Cyclin D–cdk4 activity modulates the subnuclear localization and interaction of MEF2 with SRC-family coactivators during skeletal muscle differentiation

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Prior work has indicated that D-type cyclin–cdk4 complexes, which are only active in proliferating cells, can suppress the skeletal muscle differentiation program in proliferating myoblasts. In this study, we show that cyclin D–cdk activity can block the activity of the MEF2 family of transcriptional regulators, which are crucial regulators of skeletal muscle gene expression. We have found that cyclin D–cdk activity blocks the association of MEF2C with the coactivator protein GRIP-1 and thereby inhibits the activity of MEF2. During skeletal muscle differentiation, GRIP-1 is localized to punctate nuclear structures and can apparently tether MEF2 to such structures. Cotransfection of GRIP-1 can both potentiate the transcriptional activity of a Gal4–MEF2C construct and induce MEF2C localization to punctate nuclear structures. Consistent with the absence of punctate nuclear GRIP-1 in proliferating myoblasts, we have found that ectopic cyclin D–cdk4 expression disrupts the localization of both GRIP-1 and MEF2C to these punctate subnuclear structures. Our findings indicate that cyclin D–cdk4 activity represses skeletal muscle differentiation in proliferating cells by blocking the association of MEF2 with the coactivator GRIP-1 and concomitantly disrupts the association of these factors with punctate nuclear subdomains within the cell.

[Key Words: Differentiation; myogenesis; cyclin D; MEF2; GRIP-1]

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Mammalian muscle differentiation involves a carefully ordered pattern of gene expression that is coordinated with terminal cell cycle exit. Two families of transcription factors control the differentiation of skeletal muscle cells: the myogenic bHLH family (including MyoD, Myf-5, myogenin, and MRF-4) and the MEF2 family. The myogenic bHLH transcription factors are expressed solely in skeletal muscle and heterodimerize with ubiquitously expressed bHLH proteins, termed E proteins, to form a transcriptional complex capable of activating muscle-specific genes containing a specific DNA sequence (CANNTG) termed the E box [Blackwell and Weintraub 1990; Lassar et al. 1991]. The myogenic bHLH transcription factors are able, when overexpressed, to induce the transdifferentiation of fibroblasts into myocytes [Davis et al. 1987; Weintraub 1993]. Induction of myogenesis by the myogenic bHLH proteins induces the synthesis and functional activation of MEF2C [Cserjesi and Olson 1991; Lassar et al. 1991; Wang et al. 2001]. The MEF2 family of transcription factors bind to A/T-rich DNA sequences present in the regulatory regions of many muscle-specific genes [Naya and Olson 1999]. Although MyoD, Myf-5, and MEF2D are expressed in proliferating myoblasts [Tepscott et al. 1988; Montarras et al. 1991; Breithart et al. 1993], these proteins fail to engage the differentiation program unless the cells are exposed to low mitogens and withdraw from the cell cycle. Thus, mitogen signaling regulates the activity of these transcription factors and coordinates skeletal muscle differentiation with cell cycle withdrawal.

Several hypotheses have been proposed to explain how mitogenic signals block skeletal muscle differentiation. These include PKC-mediated phosphorylation of myogenic bHLH proteins [Li et al. 1992], induction of dominant-negative HLH proteins that block E protein heterodimerization [Benezra et al. 1990], MEK1-mediated inactivation of the transcriptional activation domain of...
Cyclin D–cdk4 inhibits MEF2/GRIP-1 activity

The myogenic bHLH proteins [Perry et al. 2001], and cyclin D–cdk4-mediated inhibition of the differentiation program [Rao et al. 1994; Rao and Kohtz 1995; Skapek et al. 1995, 1996]. In the case of cyclin D–cdk4, it has been suggested that this kinase, which is active specifically in proliferating cells, can associate with the C-terminal region of MyoD and block its interaction with DNA [J.M. Zhang et al. 1999]. However, it seems likely that cyclin D–cdk4 regulates muscle differentiation via other pathways, as ectopic expression of cyclin D–cdk4 can also block the activity of myogenin [Skapek et al. 1996], which fails to directly interact with the cyclin D–cdk4 complex [Wei and Paterson 2001]. Another potential target for cyclin D–cdk4 is the tumor suppressor protein pRB, whose function is required to promote the latter stages of the skeletal muscle differentiation program [Novitch et al. 1996]. However, because expression of a form of pRB which lacked all cdk phosphorylation sites was unable to rescue cyclin D–cdk4-mediated inhibition of muscle differentiation, we have postulated that the cyclin D–cdk4 complex must block muscle differentiation by targeting other proteins in addition to pRB [Skapek et al. 1996].

In this work we explore the effects of cyclin D–cdk on MEF2 function. A number of recent studies have elegantly shown that MEF2 function is modulated by a network of transcriptional coactivators and repressors. The Histone Acetyl-Transferases (HATs) p300 and GRIP-1 have been found to associate with MEF2 [Sartorelli et al. 1997; Chen et al. 2000] and are thought to activate gene expression by inducing histone acetylation (Goodman and Smolik 2000). GRIP-1, TIF2/SRC-2, is a member of the p160-Steroid Receptor Coactivator (SRC) family, which also includes SRC-1 and TRAM1/RAC3/ACTR/pCIP/AIB-1/SRC-3 [Leo and Chen 2000]. These coactivators bind to transcription factors such as nuclear hormone receptors, to other coactivators such as p300/CREB, and to the basal transcriptional machinery such as TBP and TFIIIB [Leo and Chen 2000]. GRIP-1 binds directly to both myogenin and MEF2C, via their bHLH and MADS/MEF2 domains, respectively [Chen et al. 2000]. Repression of endogenous GRIP-1 expression by antisense RNA has been reported to block differentiation in myogenic cell lines [Chen et al. 2000].

MEF2 function is repressed by interaction with class II Histone De-Acetylases [HDACs; Miska et al. 1999; Sparrow et al. 1999; Lemercier et al. 2000; Lu et al. 2000a,b; Dressel et al. 2001], which are thought to block transcription by deacetylating histones and thereby inducing a repressive chromatin conformation [Goodman and Smolik 2000; Marmorstein and Roth 2001; McKinsey et al. 2001a]. Class II HDACs (which include HDAC4, HDAC5, and HDAC7) contain a large N-terminal domain, absent from class I HDACs [i.e., HDAC1, HDAC2, HDAC3, and HDAC8], which promotes interaction with the MADS/MEF2 domain of MEF2 factors [McKinsey et al. 2001a]. Class II HDACs inhibit MEF2 transcriptional activity but do not impair the ability of these transcription factors to either bind DNA or dimerize [Lu et al. 2000a,b]. Ectopic expression of either HDAC4 or HDAC5 inhibits C2C12 myoblast differentiation and MyoD-induced transdifferentiation of fibroblasts into muscle [Lu et al. 2000a]. This inhibition is a result of MEF2 inactivation, as MyoD-dependent transcription is not affected significantly by ectopic expression of class II HDACs [Lu et al. 2000a]. Recent findings from the Olson lab have shown that during the process of skeletal myogenesis, the Calcium/Calmodulin-dependent protein Kinase (CaMK) directly binds to and phosphorlates class II HDACs, inducing their accumulation in the cytoplasm [McKinsey et al. 2001b]. Phosphorylation of conserved serines near the nuclear localization signal of HDAC4, HDAC5, and HDAC7 promote the binding of these HDACs to 14-3-3 proteins, resulting in the sequestration of these HDACs in the cytoplasm and consequent derepression of MEF2 activity [McKinsey et al. 2001b].

In this work we show that cyclin D–cdk4 activity can block MEF2 function. Cyclin D–cdk4 activity fails to alter the cellular localization of class II HDACs, suggesting that cyclin D–cdk4 blocks MEF2 function independently of modulating the interaction of MEF2 with class II HDACs. Consistent with this idea, we have found that the interaction of MEF2C with the SRC family member GRIP-1 is blocked by ectopic cyclin D–cdk4 expression. GRIP-1 is localized to punctate nuclear structures in differentiated skeletal muscle cells [Chen et al. 2001] and is absent from these structures in proliferating myoblasts. We have found that cotransfected GRIP-1 drives accumulation of MEF2C into such punctate nuclear structures and that cyclin D–cdk4 activity inhibits the translocation of both MEF2C and GRIP-1 into these nuclear structures. Our results suggest that cyclin D–cdk4 activity in proliferating myoblasts acts to repress the skeletal muscle differentiation program by blocking the interaction of MEF2 family members with the SRC family coactivator GRIP-1 and consequently inhibits the localization of both MEF2 and GRIP-1 to punctate nuclear structures.

Results

D-type cyclins require cdk activity to repress MEF2-dependent transcription

Cotransfection of cyclin D1 and cdk4 can block the ability of either MyoD or myogenin to activate the expression of muscle reporter constructs such as muscle creatine kinase-CAT [Fig. 1A, cf. lanes 1 and 2, and lanes 5 and 6; Rao et al. 1994; Skapek et al. 1995, 1996; J.M. Zhang et al. 1999]. Maximal repression of either MyoD or myogenin activity by cotransfected cyclin D1–cdk4 requires the kinase activity of this complex as cotransfection of cdk4 together with cyclin D1-KE, a mutant form of cyclin D1 that fails to activate the cdk4 kinase [Hinds et al. 1994], restored significant expression of the MCK reporter construct [Fig. 1A, cf. lanes 2 and 3, and lanes 6 and 7]. Because Paterson and colleagues have shown that the cyclin D1–cdk4 complex can bind directly to MyoD but not myogenin [J.M. Zhang et al. 1999], we speculated that there must be another cyclin...
Figure 1. Cyclin D–cdk4 complexes can repress MEF2 transcription in a kinase-dependent manner. (A) Luciferase activity was monitored from whole-cell extracts of 10T1/2 fibroblasts cotransfected with a muscle creatine kinase [MCK]–luciferase reporter plus either empty expression vehicles [lanes 4,8], plasmids encoding MyoD [lanes 1–3], or myogenin [lanes 5–7] either in the absence [lanes 1,5] or presence [lanes 2,3,6,7] of cdk4 and either cyclin D1-WT [lanes 2,6] or the cyclin D1-KE mutant, which fails to activate cdk4 [lanes 3,7]. (B) Luciferase activity was monitored from whole-cell extracts of 10T1/2 fibroblasts cotransfected with a MEF2x3-luciferase reporter plus either empty expression vehicles [lane 1] or plasmids encoding MEF2A [lanes 2,3], MEF2B [lanes 4,5], MEF2C [lanes 6,7], or MEF2D [lanes 8,9] either in the absence [lanes 2,4,6,8] or the presence [lane 3,5,7,9] of cyclin D1 and cdk4. (C) 10T1/2 cells were cotransfected with a MEF2x3 luciferase reporter construct plus either empty expression vehicles [lane 1] or plasmids encoding MEF2C [lanes 2–5], either in the absence [lane 2] or the presence of cdk4 [lanes 3–5] and cyclin D1 [lane 3], cyclin D2 [lane 4], or cyclin D3 [lane 5]. (D) 10T1/2 cells were cotransfected with a MEF2x3 luciferase reporter construct plus either empty expression vehicles [lane 1] or plasmids encoding MEF2C [lanes 2–4], either in the absence [lane 2] or presence of cdk4-WT and cyclin D3 [lane 3] or a kinase dead mutant of cdk4 [cdk4-KM] and cyclin D3 [lane 4]. (E) 10T1/2 cells were cotransfected with a Gal4x5 reporter construct plus plasmids encoding the Gal4 DNA-binding domain [lane 1], Gal4VP16 [lanes 2,3], either in the absence [lane 2] or in the presence of cdk4-WT and cyclin D3 [lane 3]. The (−) sign indicates that an empty expression vehicle has been added instead of the corresponding expression plasmid.
The activity of MEF2C can be boosted significantly by cotransfection with an activated form of CaMK (McKinsey et al. 2000a), which induces the retention of class II HDACs in the cytoplasm (McKinsey et al. 2000b; Kao et al. 2001). To evaluate if cyclin D–cdk4 would counter the effect of CaMKIV on MEF2C function, we coexpressed the kinase with MEF2C to activate a Gal4x5-luciferase reporter (diagramed in Fig. 2A). Although activated CaMKIV boosts the activity of Gal4MEF2C (Fig. 2B, cf. lanes 1 and 2), cotransfection of cyclin D3–cdk4 significantly dampens this effect (Fig. 2B, lane 3). Cdk4 kinase activity is required to maximally repress the effects of CaMKIV on Gal4MEF2C, because cotransfection of cyclin D3KE, which fails to activate cdk4 function, together with a catalytically inactive mutant of cdk4 [cdk4KM; van den Heuvel and Harlow 1993] partially represses the activity of Gal4MEF2C (Fig. 2B, cf. lanes 3 and 4). Consistent with the notion that cdk4 catalytic activity is necessary to completely block MEF2 function, cotransfection of the cdk inhibitors p16, p21, or p27 partially reversed the inhibitory affects of cyclin D3–cdk4 on Gal4MEF2C activity (Fig. 2C, lanes 3–6).

Cyclin D3–cdk4 does not affect the ability of CaMKIV to promote the nuclear-to-cytoplasmic shuttling of class II HDAC molecules

Class II HDAC molecules are actively shuttled from the nucleus to the cytoplasm in the presence of activated CaMKI or CaMKIV (McKinsey et al. 2000b). Given that coexpression of cyclin D3 and Cdk4 can inhibit the ability of CaMKIV to potentiate Gal4MEF2C-driven transcription (Fig. 2), we were interested in determining whether active cyclin D3–cdk4 complexes could inhibit the ability of CaMKIV to shuttle class II HDACs from the nucleus into the cytoplasm. 10T1/2 cells were initially transfected with HDAC7 alone. Consistent with prior work of others (Dressel et al. 2001; Kao et al. 2001), HDAC7 was predominantly detected in the nucleus (Fig. 3B). As expected, when HDAC7 was coexpressed with an activated form of CaMKIV, HDAC7 was relocalized to the cytoplasm in 100% of the cells that expressed
both proteins (Fig. 3E). When cotransfected with exogenous cyclin D3–cdk4, CaMKIV still directed the retention of HDAC7 in the cytoplasm (Fig. 3I,J). We similarly found that activated CaMK-induced cytoplasmic retention of HDAC4 was also not altered by ectopic expression of D-type cyclins together with cdk4 (data not shown). These findings suggest that cyclinD–cdk4 complexes repress MEF2-dependent transcription by a mechanism that does not affect the nuclear export of class II HDACs by CaMK signaling.

CaMKIV and GRIP-1 synergistically activate MEF2-dependent transcription by enhancing the association of GRIP-1 with MEF2

Because GRIP-1, a member of the SRC family of coactivators, has been shown to bind to MEF2 and thereby potentiate its transcriptional activity [Chen et al. 2000], we wondered if CaMKIV and GRIP-1 would function synergistically to activate MEF2-dependent transcription. Indeed, we found that the transcriptional activity of a fragment of MEF2C containing the MADS/MEF2 domain fused to the DNA-binding domain of GAL4 [Gal4–MEF2C-(1–174); Molkentin et al. 1995] was enhanced only slightly by cotransfection with either GRIP-1 or activated CaMKIV (Fig. 4A, lanes 1–3). In contrast, cotransfection of Gal4–MEF2C-(1–174) with both GRIP-1 and activated CaMKIV led to robust transcriptional activity of this fusion protein (Fig. 4A, lane 4). Whereas activated CaMKIV could induce the activity of both Gal4–MEF2C-(1–174) or Gal4–MEF2C-full length, synergistic activation by cotransfected GRIP-1 and CaMKIV was most evident with Gal4–MEF2C-(1–174) [data not shown].

To evaluate whether CaMKIV could augment the association of MEF2C with GRIP-1, we examined the interaction of these proteins by coimmunoprecipitation in transfected COS cells [Fig. 5A]. COS cells were cotransfected with flag-tagged MEF2C [Chen et al. 2000], flag-tagged activated CaMKIV [McKinsey et al. 2000b], and HA-tagged GRIP-1 [Ma et al. 1999]. GRIP-1–HA and associated proteins were coimmunoprecipitated with an anti-HA monoclonal antibody (NM11). When GRIP-1–HA and MEF2C-Flag were coexpressed in COS cells,
MEF2C-Flag was readily detected in the GRIP-1–HA immunoprecipitation (Fig. 5A, lane 2). When these same proteins were cotransfected in the presence of an activated form of CaMKIV, the apparent association between MEF2C and GRIP-1 was augmented significantly as increased levels of MEF2C-Flag were detected in the GRIP-1–HA immunoprecipitation (Fig. 5A, lane 4). Interestingly, CaMKIV-Flag was also present in the GRIP-1–HA IP, when cotransfected in the presence (Fig. 5A, lane 4) but not in the absence (Fig. 5A, lane 3) of MEF2C-Flag. Importantly, when MEF2C-Flag and CaMKIV-Flag were cotransfected in the absence of GRIP-1–HA, they each failed to be immunoprecipitated by the anti-HA monoclonal antibody (Fig. 5A, lane 1). These findings suggest that activated CaMKIV can induce MEF2 transcriptional activity by augmenting the association of GRIP-1 with MEF2. In addition, CaMKIV may facilitate the association of GRIP-1 with MEF2 by both directing the cytoplasmic localization of class II HDACs as well as by stabilizing a ternary complex between MEF2, GRIP-1, and CaMKIV (schematically shown in Fig. 4B).

Cyclin D–cdk4 inhibits GRIP-1–MEF2C association by blocking the association of GRIP-1 and MEF2C

Because cyclin D–cdk4 blocks MEF2 function independently of altering CaMKIV-induced cytoplasmic sequestration of class II HDACs (Fig. 3), we wondered if cyclin D–cdk4 disrupts the ability of GRIP-1 to potentiate the activity of MEF2. 10T1/2 cells were cotransfected with the Gal4x5 luciferase reporter plus Gal4–MEF2C-(1–174), activated CaMKIV and GRIP-1 in either the absence or presence of the various D-type cyclins or cyclin E together with their cognate cdk (i.e., cdk4 or cdk2, respectively, schematically outlined in Fig. 4B). Whereas cotransfection of each of the D-type cyclins and cdk4 significantly reduced the expression of the Gal4 reporter by Gal4–MEF2C-(1–174) (Fig. 4C, lanes 2–5), cotransfection with cyclin E/cdk2 only slightly dampened expression of the reporter construct (Fig. 4C, lane 6). Thus D-type cyclin/cdk4 complexes specifically disrupt the synergistic activation of Gal4–MEF2C-(1–174) function by activated CaMKIV and the coactivator GRIP-1.

To evaluate if cyclin D–cdk activity disrupts the association of MEF2C with GRIP1, we cotransfected COS cells with MEF2C-Flag and GRIP-1–HA in either the absence or presence of cyclin D3 or cyclin D3–cdk4 and monitored the association of MEF2C with GRIP1 by coimmunoprecipitation. Immunoprecipitation of MEF2C-Flag with an anti-Flag monoclonal antibody led to coimmunoprecipitation of cotransfected GRIP-1–HA (Fig. 5B, lane 2). Although cotransfection of cyclin D3, in the absence of cdk4, failed to affect the amount of GRIP-1 that was coimmunoprecipitated with MEF2C-Flag (Fig. 5B, lane 3), the addition of cyclin D3 together with cdk4 led to a significant decrease in coimmunoprecipitated GRIP-1 (Fig. 5B, lane 4). Importantly, the total amounts of either MEF2C-Flag or GRIP-1–HA expressed in cells were not significantly affected by the presence of cyclin
D3–cdk4. Because cotransfection of the catalytically inactive cdk4DN together with cyclin D3KE failed to block Gal4–MEF2C function [Fig. 2B, lane 4], we investigated if cyclin D3KE and cdk4DN would affect the binding of GRIP-1 with MEF2C, like their wild-type counterparts. Cotransfection of a Gal4–GRIP-1 fusion construct and MEF2C-Flag into COS cells led to a detectable Gal4–GRIP-1–MEF2C-Flag complex as detected by the presence of MEF2C in an anti-Gal4–GRIP-1 immunoprecipitation [Fig. 5C, lane 2]. As described above, the GRIP-1–MEF2C complex was disrupted by cotransfection of cyclin D3WT and cdk4WT, as evidenced by the loss of MEF2C-Flag from the Gal4–GRIP-1 immunoprecipitation [Fig. 5C, lane 3]. In contrast, cotransfection of cdk4DN and cyclin D3KE failed to block the association of Gal4–GRIP-1 with MEF2C-Flag [Fig. 5C, lane 4]. Thus, these findings indicate that an active cyclin D–cdk4 complex can block the association of the coactivator GRIP-1 with MEF2C and may thereby repress the transcriptional activity of the latter.

**GRIP-1 interacts with both cyclin D3 and cdk4**

We wondered if cyclin D–cdk4 activity inhibits the association of GRIP-1 with MEF2 by either directly binding to or phosphorylating either of these proteins. Indeed, other SRC family members (i.e., SRC1 and SRC3) have been found to specifically associate with cyclin D1 [Neuman et al. 1997; Zwisjen et al. 1998]. Interaction of GRIP-1–HA with exogenous cyclin D3 and/or cdk4 was assayed by coimmunoprecipitation of GRIP-1–HA-associated proteins in extracts made from cotransfected COS cells. We found that when cotransfected with GRIP-1–HA, both cyclin D3 and cdk4 could be coimmunoprecipitated with GRIP-1–HA [Fig. 6, lanes 2–4]. Because kinase activity is necessary for cyclin D–cdk4 to efficiently block MEF2 function [Fig. 1B], we speculate that cyclin D–cdk4 may block the association of GRIP-1 with MEF2 by directly binding to GRIP-1 and phosphorylating sites within either this protein or MEF2.

**GRIP-1 localization to punctate nuclear structures proceeds that of MEF2C during skeletal muscle differentiation**

Because GRIP-1 and MEF2C colocalized to identical subnuclear domains [Fig. 7], we wanted to evaluate if these proteins colocalized to such
structures during skeletal muscle differentiation. Immunofluorescence was used to analyze the localization of endogenous MEF2C and GRIP-1 in both proliferating C2C12 myoblasts and in these cells after exposure to differentiation conditions (i.e., low mitogens) for either 36 or 72 h. Although both MEF2C and GRIP-1 were undetectable in most proliferating myoblasts, an occasional cell expressed relatively low levels of each protein (Fig. 8A, panels A–C). After incubation in low mitogen medium for 48 h, both MEF2C and GRIP-1 levels were observed to rise by Western analysis (Fig. 8B, lanes 1–4), and by 36 h in differentiation conditions, each accumulated in punctate nuclear structures (Fig. 8A, panels D–F). Interestingly, GRIP-1 was observed in punctate nuclear structures in cells containing only trace levels of MEF2C (arrows in Fig. 8A, panels D–F). In contrast, MEF2C association with these structures only occurred in cells showing readily detectable GRIP-1. After 72 h in differentiation conditions, while GRIP-1 remained localized exclusively to the punctate nuclear structures (Fig. 8A, panel I), MEF2C was localized diffusely throughout the nuclei as well as in the punctate structures (Fig. 8A, panel H). Although we failed to detect GRIP-1 in punctate nuclear structures in the majority of proliferating myoblasts by immunofluorescence, the protein is present in these cells as it could be readily detected by Western analysis in both proliferating myoblasts and in differentiated myotubes (Fig. 8B, lanes 1–4). These findings suggest that mitogen signaling in proliferating myoblasts somehow excludes GRIP-1 association with the punctate nuclear structures. After 36 h in differentiation medium, GRIP-1 association with the punctate nuclear structures proceeds that of MEF2C. After 72 h in differentiation medium, MEF2C expression continues to rise to levels that may saturate MEF2C binding sites in the punctate nuclear structures; consequently, MEF2C is present in both the punctate nuclear structures as well as being distributed diffusely throughout the nucleus.

![Figure 8](https://genesdev.cshlp.org)
GRIP-1 cotransfection is necessary to localize MEF2 to the punctate subnuclear domains

Although we have found that both GRIP-1 and MEF2C can be sequestered into nuclear subdomains, it is unclear which component of this complex is driving this cellular localization. In the instance of steroid hormone receptors, it is the ligand-bound receptor that translocates into these nuclear subdomains and brings the SRC family member [i.e., GRIP-1] along for the ride (Schaufele et al. 2000, Stenoien et al. 2000a,b). Thus, we wondered whether translocation of either GRIP-1 or MEF2C into such nuclear subdomains relied upon the presence of the other protein. To evaluate this, we transfected 10T1/2 cells with either MEF2C–Flag alone, GRIP-1–HA alone, or the combination of MEF2C–Flag plus GRIP-1–HA, and analyzed the cellular localization of transfected MEF2C and GRIP-1 by immunofluorescence. When transfected individually, MEF2C–Flag was present in a diffuse pattern throughout the nuclei (Fig. 9B), but GRIP-1–HA was localized to punctate nuclear subdomains in ∼30% of transfected cells [Fig. 9F,F′]. In contrast, when cotransfected together, MEF2C was now colocalized with GRIP-1 in the punctate nuclear structures (Fig. 9H,H′,I,I′). Importantly, MEF2C was only located in punctate nuclear structures in that 30% of transfected cells that contained GRIP-1 in these same nuclear subdomains. These results suggest that trafficking of MEF2C into the punctate nuclear structures requires interaction of this transcription factor with GRIP-1. Consistent with our findings that MEF2C translocates to punctate nuclear subdomains in a GRIP-1-dependent manner, we have found that during the process of skeletal muscle differentiation, GRIP-1 is first observed to localize to the punctate nuclear structures prior to the appearance of MEF2C in these subnuclear domains [see arrows in Fig. 8A, panels D–F].

D-type cyclin/cdk4 overexpression affects the subnuclear distribution of GRIP-1

Because SRC family members translocate into punctate subnuclear domains when bound to ligand-bound steroid
hormone receptors (Stenoien et al. 2000a,b), we wondered if colocalization of MEF2C and GRIP-1 into these same structures might correlate with the transcriptional activity of MEF2. To begin to address this issue, we monitored whether the subnuclear localization of GRIP-1 would be affected by cotransfection with cyclin D3–cdk4. Indeed, we found that although GRIP-1 accumulated in subnuclear structures of 25%–36% of cells transfected with GRIP-1 alone, this protein was present in such subnuclear domains in only 2%–5% of cells cotransfected with cyclin D3/cdk4 (see Table 1). We noted that the few cells that expressed GRIP-1 in subnuclear structures following cyclin D3–cdk4 cotransfection failed to express ectopic cyclin D3, suggesting that cyclin D3–cdk4 drives complete exclusion of GRIP-1 from such subnuclear structures. Cotransfection of MEF2C with GRIP-1 did not significantly alter the percentage of cells that showed punctate subnuclear staining of GRIP-1 (31%–41%, Table 1). However, cotransfection of cyclin D3–cdk4 plus MEF2C and GRIP-1 reduced the subnuclear localization of both MEF2C and GRIP-1 to 5%–8% of transfected cells [Fig. 9J–L, Table 1]. These findings indicate that exogenous GRIP-1 is localized to punctate subnuclear structures in the cell and that this localization is disrupted by cyclin D3–cdk4 overexpression. Consistent with our findings that cdk catalytic activity is necessary to disrupt MEF2C function, we observed that cotransfection of cyclin D3 with cdk4DN failed to disrupt the subnuclear localization of GRIP-1 (data not shown). Thus, the presence of GRIP-1 in punctate subnuclear structures correlates with the functional activity of MEF2C within the cell.

**Discussion**

**Cyclin D–cdk4 activity inhibits the association of MEF2 with GRIP-1 and disrupts the localization of these proteins to punctate nuclear structures**

We have found that cyclin D–cdk4 activity can block MEF2C activity without altering CaMK-induced cytoplasmic retention of class II HDACs. These findings suggest that cyclin D–cdk4 blocks MEF2C function independently of modulating the interaction of MEF2C with class II HDACs. Consistent with this idea, we have found that the interaction of MEF2C with the SRC family member GRIP-1 is inhibited by ectopic cyclin D–cdk4 expression. Because the MADS/MEF2 domain, which is conserved among all MEF2 family members (Black and Olson 1998), is able to support interaction with GRIP-1 (Chen et al. 2001), it seems likely that cyclin D–cdk4 activity can block the interaction of all MEF2 family members with this coactivator. As cyclin D1/cdk4 activity is limited to proliferating myoblasts (Wang and Walsh 1996), which are known to contain MEF2D (Breitbart et al. 1993), it seems likely that the transcriptional activity of this isoform of MEF2 is restrained in proliferating myoblasts by cyclin D1–cdk4 activity. Furthermore, because MyoD activates the expression of other MEF2 family members, including MEF2C (Wang et al. 2001) during the differentiation process, the presence of active cyclin D1–cdk4 in proliferating myoblasts would serve as a failsafe mechanism to ensure that such isoforms of MEF2 would not be functional if they were promiscuously expressed in proliferating cells.

GRIP-1 nuclear localization is modulated during the skeletal muscle differentiation process. Although the protein is present in both proliferating myoblasts and differentiated myotubes, we could detect this protein in punctate nuclear structures only in the differentiated myotubes. Muscat and colleagues have found that GRIP-1 can also be detected in punctate subnuclear structures in proliferating myoblasts (Chen et al. 2001), but both this prior work and our own findings suggest that the localization of GRIP-1 to these subnuclear structures is more prominent in differentiated myotubes (Chen et al. 2001; this work). During skeletal muscle differentiation, GRIP-1 can be detected in the punctate nuclear structures prior to MEF2C, suggesting that GRIP-1 may somehow initiate the association of MEF2 with these structures. Indeed, we have found that GRIP-1 is capable of tethering MEF2C to these punctate nuclear structures and that cyclin D–cdk4 activity inhibits the accumulation of both MEF2C and GRIP-1 into this nuclear subdomain. Our results suggest that cyclin D1–cdk4 activity in proliferating myoblasts acts to repress the muscle differentiation program by blocking both the interaction of MEF2 family members with the coactivator GRIP-1 and localization of both these pro-

**Table 1. Cyclin D3–cdk4 blocks the localization of GRIP-1 to punctate nuclear subdomains**

| Plasmid transfected | C3H10T1/2 cells showing a GRIP-1-HA nuclear staining | Punctate nuclear | Diffuse nuclear |
|---------------------|----------------------------------------------------|-----------------|----------------|
| GRIP-1-HA + vehicles | Exp 1 = 200 | 35% | 65% |
|                     | Exp 2 = 200 | 25% | 75% |
|                     | Exp 3 = 100 | 36% | 64% |
| GRIP-1-HA Cyclin D3, | Exp 1 = 200 | 2% | 98% |
| Cdk4 + vehicles     | Exp 2 = 200 | 4% | 96% |
|                     | Exp 3 = 100 | 5% | 95% |
| GRIP-1-HA MEF2C     | Exp 1 = ND* | — | — |
| + vehicles          | Exp 2 = 200 | 31% | 69% |
|                     | Exp 3 = 100 | 41% | 59% |
| GRIP-1-HA Cyclin D3, | Exp 1 = ND* | — | — |
| Cdk4 MEF2C          | Exp 2 = 200 | 5% | 95% |
|                     | Exp 3 = 100 | 8% | 92% |

10T1/2 cells were cotransfected with the indicated plasmids and subsequently fixed and immunostained for the localization of transfected GRIP-1–HA. Cells showing either punctate or diffuse nuclear staining of GRIP-1–HA were tallied in each experiment.
teins to the punctate nuclear subdomains. As cyclin D–cdk4 complexes can bind to SRC family members in vivo [Neuman et al. 1997; Zwijsen et al. 1998; this work], it seems plausible that phosphorylation of GRIP-1 by cyclin D–cdk4 may induce a conformation of GRIP-1 that is unable to associate with both MEF2 and the punctate nuclear structures.

In addition, work of others has established that mitogenic signals additionally block the activity of MyoD in proliferating myoblasts by both MEK-dependent [Perry et al. 2001] and cdk4-dependent [J.M. Zhang et al. 1999] pathways (summarized in Fig. 10). Together, these findings indicate that mitogenic signals use several distinct pathways to ensure that myogenic transcription factors are functionally inert in proliferating cells. Both inhibition of MEF2C function and release of MEF2C and GRIP-1 from the punctate nuclear structures by cyclin D–cdk4 require cdk catalytic activity. Although cyclin D3 is present at high levels in differentiated myotubes, because of the high level of cdk inhibitors in these cells [Halevy et al. 1995; Franklin and Xiong 1996; P. Zhang et al. 1999], cyclin D3–cdk4 complexes are catalytically inactive in differentiated myotubes [Cenciarelli et al. 1999] and, therefore, would not be expected to block MEF2 function in differentiated skeletal muscle.

The association of GRIP-1 and MEF2 with punctate nuclear structures may be regulated by environmental conditions

We have found that whereas ~30% of 10T1/2 cells cotransfected with GRIP-1 and MEF2 colocalize both GRIP-1 and MEF2 to punctate nuclear structures, 100% of differentiated myotube nuclei contain GRIP-1 in these nuclear subdomains [see also Chen et al. 2001]. It is unclear why only a portion of transfected 10T1/2 cells show accumulation of GRIP-1 and MEF2C in these punctate nuclear structures. However, we think it likely that physiological conditions may influence the association of GRIP-1 with the punctate nuclear structures and could potentially influence the activity of MEF2 family members. Consistent with this notion, we have found that cyclin D–cdk4 expression blocks MEF2 transcriptional activity, the association of MEF2 with the coactivator GRIP-1, and the localization of GRIP-1 and MEF2 to punctate nuclear structures. Work with nuclear steroid hormone receptors has indicated that GRIP-1 is shuttled into such nuclear subdomains upon binding to ligand-bound steroid hormone receptors, which are also shuttled into these subdomains of the nucleus when they are transcriptionally active [Schaufele et al. 2000; Stenoien et al. 2000a,b]. We are currently investigating if environmental signals that promote skeletal muscle differentiation, such as IGF-1 signaling or activated p38 [Zetser et al. 1999; Wu et al. 2000], act to promote the accumulation of GRIP-1 and MEF2 into the punctate nuclear structures in differentiated myotubes.

What is the physiological significance of GRIP-1–MEF2 association with punctate nuclear structures?

We have found that whereas all detectable GRIP-1 is associated with the punctate nuclear structures in differentiated myotubes, only a portion of MEF2C is associated with such structures, the remainder being present diffusely throughout the nuclei. During the process of skeletal muscle differentiation, low levels of MEF2C initially accumulate in the punctate nuclear structures, prior to the accumulation of high levels of MEF2C in both the punctate structures and diffusely throughout the nuclei, suggesting that MEF2C-binding sites within the punctate nuclear structures may become saturated by high levels of MEF2C. As GRIP-1 is apparently necessary to tether MEF2C to these structures, it is possible that GRIP-1 or other SRC family members are the limiting MEF2C-binding component in the punctate nuclear structures. In addition, because it is known that GRIP-1 can rapidly exchange in and out of the punctate nuclear structures when bound to steroid hormone receptors [Schaufele et al. 2000], it seems possible that the GRIP-1–MEF2 complex may similarly diffuse in and out of these structures. It will be interesting to determine what domains of GRIP-1 are necessary to tether MEF2 to the punctate nuclear structures and whether the transcriptional activity of MEF2 can be uncoupled from association with these subnuclear domains.

Materials and methods

Cell culture and transient transfection

C2C12 myoblasts, C3H-10T1/2 fibroblasts, and COS7 cells were grown in Growth Medium [GM: DMEM supplemented with 10% Fetal Bovine Serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin]. Differentiated C2C12 cells were obtained by substituting Growth Medium with Differentiation
Medium (DM: DMEM supplemented with 2% Horse Serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin). Cells were plated and transfected in 12-well plates for promoter-luciferase assays and in 10-cm plates for immunoprecipitations and indirect immunofluorescence microscopy. The cells were transfected using the FuGene 6 [Roche] reagent, according to the manufacturer's protocol. Each well of a 12-well plate received 0.5 µg of DNA, and each 10-cm plate received 10 µg of DNA.

**Promoter assays and plasmids**

The eukaryotic expression vehicles used in this study were transfected into cells as described above. Luciferase activity-based promoter assays were performed in triplicate, using the Luciferase Assay System [Promega] and the manufacturer's standard protocol. Luciferase activity was measured using a Turner Designs luminometer. The Gal4 promoter activity assays were performed with a Gal4x5LUC construct [PG5-LUC, Promega]. MEF2-responsive promoter activity was monitored using MEF2x5LUC. pRC-CMV-Cyclin E, D1, D2, and D3 wild-type and mutant constructs were obtained from Phil Hinds with the exception of the pRC-CMV-Cyclin D3-K112E, which was engineered in this laboratory. pCMV-neo-bam-cdk4 wild-type and dead kinase mutant [cdk4-KM] and pCMV-neo-BAM-cdk2 were obtained from Ed Harlow and Sander Van den Heuvel. pCMX-MEF2C-Flag was obtained from Dennis Dowhan-he and George Muscat. PSG5-GRIP-1-HA was obtained from Michael Stallcup. The pGal4-MEF2C construct was obtained from Jiahuai Han [Han et al. 1997]. pCS2-MEF2A, pcDNA1-MEF2B, pcDNA1-MEF2D, pGal4-MEF2C-1–174, pCMX-HDAC7, and an activated form of CaMKIV [pSGS-CaMK-Flag containing a codon stop in place of gln318] were obtained from Eric Olson. pRC-CMV-p16 is described in Skapek et al. [1995], and pCMV-p21cip1 was obtained from Wade Harper and Steve Eldledge [Harper et al. 1993]. pCS2-mouse-MyoD and pCS2-mouse-myogenin were obtained from M. Horwitz.

**Indirect immunofluorescence microscopy**

The cells were grown in 10-cm dishes, as described above, fixed in PBS-4% paraformaldehyde for 10 min at room temperature (extracted with methanol for 10 min if indicated), and subsequently incubated in PBS containing 4% Bovine Serum Albumin (BSA) for another 10 min. Primary antibodies were incubated in PBS-4% BSA at 37°C for 1 h at a 1/300 dilution. Cyclin D3 was detected using the C-16 rabbit polyclonal antibody [Santa Cruz Biotechnology]. Flag- and HA-tagged proteins were detected using the M2 anti-Flag [Sigma] and anti-HA [BabCO] rabbit polyclonal antibody (HA11), respectively. Endogenous MEF2C and GRIP-1 were detected using a rabbit anti-MEF2C [Cell Signaling Technology] and a monoclonal anti-TIF2 [Transduction Laboratories], respectively. Secondary antibodies were incubated in PBS-4% BSA at 37°C for 30 min at a 1/200 dilution. We used combinations of anti-rabbit or anti-mouse goat IgGs conjugated to Alexa Fluor-488 [Molecular Probes] and anti-mouse CY3 or anti-rat CY3 IgGs [Jackson Immunchemicals]. DNA was visualized by DAPI staining [Sigma].

**Immunoprecipitation and Western blotting**

Cells were collected in PBS, pelleted by gentle centrifugation at room temperature, and lysed. Typically, the pellet of one 10-cm dish was lysed in 350 µL of lysis buffer [200 mM NaCl, 50 mM Tris-HCl at pH 7.4, 2 mM EDTA, 0.5% NP-40, supplemented with anti-protease tablets; Roche] at 4°C for 15 min. After a brief sonication, insoluble materials were pelleted, and the supernatant (lysate) was collected. For each transfection, 5% of the lysate was aliquoted as an input control. Flag-tagged, Gal4-tagged, and HA-tagged proteins were immunoprecipitated using either the M2-anti-Flag [Sigma], the BK5C1-anti-Gal4 [Santa Cruz Biotechnology], or the HA11-anti-HA monoclonal antibodies [BabCO], respectively. The lysate was incubated at 4°C for 1 h on a rotating wheel, with 5 µg of the indicated antibody. Subsequently, 25 µL of A/G protein beads was added to each lysate, and the lysate was rotated at 4°C for an additional 30 min. Finally, the beads were washed three times in lysis buffer, and both immunoprecipitates and input controls were denatured at 95°C for 2 min in Laemmli Buffer. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes [Schleicher & Schuell], and analyzed by immunoblotting using the indicated antibodies at 1/500 to 1/2000 dilutions.

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