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Minireview

Regulation of MyoD function in the dividing myoblast

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Abstract Proliferating myoblasts express MyoD, yet no phenotypic markers are activated as long as mitogen levels are sufficient to keep the cells dividing. Depending upon mitogen levels, a decision is made in G1 that commits the myoblast to either continue to divide or to exit from the cell cycle and activate terminal differentiation. Ectopic expression of MyoD under the control of the RSV or CMV promoters causes 10T1/2 cells to rapidly exit the cell cycle and differentiate as single myocytes, even in growth medium, whereas expression of MyoD under the weaker SV40 promoter is compatible with proliferation. Co-expression of MyoD and cyclin D1, but not cyclins A, B, E or D3, blocks transactivation of a MyoD responsive reporter. Similarly, transfection of myoblasts with the cyclin-dependent kinase (cdk) inhibitors p16 and p21 supports some muscle-specific gene expression even in growth medium. Taken altogether, these results suggest cell cycle progression negatively regulates myocyte differentiation, possibly through a mechanism involving the D1 responsive cdks. We review evidence coupling growth status, the cell cycle and myogenesis. We describe a novel mitogen-sensitive mechanism that involves the cyclin D1-dependent direct interaction between the G1 cdks and MyoD in the dividing myoblast, which regulates MyoD function in a mitogen-sensitive manner.

Key words: Cell cycle; Myogenesis; MyoD; Cyclin D1; G1 cyclin-dependent kinase; Rb

1. Introduction

Both primary cultures of skeletal muscle as well as established muscle cell lines have been used as model systems to study the processes involved in tissue formation during development. The dividing muscle precursor cell or myoblast can be maintained as a dividing cell that will only form muscle once growth factors in the medium have been reduced. When this occurs the myoblast exits the cell cycle in G1 to a G0 state, fuses with adjacent myoblasts to form a syncitial myotube and commences to express the repertoire of skeletal muscle-specific genes [1]. However, the processes underlying this regulation have only recently come to light. With the identification of the MyoD family of skeletal muscle-specific gene regulatory proteins, known as MyoD, myogenin, myf-5 and MRF4, the study of myogenesis changed completely (reviewed in [2–6]). Committed myoblasts in the somite express MyoD and myf-5 and mark cells that will eventually give rise to vertebral muscle as well as cells that have been shown to migrate from the somite to form portions of the limb musculature (reviewed in [2,7]). Cultured myoblasts express one or both of these markers as well. Remarkably, each of these basic-helix-loop-helix (bHLH) proteins, when ectopically expressed in a variety of cell types from different germ layer origins, can convert non-muscle cells into muscle [8–10]. This result led to the hypothesis that the MyoD family of genes were involved in determining and maintaining the myogenic lineage during development. Although gene targeting studies in the mouse have essentially established the regulatory hierarchy for the MyoD family of proteins (see review, [7]), very little is known about the regulation of the myogenic bHLH proteins themselves or how their function is regulated in the dividing myoblast [4].

The MyoD family of bHLH proteins are transcription factors that form heterodimers with ubiquitously expressed members of the E-protein family, E12/47, E2-2, E2-5 and HEB, and bind to a DNA consensus CANNTG, known as an E-box, found in the promoters of several muscle-specific genes [11,12]. Even though MyoD and myf-5 are expressed in dividing myoblasts [13], their function is kept in check and only when the myoblast has withdrawn from the cell cycle upon mitogen reduction are muscle-specific genes activated. This implies that there are regulatory mechanisms that control myogenic factor function by sensing the cell cycle status of the myoblast as it responds to growth factors and external signals.

Here we discuss evidence from our own work and others as to how the myogenic bHLH proteins are functionally regulated during myoblast proliferation and how the activation of MyoD and the myogenic program is coupled to exit from the cell cycle.

2. MyoD function and growth control

In the initial characterization of the MyoD protein Lassar and coworkers reported that MyoD appeared to antagonize cell growth since the G418 resistant transformation frequency for 10T1/2 fibroblasts co-transfected with a MyoD expression plasmid was about 1/10 that of the controls [14]. It was concluded that the reduced transformation frequency was due to the fact that MyoD committed recipient cells to myogenesis and withdrawal from the cell cycle. This interpretation was further supported by the observation that basic fibroblast

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growth factor (βFGF), included in the medium to promote continued proliferation, increased transformation frequency to control levels. It was later shown that MyoD could induce growth arrest in normal and transformed cells independent of differentiation and that the efficiency of growth arrest was dependent upon the level of MyoD expression in the target cells [15,16]. In these initial studies the growth inhibitory effect was mapped to the DNA-binding and dimerization region of MyoD (the bHLH domain) but mutants with alterations in the basic region that did not bind DNA were also capable of inhibiting cell growth almost as efficiently as normal MyoD. This result was not explained at the time but suggested that specific DNA-binding and gene activation were not necessary to effect growth arrest. We had also shown that the myogenic conversion of 10T1/2 cells by avian MyoD (CMD1) was dependent upon the level of MyoD expression: overexpression of MyoD with the Rous sarcoma virus (RSV) promoter resulted in differentiated single myocytes whereas expression of MyoD under the control of the weaker SV40 promoter produced a myogenic cell line that could divide while expressing low levels of MyoD [8–10].

A great deal of work has focused on the transition from the G0 to G1 state and the response of the quiescent cell to growth signals that trigger replication (see review, [17]). This transition involves the activation of the G1 cyclins (D1, D2, D3, A and E) and the regulatory subunits for the G1 cyclin-dependent kinases (cdks) (cdk4, cdk6 and cdk2) (see review, [18]). The activation of the cyclins is a primary response in the initiation of the cell cycle. Previous work has established that cyclin D1 is the predominant cyclin that controls the rate of progression through the G1 phase of the cell cycle and that virtually all of the cyclin D1-dependent kinase activity is attributable to cdk4 in mouse fibroblasts [19,20].

The myogenic bHLH factors activate muscle-specific gene transcription only in cells cultured in low-mitogen medium. Their activity is suppressed when myoblasts are cultured in high serum medium or medium containing peptide growth factors, such as transforming growth factor-β (TGF-β) or βFGF [21–25]. The underlying mechanisms responsible for the inhibition of differentiation by these agents are complex and depend a great deal on cell background. Although myogenic cultures inhibited from differentiating by treatment with βFGF and TGF-β show reduced expression from the MyoD gene, additional regulatory pathways must be involved since ectopic expression of MyoD does not override this inhibition induced by the growth factors [26]. This result was interpreted to mean that some sort of post translational mechanism controls the activity of the MyoD family of muscle-specific gene regulators. In a similar fashion the oncogene Ras p21-val also inhibits myogenesis without affecting the DNA-binding or transcriptional activity of either MyoD or MRF4 towards an E-box reporter [27]. Quail primary muscle cells transformed with RSV are blocked for muscle structural gene expression but continue to express MyoD mRNA, again implying a post transcriptional regulation of MyoD function in the presence of v-src [28]. Likewise c-myc inhibits the MyoD and myogenin initiated differentiation of NIH3T3 even in the presence of ectopic MyoD expression so post translational regulation of MyoD function is again implied [29].

Several models based upon various experimental approaches have been put forth to explain how the activity of MyoD might be suppressed in the dividing myoblast [30–33]. The first model, proposed by Olson and colleagues, relied on the observation that a conserved threonine residue in the basic region of all the myogenic factors was essential for activity. This threonine was also a consensus site for protein kinase C (PKC) phosphorylation in vitro, a kinase activated by βFGF [32]. This threonine in the myogenin basic region could be phosphorylated in COS cells co-transfected with a PKC expression vector and mutation of this threonine to an asparagine, which did not completely inactivate myogenic activity, resulted in a myogenin that was now refractile to βFGF inhibition. This result was consistent with the idea that βFGF triggered the phosphorylation of the basic region's essential threonine to block myogenin DNA-binding activity, and by extension, activity of all the myogenic factors. However, subsequent work by Konieczny's group demonstrated that ectopically expressed MRF4 isolated from myoblasts treated with βFGF, TGF-β or co-transfected with a PKC expression vector contained no detectable phosphothreonine [34]. Given these results, it is currently considered unlikely that βFGF and TGF-β inhibit the activity of the myogenic factors through the direct PKC-dependent phosphorylation of the basic region but rather block activity via an indirect mechanism. An alternative explanation would require that myogenin is regulated differently than MRF4 even though they have the identical threonine in the basic region that is phosphorylated by PKC but this has not been resolved.

Benezera and colleagues identified the first member of a novel class of dominant negative HLH proteins lacking the basic region, known as Id1 for inhibitor of differentiation, that was shown to dimerize preferentially to the ubiquitously expressed bHLH E-proteins (E12/47, E2-2, E2-5, and HEB) to block heterodimer formation and myogenic factor activity [30]. Id1 is expressed in dividing myoblasts where it presumably binds to the E-proteins to prevent their interaction with the myogenic factors MyoD or myf-5. Id1 mRNA expression is greatly reduced in differentiating muscle cultures so it was proposed that the relative levels of Id1 and E-protein could control the activity of the myogenic factors through competitive dimerization. Unexpectedly, forced expression of Id1 in myoblasts attenuated differentiation but did not prevent myogenesis as Id protein is apparently degraded under differentiation conditions. A complication with the Id/E-protein stoichiometry model comes from the observation that the protein levels for Id1–4, as determined by Western blot analysis, are substantially lower than for the total E-proteins expressed in C2C12 myoblasts (Paterson, unpublished observations) making a direct titration effect unlikely. Although both Id1 and Id3 are required for neurogenesis (as reported in the double knockout mouse) any corresponding defects in muscle development were not noted, so the role for Id1 and Id3 in myogenesis is not clear [35]. Unlike Id1 and Id3, Id2 is a retinoblastoma protein (Rb) target that overrides the inhibitory activities of Rb when activated by the Myc oncoproteins and participates in neural tumor formation [36]. However, Id2 is not expressed in differentiating skeletal muscle during embryogenesis or in adult muscle so it is unlikely to be an Rb or E-protein regulator in myogenesis [36,37]. Peptide growth factors and phorbol ester induce Id expression in C2C12 myoblasts so the Id proteins may play some role in the regulation of MyoD function in the dividing myoblast but their exact role will require further study [38].

Genes that respond rapidly to growth stimuli, the immedi-

ate early genes (myc, jun and fos), also repress myogenic factor function and expression [29,39–41]. In addition, peptide growth factors and phorbol esters induce PKC, an activator of cJun and cFos [42]. Initial studies suggested that the leucine zipper of the bZip protein cJun interacted directly with the bHLH domain of MyoD to suppress myogenic activity [39]. Subsequent studies, however, clearly demonstrated the amino-terminal region of cJun without the leucine zipper inhibited myogenesis as efficiently as full size cJun so the bZip domain was not involved in the inhibitory interaction [41].

The discovery that MyoD function involved the participation of the histone acetylase (HAT) and co-activator p300 suggested that p300 itself could be a target for the regulation of MyoD activity [43–45]. E12 alone also interacts with p300 in reporter and protein interaction assays, consistent with the idea that the heterodimer is subject to co-activation by p300 in vivo [46]. In fact, antibodies to p300 injected into myoblasts blocked the MyoD activation of muscle-specific reporters, cell cycle withdrawal and myogenesis [43,44]. However, it now appears that p300 acts as a scaffold for another HAT, P/CAF, and it is the HAT activity of P/CAF that is important for the myogenic activity of MyoD [47–49]. Injection of P/CAF antibodies into C2C12 muscle cells also inhibited differentiation. Only deletions in P/CAF that remove HAT activity blocked myogenesis but HAT deletions in p300 had no effect. P/CAF also acetylates MyoD in vitro and replacement of the acetylated lysine residues with arginines reduced reporter activation substantially but this had little effect on DNA-binding in vitro with or without E12 [47]. The disparity between DNA-binding and reporter activation suggests the acetylated domain adjacent to the MyoD basic region may interact with additional transcriptional activators yet to be identified. p300 also serves as a co-activator for another class of transcription factors essential for myogenesis, the serum response factor-related myocyte enhancer factor 2 (MEF2) family of proteins [46,50]. Four members of this family have been identified in the vertebrates to date, MEF2A–D (reviewed in [51]). Mutational studies in *Drosophila*, where there is a single related gene called DMEF2, have shown that DMEF2 is essential for myogenesis but its interaction with *Drosophila* p300 has not been formally described as a prerequisite for myogenesis in the fly [52,53].

Induction of the proto oncogenes cJun and Fos, either by peptide growth factors or by the co-transfection of cJun and Fos expression plasmids during myogenesis, blocks terminal muscle cell differentiation by inhibiting the activity of MyoD. This does not involve a direct interaction with the myogenic factor, as mentioned earlier. The adenovirus oncoprotein E1A inhibits the activities of cJun and JunB by targeting their physical interaction with p300 [54]. We have recently determined that a similar mechanism is involved in the inhibition of myogenesis by cJun (Zhao, Wei, and Paterson, unpublished observations). The MyoD transactivation of muscle-specific reporters can be suppressed by a 78 amino acid domain from the N-terminus of cJun through direct physical interaction with p300 on a site shown to also bind MyoD (amino acids 1514–1922). This N-terminal region in cJun does not include either the delta domain [55] or the JNK phosphorylation sites on serines 63 and 73 [56] and is absent from JunD, a member of the jun family that does not inhibit myogenesis [41]. Ectopic expression of the 78 amino acid domain disrupts MyoD interaction with p300 in the mammalian two-hybrid system and blocks activation of muscle-specific reporters, myogenic conversion of 10T1/2 cells by MyoD, and myogenesis in C2C12 muscle cells. Co-expression of p300 can restore normal reporter activity, efficient myogenic conversion and normal myogenesis in C2C12 cells, suggesting p300 itself is the limiting factor in the cJun–p300 interaction. cJun residues Ser 63/73 are apparently not required for interaction of this 78 amino acid peptide with p300, as reported for cJun interaction with CBP [57], highlighting possible differences in the co-factor function of CBP and p300. Therefore, peptide growth factors cannot only regulate myogenesis by modulating cell cycle decisions but also by disrupting the essential interaction between MyoD and it co-activator, p300, through the induction of the proto oncogene cJun.

3. MyoD function and the cell cycle

A great deal of evidence from in vitro studies as well as from the characterization of naturally occurring mutations has demonstrated that the Rb is a key regulator of the cell cycle and in the ability of cells to enter and remain in the G0 state (see review, [58]). The phosphorylation status of Rb and the cycling state of the cell are directly correlated. Hypophosphorylated Rb is correlated with lack of cell growth, repression of genes involved in DNA replication and the differentiated state of a variety of cell types. Hyperphosphorylated Rb is correlated with cell growth. The viral oncoproteins from adenovirus (E1A), SV40 (T-antigen), and papillomavirus (E7) interact with hypophosphorylated Rb to block growth suppression and differentiation in a variety of systems, including muscle [59–61]. The HLH protein Id2 also binds to hypophosphorylated Rb to block Rb function but its mRNA is apparently not expressed in developing muscle, suggesting the protein is also absent [36,62]. In Rb−/− Saos cells MyoD does not arrest proliferation, but once wild-type Rb is restored to these cells, MyoD arrests growth [31]. This was interpreted to suggest that MyoD and Rb were acting in the same regulatory pathway. In vitro binding and immunoprecipitation studies demonstrated a MyoD/Rb interaction. This result suggested that a direct MyoD/Rb interaction blocked Rb phosphorylation and led to the suppression of myoblast cell growth, cell cycle exit and differentiation. The fact that T-antigen peptide can compete the observed MyoD/Rb interaction and that MyoD/Rb complexes can also be immunoprecipitated from muscle cell extracts supported this interpretation [31]. Lassar and associates have recently further defined the role of Rb in cell cycle arrest during skeletal muscle differentiation [63]. Their findings indicate that Rb promotes myogenesis by inhibiting cell cycle progression and cooperating with MyoD to activate the transcriptional activation domain (TAD) of MEF2. Although it is unclear how MyoD activates the MEF2 TAD, the process requires Rb and the phosphorylation of Ser 387 in the MEF2 TAD. This synergy between MyoD and MEF2 does not require the MADS domain, a region in MEF2 previously suggested to interact with MyoD in transcriptional activation [64]. In cell cycle control during myogenesis Rb emerges as a key player and the function of Rb is regulated by the G1 cyclins and their associated kinases, cdk4 [65] and cdk6 [18]. However, the physiological targets for Rb in muscle have yet to be identified.

A key set of experiments reported by Kohtz and associates demonstrated that the activation of muscle gene transcription

in differentiating skeletal muscle myoblasts could be inhibited by the ectopic expression of cyclin D1, but not cyclins A, B1, B2, C, D3 and E [66]. Cyclin D1 also inhibited reporter activation by myogenin mutated in threonine 87, the PKC phosphorylation site conserved in all the myogenic bHLH factors, indicating phosphorylation of this site is not relevant to the mechanism of cyclin D1 inhibition. As mentioned earlier, virtually all of the cyclin D1 kinase activity is attributable to cdk4 in cultured mouse fibroblasts and this is likely to be the case in muscle cells. Experiments along similar lines by Lassar and coworkers confirmed that forced expression of cyclin D1 inhibited myogenesis and this correlated with the apparent increased phosphorylation of MyoD, independently of Rb phosphorylation [33,67]. In this instance ectopic expression of the cdk inhibitors (CKIs) p16 and p21 could augment the differentiation of subconfluent C2C12 myoblast cultures and suggested a block of cdk kinase activity would prevent inhibition of MyoD function in proliferating cells. This lead the authors to conclude MyoD might be a direct target for G1 cdk kinases, an area of interest to our own studies on the role of phosphorylation in the regulation of MyoD heterodimer formation with E12 [68].

4. G1 cdks and the regulation of MyoD

The implied phosphorylation of MyoD by cdk4 suggested these two proteins might interact directly. To determine whether MyoD bound cdk4 we initially looked at interactions between cdk4/cyclin D1 produced in the baculovirus system and MyoD produced in *Escherichia coli* [69]. The cdk4/cyclin D1 produced in baculovirus-infected sf9 cells specifically phosphorylates a GST Rb target and this phosphorylation is inhibited by the cdk4-specific inhibitor p16 [70]. Using GST fusions of all the myogenic factors, only baculovirus produced cdk4/D1 specifically bound to MyoD. GST fusions of E12 or E47, the heterodimeric partner proteins for these factors in vivo, did not bind cdk4/D1. Although MyoD bound to the cdk4/cyclin D1 complex, it did not bind to cyclin D1 alone but only to cdk4. This suggested MyoD did not dissociate the active kinase and bound only to the catalytic subunit. More importantly, it suggested the active kinase was not required for interaction with MyoD. Co-expression of various combinations of MyoD, cdk4 and cyclin D1 in the baculovirus system and immunoprecipitation analysis of the complexes formed confirmed the result that only MyoD and the catalytic subunit of cdk4 were necessary for interaction and this could occur in vivo as well. Further in vivo studies using the mammalian two-hybrid system and direct co-immunoprecipitations from myoblast extracts confirmed that MyoD, but not myogenin, interacts with the catalytic subunit of cdk4 and that a MyoD/cdk4/cyclin D1 complex could be isolated from dividing myoblasts but not myotubes, respectively.

To determine if a MyoD cdk4 interaction could disrupt MyoD function we first looked at MyoD DNA-binding activity. Surprisingly, increasing amounts of cdk4 could specifically disrupt DNA-binding of both MyoD homodimers and MyoD/E12 heterodimers [69]. Importantly, cdk4 did not disrupt the interaction of myogenin or myogenin/E-protein complexes with DNA under the same conditions. Disruption of MyoD DNA-binding did not require the active kinase since cdk4 alone could inhibit the interaction with DNA. Since cdk4 is expressed at similar levels in both myoblasts and myotubes [33,66] this result suggested that cdk4 would likely not be present in myotube nuclei to potentially disrupt MyoD function. In dividing myoblasts both cyclin D1 and cdk4 are nuclear, but in well-formed myotubes cyclin D1 is absent and cdk4 is in the cytoplasmic compartment of newly differentiated muscle, as postulated. Cyclin D1 is the mitogen sensor and the limiting factor in the assembly of active cdk4/cyclin D1 complexes and it is absent in differentiated myotubes [71]. Ectopic expression of a stable cyclin D1 (T286A) was induced in myotubes to see if cdk4 would relocate to myotube nuclei [72]. Induction of stable cyclin D1 resulted in the nuclear compartmentalization of cdk4 in myotubes. This was the first hint that the regulation of cyclin D1 levels by mitogens could possibly impact nuclear MyoD function by regulating the cellular localization of cdk4. This hypothesis turned out to be correct since the forced nuclear localization of cdk4 in the absence of cyclin D1 blocked expression from a MyoD activated reporter but not activation of the same reporter by myogenin. In addition, reporter inhibition did not depend upon cdk4 kinase activity since cdk4 that was inactive (i.e. it could not be phosphorylated by cdk activating kinase or CAK) [70] also blocked reporter activation by MyoD but not myogenin. Consistent with this result, conversion of 10T1/2 fibroblasts by MyoD was also inhibited in a dose-dependent fashion by ectopic expression of both wild-type and inactive cdk4 with a nuclear localization signal (NLS). Active cdk4 without an NLS only blocked myogenesis in growth medium since nuclear localization under these conditions depends upon mitogens and cyclin D1. This result coupled the cell cycle and myogenesis through a cyclin D1-dependent interaction between MyoD and cdk4 and provided a mechanism to keep MyoD inactive in the dividing myoblast. The specific MyoD–cdk4 interaction in dividing myoblasts, coupled with the cyclin D1-dependent nuclear targeting of cdk4, suggested a mitogen-sensitive mechanism whereby cyclin D1 can regulate MyoD function and the onset of myogenesis by controlling the cellular location of cdk4 rather than the phosphorylation status of MyoD [69].

Using the mammalian two-hybrid system in C2C12 cells we were unable to detect any interaction between Rb and either MyoD or myogenin in dividing or differentiated cells [73]. However, both MyoD and Rb interacted strongly with E12 and E2F, respectively, under both conditions. Therefore, in this type of in vivo assay no evidence for an Rb interaction with the myogenic factors could be detected, in contrast to the in vitro studies by Gu, Nadal-Ginard and coworkers [31]. It is clear, however, that Rb promotes the expression of late myogenic markers by cooperating with MyoD to switch on the TAD of MEF2 and to inhibit cell cycle progression [63]. With regard to the latter issue, Caruso and associates reported that MyoD activates the Rb promoter in differentiating myoblasts and have proposed this as an additional mechanism to provide excess hypophosphorylated Rb to block cell growth [74]. It is interesting to speculate that hypophosphorylated Rb may transcriptionally activate additional genes in the post mitotic myoblast, which in turn may be necessary to trigger the late myogenic program. It is still not known if all Rb in differentiated myotubes is inactive or in association with the histone deacetylase transcriptional repressors such as HDAC1 [75].

MyoD interacts with cdk4 through a conserved 15 amino acid domain in the C-terminus of MyoD [73]. Surprisingly,

full MyoD, the C-terminus beyond the bHLH region, or just the 15 amino acid cdk4-binding domain all inhibit cdk4 phosphorylation of an Rb target in vitro, whereas myogenin does not. Expression of only the MyoD 15 amino acid cdk4-binding domain in vivo fused either to GFP or GST with an NLS blocks Rb phosphorylation, inhibits cell growth and promotes differentiation of C2C12 cells in growth medium. In a functional assay, MyoD deleted in the C-terminus beyond the bHLH region cannot rescue the defective myogenic phenotype in BC3H1 cells but the addition of just the 15 amino acid cdk4-binding domain to the deleted MyoD restores the normal myogenesis seen with full MyoD. Rescue is not seen with p16 or p21, thus inhibition of cdk4 is not the mechanism responsible for the rescue observed with the addition of the MyoD cdk4-binding region. This domain has some other function in the context of MyoD yet to be determined. We have proposed that a regulatory check point in the terminal cell cycle arrest of the myoblast during differentiation involves the modulation of the cyclin D1 cdk-dependent phosphorylation of Rb through the opposing effects of cyclin D1 and MyoD. This modulation is critically dependent upon the regulation of the cyclin D1 gene through various pathways that respond to cellular growth signals [69,73].

5. Cyclin D1 regulation and myogenesis

A key factor in the regulation of cell cycle withdrawal during terminal differentiation of the myoblast that is implicit from the MyoD–cdk4 interaction we have described is the control of the cyclin D1 gene and the concomitant regulation of nuclear cdk levels. Peptide growth factors or high concentrations of serum in growth medium increase cyclin D1 mRNA and protein levels but the precise molecular mechanisms underlying this activation have only recently come to light [18]. Members of the AP-1 family of transcription factors, cJun and fos, are well-documented in their role as transcriptional mediators in various cellular contexts after mitogenic stimulation or exposure to stress but their role in normal cell growth is less understood [42]. Studies of the cJun deficient mouse have shown that the basal levels of AP-1 (a cJun and fos heterodimer) activity are essential for survival and mediate not only response to environmental stimuli but also play a critical role in normal cell cycle progression [76–78]. In this last instance recent studies have shown that AP-1 regulates G1 progression through its control of cyclin D1 gene expression [77] and that p300 is a co-activator for AP-1 in the context of the cyclin D1 promoter [76]. Using cJun−/− fibroblasts, it has been shown recently that cJun negatively regulates the transcription of p53 and its target gene, the CKI, p21, providing a further link between mitogenic signaling and cell cycle control [79]. Thus cJun not only inhibits MyoD interaction with its co-activator, p300, but it also is required to activate the cyclin D1 gene and reduce levels of the CKI, p21, to promote continued growth.

NF-κb is also a positive mediator of cell growth and has recently been shown to directly regulate the cyclin D1 promoter in C2C12 myoblasts and embryonic fibroblasts [80,81]. NF-κb expression can inhibit myogenic differentiation by directly activating cyclin D1 expression whereas myoblasts that lack NF-κb, induced in this instance by NF-κb antisense methods, display a reduction in proliferation and exit the cell cycle more rapidly than control cells [80]. Thus the ability of NF-κb to control cellular proliferation and differentiation are tightly coupled to the regulation of the cyclin D1 gene.

β-Catenin is not only a structural component of adherence junctions but is also a co-factor in transcription activation complexes with members of the lymphoid enhancing factor family (LEF-1). Elevated β-catenin levels in colorectal cancer caused by mutations in either β-catenin or in the adenomatous polyposis coli protein, which regulates the degradation of β-catenin, result in the increased formation of transcriptionally active β-catenin/LEF-1 complexes [82,83]. The increased transcription of unknown target genes results in uncontrolled cell growth. The cyclin D1 gene was shown recently to be a target for β-catenin/LEF-1 activation through a LEF-1-binding site in the promoter [84,85]. This is the same region that is transcriptionally repressed by caveolin-1, a protein that has recently been shown to inhibit the cyclin D1 promoter [86]. Thus regulation of MyoD function during development through the wingless/wnt pathway [87] could involve the regulation of the cyclin D1 gene via the activity of β-catenin, a known target for wingless/wnt activation [88].

Increasing evidence, as briefly described here, points to the conclusion that the regulation of the cyclin D1 gene plays a central role in modulating cell growth during development and differentiation as well as in the onset of various types of cancer [89].

6. Myogenesis and the CKIs

Progression through the G1 phase of the cell cycle is closely modulated by a family of cdks, cdk4 and cdk6, whose activities are in turn constrained by a group of proteins known as the CKIs (see review, [90]). The CKIs are divided into two groups based upon their structure and cdk targets. The first group is know as the INK4 proteins since they bind specifically to and inhibit the catalytic subunit of cdk4 and cdk6 and include p15, p16, p18, and p19. The second group, the cip/kip family, bind to the cyclin D-, A- and E-dependent kinases via both the cyclin and catalytic subunits and include p21, p27 and p57. It has been shown in transfection studies that MyoD can upregulate the promoter for the CKI p21, implying similar regulation during cell cycle exit in myogenesis [91]. However, mice lacking both MyoD and myogenin genes have normal expression patterns of p21 and mice lacking the p21 gene develop normally, suggesting that the role of p21 in myogenesis is either redundant or minor [92]. Interestingly, mice lacking both p21 and p57 fail to form myotubes and myoblasts show increased proliferation and apoptosis, suggesting that p21 and p57 redundantly play a role in skeletal muscle terminal cell cycle withdrawal [93]. p57 is predominantly expressed in differentiated tissues, and the 7 kb mRNA for this inhibitor is only detectable in skeletal muscle and heart, so its importance in cdk regulation in muscle may be dominant compared with p21 [94]. Unexpectedly, the p21 and p27 CKIs are also essential activators of cyclin D-dependent kinases in mouse fibroblast [95]. p21/p27 remain associated with cyclin D–cdk4 in an active kinase complex and are liberated later in the cell cycle to inhibit cyclin E/cdk2. Both p21 and p27 are required for the assembly and nuclear import of cyclin D1/cdk4 and their participation in this process is though to provide the nuclear import signal that is lacking in the kinase.

The calcium-binding protein calmodulin is also a key regulator of the cell cycle [96]. Recent studies have shown that

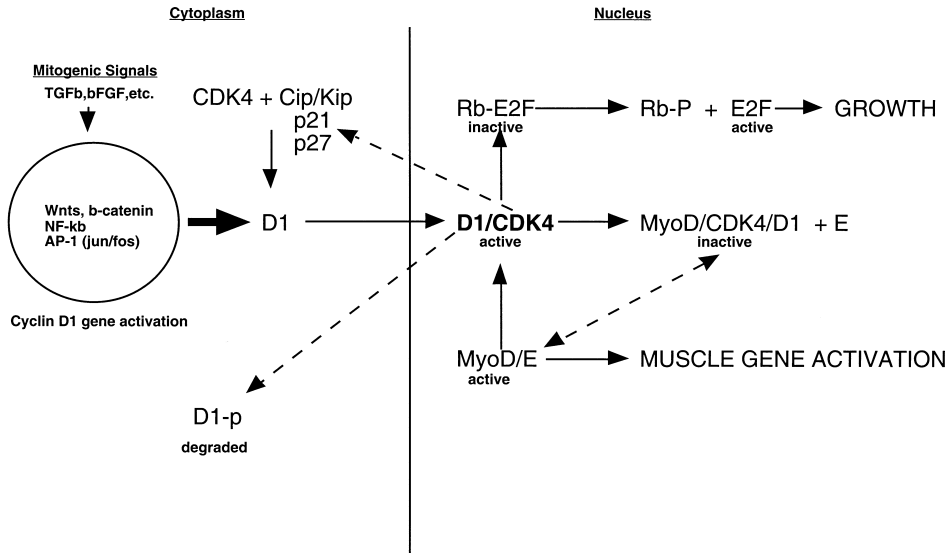

Fig. 1. The activation of the cyclin D1 gene (bold arrow) in response growth signals from peptide growth factors (such as βFGF or TGF-β) or through cell–cell contact (the wnts) determines the nuclear levels of the G1 cdk, cdk4. Cdk4 and other G1 cdks sequester MyoD and keep it inactive in the dividing myoblast while the excess cdk4 drives growth through the phosphorylation of Rb and the activation of E2F family members. Once cyclin D1 levels decrease (degraded after phosphorylation) in response to low mitogens or growth signals (dashed arrow), cdk4 is translocated to the cytoplasmic compartment (dashed arrow) allowing MyoD to form an active complex with the E-proteins in the nucleus to activate muscle gene expression during terminal differentiation. The dynamic interaction between MyoD and cyclin D1-dependent nuclear cdk4 (denoted by double-headed dashed arrow) regulates transcriptional activation by MyoD and the cdk-dependent phosphorylation of Rb.

calmodulin is essential for cdk4 activity and the nuclear accumulation of cyclin D1–cdk4 complexes during G1 [97] and that this accumulation likely involves Hsp90.

7. Conclusion

Although the identification and characterization of the MyoD bHLH family of myogenic regulators has enhanced our understanding of muscle formation during development, little is known about transcriptional control or the functional regulation of these factors during this process: what activates the myogenic regulatory genes at the appropriate time during development and how is their functional activity regulated by environmental cues that modulate cell growth and trigger differentiation? Here we have presented a brief overview of some of the regulatory mechanisms that regulate skeletal muscle cell growth, cell cycle withdrawal and differentiation and how these various stages in development impact the regulation of MyoD function (Fig. 1). The new observation that MyoD interacts with the G1 cdks to regulate Rb function and cell growth provides an explanation for several results that have raised more questions than answers in earlier studies. Now it is clear that signaling pathways that induce cyclin D1, a key regulator of cell cycle progression (see review, [89]), also induce increased levels of nuclear cdk4 which, in turn, inhibits MyoD function in the dividing cell. Functional MyoD, liberated from cdk inhibition upon the reduction in cyclin D1 levels in response to decreased mitogen signaling, activates myogenesis as well as genes that maintain cell cycle exit, such as p21 and Rb. Overexpression of MyoD blocks existing cdk4 to trigger growth arrest and this only depends upon the 15 amino acid cdk4-binding domain in the C-terminus of MyoD. Our preliminary studies indicate that the MyoD cdk4-binding domain also interacts with cdk6 [98] and cdk2 [99,100], the other major G1 cdks, so inactivation of MyoD by sequestration throughout G1 to get past the check point can certainly occur in the dividing myoblast. It is not yet known if MyoD interaction with cdk2 and cdk6 behaves exactly like cdk4 but cdk6 is cytoplasmic in myotubes and can be relocated to nuclei in response to the induction of stable cyclin D1 in myotubes just like cdk4. This is not unexpected since cdk4 and cdk6 are $>70\%$ identical at the amino acid level. The direct interaction between MyoD and the G1 cdks provides a novel mechanism for the regulation of MyoD function that is tightly regulated by the growth status of the cell through the expression of the cyclin D1 gene. Clearly there are redundant mechanisms involved in the regulation of differentiation and exit from the cell cycle and no doubt different themes will emerge as this process is explored further.

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