Myogenin Expression, Cell Cycle Withdrawal, and Phenotypic Differentiation Are Temporally Separable Events that Precede Cell Fusion upon Myogenesis

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Abstract. During terminal differentiation of skeletal myoblasts, cells fuse to form postmitotic multinucleated myotubes that cannot reinitiate DNA synthesis. Here we investigated the temporal relationships among these events during in vitro differentiation of C2C12 myoblasts. Cells expressing myogenin, a marker for the entry of myoblasts into the differentiation pathway, were detected first during myogenesis, followed by the appearance of mononucleated cells expressing both myogenin and the cell cycle inhibitor p21. Although expression of both proteins was sustained in mitogen-restimulated myocytes, 5-bromodeoxyuridine incorporation experiments in serum-starved cultures revealed that myogenin-positive cells remained capable of replicating DNA. In contrast, subsequent expression of p21 in differentiating myoblasts correlated with the establishment of the postmitotic state. Later during myogenesis, postmitotic (p21-positive) mononucleated myoblasts activated the expression of the muscle structural protein myosin heavy chain, and then fused to form multinucleated myotubes. Thus, despite the asynchrony in the commitment to differentiation, skeletal myogenesis is a highly ordered process of temporally separable events that begins with myogenin expression, followed by p21 induction and cell cycle arrest, then phenotypic differentiation, and finally, cell fusion.

As in many cell lineages, proliferation and phenotypic differentiation of skeletal myoblasts are mutually exclusive events. Skeletal myogenesis can be induced in culture by depriving cycling myoblasts of serum, which then form multinucleated myotubes that are irreversibly postmitotic (20, 37). A coordinate induction of muscle-specific gene products occurs concomitantly with these morphological changes (2, 40). Members of the MyoD family of muscle-specific transcription factors (MyoD, myogenin, Myf-5, MRF4) are involved in these processes, and their ectopic expression in a variety of nonmuscle cells can promote myogenesis (7, 17, 38, 46). Although MyoD can inhibit cell cycle progression in vitro independently of differentiation (12, 43), the basis for the antagonism between cell proliferation and differentiation has remained obscure (28, 36). Recently, the reported induction of the cell cycle inhibitor p21 during myogenesis has provided insight into the mechanism that might couple cell cycle arrest and myogenic differentiation (21, 22, 39). The induction of p21 can account, at least in part, for the decrease in cyclin-dependent kinase (Cdk)[1] activity in differentiating myocytes, and its ectopic expression in myoblasts is sufficient for cell cycle arrest in mitogen-rich medium (21).

Although some aspects of muscle differentiation and cell cycle control have been elucidated, the temporal relationships among the events that govern the transition from proliferative myoblasts to the terminally differentiated multinucleated myocytes have remained controversial. Several studies have suggested that the postmitotic state is not acquired until myocytes fuse (8, 9, 15, 16, 19, 26, 31, 33–35). However, other studies support the contention that cell cycle withdrawal occurs before the expression of contractile proteins and cell fusion (3–5, 10, 18, 23, 24, 29, 30, 32, 42, 44, 50). Using molecular probes that were not available when these prior studies were performed, we show here that myoblasts induce the synthesis of myogenin before the establishment of the postmitotic state. After myogenin expression, skeletal myogenesis proceeds through a highly ordered sequence of events that begins with p21 induction and cell cycle withdrawal, followed by the expression of the contractile apparatus, and finally, cell fusion to form multinucleated myotubes.

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1. *Abbreviations used in this paper:* BrdU, 5-bromodeoxyuridine; Cdk, cyclin-dependent kinase; MHC, myosin heavy chain.

Materials and Methods

Cell Culture

C2C12 mouse skeletal myoblasts (American Type Culture Collection, Rockville, MD) were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were maintained at subconfluent densities in DME supplemented with 20% FCS (growth medium). Myogenic differentiation was induced by changing subconfluent (60–70%) cells to DME containing 2% heat-inactivated horse serum (differentiation medium).

Antibodies

The following antibodies were used in this study: rabbit polyclonal antibodies C-19 (anti-p21) and C-22 (anti-Cdk4) (Santa Cruz Biotech., Santa Cruz, CA), mouse mAb MF-20, which stains all sarcomeric myosin heavy chain (MHC) isoforms (1), and mouse mAb F5D, which reacts with recombinant myogenin but not with MyoD, Myf-5, or MRF4 (13, 48).

Immunoblot Analysis

Whole cell extracts were prepared as previously described (21). Proteins (40 μg) were separated by electrophoresis through SDS-PAGE (27) and transferred by semidry blotting to Immobilon-P membranes (0.45 μm; Millipore Corp., Bedford, MA). Membranes were blocked for 1 h with TBST (25 mM Tris-Cl, pH 8.0, 125 mM NaCl, 0.05% Tween-20) containing 4% nonfat dry milk and then incubated for 1 h with primary antibodies diluted in 2% nonfat dry milk, TBST (MF-20 and F5D, 1:10; C-19, 1:50; C-22, 1:200). After washes with TBST, membranes were incubated for 30 min with species-appropriate HRP-conjugated secondary antibodies (diluted 1:3,500; Amersham Corp., Arlington Heights, IL), washed with TBST, and finally, washed with PBS. Visualization of the immune complexes was carried out with an enhanced chemiluminescent system (Amersham Corp.).

Immunofluorescence Microscopy

For immunofluorescence analysis, C2C12 cells were seeded onto sterile glass coverslips that had been soaked for 5–10 min in 70% ethanol/0.4% HCl, rinsed extensively with water, washed in 95% ethanol, and air dried. Myogenic differentiation was induced as indicated above. Cells were washed three times with PBS before fixation and immunostaining. Unless otherwise specified, all manipulations were at room temperature, all dilutions of antibodies were prepared in 2% normal goat serum, 0.1% NP-40 (in PBS), and incubations were followed by washes with 0.1% NP-40 in PBS. Cells were fixed for 5–10 min with cold methanol (−20°C), rehydrated with PBS, blocked for 15 min in 10% normal goat serum (in PBS), and incubated for 1 h with primary antibody mixtures prepared for double immunofluorescence labeling. For p21/MHC and p21/myogenin costaining, the C-19 antipeptide rabbit polyclonal antibody was diluted 1:25 in MF-20 and F5D hybridoma tissue culture supernatants, respectively. For peptide competition, 40 μg/ml of either p21 immunogenic peptide or an unrelated peptide corresponding to amino acids 282–303 of human Cdk4 (both from Santa Cruz Biotech.) was added to the C-19/MF-20 antibody mixture 1–2 h before incubation of coverslips. After extensive washes, cells were incubated for 45 min with biotinylated anti–rabbit IgG (dilution 1:200; Amersham Corp.), followed by incubation for 45 min with a cocktail of streptavidin-Texas red (dilution 1:500; Amersham Corp.) and FITC-conjugated anti–mouse IgG (dilution 1:250; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). No cross-reactivity of the secondary antibodies was observed in control experiments in which either primary antibody was omitted (data not shown). After washes, nuclei were counterstained with 0.05 μg/ml Hoechst 33258 (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1–2 min before mounting.

For 5-bromodeoxyuridine (BrdU) incorporation experiments, asynchronously growing C2C12 cells were changed to differentiation medium and labeled for the indicated times with 10 μM BrdU (Amersham Corp.). After labeling, coverslips were processed for indirect immunofluorescence essentially as above. Briefly, cells were incubated with either C-19 (anti-p21) or F5D (anti-myogenin) antibodies, followed by species-appropriate biotinylated secondary antibodies and streptavidin-Texas red. Cellular DNA was denatured for 30 min at 37°C in 2 N HCl and 0.5% Triton X-100. After extensive washes first with 0.1 M sodium tetraborate, pH 8.5, and then with PBS, cells were stained with FITC-conjugated anti-BrdU mAb (dilution 1:20; Boehringer Mannheim Biochemicals), counterstained with Hoechst 33258, and mounted for examination.

Coverslips were mounted on glass slides (mounting medium for fluorescence, Kirkegaard and Perry Laboratories, Inc.). Specimens were examined and photographed on a Diaphot microscope (Nikon Inc., Garden City, NY) equipped with phase-contrast and epifluorescence optics (×100 lens). Pictures were recorded on Kodak Gold Plus film (Eastman Kodak Co., Rochester, NY).

Results

We first examined by indirect immunofluorescence the subcellular localization of p21 in C2C12 cells, a well-characterized murine skeletal muscle cell line. Cells were stained simultaneously for p21 (red) and MHC (green), a marker of myogenic differentiation. Although undifferentiated myoblasts revealed a low level of red fluorescent signal (Fig. 1 *a*), this signal was also detected in control experiments in which the anti-p21 antibody was omitted (data not shown), indicating that myoblasts are essentially devoid of p21. In contrast, terminally differentiated myotubes displayed a strong nuclear p21 signal (Fig. 1 *b*) that could be abrogated by preincubating the anti-p21/anti-MHC antibody mixture with the p21-immunogenic peptide, but not with an unrelated peptide (Fig. 1, *c* and *d*). In agreement with the results with C2C12 cells, p21 was also detected within the nuclei of myotubes obtained from passaged cultures of nonimmortalized diploid human skeletal muscle cells (data not shown).

Myogenin Expression and Cell Cycle Withdrawal Are Temporally Separable Events

The above results demonstrate the expression of p21 in postmitotic myotubes, consistent with the suggested role of p21 in coupling cell cycle withdrawal and myogenic differentiation (21, 22, 39). It has been previously suggested that expression of myogenin, an early marker for the entry of myoblasts into the differentiation pathway (16, 47), only occurs in postmitotic myocytes (6, 38, 49). Thus, our initial interest was to determine whether myogenin expression and cell cycle withdrawal are separable or concurrent events. To this end, C2C12 myoblasts were induced to differentiate for 19 h and then examined simultaneously for myogenin expression and BrdU incorporation into nascent genomic DNA. No multinucleated myotubes were observed in these cultures at this time point. As shown in Fig. 2, a significant fraction of myogenin^{+} cells were

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Figure 1. Expression and subcellular localization of p21 in C2C12 myotubes. Indirect immunofluorescence of C2C12 cells stained simultaneously with anti-p21 and anti-MHC antibodies. Cells were examined using a dual filter for red (p21) and green (MHC) fluorescence. Representative fields are shown (*top*, immunofluorescence; *bottom*, Hoechst 33258 nuclear staining). (*a*) Cells maintained in growth medium (*myoblasts*). Similar pattern was observed in control experiments in which the anti-p21 antibody was omitted (not shown). (*b–d*) Cells maintained for 3 d in differentiation medium (*myotubes*). For peptide competition, the anti-p21/anti-MHC antibody mixture was preincubated with an excess of p21 immunogenic peptide (*c*) or with an unrelated peptide (*d*) before incubation of cells.
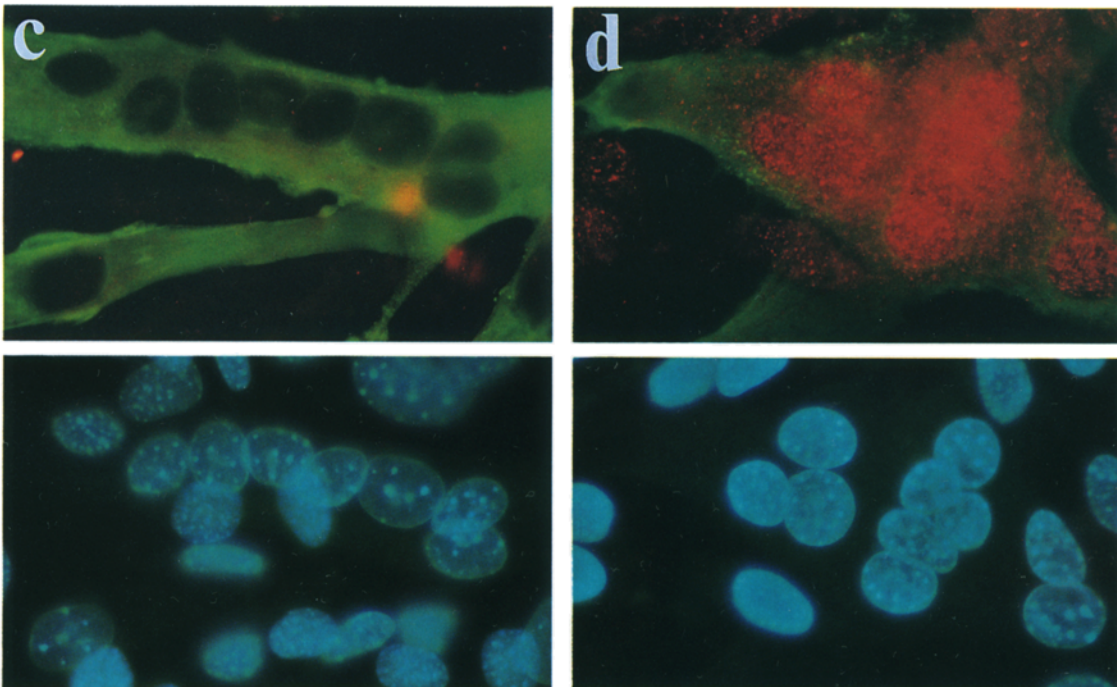
α-p21/α-MHC
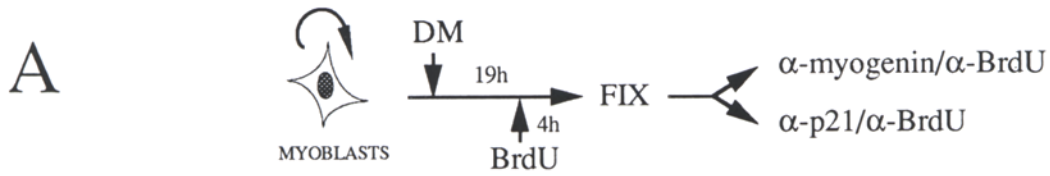
Myoblasts

Myotubes
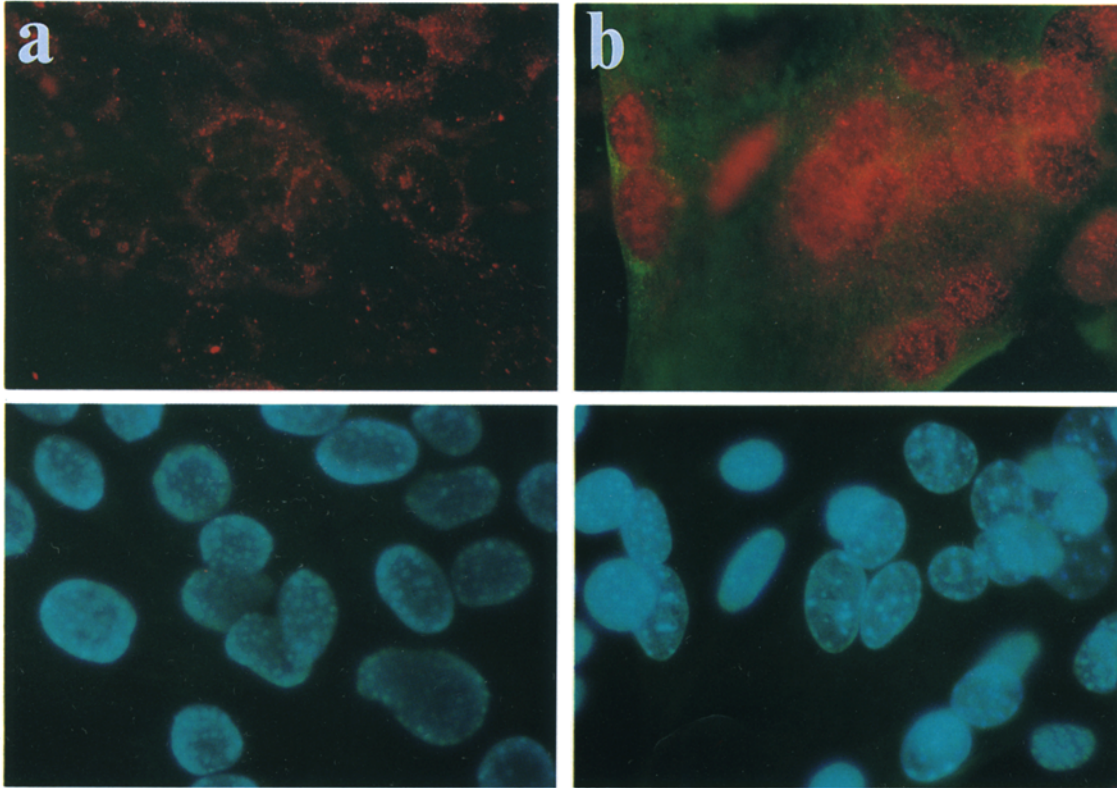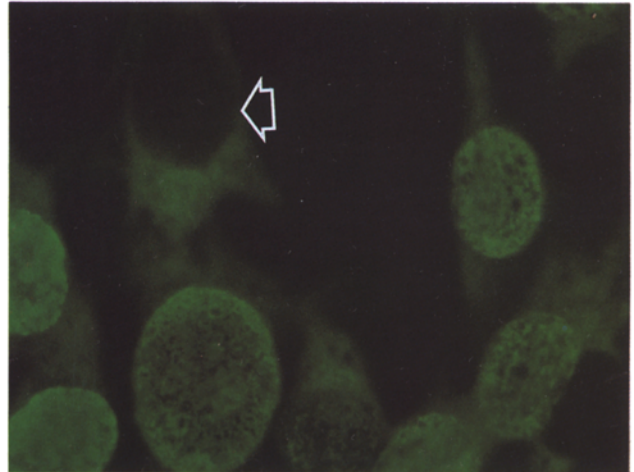
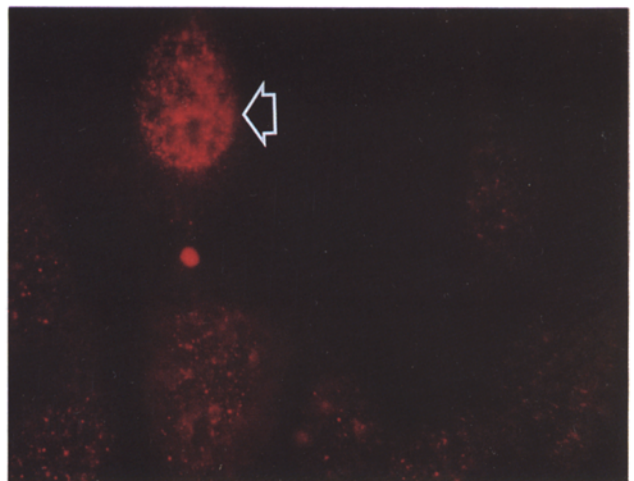## PEPTIDE COMPETITION

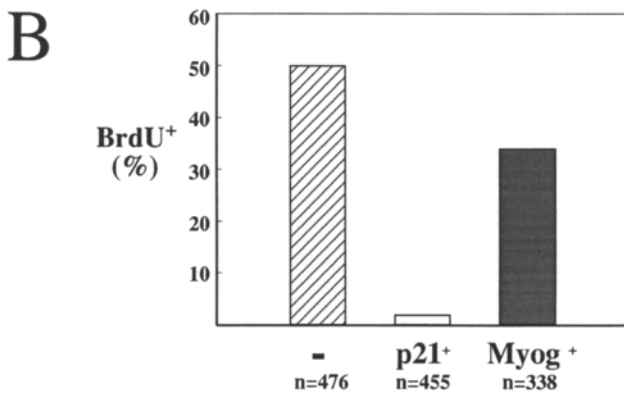**p21 Immunogenic Peptide**

Unrelated Peptide
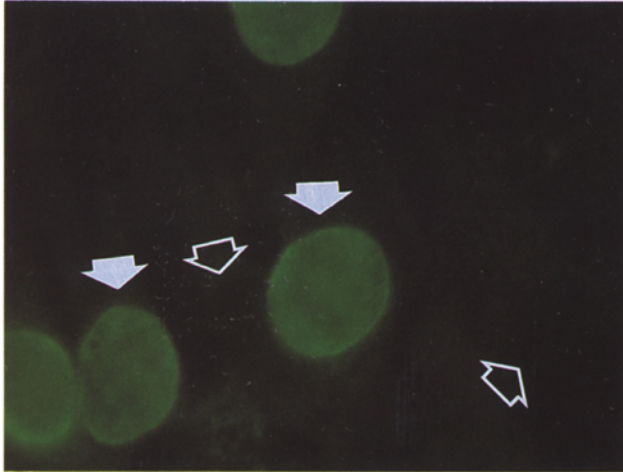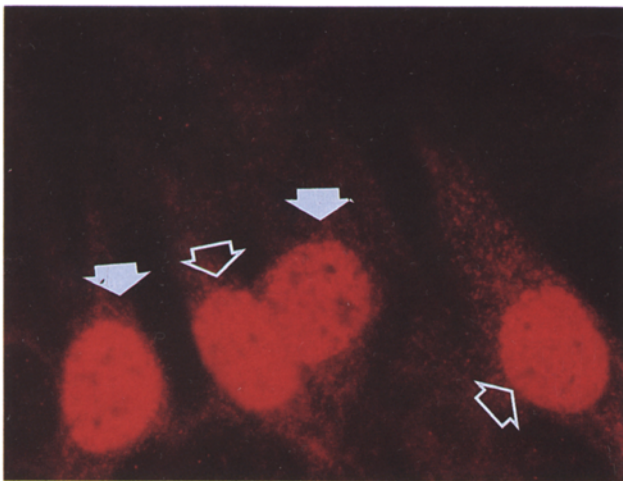
Figure 2. Expression of p21, but not myogenin, correlates with cell cycle withdrawal in differentiating myocytes. (*A*) C2C12 cells were exposed to differentiation medium (*DM*) for 19 h and labeled with 10 μM BrdU for the last 4 h before double immunostaining for BrdU (green) and either myogenin or p21 (red). Open arrowheads, myogenin$^+$/BrdU$^-$ or p21$^+$/BrdU$^-$ cells. Solid arrowheads, two myogenin$^+$/BrdU$^+$ cells. (*B*) Relative frequency of BrdU$^+$ nuclei among p21-positive cells (*p21$^+$*), myogenin-positive cells (*Myog$^+$*), or cells that did not express either p21 or myogenin (−). *n*, number of cells analyzed in each case.
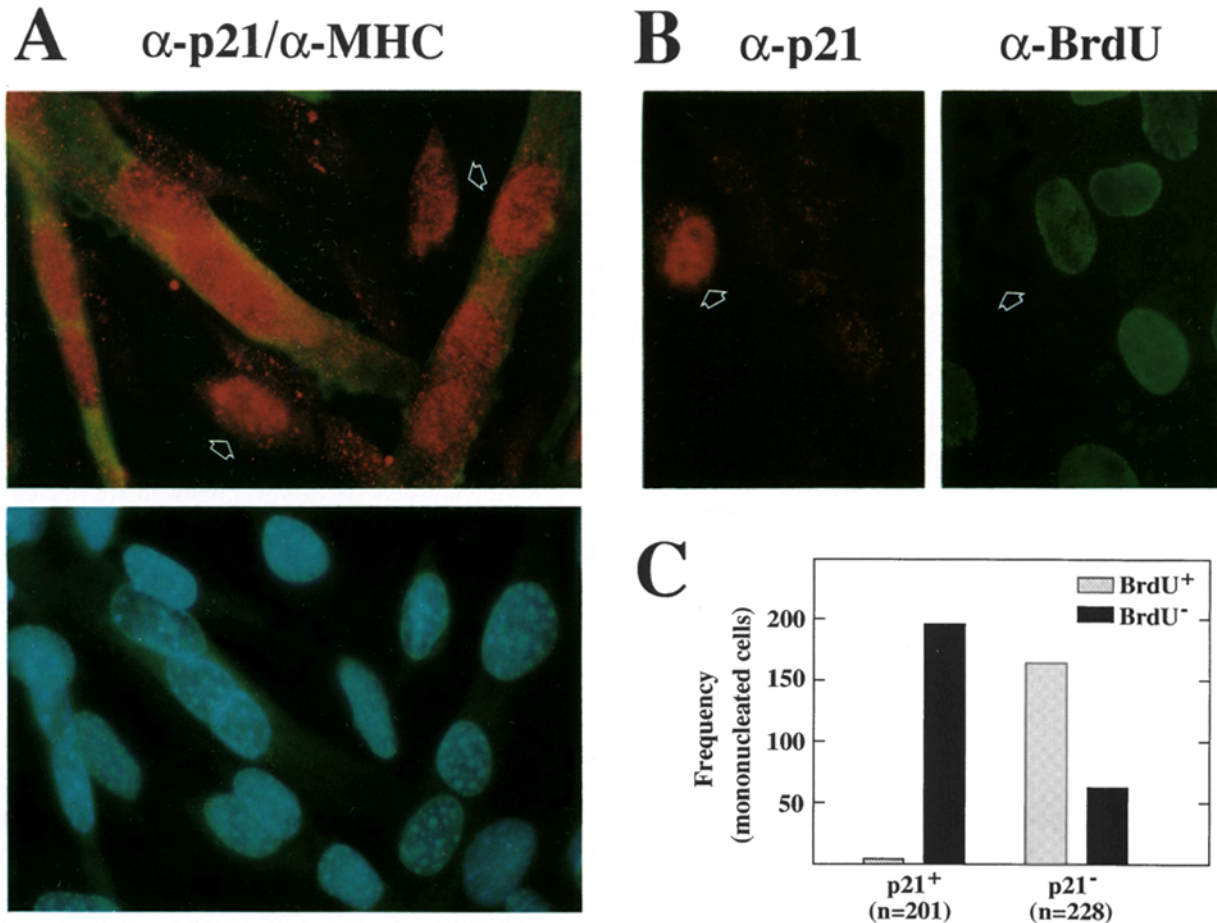
Figure 3. Maintenance of the postmitotic state in differentiating myocytes correlates with p21 expression. C2C12 cells were exposed to sustained differentiation medium for 4 d and then serum-restimulated with growth medium for 20 h (A) or 16 h (B and C). (A) Cells were stained simultaneously for p21 and MHC and examined using a dual filter for red (p21) and green (MHC) fluorescence. A representative field is shown (top, immunofluorescence; bottom, Hoechst 33258 staining). p21 is expressed in both mononucleated MHC⁻ cells (arrowheads) and syncytial MHC⁺ myotubes. (B) Cells were continuously labeled with 10 μM BrdU during serum restimulation and then double stained for p21 (red) and BrdU (green). (C) Frequency of BrdU⁻ and BrdU⁺ nuclei among mononucleated p21⁻ and p21⁺ cells. n, number of cells analyzed in each case.

BrdU⁺ (34%, $n = 338$), indicating that myoblasts expressing myogenin remain capable of replicating DNA. In striking contrast, the great majority of p21⁺ cells in these differentiating cultures were BrdU⁻ (98%, $n = 455$). These results demonstrate that expression of the cell cycle inhibitor p21, but not myogenin, correlates with the establishment of the postmitotic state in differentiating myoblasts.

Further indication that p21 expression reflects the postmitotic state was provided by analyzing cultures of serum-restimulated myocytes, which cannot reinitiate DNA synthesis in response to mitogens (11, 24, 25, 44). We reasoned

that, if p21 is involved in the maintenance of the postmitotic state, its expression would be sustained in serum-restimulated myotubes. In this study, cells were induced to differentiate for 4 d, then serum restimulated for 20 h, and double stained for p21 and MHC. Expression of p21 in these serum-restimulated cultures was detected both in mature myotubes and in MHC⁺ and MHC⁻ mononucleated cells (Fig. 3 A). In another experiment where cells were serum starved for 2 d and serum restimulated for 20 h, the frequency of p21⁺ nuclei before and after mitogen restimulation was 33% ($n = 303$) and 32% ($n = 307$), respec-

tively. These data indicate that p21 expression cannot be reversed by growth factor reexposure even in undifferentiated mononucleated myoblasts. Furthermore, p21 expression in serum-restimulated cultures correlated with the absence of DNA synthesis in all myotubes (data not shown) and in nearly all p21$^+$ mononucleated cells (98%, $n = 201$; Fig. 3, B and C). In contrast, p21$^-$ (mononucleated) cells had entered S phase at a high frequency under the same conditions (72%, $n = 228$; Fig. 3, B and C). Thus, the expression properties of p21 in mononucleated myocytes and syncytial myotubes are consistent with its possible involvement in both the maintenance as well as the establishment of the postmitotic state. Similar experiments analyzing myogenin expression after serum restimulation were also performed. The frequency of myogenin$^+$ nuclei in cultures that were serum-starved for 1 d or serum restimulated for 19 h after serum starvation was 13% ($n = 1013$) and 11% ($n = 1064$), respectively. The sustained expression of myogenin in serum restimulated myocytes indicates that it is an early marker of cells that are irreversibly committed to terminal differentiation; however, the high frequency of BrdU$^+$/myogenin$^+$ cells demonstrates that myogenin is not a marker of the postmitotic state.

Myogenin Expression, Cell Cycle Withdrawal, Phenotypic Differentiation, and Cell Fusion Are Highly Ordered Events during Skeletal Myogenesis

The ability of myogenin$^+$ myocytes to replicate DNA and the detection of postmitotic mononucleated MHC$^-$ cells in differentiating cultures provided a first indication that myocytes might withdraw from the cell cycle after induction of myogenin but before phenotypic differentiation and fusion. In view of the above data demonstrating that p21 is a marker of cell cycle withdrawal (Figs. 2 and 3), we examined by immunoblot analysis the timing of p21 induction relative to myogenin and MHC expression. As shown in Fig. 4, expression of myogenin preceded the induction of p21, which in turn preceded the expression of MHC. Whereas p21 appeared to reach maximum levels of expression by day 2 in differentiation medium, MHC steadily increased up to day 4. Although Western blot analysis revealed low levels of p21 in proliferating myoblasts, no specific nuclear signal was detected in myoblasts immunostained for p21 (Fig. 1 a), suggesting that the immunofluorescence method was unable to detect the low levels of p21 protein that occur in myoblasts. The specificity of the p21 antibody used in the immunoblot analysis was demonstrated in competition experiments with immunogenic peptide, which resulted in a decreased signal. The level of expression of the cell cycle regulator Cdk4 did not apparently change
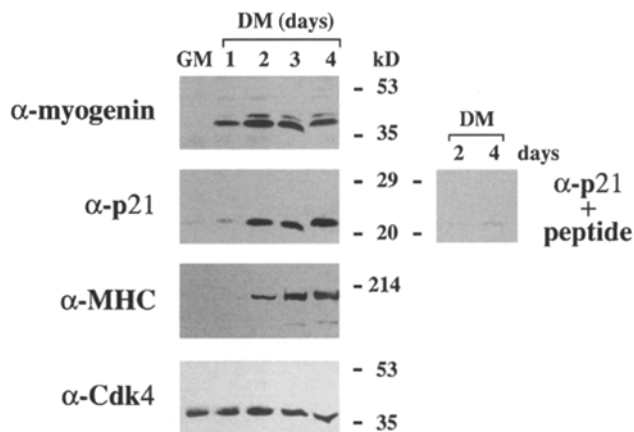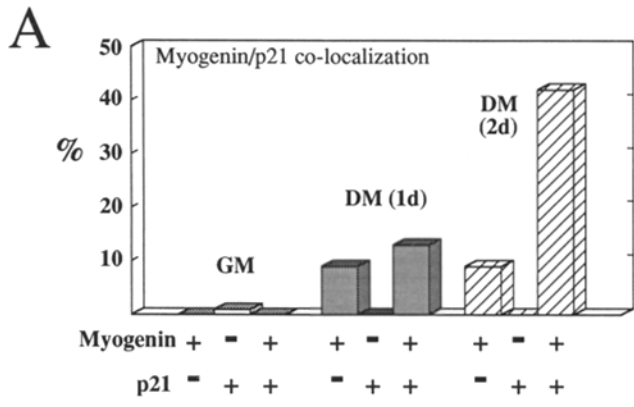

Figure 4. Time course of expression of myogenin, p21, and MHC during myogenesis. Western blot analysis on cell extracts prepared from C2C12 cells maintained in growth medium (*GM*) or after the indicated periods of time in differentiation medium (*DM*). For peptide competition (*α-p21 + peptide*), the polyclonal anti-p21 antibody was preincubated with an excess of immunogenic peptide before probing the membrane. The molecular weight of protein markers is shown (*kD*).
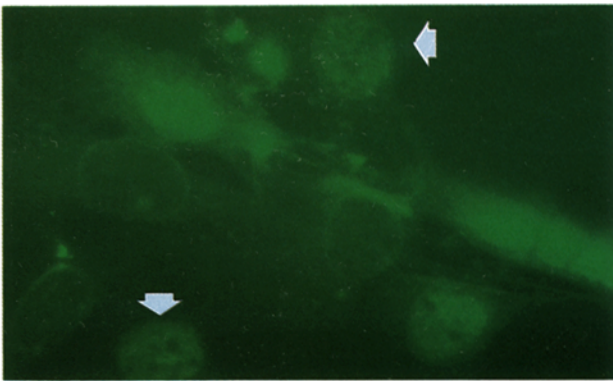
in differentiating cultures, in agreement with previous studies (41).

The above results suggest an overall order of marker expression during myogenesis; however, they do not rule out that these processes are stochastic at the level of the individual cell. Thus, to investigate on a cell-by-cell basis the temporal relationships among myogenin expression, cell cycle withdrawal, phenotypic differentiation, and cell fusion, double immunofluorescence experiments were performed on cultures of C2C12 cells at different time points after serum deprivation. To determine the temporal relationship between myogenin expression and cell cycle withdrawal, cells were double stained for myogenin and p21 (Fig. 5 A). Myogenin$^+$/p21$^-$ (mitotic) cells, as well as myogenin$^+$/p21$^+$ (postmitotic) cells were detected in these cultures. However, myogenin$^-$/p21$^+$ (postmitotic) cells were not observed in differentiating cultures, suggesting that differentiating myoblasts withdraw from the cell cycle after induction of myogenin. Consistent with these observations, the relative frequency of p21$^+$ cells among the population of myogenin$^+$ cells increased from 58 to 82% at days 1 and 2 in differentiation medium, respectively. Expression of myogenin and p21 was initially detected in mononucleated cells, and all multinucleated myotubes were myogenin$^+$/p21$^+$. Collectively, these results indicate that myocytes withdraw from the cell cycle after the onset of myogenin expression but preceding cell fusion.
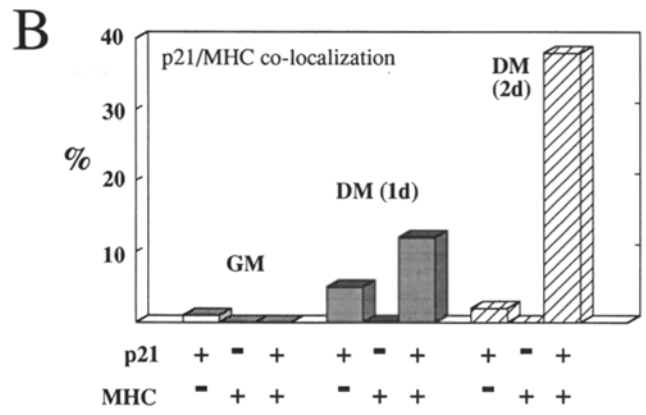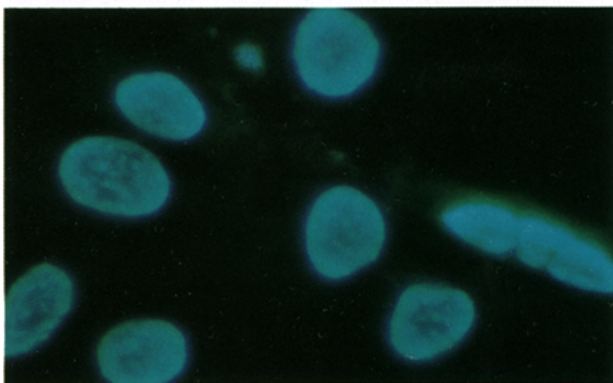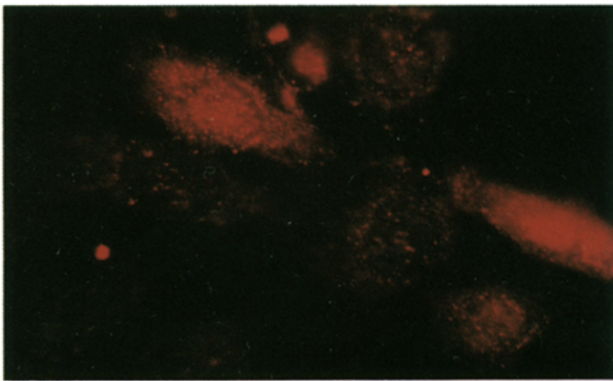
Figure 5. Ordered timing of induction of myogenin, p21, MHC, and cell fusion during myogenesis. C2C12 myoblasts were maintained in growth medium (*GM*) or exposed to differentiation medium (*DM*) for 1–2 d and then double stained for the indicated proteins. The frequency of each observed phenotype is expressed as percentage of the total number of cells analyzed at each time point. Cells negative for the two markers analyzed are not plotted. Representative fields of cells exposed to DM for 2 d are shown. (*A*) myogenin/p21 double staining. The frequency of myogenin$^-$/p21$^-$ cells and the total number of cells analyzed in GM, DM (*1 d*), and DM (*2 d*) were 99% ($n = 203$), 77% ($n = 430$), and 49% ($n = 461$), respectively. Top and middle panels, immunofluorescence; bottom, Hoechst 33258 staining. Note the presence of myogenin$^+$/p21$^-$ cells (*arrows*) and myogenin$^+$/p21$^+$ cells. (*B*) MHC/p21 double staining. The frequency of MHC$^-$/p21$^-$ cells and the total number of cells analyzed in GM, DM (*1 d*), and DM (*2 d*) were 99% ($n = 204$), 83% ($n = 453$), and 60% ($n = 406$), respectively. Top panel, p21 (red) and MHC (green) immunostaining; bottom, Hoechst 33258 staining.

A

Myogenin/p21 co-localization

	GM			DM (1d)			DM (2d)		
Myogenin	+	−	+	+	−	+	+	−	+
p21	−	+	+	−	+	+	−	+	+

α-myogenin

α-p21

B

p21/MHC co-localization

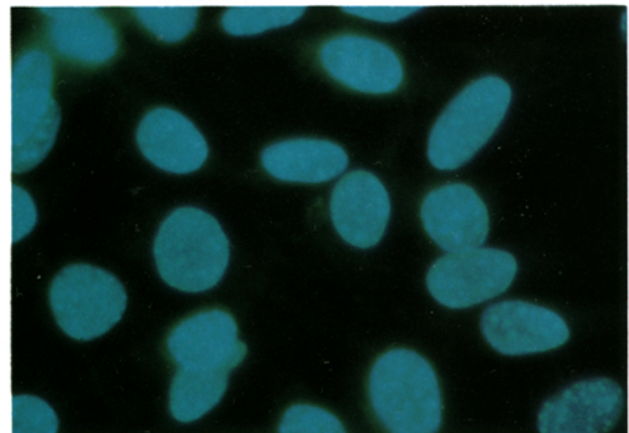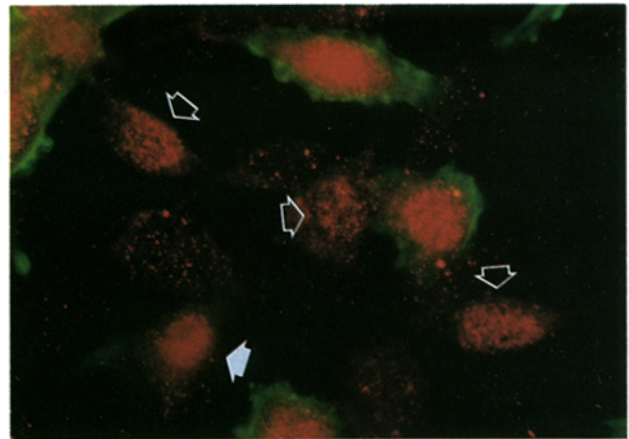|	GM			DM (1d)			DM (2d)		
p21	+	−	+	+	−	+	+	−	+
MHC	−	+	+	−	+	+	−	+	+

α-p21/α-MHC

To determine the temporal relationship between cell cycle withdrawal and phenotypic differentiation, cells were double stained for p21 and MHC (Fig. 5 *B*). Postmitotic undifferentiated cells (MHC$^-$/p21$^+$) and postmitotic differentiated cells (MHC$^+$/p21$^+$) expressing different levels of MHC were detected in differentiating cultures. However, differentiated mitotic cells (MHC$^+$/p21$^-$) were not observed, suggesting that induction of the contractile phenotype only occurs in postmitotic myocytes. Consistent with this hypothesis, the relative frequency of MHC$^+$ cells among the population of p21$^+$ cells increased from 74 to 95% at days 1 and 2 in differentiation medium, respectively. Moreover, MHC expression was first detected in mononucleated cells, and all myotubes that occurred at the later time points were MHC$^+$/p21$^+$. As shown in Table I, these double immunostaining experiments revealed that myogenin$^+$ myocytes occurred at the highest frequencies in differentiating cultures, followed by p21$^+$ and MHC$^+$ cells, in agreement with results from the immunoblot analyses (Fig. 4). Syncytial nuclei, an index of cell fusion, occurred at the lowest frequency (Table I). These observations demonstrate that skeletal myogenesis is a highly ordered process within a given cell. After the onset of terminal differentiation, as indicated by the expression of myogenin, a myoblast withdraws from the cell cycle, then phenotypically differentiates, and finally, fuses to form a syncytial myotube.

Discussion

This study demonstrates that skeletal myogenesis is a highly ordered process and elucidates the temporal relationships among the events that govern the transition from the proliferative myoblast to the terminally differentiated myocyte. Whereas the expression of myogenin is not sufficient for cell cycle exit, subsequent induction of p21 in mononucleated myogenin$^+$ myoblasts correlates both with the establishment and the maintenance of the postmitotic state. Later during myogenesis, postmitotic (p21-positive) myoblasts activate the expression of contractile proteins and then fuse into multinucleated myotubes.

Myogenin Expression and Cell Cycle Withdrawal Are Separable Events during Myogenesis

Our initial interest was to determine the temporal rela-

Table I. Number of Syncytial Nuclei and Cells Positive for Myogenin, p21, and MHC in Cultures of Undifferentiated and Differentiating C2C12 Myocytes

		Differentiation Medium	
	Growth medium	1 d	2 d
Myogenin	0% ($n = 203$)	22% ($n = 430$)	51% ($n = 461$)
p21	1% ($n = 407$)	15% ($n = 883$)	41% ($n = 867$)
MHC	0% ($n = 204$)	12% ($n = 453$)	40% ($n = 406$)
Syncytial nuclei	0% ($n = 204$)	3% ($n = 453$)	23% ($n = 406$)

The frequency of each marker was calculated from the double immunofluorescence experiments of Fig. 5. C2C12 myoblasts were maintained in growth medium or exposed to differentiation medium for 1–2 d. To determine the frequency of syncytial nuclei, an index of cell fusion, the number of nuclei contained within multinucleated MHC$^+$ myotubes was expressed relative to the total number of nuclei in the corresponding microscope field.

tionship between myogenin expression and cell cycle withdrawal during skeletal myogenesis. While MyoD and Myf-5 are thought to be involved in myoblast determination, myogenin is an early marker for the entry of myoblasts into the terminal differentiation pathway (16, 38, 45, 47). Based on immunolocalization experiments, it has been suggested that myogenin expression only occurs in postmitotic myocytes (6, 38, 49). Consistent with this notion, it has been previously reported that the induction of myogenin mRNA either coincides with or follows that of p21 during C2 myoblast differentiation (22, 39). However, our immunodetection and immunoblot analyses show that the induction of myogenin protein precedes that of p21 (see below). Although we detected both proteins within the nuclei of myotubes and differentiating mononucleated myocytes, a functional distinction can be made when mononucleated cells are examined with respect to their ability to synthesize DNA. Whereas a significant fraction of mononucleated myogenin$^+$ cells were still capable of DNA synthesis, p21-expressing mononucleated myocytes failed to incorporate BrdU under identical conditions. Further indication that p21 is a bona fide marker of the postmitotic state of myocytes was provided by analyzing cultures of serum-restimulated differentiating myocytes. In these cultures, expression of p21 was maintained in both myotubes and mononucleated myocytes, and its expression correlated with the absence of new DNA synthesis under conditions where p21$^-$ cells were actively replicating DNA. These results indicate that myogenin expression, which precedes p21 induction, is not sufficient to promote cell cycle arrest. In contrast, subsequent expression of p21 in myogenin$^+$ myoblasts correlates with the establishment as well as the maintenance of the postmitotic state.

Skeletal Myogenesis Is a Highly Ordered Process

Prior studies have suggested that cell fusion is a prerequisite for the establishment and maintenance of the postmitotic state (8, 9, 15, 16, 19, 26, 31, 33–35). However, other investigators have suggested that cell cycle withdrawal occurs before phenotypic differentiation (3–5, 10, 18, 23, 24, 29, 30, 32, 42, 44, 50). The present study demonstrates not only that skeletal myogenesis is a highly ordered process, but that there exists a striking distinction between myogenin expression and cell cycle withdrawal and phenotypic differentiation. Based on these observations, we propose the ordered model of skeletal myogenesis schematized in Fig. 6. After growth factor removal, proliferating myoblasts asynchronously activate the expression of myogenin yet remain capable of synthesizing DNA. Subsequently, myogenin$^+$ myoblasts become postmitotic, mononucleated cells that express p21. These postmitotic cells then initiate the synthesis of contractile proteins and finally fuse into multinucleated myotubes. Several lines of evidence lend support for this model. First, the induction of myogenin preceded cell cycle arrest and p21 induction, which in turn preceded that of MHC (phenotypic differentiation); second, myogenin$^-$/p21$^+$ and MHC$^+$/p21$^-$ cells were not observed in differentiating cultures; third, all syncytial myotubes expressed myogenin, p21, and MHC; finally, the relative frequencies of myogenin$^+$/p21$^+$ versus myogenin$^+$/p21$^-$ and MHC$^+$/p21$^+$ versus MHC$^-$/p21$^+$
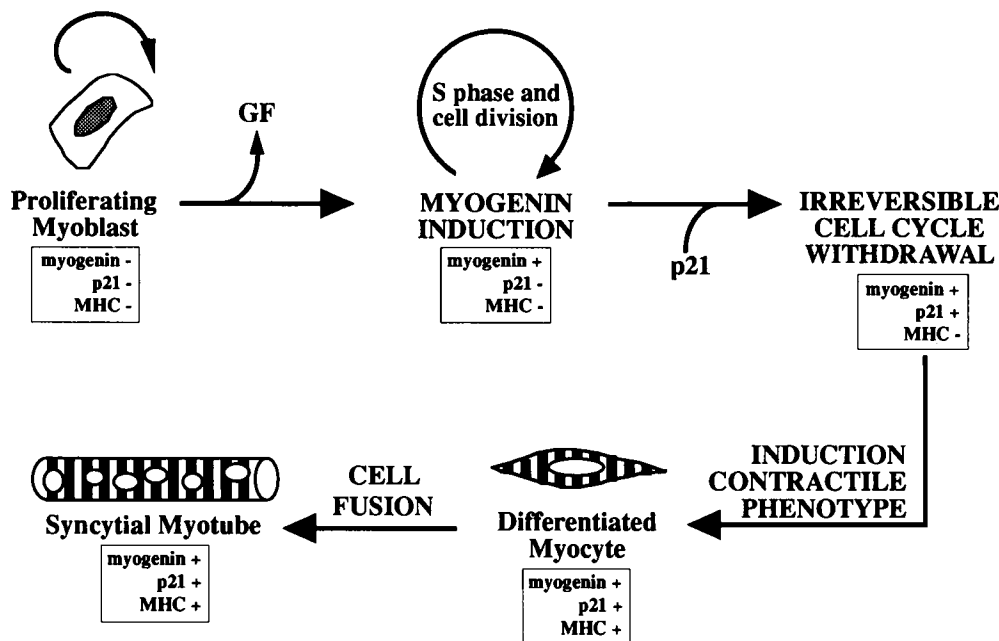
Figure 6. Model for the myogenic differentiation of C2C12 cells. This model shows the temporal relationship between myogenin expression and the subsequent myogenic events, but does not imply any causal relationship. The phenotype associated with each of the stages of myoblasts and differentiating myocytes is boxed. Myoblasts asynchronously induce myogenin expression after removal of growth factors yet remain capable of replicating DNA. Subsequently, myogenin⁺ myocytes withdraw from the cell cycle, then phenotypically differentiate, and finally fuse into syncytial myotubes (see text for details).

cells progressively increased during myogenesis. Taken together, these observations strongly suggest that differentiating C2C12 myocytes induce myogenin before the induction of p21 and cell cycle withdrawal, and then undergo phenotypic differentiation and cell fusion. In this regard, it has been suggested previously that myoblasts exposed to differentiation medium enter a latent period during which cells remain ambivalent toward proliferation or differentiation (10, 30, 32). This latent period might correspond to the gap between myogenin expression and p21 expression.

In summary, here it is shown on a cell-by-cell basis that in vitro myogenesis involves at least four temporally separable events: (1) entry of myoblasts into the differentiation pathway, as indicated by the induction of myogenin; (2) irreversible cell cycle withdrawal, as indicated by the expression of p21; (3) phenotypic differentiation, as indicated by the induction of MHC; and (4) cell fusion. Despite a strict temporal order of expression, direct causal relationships among myogenin, p21, and MHC are unlikely to exist. For example, presumptive myoblasts in *myogenin(−/−)* knockout mice express p21 mRNA and withdraw from the cell cycle normally (39). Moreover, mice lacking p21 appear to develop normally (14), indicating that p21 expression is not essential for phenotypic differentiation and cell cycle withdrawal. These observations suggest that functionally redundant and/or independent regulatory circuits control the highly ordered myogenic program. The present study provides a temporal framework that will permit further dissection of the regulatory networks that couple myogenin expression, cell cycle withdrawal, and phenotypic differentiation during skeletal myogenesis.

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