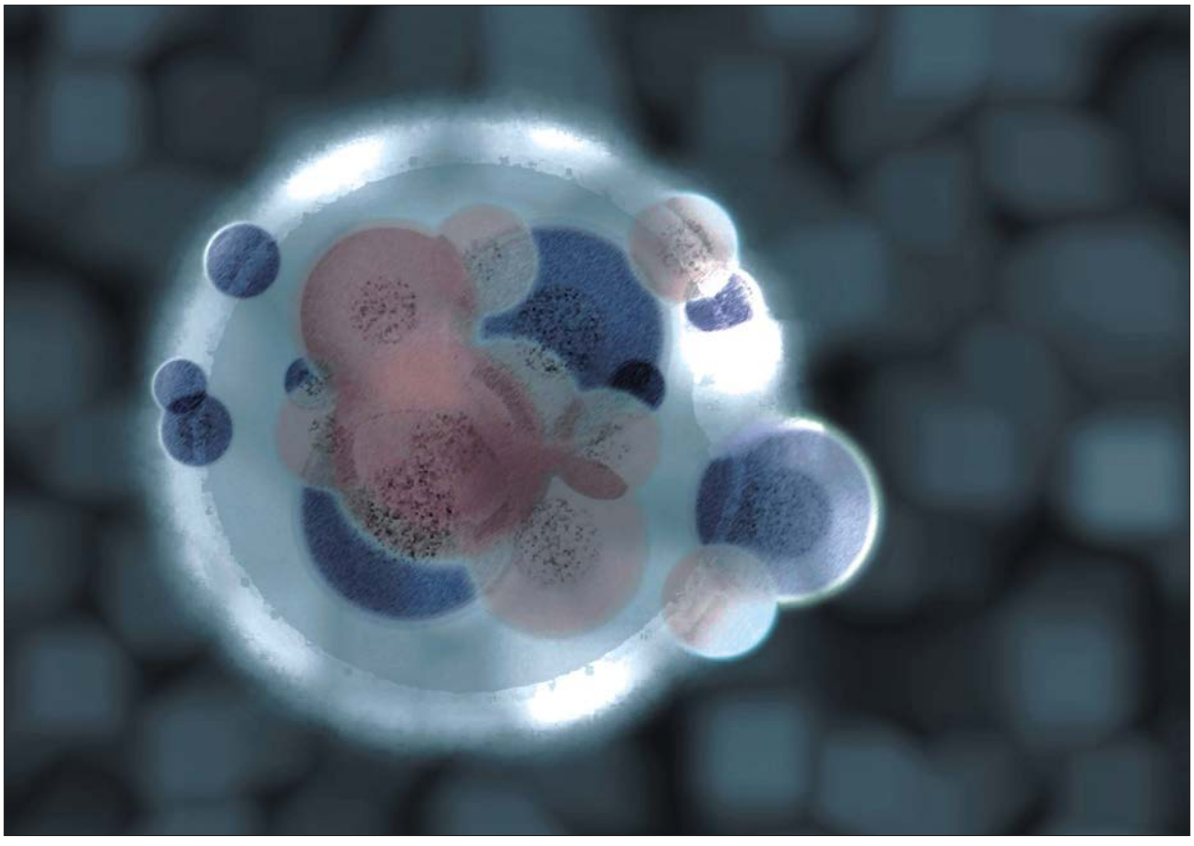

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Rapamycin Regulates Myogenesis by Inhibiting Myogenin Downregulation in BC3H1 Muscle Cells

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Abstract: In higher organisms most cell types exist in a quiescent state, G_0. Upon activation, cells exit from G_0 and enter the proliferative phases of the cell cycle. Rapamycin-FKBP12 is a potent inhibitor of proliferation of many cell types. In BC3H1 myogenic cells, rapamycin induces differentiation while inhibiting cellular proliferation. Since terminal differentiation requires withdrawal from the cell cycle, we have studied various cell-cycle regulators, the differentiation factor myogenin and the inhibitor of differentiation, which might be involved in this process. In the present study, we show that myogenic differentiation in the presence of rapamycin is coupled with the inhibition of phosphorylation of retinoblastoma protein (pRB) and sustenance of myogenin without affecting inhibitor of differentiation (Id) protein.

Key words: Rapamycin, differentiation, proliferation, myogenin, myogenesis, cdks

INTRODUCTION

The murine muscle cell line, BC3H1[1], lacks MyoD and does not fuse[2], but is capable of reversible differentiation[3] and does express myogenin, a related muscle-specific transcription factor[4]. We have reported that rapamycin-FKBP12 inhibited proliferation and induced α-actin expression, a differentiation marker in BC3H1 cells[5]. However, the signaling components that are involved during rapamycin-induced differentiation of these non-fusing muscle cells are not known.

In muscle cells, proliferation and differentiation are regulated by interaction between myogenic factors and regulators of cell-cycle progression[6]. Cyclin E (CyE) is necessary and rate limiting for the passage of mammalian cells through the G1 phase of the cell cycle and functions by regulating cyclin dependent kinase 2 (cdk2) activity. pRb is a nuclear protein and its phosphorylation is required for proliferation[7]. Cells in early G1 contain exclusively unphosphorylated or underphosphorylated pRb. At some point in late G1, pRb is hyperphosphorylated and remains in this state until M phase. Hypophosphorylated pRb suppresses the progression from G1 to S phase[8]. A major discovery that facilitated progress in understanding cell determination and differentiation came with the discovery of a family of myogenic regulatory factors (MRFs) genes[9], whose expression initiates a cascade of events that initiate muscle-cell differentiation. Gene-targeting experiments indicate that myogenin plays an essential *in vivo* role in the terminal differentiation of myotubes[10]. The expressions of inhibitor of differentiation proteins (Id) are also involved in cell cycle control and prevent differentiation of myoblasts[11]. To elucidate the molecular mechanisms underlying myogenic proliferation and differentiation, we examined the effects of rapamycin-FKBP12 on pRb phosphorylation, cell-cycle regulators cdk2 and E2F-2, the myogenic regulatory proteins myogenin and inhibitor of differentiation 1 (Id1).

MATERIALS AND METHODS

Cell culture: BC3H1 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 20% fetal bovine serum [FBS, growth medium (GM)], 100 units mL^{-1} penicillin and streptomycin. To initiate differentiation, GM was replaced with DMEM containing 0.5% FBS [differentiation medium (DM)]. The immunosuppressive drugs rapamycin and FK506 were added at the initiation of the cultures as indicated.

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Immunoblotting: Anti-pRb and α-myogenin monoclonal antibodies were obtained from Pharmingen and polyclonal antibodies to cdk2, Id1 and E2F-2 were purchased from Upstate Biotechnology Inc. and SantaCruz Biotechnology, respectively. BC3H1 cells were made quiescent by plating in DM (containing 0.5% FBS) for 48-72 h. The cells were then switched to GM (containing 20% FBS) in the presence and absence of rapamycin and FK-506. Cell lysates were prepared from cells at indicated time periods. Immunoblotting was performed as described[12].

Kinase assays: Quiescent BC3H1 cells were treated with either no drugs (controls) or 100 ng mL^{-1} rapamycin or FK506 in growth medium. At the described time points, cells were lysed in pRb lysis buffer [(50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1mM EDTA, 0.1 mM NaF, 0.2 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin, 10 μg mL^{-1} soybean trypsin inhibitor and 0.5% NP-40)] and protein concentration was measured using the Bradford reagent. The protein extract (100 μg) was diluted to 500 μL in RIPA buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1% SDS and 5 mM dithiothreitol) and incubated with anti-cdk2 antibody for 2 h. Protein A-sepharose (40 μL) was added and gently rocked for 1 h at 4°C. After washing, kinase reactions were carried out using histone H1 (Boehringer Mannheim) and 50 μM γ^{32}P[ATP]. Samples were analyzed by electrophoresis on 12% SDS-polyacrylamide gels. Gels were dried for 2 h and quantitated using a phosphorimager and Quant Image 1.44 software.

RESULTS

In order to further understand the signaling components involved during myogenic differentiation, we have analyzed the effect of rapamycin on Rb phosphorylation *in vitro* using BC3H1 cell cultures. In BC3H1 cells, rapamycin-FKBP12 delayed pRb phosphorylation (Fig. 1). Upon addition of growth medium (GM, containing 20% FBS) to quiescent cells, the phosphorylated form of pRb was first detected at 12 h and by 16 and 20 h essentially only phosphorylated pRb was detected (Fig. 1). Whereas in rapamycin treated cultures, significant amount of hypo-phosphorylated pRb is present. Unlike rapamycin-FKBP12, FK506 did not inhibit pRb phosphorylation and by 16 h, as in control, only hyperphosphorylated pRb was detected (Fig. 1). FK506, like rapamycin, also binds to FKBP12, but does not inhibit BC3H1 proliferation or induce differentiation. These

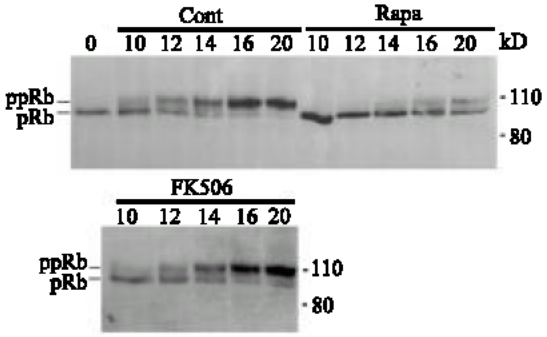

Fig. 1: Serum induced pRb Phosphorylation is blocked by rapamycin. 75 μg of protein lysate from each time point was size-fractionated on a 7.5% SDS-PAGE followed by transfer to nitrocellulose and immunoblotting with an antibody against pRb (human). The indicated times are in hours after switching from DM to GM. The positions of hyperphosphorylated ("ppRb") and underphosphorylated ("pRb") in each case only relevant portions of the gels are shown

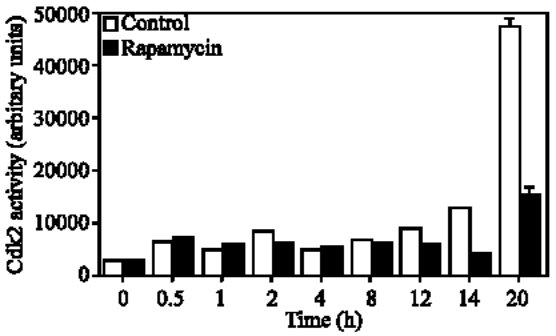

Fig. 2: Inhibition of cdk2 kinase activity by rapamycin. Cdk2 kinase activity was examined in the presence (filled bars) or absence (open bars) of rapamycin at the indicated times. Rapamycin prevented the significant rise in cdk2 activity seen after 12 h in control BC3H1 cells (and associated with pRb phosphorylation). The results are representative of two independent experiments

results suggest that rapamycin prevents cell cycle entry by blocking pRb phosphorylation.

We next examined the activity of the cyclin E-associated kinase, $p33^{cdk2}$. There was no marked difference in cdk2 kinase activity prior to 12 h (Fig. 2) in cells cultured in the presence and absence of rapamycin. However, compared to controls, cdk2 kinase activity was significantly decreased after 12 h in rapamycin-treated cultures. These results show that the inhibition of pRb phosphorylation is associated with a decrease in cdk2 kinase activity.

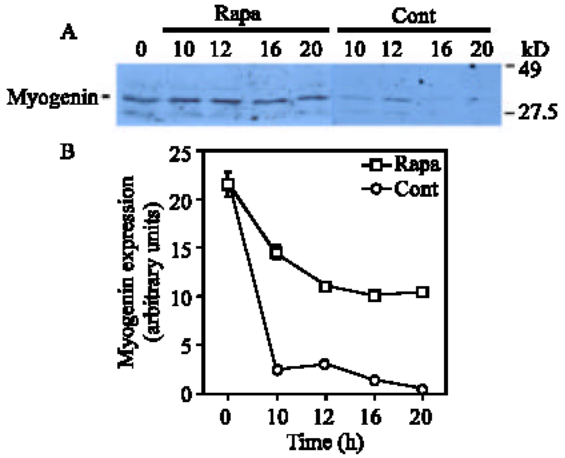

Fig. 3: Myogenin downregulation is prevented by rapamycin. A) Myogenin expression was determined at the indicated times in BC3H1 cells cultured in the presence and absence of rapamycin. B) Densitometry analysis of myogenin expression by NIH image software

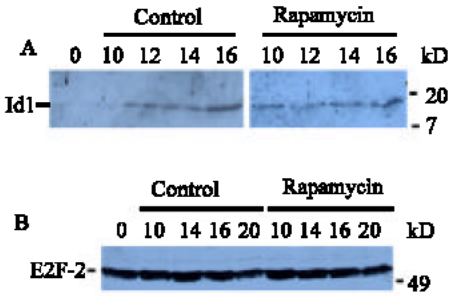

Fig. 4: Id1 and E2F-2 are not affected by rapamycin. Id1 (A) and E2F-2 (B) expression were determined in BC3H1 cells cultured in the presence and absence of rapamycin after the indicated times

Myogenin expression is required for BC3H1 cell differentiation. We therefore performed immunoblot analysis to study myogenin expression in BC3H1 cells cultured in the presence and absence of rapamycin. Present results show that serum starved quiescent cells, as expected, expressed higher levels of myogenin expression, which was greatly reduced upon serum addition. Indeed, no myogenin was detected 20 h after serum addition. On the other hand, myogenin expression was readily detectable in BC3H1 cells treated with rapamycin even at 20 h and was higher at all times as compared to cells cultured with serum alone (Fig 3A and B). These results suggest that presence of rapamycin in the culture inhibits serum-induced downregulation of myogenin.

We next investigated whether or not the prolonged expression of myogenin is due to alteration of negative modulators of differentiation. Figure 4 shows that BC3H1 cells at a quiescent state express very low levels of Id1 and addition of serum induces Id1 expression in control skeletal muscle cells. The proliferative stages of cells (i.e. 16 h after serum addition) had the highest levels of Id1 expression. In contrast, rapamycin-treated cultures had comparable levels of Id1 expression except for at the 10 h time point. A recent finding that lymphocytes from E2F-2 null mice have increased proliferation indicates that E2F-2 acts as a transcriptional repressor. We therefore investigated to see whether E2F-2 expression is increased after rapamycin treatment. However, we have not found any significant differences in E2F-2 expression between control and rapamycin-treated BC3H1 cells. These results suggest that rapamycin does not have any effect on E2F-2 expression.

DISCUSSION

In this study, we provide an insight into the molecular mechanism of rapamycin induced differentiation. First, the rapamycin inhibition of pRb phosphorylation in BC3H1 cells is correlated with cell-cycle progression[13]. The fact that FK506 did not block pRb phosphorylation and prevent cell cycle progression[14] though it binds to the same cytosolic protein as rapamycin suggest the divergence of signaling pathways by rapamycin. Since pRb phosphorylation was unaffected after 10 h into cell cycle progression and cdk2 is known to regulate pRb function via phosphorylation[1,5], we assessed cdk2 activity. Cdk2 activity is increased at 12 h and is maximal at 16 h after serum addition in consistent with the time course of the inhibition of pRb phosphorylation.

Second, myogenin expression as assessed by immunoblot analysis, was higher in quiescent cells and was repressed upon serum addition. Whereas, myogenin protein expression was higher in rapamycin treated BC3H1 cells than in serum induced cells. However, we do not know if this is due to either increased myogenin synthesis or decreased myogenin degradation as myogenin mRNA expression was unaffected by rapamycin treatment (data not shown). Myogenin is very closely related member of the MyoD family, which is degraded by cdk2 via phosphorylation dependent mechanism. The down regulation of proteins is blocked by cdk inhibitors, roscovitine and p57[14,16]. Because of its similarity with MyoD, it is tempting to speculate that myogenin is also regulated by cdk2 via phosphorylation. Interestingly,

there are four putative cdk-like phosphorylation sites (S/TPXX) present in myogenin at aminoacids 7, 43, 57 and 170. A particularly appealing application of this concept is the possibility of myogenin expression is linked to cell cycle regulators as serum ->increased cdk2 activity ->increased myogenin degradation. Rapamycin prevents myogenin degradation by blocking cdks activity. Additional experimental are required to test if any of these sites are involved in myogenin degradation. Since rapamycin-treated cells displayed relatively higher expression at 10 h as compared to control, it is conceivable kinases other than cdk2 that are regulated by rapamycin might also participate in myogenin degradation.

Conflicting reports show that rapamycin regulates myogenesis via kinases dependent[17,18] and independent pathway[19]. Present results suggest that rapamycin induced differentiation is coupled with inhibition of cdk2 activity. These discrepancies may arise partially due to the use of different cell lines as well as the mode of rapamycin treatment. For instance, we used non-fusing BC3H1 cells, while others have used fusing myoblast cell lines. Second, we used α-actin expression while others have used myoblast fusion as an indicator of differentiation. Third, we added rapamycin to cells cultured in a growth medium containing 20% FBS, whereas rapamycin was added to differentiated cells after serum starvation in other studies. While presence of many growth factors in FBS may impart different signals on SMCs growth in an intact cell system, experiments using individual growth factors on SMCs cannot easily replicate the signaling induced by multiple growth factors acting in tandem *in vivo*.

We next investigated the possibility that rapamycin could alternatively induce differentiation by decreasing either inhibitor of differentiation proteins or by acting independent of inhibitory factors. Since there was no significant difference in Id1 expression between control and rapamycin-treated cells, our results indicate that rapamycin induced differentiation is independent of Id1 expression.

E2F-1, -2 and -3, at a physiological level, associate exclusively with pRb and are sufficient to induce S-phase. While loss of E2F-2 has been reported to enhance lymphocyte proliferation[20], E2F-2 expression was not altered in our study after rapamycin treatment suggesting it does not participate in inhibition of proliferation. Additional experiments are required to determine the involvement of other members of E2F family in the differentiation of these cells.

In conclusion, present data indicate that the sustaining myogenin expression after rapamycin treatment might possibly be due to inhibition of cdk2 activity and myogenin degradation independent of Id1 and E2F-2. Rapamycin, thus, could be a useful agent for studying myogenic factors and differentiation pathways.

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