A single cdk inhibitor, p27\textsuperscript{Xic1}, functions beyond cell cycle regulation to promote muscle differentiation in \textit{Xenopus}

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Acceptor 28 September 2002

SUMMARY

The molecular basis of the antagonism between cellular proliferation and differentiation is poorly understood. We have investigated the role of the cyclin-dependent kinase inhibitor p27\textsuperscript{Xic1} in the co-ordination of cell cycle exit and differentiation during early myogenesis in vivo using \textit{Xenopus} embryos. In this report, we demonstrate that p27\textsuperscript{Xic1} is highly expressed in the developing myotome, that ablation of p27\textsuperscript{Xic1} protein prevents muscle differentiation and that p27\textsuperscript{Xic1} synergizes with the transcription factor MyoD to promote muscle differentiation. Furthermore, the ability of p27\textsuperscript{Xic1} to promote myogenesis resides in an N-terminal domain and is separable from its cell cycle regulation function. This data demonstrates that a single cyclin-dependent kinase inhibitor, p27\textsuperscript{Xic1}, controls in vivo muscle differentiation in \textit{Xenopus} and that regulation of this process by p27\textsuperscript{Xic1} requires activities beyond cell cycle inhibition.

Key words: Cell cycle, Cdk inhibitor, Muscle, Xenopus

INTRODUCTION

The decision to divide or differentiate is determined by a fine balance of opposing developmental signals. During differentiation, multi-potential cells initiate genetic programs that commit them to progressively restricted lineages. In muscle, the cascade of myogenic helix-loop-helix (mHLH) proteins, which comprises MyoD, Myf5, myogenin and MRF4, signals cells to undergo myogenic commitment, to express muscle-structural genes and, finally, to become functional myofibers. To terminally differentiate, cells must not only receive the appropriate differentiation cues, but must also exit the cell cycle. While the molecules and mechanisms involved in early differentiation of muscle are relatively well understood, the link between promoters of terminal differentiation and regulators of cell cycle arrest has been extensively studied primarily in tissue culture systems.

Cell cycle arrest can be mediated through the inactivation of cyclin-dependent kinases (cdks) by cdk inhibitors (cdkis), of which there are two families. The p16\textsuperscript{Ink4a} family (p15\textsuperscript{Ink4b}, p16\textsuperscript{Ink4a}, p18\textsuperscript{Ink4c} and p19\textsuperscript{Ink4d}) specifically inhibits cdk4 and cdk6, while the p21\textsuperscript{Cip1} family (p21\textsuperscript{Cip1}, p57\textsuperscript{Kip2} and p27\textsuperscript{Kip1}) inhibits all cdks involved in the G1/S transition (reviewed by Sherr and Roberts, 1999). In myogenic tissue culture systems, the link between proliferation and differentiation has been proposed to be via MyoD and p21\textsuperscript{Cip1}. Upon serum withdrawal, 10T1/2 cells transfected with MyoD can transcriptionally upregulate p21\textsuperscript{Cip1} expression, causing cell cycle arrest and subsequent myotube fusion (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995).

This straightforward in vitro model has not been supported by the creation of mice with homozygous deletions for p21\textsuperscript{Cip1} (Deng et al., 1995). Mice that lack p21\textsuperscript{Cip1} develop normally, with no signs of defective muscle differentiation (Deng et al., 1995). Additionally, p21\textsuperscript{Cip1} expression in myogenic cells of mice lacking the genes encoding MyoD and myogenin is normal, indicating that p27\textsuperscript{Xic1} expression does not require these mHLH factors (Parker et al., 1995; Sabourin et al., 1999). Nevertheless, mice lacking both p21\textsuperscript{Cip1} and a second cdki, p57\textsuperscript{Kip2}, fail to form myotubes and display increased proliferation and apoptosis of myoblasts, demonstrating that p21\textsuperscript{Cip1} and p57\textsuperscript{Kip2} redundantly control differentiation of mouse skeletal muscle (Zhang et al., 1999). Therefore, while confirming their importance and providing useful insight into their in vivo roles during muscle differentiation, redundancy makes information about the mode of action of cdkis from knockout mouse models difficult to interpret. In contrast to the redundancy observed in cdkis in mice, there is only one known cdki in \textit{Xenopus}, p27\textsuperscript{Xic1}, and it exhibits structural and functional characteristics of all three p21\textsuperscript{Cip1} family members (Su et al., 1995; Shou and Dunphy, 1996).

Recent reports have shown that p27\textsuperscript{Xic1}, p21\textsuperscript{Cip1} and p57\textsuperscript{Kip2} have a role in neural/glial cell fate determination that is distinct and separable from their regulation of the cell cycle (Ohnuma et al., 1999; Dyer and Cepko, 2000; Zezula et al., 2001). Previous work has demonstrated that the capacity of p27\textsuperscript{Xic1} to bias committed neuroblasts towards a glial fate lies in a distinct region of the N terminus of the molecule but is independent of cell cycle exit and overall cdk2 kinase inhibition (Ohnuma et al., 1999). These findings indicate the possibility of a
differentiation function for p27Xic1 in myogenesis, which is distinct from its regulation of the cell cycle.

We show that p27Xic1 is first expressed in the embryonic myotome at stage 11, after MyoD expression, but prior to muscle structural gene expression. Its early myotomal expression makes p27Xic1 a prime candidate molecule for coordinating cell cycle exit and myogenic differentiation. Consequently, we have used the many advantages of the Xenopus system to study the role of cdkis during in vivo muscle differentiation and cell cycle exit during the early stages of embryogenesis.

In this report we demonstrate that, whereas initial expression of MyoD is uniform, cell cycle exit in the myotome occurs in a wave from the front to the back of the embryo. Although MyoD does not cause cell cycle arrest by upregulating p27Xic1 in vivo, p27Xic1 does synergize with MyoD to promote muscle differentiation. Furthermore, the ability of p27Xic1 to promote myogenesis is separable from its role in regulating the cell cycle. Finally, we provide evidence that p27Xic1 is not needed for myogenic commitment, but is required for differentiation of muscle in Xenopus.

MATERIALS AND METHODS

Xenopus embryos, fixation and β-galactosidase staining

Xenopus laevis embryos obtained by hormone induced laying were in vitro fertilized, dejellied in 2% cysteine pH 7.8-8.0, washed and incubated in 0.1x MBS. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994), fixed and stained for β-galactosidase (200-300 pg injected per embryo) as described (Sive et al., 2000).

mRNA injection and morpholino antisense oligo procedures

Capped RNAs were synthesized in vitro from nucleotides (Chitnis et al., 1995), MyoDb, MyoD-enR (Wittenberger et al., 1999), p27Xic1 (Su et al., 1995), p27Xic1 NT, p27Xic1 CT, p27Xic1 NT 35-96 (Ohnuma et al., 1999), p21Cip1 (Harper et al., 1993), using the SP6 Message Machine kit (Ambion). Embryos were injected in 0.2x MBS supplemented with 6% Ficoll.

The base composition of the p27Xic1 antisense morpholino oligodeoxynucleotide is 5'-GCAAGGGCGATGTTGAAAACGAGCCA-T-3' (Gene Tools LLC). The control morpholino is a random sequence of the same length.

Explants and RT/PCR

Animal caps were dissected at stage 8 and ventral marginal zones were dissected at stage 10. Explants were incubated in 0.7xMBS until collection. RNA was isolated using RNAzolB (Tel-Test), oligo-dT primed and reverse transcribed into cDNA using standard methods. RT-PCR primers were calibrated to yield linear results that directly correlate template abundance and PCR products (Steinbach et al., 1998). The PCR primers used were: p27Xic1, 5'-GTGGGACCCCC-TCTTAAGGGC-3' (forward) and 5'-TTCAGTGGGCAACTAGGT-3' (reverse); MA (Stutz and Spohr, 1986), MHC, 5'-TTCCAGCTAGTCTAAAC-3' (forward) and 5'-TCTGTTGCGATGCTCTCC-3' (reverse) and ODC (Agris et al., 2000).

Immunohistochemistry

Monoclonal anti-sarcomeric actin (5C5) (Sigma) applied at a dilution of 1:500 for 2 hours at room temperature was recognized with either a goat anti-mouse IgM cy3 (1:800) (Jackson Immunoresearch) or a goat anti-mouse IgM-AP, using NBT/BCIP as color substrates. Tissue culture supernatant from the hybridoma D7F2 (anti-MyoD) was applied at a dilution of 1:4, recognized with a sheep anti-mouse Ig-AP secondary (1:150) (Jackson Immunoresearch) and amplified using an anti-AP antibody (APAAP complex, 1:50, Serotec). NBT/BCIP were used as color substrates. BrdU incorporation and detection was performed essentially as described in the Boehringer Mannheim instructions for the 5-bromo-2'-deoxy-uridine Labeling and Detection kit 1 (1296 736). Embryos were injected with 1 nmol of BrdU 2 hours before MEMFA fixation. Anti-BrdU (1:10) was applied to 10 μM parafin wax embedded sections for 30 minutes at 37°C, washed and detected with an anti-mouse Ig-fluorescein (1:100) for 30 minutes at 37°C.

Whole mount in situ hybridization, antibody staining and TUNEL assay

Whole-mount in situ hybridization was performed as described (Shimamura et al., 1994). Linearized plasmid from MyoD (HindIII/T7), MA (HindIII/T7), MHC (NcoI/Sp6) and p27Xic1 (BamHI/T7) was used to generate digoxigenin-11-UTP-labeled (Boehringer Mannheim) antisense RNA probes from the polymerases indicated. Double in situ hybridization was performed as described (Sive et al., 2000). BM Purple, NBT/BCIP and BCIP (Roche) were used as substrates.

Whole-mount antibody staining was performed as described (Sive et al., 2000) using an anti-phospho-histone H3 (TCS Biologicals) at 1:500 or an anti-muscle ATP-ase 12/101 (DSHB) at 1:400, and detected with an alkaline phosphatase-conjugated secondary using NBT/BCIP as substrates.

TUNEL staining was performed as described (Hensey and Gautier, 1998).

Western blotting

Protein extracts were prepared as described (Philpott and Friend, 1994). Total protein was separated by SDS-PAGE and western blotted to nitrocellulose by standard methods. p27Xic1 was detected using a polyclonal antibody (Ohnuma et al., 1999). Antibody binding was detected using the Pierce SuperSignal chemiluminescence detection system. Blots were stripped (Chemicon International) and probed with anti-β-tubulin antibody (1:400) (Santa Cruz Biotechnology).

RESULTS

Pattern of cell cycle exit is directly related to myogenic differentiation

The highly ordered events in the process of skeletal myogenesis can be temporally separated in cultured cells (Andres and Walsh, 1996). The onset of C2C12 myoblast differentiation is signified by myogenin expression, followed by p21Cip1 induction, cell cycle arrest, and, finally, cell fusion (Andres and Walsh, 1996). In Xenopus, a temporal cascade of the mHh transcription factors MyoD, Myf5, and later, MRF4 regulate the progression of uncommitted, proliferating cells to determined myoblasts that exit the cell cycle and terminally differentiate into myotubes (Hopwood et al., 1989; Hopwood et al., 1991; Jennings, 1992). Myogenin is expressed only in the adult skeletal muscle of Xenopus (Jennings, 1992; Nicolas et al., 1998; Charbonnier et al., 2002). The initial broad and ubiquitous expression of MyoD is restricted to committed muscle cells (Harvey, 1990; Scales et al., 1990; Harvey, 1991; Rupp and Weintraub, 1991) shortly after the mid-blastula transition (MBT), when zygotic transcription begins (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). MyoD protein synthesis is synchronous across the future somite (Fig.
p27Xic1 localizes to the presomitic mesoderm (Fig. 2B, arrow), band of presumptive mesoderm (Keller, 2000). By stage 11, from the area above the blastopore, which corresponds to the (Fig. 2A). Importantly, its expression is specifically excluded transcriptionally upregulate p21Cip1 (Guo et al., 1995; Halevy that, in these systems, MyoD can drive cell cycle exit and differentiation. However, evidence from tissue culture cells suggests that, despite equal levels of MyoD protein, cells exit the cell cycle first in the front of the embryo and thus differentiate earlier than those in the rear of the embryo. We investigated this possibility by injecting BrdU into the blastocoel of stage 12-12.5 embryos and allowing them to develop for 2 hours until they reached mid-neural plate stage (stage 15), the stage at which muscle structural genes are first expressed (Gurdon et al., 1997). Longitudinal sections of these embryos were stained for the early myogenic marker, muscle actin (MA), and for BrdU, a marker of S-phase cells. As expected at this stage, MA is intensely expressed in the front of the embryo, but is virtually absent from the rear, reflecting the anteroposterior wave of differentiation (Fig. 1B) (Harvey, 1992). We observed that while cells in the differentiated anterior region do not stain for BrdU, many BrdU-positive cells are located in the rear of the myotome (Fig. 1B, arrows). Thus, anterior muscle cells exit the cell cycle before cells in the posterior of the embryo, confirming that proliferation and differentiation are mutually exclusive events and that temporospatially regulated cell cycle exit is likely to contribute to the front-to-back wave of muscle differentiation.

p27Xic1 and MyoD expression in the early embryo

Data presented in Fig. 1 indicates that MyoD expression alone is insufficient to cause cell cycle exit and differentiation in the myotome. However, evidence from tissue culture cells suggests that, in these systems, MyoD can drive cell cycle exit and transcriptionally upregulate p21Cip1 (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). Because of these data, we wished to determine whether MyoD expression temporally and spatially overlaps with that of p27Xic1 during early Xenopus development.

Using whole-mount in situ hybridization, we found that p27Xic1 is first detected throughout the animal pole at stage 10 (Fig. 2A). Importantly, its expression is specifically excluded from the area above the blastopore, which corresponds to the band of presumptive mesoderm (Keller, 2000). By stage 11, p27Xic1 localizes to the presomitic mesoderm (Fig. 2B, arrow), and is also found in the developing notochord and forming primary neurons. Additionally, p27Xic1 is transiently expressed in the epidermis between stages 10.5 and 15, demonstrated at stage 13 in Fig. 2L. At mid-neural plate stage (stage 15), p27Xic1 expression in the myotome is greatly intensified (Fig. 2C), obscuring staining in the medial and intermediate neural stripes. However, p27Xic1 is still visible in the lateral stripe, placodal regions (Fig. 2C, arrows) and notochord. By early tailbud stages (stage 22), p27Xic1 is expressed most strongly in the muscle, in a gradient with the most intense staining in the posterior myotome (Fig. 2D) (Ohnuma et al., 1999). By stage 26, p27Xic1 is diminished in the differentiated anterior myotome, but is still prominent in the posterior muscle, as well as being expressed in the eye and brain (Fig. 2I) (Ohnuma et al., 1999).

We then compared expression of p27Xic1 with that of MyoD. Early gastrula stage embryos (stage 10-11) show substantial differences between MyoD and p27Xic1 expression (compare Fig. 2A,B with 2E,F). While MyoD localizes to mesodermal precursors above the invaginating blastopore lip (Fig. 2E) (Hopwood et al., 1992), p27Xic1 expression is strikingly excluded from this region, with strong staining only in the animal pole ectoderm (Fig. 2A). At later gastrula stages, in contrast to p27Xic1, MyoD is found in both the lateral and ventral marginal zones in a horseshoe-like pattern around the blastopore (Fig. 2F), and is excluded from the dorsal region corresponding to the Spemann organizer and forming notochord (Fig. 2F, asterisk) (Frank and Harland, 1991). However, expression of MyoD in the ventral marginal zone, containing cells fated for non-somatic lineages, such as lateral plate mesoderm and blood (Keller, 1975; Keller, 1976; Dale and Slack, 1987a; Keller, 1991), is transient and is insufficient to convert these cells to muscle (Frank and Harland, 1991). During late gastrulation (stage 13), p27Xic1 is only detected in the anterior subset of MyoD-expressing cells in the lateral marginal zone that are destined to a somitic fate (Fig. 2K), but at stage 15, p27Xic1 and MyoD are both expressed throughout the entire myotome (Fig. 2C,G). Although MyoD expression is fairly uniform throughout the myotome at stage 22 (Fig. 2H), p27Xic1 is expressed in a gradient with the most intense staining in the rear of the embryo (Fig. 2D). By stage 26, MyoD is also reduced anteriorly and is expressed in an anterior to posterior gradient (Fig. 2J) similar to that of p27Xic1 (Fig. 2I).

p27Xic1 protein is also observed in the developing myotome at stage 15, using a p27Xic1-specific antibody (Fig. 2M). This staining appears to be more intense in the anterior than the
posterior of the myotome, mirroring the wave of cell cycle exit and differentiation in this tissue (Fig. 1B). Co-staining bisected embryos with DNA-specific Hoechst and p27Xic1 demonstrates that p27 Xic1 protein is nuclear (Fig. 2N,O), similar to MyoD protein at this stage (Rupp et al., 1994).

Thus, the early expression of p27Xic1 and MyoD is markedly different, suggesting that MyoD may not transcriptionally regulate the onset of p27Xic1 expression (compare Fig. 2A with 2E). However, MyoD and p27Xic1 do share regions of overlap in cells committed to a somitic fate beginning at stage 11 (Fig. 2B,F), and p27Xic1 is highly expressed in the myotome at stage 15 (Fig. 2C,M) when overt signs of muscle differentiation are first detected (Gurdon et al., 1997). Therefore, p27Xic1 is appropriately temporally and spatially expressed to function during myogenesis and could be regulated by MyoD later in development. Moreover, the subsequent downregulation of MyoD and p27Xic1 in terminally differentiated muscle indicates that although both may be important in the initiation of muscle differentiation, they are not required for its maintenance.

**MyoD does not upregulate p27Xic1**

Data from tissue culture systems suggests that MyoD can transcriptionally upregulate the cdki, p21Cip1, causing cell cycle arrest and differentiation upon serum withdrawal (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). The conspicuous differences in the initial expression patterns of MyoD and p27Xic1 revealed by our in situ hybridization experiments indicate that MyoD may not be regulating p27Xic1 at the earliest stages. Later, however, both MyoD and p27Xic1 are very highly expressed in the embryonic myotome during muscle differentiation. By analogy with cell culture, we wanted to determine whether MyoD could upregulate transcription of the *Xenopus* cdki, p27Xic1, during in vivo development.

To investigate this question, first we used ectodermal explants (animal caps) isolated from late blastula stage embryos, which normally differentiate into ciliated epidermis (Chang and Hemmati-Brivanlou, 1998). However, overexpression of MyoD can induce MA expression in this tissue (Hopwood and Gurdon, 1990). To determine whether MyoD could upregulate transcription of the Xenopus cdki, p27Xic1, during in vivo development.

![Fig. 2.](image-url) p27Xic1 is expressed in cells destined to a somitic fate. Embryos were analyzed by whole-mount in situ hybridization at the indicated stages for the expression of p27Xic1 (A-D,1L) and MyoD (E-H,J).

(A) p27Xic1 expression in the animal pole at stage 10. Lateral view with dorsal towards the left, asterisk marks the involuting dorsal lip. (B) p27Xic1 localizes to the presomitic mesoderm at stage 11 (arrow). Dorsal view, anterior is downwards. (C) Stage 15 embryo with p27Xic1 in the myotome, notochord, primary neurons and anterior placodes (arrows). Dorsal view with anterior downwards. (D) By stage 22, p27Xic1 is downregulated in the more mature, anterior somites. Lateral view, anterior left. (E) Lateral view of an embryo stained for MyoD at stage 10 with dorsal leftwards and animal pole upwards. V, ventral marginal zone; L, lateral marginal zone; asterisk, involuting dorsal lip. (F) MyoD is expressed in a horseshoe around the blastopore at stage 11. Dorsal view,
in both injected and uninjected caps, and so may still be required for MyoD-induced myogenesis.

To confirm that MyoD alone cannot upregulate p27Xic1 in vivo, we injected 100 pg of MyoD into one cell of two-cell stage embryos, along with β-gal as a tracer (light blue, injected side to left) and allowed to develop until stage 15 (A-D) or 22 (E-H). Dorsal views with anterior downwards (midline indicated by broken white line) demonstrate that phospho-histone-H3 staining (purple) is reduced after injection of all four p27Xic1 constructs (A-D). Transverse sections stained with an antibody against muscle actin (dark blue) indicate that full-length p27Xic1 (A) and p27Xic1 NT (B) can increase the size of the myotome (arrows), while p27Xic1 CT (C) and p27Xic1 35-96 (D) have no effect.

Although MyoD alone does not upregulate p27Xic1, it may be responsible for its maintenance. To investigate this possibility, we injected a construct of MyoD fused to the repressor domain of the Drosophila Engrailed protein (DNMyoD) (Wittenberger et al., 1999), along with β-gal, into one cell of two-cell stage embryos and assayed for MA and p27Xic1 expression by whole-mount in situ hybridization (Fig. 3D,E). Seventy-eight percent of embryos exhibited region staining for p27Xic1 (data not shown, 33% of embryos, n=39). However, this upregulated expression is contiguous with the domain where p27Xic1 is normally expressed and does not correspond with the ventral areas of ectopic MA expression that can be induced by high-level MyoD overexpression (data not shown).

Fig. 3. MyoD is unable to upregulate expression of p27Xic1 in vivo. (A) Quantitative RT-PCR for MA, p27Xic1 and ODC on cDNA from animal caps of embryos injected with 100 pg MyoD. (B-F) Embryos were injected with 100 pg MyoD (B,C,F,F’) or 50 pg DNMyoD (D,E) into one cell of two-cell stage embryos, along with β-gal as a tracer (light blue, injected side towards the left in B-E or upwards in F,F’). Dorsal views of embryos analyzed for (B,D) MA or (C,E) p27Xic1 expression by whole-mount in situ hybridization at stage 15 (F,F’). (F,F’) MyoD-injected embryos allowed to develop to stage 21 were stained for phospho-histone-H3 then longitudinally sectioned and stained for MA (red) and Hoechst (blue, DNA specific). MyoD overexpression enlarges the area staining for MA (F) and also causes extra proliferation (F’, arrows).

Fig. 4. p27Xic1 can enlarge the myotome independently of its ability to arrest the cell cycle. One cell of two-cell stage embryos were injected with (A,E) 45 pg p27Xic1, (B,F) 15 pg p27Xic1 NT, (C,G) 50 pg p27Xic1 CT or (D,H) 50 pg p27Xic1 35-96 and β-gal as a tracer (light blue, injected side to left) and allowed to develop until stage 15 (A-D) or 22 (E-H). Dorsal views with anterior downwards (midline indicated by broken white line) demonstrate that phospho-histone-H3 staining (purple) is reduced after injection of all four p27Xic1 constructs (A-D). Transverse sections stained with an antibody against muscle actin (dark blue) indicate that full-length p27Xic1 (A) and p27Xic1 NT (B) can increase the size of the myotome (arrows), while p27Xic1 CT (C) and p27Xic1 35-96 (D) have no effect.
downregulated MA (n=131), while 62% of embryos had decreased p27Xic1 (n=85). Thus, loss of MyoD activity leads to a decrease in p27Xic1 expression and a concomitant loss in muscle differentiation, indicating that MyoD may be required for p27Xic1 maintenance.

Knockout mouse studies demonstrate that multiple cdkis can redundantly control myogenic differentiation. Mice homozygous for a deletion in p21Cip1 have no obvious defect in myogenesis (Deng et al., 1995; Brugarolas et al., 1998). p57Kip2 knockout mice have altered cell proliferation in several tissues, again with no gross myogenic defect (Yan et al., 1997; Zhang et al., 1997). However, mice lacking both p21Cip1 and p57Kip2 experience complete failure in muscle differentiation (Zhang et al., 1999). Therefore, MyoD could perhaps be acting through an unidentified cdki to bring about cell cycle arrest in *Xenopus*. To examine this possibility, we tested the ability of injected MyoD to induce cell cycle arrest. We injected MyoD into one cell of two-cell stage embryos and allowed them to develop until stage 21. These embryos were then stained for phospho-histone-H3 (ph3), a marker of mitosis (Saka and Smith, 2001), longitudinally sectioned and stained for MA. The MyoD-injected side of the embryo displayed a dorsoskeletal bulge, comprising an enlarged myotome and, in addition, a large area between the differentiated myotome and the epidermis that does not stain for muscle or neural markers (Fig. 3F, data not shown) (Ludolph et al., 1994). Strikingly, there were more ph3-positive cells on the injected side than the uninjected side of the embryo, indicating that MyoD overexpression can promote proliferation under some circumstances (100% of embryos, n=9) (Fig. 3F, arrows), and making it unlikely that it directly upregulates an unknown cdki. However, note that no ph3-positive cells are found in the expanded muscle (Fig. 3F), indicating that MyoD does not substantially extend the proliferative period of this tissue.

### p27Xic1 overexpression enlarges the myotome

Does p27Xic1 have a role in myogenesis? MyoD can apparently induce ectopic MA expression without upregulating p27Xic1 in animal caps and in whole embryos (Fig. 3A-C). To investigate directly whether p27Xic1 expression can promote myogenesis, we overexpressed p27Xic1, along with β-gal, in one cell of two-cell-stage embryos, allowed them to develop until stage 22 and stained transverse sections for MA (Fig. 4E). Injecting between cell-stage embryos, allowed them to develop until stage 22 and we overexpressed p27 Xic1, along with p27 Kip1 and p57 Kip2. The C terminus contains both a cdk/cyclin binding motif similar to that found in p21Cip1, p27Kip1 and p57Kip2. The C terminus of p27Xic1 is unable to induce Müller glia cells. ph3 staining demonstrates by inhibition of cdks via its N terminus or through DNA replication inhibition via its C terminus (Su et al., 1995).

**Fig. 5.** p27Xic1 synergizes with MyoD to promote muscle differentiation. (A) Embryos were injected in both cells of two-cell stage embryos with MyoD (100 pg), p27Xic1 (45 pg), p27Xic1 NT (15 pg), p27Xic1 CT (50 pg), p27Xic1 35-96 (50 pg) or a combination thereof. Ventral marginal zones dissected from injected embryos at stage 10 were allowed to develop until parallel embryos reached stage 19 and analyzed by RT-PCR for expression of muscle actin (MA), myosin heavy chain (MHC) and ornithine decarboxylase (ODC) as an internal control. (B) Quantification of the synergistic upregulation of MA by MyoD and p27Xic1 mutant overexpression in ventral marginal zones. The relative induction of MA was determined by measuring the incorporated [32P]dATP and normalizing to ODC. The standard error of the mean was obtained by performing this typical experiment in triplicate.
that overexpressing similar doses of these three p27Xic1 mutants downregulates proliferation in the myotomal region, but does not cause excessive apoptosis or death (NT 62% decrease; CT 60% decrease; 35-96 49% decrease of ph3-expressing cells, n=10) (Fig. 4B-D). After developing to stage 22, injected embryos were transversely sectioned and stained for MA (Fig. 4F-H). While p27Xic1 NT was able to expand significantly the region staining for MA (injected side 1.46±0.016 times larger than un.injected side), neither p27Xic1 CT nor p27Xic1 35-96 had any significant effect (injected sides were 1.02±0.0 and 1.07±0.024 times larger than un injected side, respectively) (Fig. 4F-H). Therefore, cell cycle inhibition by the CT and 35-96 is insufficient for myotomal expansion, demonstrating that the N terminal differentiation domain of p27Xic1 is required for this function.

**p27Xic1 and MyoD act together to promote myogenic differentiation**

The increase in myotome size caused by overexpression of p27Xic1 and p27Xic1 NT was only observed within and adjacent to the region where embryonic muscle normally forms (i.e. ectopic myogenesis in lateral or ventral regions was not observed). Several reports indicate that muscle creatine kinase transcription is enhanced by co-expression of the cdki, p21Cip1 and MyoD in 10T1/2 cells (Skapek et al., 1995; Guo and Walsh, 1997; Reynaud et al., 1999). These findings, combined with our overexpression results, led us to hypothesize that p27Xic1 must be working to promote muscle differentiation within the population of cells that already express a threshold level of myogenic factors such as MyoD. To test this hypothesis more quantitatively, we injected both cells of two-cell stage embryos with p27Xic1 Mo and MyoD in 10T1/2 cells (Skapek et al., 1995; Guo and Walsh, 1997; Reynaud et al., 1999). These findings, combined with our overexpression results, led us to hypothesize that p27Xic1 must be working to promote muscle differentiation within the population of cells that already express a threshold level of myogenic factors such as MyoD. To test this hypothesis more quantitatively, we injected both cells of two-cell stage embryos with MyoD alone or in conjunction with full-length p27Xic1, p27Xic1 NT, p27Xic1 CT or p27Xic1 35-96. At stage 10, ventral marginal zone explants (VMZs) were dissected and allowed to develop until parallel explants reached stage 19. Quantitative RT-PCR was performed using primers for MA with ODC as a loading control (Fig. 5). Ventral marginal zone tissue is not specified at stage 10 and develops into non-somitic ventral structures in isolation (Dale and Slack, 1987b), but we find that MyoD overexpression will induce MA expression in this tissue.

When overexpressed alone, neither full-length p27Xic1 nor any of the p27Xic1 mutants induces MA expression in VMZs (Fig. 5A, lanes 3-6). MyoD alone induces minimal MA expression, and this is unchanged by co-expression of p27Xic1 CT or p27Xic1 35-96 (Fig. 5A, lanes 2, 9 and 10). However, when MyoD+full-length p27Xic1 or MyoD+p27Xic1 NT are injected together, a much greater upregulation of MA expression is observed (Fig. 5A, lanes 7 and 8) (3.32 times and 2.39 times more, respectively). This synergy between MyoD and full-length p27Xic1 or p27Xic1 NT (but not between MyoD and p27Xic1 CT or p27Xic1 35-96) was also seen in animal caps and dorsal marginal zone explants tested at various stages (data not shown). Thus, MyoD and p27Xic1 synergize to promote muscle differentiation. Furthermore, the synergistic property of p27Xic1 resides in the N-terminal differentiation domain and, therefore, this property can be distinguished from the ability of p27Xic1 to inhibit overall cdk2 kinase activity and arrest the cell cycle.

Although able to induce the early muscle differentiation marker MA, MyoD is unable to promote terminal differentiation of muscle both in animal caps and in whole embryos (data not shown) (Hopwood and Gurdon, 1990), suggesting a requirement for complementary regulatory factors. As cells committed to the myogenic lineage must withdraw from the cell cycle to differentiate, and mHLH factors alone do not induce cell cycle withdrawal at physiological concentrations in vivo (Davis et al., 1987; Tapscott et al., 1988; Braun et al., 1989; Olson, 1992), we hypothesized that co-expression of MyoD and p27Xic1 might promote expression of terminal muscle markers. Therefore, we used primers for the terminally differentiated muscle marker, myosin heavy chain (MHC), and performed RT-PCR on the VMZs co-injected with MyoD, p27Xic1 and the p27Xic1 mutants described above. However, none of the combinations of MyoD, p27Xic1 or p27Xic1 mutants was able to induce expression of MHC in the VMZs (Fig. 5) or in whole embryos, as assayed by in situ hybridization (data not shown). MyoD, p27Xic1 and the p27Xic1 mutants were also unable to upregulate expression of the late muscle marker 12/101 in VMZs at stage 19 (data not shown). Thus, although MyoD and p27Xic1 promote the early stages of muscle differentiation, additional factors must be present or active for these explants to terminally differentiate.

**p27Xic1 is required for myogenic differentiation**

Overexpression of p27Xic1 causes a modest increase in the size of the embryonic myotome. The ability of p27Xic1 to enlarge the myotome and to synergize with MyoD is separable from its ability to arrest the cell cycle. However, we wanted to know whether p27Xic1 was required for myogenesis or whether it simply promoted differentiation of cells already committed to a myogenic fate when overexpressed. To address this question, we used antisense morpholino oligonucleotides which bind over the translation initiation site of RNA and prevent accumulation of the targeted protein (Heasman et al., 2000). The production of p27Xic1 protein was completely inhibited by injection of p27Xic1 morpholino (p27Xic1 Mo), while injection of a control morpholino (Con Mo) had no effect (Fig. 6A).

To determine if p27Xic1 is required for myogenesis and, if so, where in the myogenic pathway it acts, we injected 20ng of either Con Mo or p27Xic1 Mo, along with β-gal as a tracer, into one cell of two-cell stage embryos and performed in situ hybridization for MyoD, Myf5, MA and MHC at stage 15 (Fig. 6). Interestingly, loss of p27Xic1 had no effect on the expression of MyoD (93% of embryos, n=136) (Fig. 6B) or Myf5 (86% of embryos, n=66) (Fig. 6C). However, MA expression was greatly reduced (69% of embryos, n=111) (Fig. 6D) and the terminal differentiation marker, MHC, was almost entirely absent (96% of embryos, n=100) (Fig. 6F). Furthermore, whole-mount antibody staining revealed significantly reduced expression of the muscle ATPase, 12/101 (75% of embryos, n=24) (Fig. 6H,1). Injection of the Con Mo had no effect on any of the markers tested (Fig. 6E and data not shown). Ohnuma et al. (Ohnuma et al., 1999) have previously demonstrated that p21Cip1 can substitute for p27Xic1 in Müller glial cell induction in the retina. The loss of myogenic differentiation caused by injection of p27Xic1 Mo is specific to loss of cdki function because it can be rescued by co-injection with p21Cip1 (Fig. 6G). While 96% of embryos injected with p27Xic1 Mo significantly downregulated MHC staining (Fig. 6F), 84% of embryos co-injected with p27Xic1 Mo and p21Cip1...
had nearly normal MHC expression (n=149) (Fig. 6G). This data indicates that although p27Xic1 is not required for myogenic determination (i.e. MyoD and Myf5 expression), it is absolutely required for muscle differentiation.

One predicted phenotype resulting from preventing translation of a cdki required for muscle differentiation is increased myotomal proliferation. To test this hypothesis, we injected 20 ng of either Con Mo or p27Xic1 Mo into one cell of two-cell stage embryos, allowed them to develop until stage 21 and stained for ph3. These embryos were then longitudinally sectioned and stained with an antibody against MA (Fig. 7). Injection of the Con Mo had no effect on proliferation or MA expression (100% of embryos, n=7) (Fig. 7A,A’). However, in accordance with the data from our whole-mount in situ hybridization, loss of p27Xic1 led to a dramatic decrease in MA expression (100% of embryos, n=11) (Fig. 7B). These embryos also had ph3-positive cells within the area that would normally stain for MA and an increase in the number of dividing cells throughout the mesenchyme and epidermis (82% of embryos, n=11) (Fig. 7B’, arrows).

Cell cycle exit is a prerequisite for muscle differentiation. Therefore, we wished to determine whether the reduced muscle phenotype observed upon loss of p27Xic1 is the result of a failure to undergo cell cycle arrest, or whether a separate p27Xic1 function is required for muscle differentiation. To address this question, we treated p27 Xic1 Mo- or Con Mo-injected embryos with hydroxyurea and aphidicolin (HUA), reagents that arrest cells at the G1/S phase transition of the cell cycle. Even after HUA treatment, p27Xic1 Mo-injected embryos failed to develop normal, differentiated muscle (Fig. 6H) when compared with both the uninjected side of the embryo (Fig. 6I) and the Con Mo-injected controls (data not shown). Therefore, in agreement with our overexpression data (Fig. 4), p27Xic1 is required for myogenesis independent of its ability to arrest the cell cycle.

What is the fate of the myocytes that fail to differentiate in the absence of p27 Xic1? When deprived of mitogenic stimulation, a large proportion of differentiating myocytes undergo programmed cell death (Wang and Walsh, 1996). However, differentiated myotubes remain viable in low-serum culture for more than 2 weeks (Wang and Walsh, 1996). The acquisition of this apoptosis-resistant phenotype correlates with the induction of p21Cip1, and ectopic expression of p21Cip1 confers apoptotic protection to myocytes (Wang and Walsh, 1996). To determine whether loss of the cdki p27Xic1 resulted in excessive programmed cell death in vivo, embryos were injected in one of two cells with 20 ng Con Mo or p27Xic1 Mo and allowed to develop until stage 15. Injected embryos were analyzed for apoptotic cells by whole-mount TUNEL assay staining (Hensey and Gautier, 1998). Overall, Con Mo-injected embryos demonstrated very few apoptotic cells and there was no observable difference between the injected and uninjected sides of the embryo (no change in 100% of embryos, n=31) (Fig. 6J). However, the number of apoptotic cells increased upon injection of p27Xic1 Mo (47% of embryos, n=30) (Fig. 6K, representative embryo). Despite the increase in apoptotic figures observed upon injection of p27Xic1 Mo, the decrease in myogenic differentiation cannot be attributed entirely to extensive programmed cell death of the prospective muscle cells because MyoD and Myf5 appear unaffected by the absence of p27Xic1 (Fig. 6B,C). These data demonstrate that although loss of p27Xic1 increases the incidence of apoptosis, a significant proportion of the affected cells must either remain undifferentiated myotomal cells or adopt alternative differentiation fates.

Embryos injected with 20 ng of p27Xic1 Mo were allowed...
to develop until stage 26 and longitudinally sectioned to
determine whether the downregulation of MA expression
persisted and what effect this had on embryonic development.
At this stage, p27Xic1 translation is still inhibited by injection
of the p27Xic1 Mo (data not shown). The p27Xic1 Mo-injected
embryos are bent, possibly because of the failure to develop
muscle (Kopan et al., 1994). The injected side of these embryos
completely fails to form somitic structures, has little or no MA
expression and, indeed, appears to have more cells (Fig.
7C,C'). Thus, loss of p27Xic1 leads to a total failure in muscle
formation, somitogenesis and differentiation that persists at
least until late tailbud stages.

DISCUSSION

The coordinate regulation of the cell cycle with myogenic
differentiation has most extensively been studied using cell
culture techniques (Guo et al., 1995; Halevy et al., 1995; Parker
et al., 1995). These systems are somewhat artificial, though, as
an exceptionally high level of MyoD expression is needed to
inhibit the cell cycle and serum withdrawal is required to
induce differentiation (Crescenzi et al., 1990; Sorrentino et al.,
1990; Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995).
Mice with homozygous deletions for a number of cdkis have
also been employed to investigate the importance of these
molecules in the process of myogenesis in vivo (Deng et al.,
1995; Kiyokawa et al., 1996; Nakayama et al., 1996; Yan et
al., 1997; Zhang et al., 1997; Brugarolas et al., 1998; Fero et
al., 1998; Zhang et al., 1998; Zhang et al., 1999). However,
despite all three mammalian p21Cip1 family members being
highly expressed in differentiating skeletal muscle (Polyak et
al., 1994; Matsuoka et al., 1995; Parker et al., 1995; Nakayama
et al., 1996; Yan et al., 1997; Zabludoff et al., 1998), their
individual importance in the process of myogenesis is difficult
to determine because of a high level of redundancy. Therefore,
we have examined the interactions between myogenic
determination factors and the cell cycle in vivo using Xenopus
laevis as a model organism. This highly accessible, readily
manipulable system is attractive because several myogenic
genes have been cloned, the timing of differentiation has been
defined, and there is little apparent redundancy as Xenopus
has only one known cdki, p27Xic1. We present here clear in vivo
evidence demonstrating the importance of this single cdki in
coordinating the processes of cell cycle exit and differentiation.

Our whole-mount in situ hybridization revealed that p27Xic1
is highly expressed in the developing myotome, making it a
prime candidate molecule in the coordination of cell cycle exit
and differentiation (Fig. 2). During early development, p27Xic1
and the mHLH transcription factor, MyoD, are expressed in
substantially different embryonic regions (Fig. 2A,E). However,
when muscle differentiation begins, the mesodermal
staining patterns of p27Xic1 and MyoD are indistinguishable
(Fig. 2C,G) and both p27Xic1 and MyoD protein localize in the
nuclei of differentiating muscle cells (Fig. 2M-O) (Rupp et al.,
1994). The dynamic temporal and spatial expression of p27Xic1
is consistent with a role in the maintenance and promotion of
the myogenic differentiation program in synergy with MyoD.

Data from tissue culture systems have suggested that MyoD
can transcriptionally upregulate expression of the cdki, p21Cip1
and, upon serum withdrawal, induce myogenic differentiation

Fig. 7. Ablation of p27Xic1 causes excess proliferation and loss of
differentiated muscle. Embryos were injected in one cell of two-cell
stage embryos with (A,A') 10 ng Con Mo or (B-C') 10 ng p27Xic1
Mo and β-gal (light blue in A',B') (injected side upwards) and
allowed to develop until stage 21 (A-B') or stage 26 (C,C'). Embryos
were stained for phospho-histone-H3 (purple in A',B'),
longitudinally sectioned and stained with an antibody against muscle
actin (MA) (red in A,B,C'). DNA is stained with Hoechst (blue,
A,B,C).

(Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995).
However, the in vivo relevance of this finding is questionable,
as cells cultured from MyoD-null mice do not show altered
p21Cip1 expression (Parker et al., 1995; Sabourin et al., 1999).
We have directly investigated whether MyoD can regulate
expression of the Xenopus cdki, p27Xic1, in both in vivo and in
embryonic tissue explants (Fig. 3). When overexpressed at
levels capable of upregulating MA expression, MyoD alone, or
in combination with Myf5, is unable to upregulate p27Xic1
expression (Fig. 3 and data not shown). Thus, MyoD
overexpression can induce myogenesis laterally and ventrally
without inducing p27Xic1. However, p27Xic1 is expressed in the
ectoderm outside the neural plate between stages 10.5-15 (Fig.
2L), and is therefore available to synergize with injected MyoD
to promote muscle differentiation in these areas. Moreover,
MyoD is unlikely to be acting through an unidentified cdki to
arrest the cell cycle and initiate muscle differentiation, because
when MyoD is overexpressed at a level capable of enlarging
the myotome, we observed an upregulation of the mitotic
marker ph3 (Fig. 3F')

Interestingly, the increase in ph3 staining upon MyoD
overexpression was not seen in the myotome, but rather in the
epidermis and in the mesenchymal tissue between the skin and
the myotome (Fig. 3F), Ludolph et al. (Ludolph et al., 1994) have previously reported that MyoD overexpression enlarged the myotome even in the absence of post-gastrulation cell division. However, the effect of blocking proliferation on the dorsoanterior mass, enlarged brain and epidermal thickening induced by MyoD overexpression was not discussed (Ludolph et al., 1994). Therefore, although the enlarged myotome may be due to increased myoblast recruitment, the mesenchymal mass may be attributable to enhanced proliferation. In any case, our results indicate that cell cycle exit in the myotome is unlikely to result from MyoD-mediated upregulation of p27Xic1 or another undiscovered cdki.

MyoD is thought to be involved in several regulatory feedback loops (Thayer et al., 1989; Steinbach et al., 1998). Therefore, we wanted to determine whether MyoD is necessary for p27Xic1 expression, even though it appears to be insufficient to initiate its transcription. Injection of a DNMyoD construct inhibited expression of both MA and p27Xic1, indicating that MyoD may be necessary for maintenance of p27Xic1 (Fig. 3D,E). However, p27Xic1 may be upregulated by an unidentified bHLH factor that requires the activating partner, E12 (Rashbass et al., 1992). Therefore overexpression of DNMyoD could be inhibiting both MA and p27Xic1 by binding up this molecule. Additionally, as DNMyoD reduces the size of the myotome, the observed effect on p27Xic1, which is highly expressed in the myotome, may be indirect.

p27Xic1 is highly expressed in the differentiating myotome, consistent with an essential role in myogenesis. Using an antisense morpholino oligonucleotide directed at p27Xic1, we found that p27Xic1 is absolutely required for myogenic differentiation (Fig. 6). Inhibiting expression of the p27Xic1 protein had no effect on the expression of the determination markers MyoD and Myf5 (Fig. 6B,C), but significantly downregulated MA and 12/101 expression (Fig. 6D,H,I) and completely inhibited expression of the terminal muscle marker, MHC (Fig. 6F). These data indicate that p27Xic1 acts downstream of myogenic commitment, but prior to terminal differentiation. The block in myogenic differentiation caused by loss of p27Xic1 is specific as it can be rescued by injection of p21Cip1 (Fig. 6G). Embryos allowed to develop until stage 26 still exhibited near total loss of somitic muscle formation. In our experience, translation of p27Xic1 is inhibited by injection of p27Xic1 Mo to at least stage 32 (data not shown). An interesting question to investigate would be whether the ability to differentiate is recovered upon loss of the p27Xic1 Mo and re-accumulation of p27Xic1 protein or whether the window of competence for muscle differentiation has been lost.

In some instances, myogenic repression is a consequence of de-regulated growth control, while in others it is independent of cell proliferation (reviewed by Olson, 1992). Longitudinal sections of embryos injected with p27Xic1 Mo and stained for both ph3 and MA revealed an upregulation in mitotic cells and a downregulation of differentiation (Fig. 7B,B'). To investigate whether this failure to exit the cell cycle was responsible for the lack of differentiated muscle seen in the absence of p27Xic1 protein, we blocked cell division in p27Xic1 Mo-injected embryos by incubating them in HUA. Even when cells were arrested, they failed to differentiate into muscle without functional p27Xic1 (Fig. 6H,I). Therefore, we propose that, in vivo, a combination of cell cycle exit failure and lack of the N-terminal function of p27Xic1 contributes to the loss of myogenic differentiation observed upon loss of p27Xic1. Although p27Xic1 is likely to be crucial for proper cell cycle regulation, it plays a separate distinct role in promoting differentiation, as described below.

We have shown that p27Xic1 overexpression blocks cell proliferation, actively promotes myogenesis and enlarges the size of the myotome, suggesting that p27Xic1 is a limiting factor during muscle differentiation (Fig. 4). Xenopus embryonic cells do not grow before independent feeding stages, but rather subdivide existing tissue. Therefore, the p27Xic1-mediated increase in myotome size is probably due to enhanced recruitment into the muscle lineage rather than an increase in cell number or enhanced muscle cell growth. This hypothesis is supported by the finding that co-injection of MyoD and p27Xic1 enhances the expression of the early muscle structural gene, MA, in ventral marginal zones and in whole embryos (Fig. 5; data not shown). However, neither VMZs nor whole embryos co-injected with MyoD and p27Xic1 upregulated expression of the terminal differentiation markers MHC or 12/101 (Fig. 5; data not shown). These data also indicate that although p27Xic1 can promote early myogenic differentiation in synergy with MyoD, further regulatory molecules are required to promote the full differentiation program.

Most interestingly, the ability of p27Xic1 to promote myogenic differentiation is complementary to, but separable from, its ability to inhibit the cell cycle. Recent studies have revealed that cdks can act as dual-function molecules that participate in the regulation of both the cell cycle and differentiation (Ohnuma et al., 1999; Dyer and Cepko, 2000; Zezula et al., 2001). However, these analyses have been performed in determined neural systems such as the PC12 neural cell line (Erhardt and Pittman, 1998), oligodendrocytes (Zezula et al., 2001) or the retina (Ohnuma et al., 1999; Dyer and Cepko, 2000). As the processes of myogenesis and neurogenesis are highly analogous (reviewed by Jan and Jan, 1993; Relaix and Buckingham, 1999), we wanted to extend these observations and investigate whether a similar differentiation role for cdks existed during myogenesis in the...
early embryo. We have shown that overexpression of full-length p27Xic1 and p27Xic1 NT can enlarge the size of the myotome, but p27Xic1 CT and p27Xic1 NT 35-96, although still capable of cell cycle inhibition, cannot (Fig. 4). As a more quantitative method of analysis, we chose to examine this phenomenon further in ventral marginal zone explants (VMZs). MyoD overexpression alone induces minimal MA expression that is unchanged by co-expression with p27Xic1 CT or p27Xic1 NT 35-96 (Fig. 5). However, co-expression of MyoD with p27Xic1 FL or p27Xic1 NT greatly enhances expression of MA over that of MyoD alone, demonstrating that the ability of p27Xic1 to synergize with MyoD is distinct from its ability to inhibit overall cdk2 kinase activity or to arrest the cell cycle and encompasses an N-terminal region upstream of amino acid 35 (Fig. 5).

p27Xic1 overexpression increases the size of the myotome and synergizes with MyoD to promote muscle differentiation. Moreover, p27Xic1 is required in parallel with, or downstream of, MyoD and Myf5 for muscle formation, and is, therefore, well placed to play a dual role in myogenesis. In Fig. 8, we propose that, to differentiate into myocytes, cells must express a threshold level of MyoD and p27Xic1, as well as exit the cell cycle. p27Xic1 clearly performs two functions: cell cycle arrest and promotion of differentiation via its N terminus. Both functions are essential for development to occur normally. The existence of a single molecule that is responsible for both of these crucial functions, allows elegant, coordinated control of division and differentiation.

Reynaud et al. (Reynaud et al., 1999) have demonstrated that p57Kip2 can stabilize MyoD protein and this ability is dependent upon the N terminus of the molecule. Although MyoD overexpression has been shown to induce growth arrest in a number of cell lines, myoblasts are able to proliferate despite its expression (Davis et al., 1987; Tapscott et al., 1988; Olson, 1992), indicating that its activity must be regulated in proliferating myoblasts. Several methods of inhibitory regulation of MyoD have been suggested, including modulation by binding partners such as Id, phosphorylation and either direct or indirect inhibition by the cyclin D-dependent kinases (Benezra et al., 1990; Jen et al., 1992; Rao et al., 1994; Skapek et al., 1995; Song et al., 1998; Kitzmann et al., 1999). The ability of p57Kip2 to extend the half-life of MyoD protein was originally postulated to be due to its inhibition of cdk-dependent phosphorylation of MyoD (Reynaud et al., 1999). However, the activity and stability of a non-phosphorylatable form of MyoD (MyoDAla200) is also enhanced by co-expression of p57Kip2 (Reynaud et al., 2000). Further data from this report implicates an N-terminal helix domain in p57Kip2 in direct binding with the basic domain of MyoD and masking potential degradation signals (Abu Hatoum et al., 1998). Although our initial experiments do not demonstrate direct binding between an overexpressed Myc-tagged MyoD and p27Xic1, nor do we see p27Xic1-mediated stabilization of overexpressed MyoD, how p27Xic1 is able to synergize with MyoD to promote myogenesis is an important issue. Future investigations into the ability of p27Xic1 to stabilize MyoD either by inhibition of phosphorylation by cyclins/cdkks, regulation of nuclear localization, alteration of transcriptional activity through regulation of DNA binding or a combination of these mechanisms may prove interesting. Such data may reveal exciting paradigms for how cdkis might regulate cell fate determination and differentiation in several developmental contexts.

We thank Shinichi Ohnuma and Ralph Rupp for generously supplying plasmids, Michael Zuber for supplying the MHC primers, Phil Jones for support with statistical analysis and William Harris for helpful discussions. The anti-p27Xic1 antibody was a very generous gift from Dr Rence Yew. This work was funded by the Cancer Research Campaign (Grant SP2476/0101) and A. E. V. is supported by a Howard Hughes Medical Institute Predoctoral Fellowship.

REFERENCES


Guo, K. and Walsh, K. (1997). Inhibition of myogenesis by multiple cyclin-
Cdk complexes. Coordinate regulation of myogenesis and cell cycle activity at the level of E2F. J. Biol. Chem. 272, 791-797.


