in differentiation medium (Fig. 7*C*). In contrast, when LY294002 was added together with the differentiation medium, Id mRNA level was not down-regulated. This absence of Id down-regulation could explain at least in

part the inability of MyoD to induce cell fusion and myogenin and p21 mRNA expression in LY294002-treated cells.

DISCUSSION

In this study we have shown that two structurally different inhibitors of phosphatidylinositol 3-kinase, *i.e.* LY294002 and wortmannin, blocked both the morphological and the biochemical differentiation of two skeletal muscle models, L6E9 myoblasts and 10T1/2 cells stably transfected with MyoD (10T1/2-MyoD).

In a previous report, we showed that PI 3-kinase activity and insulin-stimulated glucose transport in L6E9 muscle cells were inhibited by 1 h treatment with wortmannin. The inhibition of insulin action was dose-dependent with an ED_{50} around 10–20 nM (32). Here we show that chronic treatment with LY294002 or wortmannin blocked both the morphological and the biochemical differentiation of L6E9 cells. PI 3-kinase inhibitors did not interfere with myoblast proliferation at the doses used (data not shown) and they did not cause any alteration in the elongation or alignment of myoblast observed during the first 24 h after serum deprivation. However, whereas untreated cells began to fuse a few hours after alignment, wortmannin- or LY294002-treated cells remained aligned but did not fuse into myotubes. PI 3-kinase also appears to be essential for signaling leading to the onset of muscle-specific gene expression. Thus, both wortmannin and LY294002 blocked the induction of myogenin mRNA levels and the exit from the cell cycle as measured by the lack of p21 mRNA expression. The induction of the skeletal muscle marker GLUT4 was also impaired without changes in the expression of two non-muscle-specific proteins: β_1-integrin, an integral plasma membrane protein and the p85 regulatory subunit of PI 3-kinase. This suggests that PI 3-kinase inhibitors only affected differentiation-associated events.

Similar results were obtained when 10T1/2-MyoD cells were allowed to differentiate in the presence of the phosphatidylinositol 3-kinase inhibitor LY294002: cells elongated but they were unable to fuse. Upon serum deprivation, untreated 10T1/2-MyoD cells formed multinucleate myotubes and induced the expression of myogenin and p21 mRNAs. In contrast, none of these myogenic characteristics was observed when 10T1/2-MyoD myoblasts were allowed to differentiate in the presence of LY294002.

Our results seem to indicate that PI 3-kinase inhibitors prevented the activation of other muscle regulatory factors such as myf5 or MyoD, already expressed during the myoblast phenotype (43). Indeed, this was the case in 10T1/2-MyoD cells where MyoD overexpression in undifferentiated cells did not bypass the inhibitory action of LY294002. It has been reported that the activity of muscle regulatory factors can be prevented by increasing the levels of Id protein or by silencing their transcriptional activity as a result of phosphorylation catalyzed by different kinases (15, 16). In this study, we focused our attention on the dominant negative bHLH protein Id, which is normally down-regulated after serum deprivation but which remained expressed at high levels in LY294002-treated 10T1/2-MyoD cells. This blockade of Id down-regulation could explain, at least in part, the inability of overexpressed MyoD to induce differentiation.

In all, our data implicate PI 3-kinase at an early step in the terminal differentiation of muscle cells and rule it out as an essential component in the control of proliferation of myoblast cells or the initial morphological changes associated with differentiation such as alignment and elongation of myoblasts. A schematic summary of our results is presented in Fig. 8.

PI 3-kinases have been associated with many signal transduction pathways, indicating that this family of enzymes plays a variety of roles in response to different stimuli. Here we

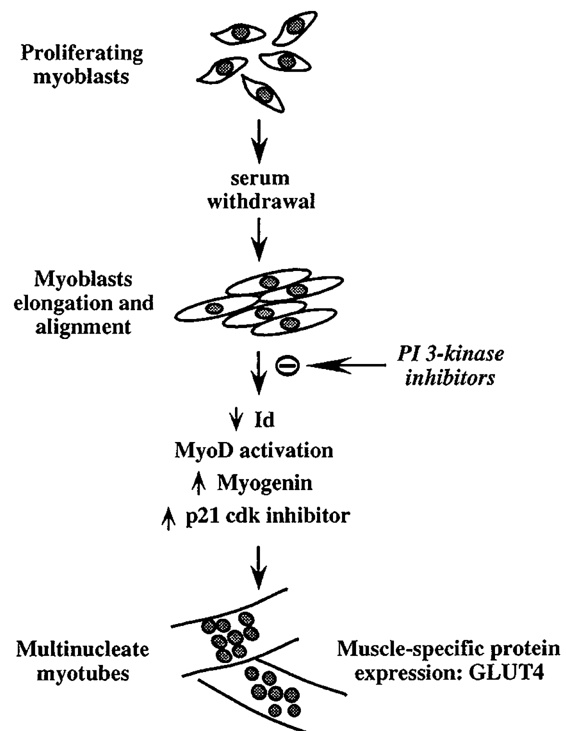

FIG. 8. **Model for a role of PI 3-kinase in differentiation of muscle cells.** Upon serum withdrawal, myoblasts differentiate. At the morphological level, myoblasts initially elongate and align. A few hours later, cells begin to fuse to each other to give rise to multinucleate myotubes. When PI 3-kinase inhibitors are added together with the differentiation medium and maintained throughout the differentiation period, myoblasts are able to proceed through the first differentiation events, *i.e.* elongation and alignment, but the rest of the differentiation program appeared to be blocked: (i) Id down-regulation is inhibited and it is known that high levels of Id inactivate MyoD; (ii) MyoD remains inactive; (iii) myogenin gene expression is not induced and (iv) the gene expression p21 cdk inhibitor is blocked, suggesting that myoblasts are unable to exit the cell cycle, an essential step for full differentiation. These events finally lead to the inability of myoblasts to fuse into multinucleate myotubes and to express muscle-specific proteins such as GLUT4.

present results that strongly implicate PI 3-kinase activity in myogenesis. However, the precise myogenic mechanism(s) that are blocked by the PI 3-kinase inhibitors remain to be defined. At the moment, there is very scarce information regarding the signaling pathways by which the microenvironment influences myogenesis. Among the non-exclusive positive regulators of myogenesis that have been described are the insulin growth factors IGF-I, IGF-II, and their specific receptors, and also, the occupation of the extracellular matrix receptors integrins. The following observations allow us to postulate the involvement of PI 3-kinase(s) in myogenic signaling pathways.

(i) Growth factors are generally considered to inhibit myogenesis (reviewed in Ref. 1). However, it has been reported that myoblasts in low serum medium initiate the expression of IGF-II which is secreted in significant amounts to the medium (44). It has been proposed that the insulin-like growth factors IGF-I and -II act in an autocrine and/or paracrine manner on myoblasts to promote myogenic differentiation by interacting primarily with the IGF-I receptor (45). In this regard, IGF-binding proteins from muscle cells inhibit IGF-I-induced differentiation of L6E9 myoblasts (46).

The intracellular myogenic signaling process dependent on IGFs is poorly understood but it is known that IGF-I receptors associate through IRS-I with the p85 regulatory subunit of PI 3-kinase. This interaction results in the activation of the enzyme (47, 48) and the phosphorylation of IRS-1 by the serine

kinase activity of PI 3-kinase (49, 50). If such an association were also essential for myogenic signaling, PI 3-kinase inhibitors may block terminal differentiation at this level. On the other hand, IGF-II receptors have also been implicated in myogenesis by using the IGF-II receptor-selective (Leu-27) IGF-II analog (51) and, interestingly, wortmannin has been shown to block exocytosis of IGF-II receptors in 3T3-L1 adipocytes (52).

(ii) Integrins seem to play an important role in mediating signals from the extracellular matrix to influence myogenesis. Indeed, in the presence of a monoclonal antibody which blocks the function of the integrin β-subunit, myoblasts continue to replicate but do not fuse or express muscle-specific markers (17). Myotube formation is also inhibited by antibodies against the integrin VLA-4 and its counter-receptor VCAM-1 (18). Interestingly, an integrin-dependent translocation of PI 3-kinase to the cytoskeleton which involves specific interactions of p85α with actin filaments and focal adhesion kinase has been recently demonstrated in thrombin-stimulated platelets (53). Moreover, integrin and platelet-derived growth factor receptor regulate the association of PI 3-kinase with focal adhesion kinase in NIH3T3 cells (54). An analogous mechanism for PI 3-kinase activation via integrins in muscle cells would provide an explanation for the effect of PI 3-kinase inhibitors on myogenic differentiation.

(iii) Many reports have recently proposed a role for a mammalian PI 3-kinase in membrane trafficking. Indeed, wortmannin and/or LY294002 have been shown to disrupt GLUT1 trafficking in L6E9 myoblasts (32), to block exocytosis of IGF-II receptors in 3T3-L1 adipocytes (52), and to inhibit the sorting and transport of lysosomal enzymes (55, 56). Moreover, a mammalian homolog of the yeast PI 3-kinase vacuolar protein sorting Vps34p has been recently cloned (27). Thus, PI 3-kinase inhibitors could be blocking myogenesis by disrupting the intracellular trafficking of proteins involved in the myogenic signaling through IGFs and/or integrins although other regulatory mechanisms cannot be ruled out.

In conclusion, these data suggest that one or more PI 3-kinase isoforms might be involved in the myogenic signaling through different positive effectors. We are now attempting to define the myogenic pathway(s) that depend on PI 3-kinase activity and to identify the isoform(s) of PI 3-kinase involved in myogenesis.

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