Rapamycin-FKBP12 Blocks Proliferation, Induces Differentiation, and Inhibits cdc2 Kinase Activity in a Myogenic Cell Line*

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Thottala Jayaraman and Andrew R. Marks†
From the Molecular Medicine Program, Department of Medicine and Brookdale Center for Molecular Biology, Mount Sinai School of Medicine, New York, New York 10029

Rapamycin is a potent immunosuppressant that binds to the cytosolic protein, FKBP12, and blocks T cell activation. Here we report that rapamycin also blocks myogenic proliferation and induces differentiation, associated with a decrease in p34\sup{cdk2} activity and cyclin A levels. In yeast and mammals, rapamycin blocks cell cycle progression by causing G1 arrest, arguing for a conserved signaling pathway governing the G1 to S transition. p34\sup{cdk2} has been shown to play a role in both the transition from G1 to S and from G2 to M in yeast. In higher eukaryotes the role of p34\sup{cdk2} in G1 to S transition is less clear. Rapamycin and the structurally related macrolide antibiotic FK506 both bind to a cytosolic protein, the FK506-binding protein (FKBP12). We show that inhibition of myogenic proliferation is achieved at low doses of rapamycin (<1 ng/ml) and is competed by a molar excess of FK506, indicating specificity for FKBP12. The distinct FK506-calcineurin pathway did not affect myogenic proliferation, differentiation, or p34\sup{cdk2} kinase activity. Thus, the rapamycin-FKBP12 signaling pathway involves a specific and direct effect on p34\sup{cdk2} kinase activity at the G1 to S transition and identifies a regulatory step during myogenic differentiation.

The structurally related immunosuppressants, FK506 and rapamycin, both bind FKBP12, a ubiquitously expressed cytosolic protein (1). FKBP12 is a cis-trans peptidyl-prolyl isomerase, but this enzymatic activity has been dissociated from the immunosuppressant properties of the two drugs (2). FK506-FKBP12 complex binds to and inhibits the activity of calcineurin, a Ca2+-calmodulin-dependent serine-threonine phosphatase (3). The actions of these drugs have been well characterized in T lymphocytes. FK506-FKBP12-calcineurin inhibits a Ca2+-dependent signaling pathway in a gain of function manner, blocking translocation of the cytoplasmic component of the transcription factor NF-AT required for interleukin-2 gene transcription and subsequent T cell activation (4). Rapamycin-FKBP12 inhibits a Ca2+-independent pathway and blocks the transition from G1 to S required for T cell proliferation (5). Rapamycin has been shown previously to inhibit proliferation in Swiss 3T3 cells (6), T cells (7-9), and in a hepatoma cell line, H4 (10). Rapamycin-FKBP12 inhibits activation of p70 S6 kinase, but does so indirectly, indicating that an upstream target(s) for the complex must exist (5, 6, 10). A recent study has shown a decrease in p34\sup{cdk2} kinase activation in T lymphocytes (11); however, the mechanism of rapamycin inhibition of p34\sup{cdk2} kinase activation remains unclear.

In the present study we have examined the role of the rapamycin-FKBP12 signaling pathway during myogenic proliferation. The basic helix-loop-helix proteins, including MyoD and myogenin, induce myogenic differentiation and block cell cycle progression (12, 13). Although it is still not known how the basic helix-loop-helix proteins achieve this regulation of myogenesis, it has been reported recently that the tumor suppressor retinoblastoma protein (pRB) interacts directly with MyoD and that pRB is required for the cell growth inhibitory activity of MyoD (14).

As a model for studying myogenesis, we have used a well characterized murine muscle cell line, BC3H1 (15), which lacks MyoD and does not fuse (16), but is capable of reversible differentiation. We have found that rapamycin-FKBP12 causes a cell cycle arrest at the G1/S transition in proliferating BC3H1 cells. Moreover, rapamycin-FKBP12 decreased the levels of cyclin A directly associated with inhibition of p34\sup{cdk2} kinase activation. In addition to the proliferative arrest, differentiation was induced, suggesting that rapamycin-FKBP12 targets a signaling pathway involved in the regulation of both myogenic proliferation and differentiation.

**EXPERIMENTAL PROCEDURES**

Materials—Immunosuppressive drugs FK520, rapamycin, and 818 were kindly provided by Dr. John Siekierka (Merck). [3H]Thymidine was from ICN Radiochemicals. Polyclonal anti-cyclin A antibody was from Upstate Biotechnology Inc. (New York), and polyclonal anti-p34\sup{cdk2} kinase antibody was a gift from Dr. Hiroaki Yikokawa (Memorial Sloan-Kettering Cancer Institute, New York). All other chemicals were from Sigma, and tissue culture reagents were from Life Technologies, Inc.

Cell Lines and Cultures—BC3H1 mouse cell line (15) was from the ATCC. Cultures were maintained in DMEM medium containing 20% fetal bovine serum, 100 unit/ml penicillin, and 100 ug/ml streptomycin. The medium was changed every 48 h. Microcultures were established as quadruplicates in flat bottom 96-well micratiter plates. 5 x 10^3 cells were plated in each well in the presence and absence of varying concentrations of drugs. After indicated time periods, cells were harvested, and [3H]thymidine incorporation was measured using a liquid scintillation counter.

Flow Cytometric Analysis—Cells were treated with either 100 ng/ml rapamycin or FK520 for 24 h, harvested, and washed in ice-cold phosphate-buffered saline, fixed in 70% ethanol, and stained overnight at 4 °C prior to analysis. Cells were then washed once with ice-cold phosphate-buffered saline, treated with RNase (1 h at 37 °C, 500 units/ml), and after washing, cellular DNA was stained with propidium iodide (50 μg/ml). Cell cycle determination was performed using a Coulter analyzer. Results represent a minimum of 3000 cells assayed for each determination.

Northern Analysis—Total RNA was prepared from BC3H1 cultures after indicated time periods by guanidinium isothiocyanate procedure.

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†Established Investigator of the American Heart Association. To whom correspondence should be addressed: Molecular Medicine Program, Box 1289, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY. Tel.: 212-241-0306; Fax: 212-860-9279.

1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum.

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as described previously (17). Twenty µg of RNA from each sample was size-fractionated by formaldehyde-agarose gel electrophoresis, followed by transfer to nitrocellulose overnight in 20 x SSC (saline, sodium citrate). The cDNA probes used for this study were as follows: 1) an 800-base pair cDNA (pAC 269) from chicken skeletal muscle a-actin (18) which recognizes both muscle specific (smooth, skeletal, and cardiac) and embryonic (α and γ) actin isoforms; 2) glyceraldehyde-3-phosphate dehydrogenase probe, a 1.3-kb PstI fragment from clone pUC-GAPDH13 containing the entire coding region and part of the 3'-untranslated region of the rat glyceraldehyde-3-phosphate dehydrogenase (19). Probes were labeled by random hexamer priming to a specific activity of >10⁶ cpm/µg. Hybridization was performed as described previously (17). For normalization, blots were stripped by heating to 95 °C in 0.2 x SSC with 0.1% SDS buffer for 5 min and then rehybridized with a probe prepared from GAPDH cDNA. Autoradiography was performed by exposing blots after hybridization with one intensifying screen at ~80 °C for the indicated time period. Gels were scanned using a Macintosh computer and Adobe Photoshop, and densitometry was performed with Image 1.44, or with a Phosphorimage, and results expressed in arbitrary units for a representative experiment.

Protein Kinase Assay—BC3H1 cells growing in log phase were treated with either no drugs (controls) or 100 ng/ml rapamycin or 100 ng/ml FK520 for 1 h at 37 °C, washed, and resuspended in medium containing 20% FCS. After the indicated time periods, cells were harvested and lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na2VO4, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml N-tosyl-L-phenylalanine chloromethyl ketone, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). Protein concentration was measured using the Bradford reagent (Bio-Rad) with bovine serum albumin as standard. Protein extract (100 µg) was diluted to 800 µl in RIPA buffer and incubated with anti-cdk2 antibody for 2 h. Twenty µl of protein A-Sepharose was added and gently rocked for 1 h at 4 °C. Samples were centrifuged in a table top microcentrifuge for 15 s, and the protein A-Sepharose beads were washed three times with ice-cold RIPA and then once with kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and 1 mM dithiothreitol). Phosphorylation was carried out in 25 µl of kinase assay buffer with addition of 0.1 µg/ml histone H1 (Boehringer Mannheim) and 50 µM [γ-32P]ATP for 10 min at 30 °C. The reaction was terminated with 9 µl of 4 x Laemmli's sample buffer and boiled for 5 min. Samples were analyzed by electrophoresis on 12% SDS-polyacrylamide gels. Gels were dried for 2 h, and autoradiography was performed at room temperature. Autoradiograms were scanned using Adobe Photoshop and a Kodak Silverscan and Image 1.44 software on a MacIntosh IICx. Radioactivity was scanned using a MacIntosh computer and Adobe Photoshop, and densitometry was expressed in arbitrary units for a representative experiment.

RESULTS

Rapamycin Inhibits the Proliferation of BC3H1 Cells—We had reported previously that FKBP12 was expressed at high levels in all types of muscles examined, including skeletal, cardiac, and smooth muscles (20). To examine the cellular role of FKBP12 in muscle, we chose BC3H1 cells as a model, because they express FKBP12 and have been well characterized in terms of myogenic differentiation (17, 21–25). BC3H1 cells reversibly differentiate to a mature muscle phenotype but do not fuse, allowing withdrawal of serum growth factors in low serum medium (0.5% FCS). When cultured in 20% FCS the cells double approximately every 16 h and maintain a proliferative phenotype at subconfluent cultures. Rapamycin (as low as 1 ng/ml), but not FK520 (a drug with identical properties to FK506) or 818 (a drug which inhibits isomerase activity but is not immunosuppressive), inhibited BC3H1 cell proliferation as determined by [3H]thymidine incorporation after 48 h of culture. Rapamycin inhibited proliferation in BC3H1 cells in a dose-dependent manner (Fig. 1). Drugs were added at the initiation of BC3H1 cell culture (cells were plated at low confluence (~30%) in DMEM medium containing 20% FCS. Cells were harvested at indicated time periods, and [3H]thymidine incorporation was determined. The inhibitory effect of rapamycin on proliferation persisted for at least 72 h after addition of drug (Fig. 2). FK520 caused a nonsignificant increase in proliferation. Similar effects were found in C2C12 cells, a murine skeletal muscle cell line (data not shown).

Specificity for FKBP12—Both FK506 and rapamycin bind FKBP12 and interfere with T cell activation. However, the effects on cell cycle progression are distinct, with FK506-FKBP12 acting on Ca2+-dependent G0 to G1 and rapamycin on Ca2+-independent G1 to S transitions. Since only rapamycin inhibits proliferation, we wished to see whether addition of excessive FK506 reverses the inhibition of cells with rapamycin by competing for FKBP12. At molar excesses (100-fold), FK520 blocked the inhibitory effect of rapamycin on myogenic proliferation (Fig. 3) similar to the effect reported previously in T lymphocytes (5). These results indicate that the antiproliferative effect of rapamycin in BC3H1 cells occurs via binding to FKBP12.

Rapamycin Induces Differentiation—The antiproliferative effect of rapamycin-FKBP12 was associated with arrest of cell cycle progression at the G1 to S transition (Table 1). Rapamycin treatment increased the fraction of cells in G0/G1 from 45.5 to 69.2% and reduced the percentage of cells in S from 7.4 to 3.9%. FK520 did not effect cell cycle progression. This cell cycle arrest was accompanied by an induction of myogenic differentiation manifested by an increase in muscle-specific α-actin expression (Fig. 4). BC3H1 cell differentiation is marked by dramatic changes in cell shape which accompany increased expression of muscle-specific gene products, and similar cell shape changes were observed upon addition of rapamycin. FK520 had no such effect on myogenic differentiation. These results indicate that rapamycin-FKBP12 does not simply block DNA synthesis, but it also activates myogenic differentiation pathways. Interestingly, β-actin mRNA was also increased after exposure to rapamycin, indicating that the drug was inducing differentiation via a signaling pathway distinct from mitogen withdrawal.

Fig. 1. Effect of immunosuppressive drugs on the proliferation of BC3H1 cells. 5 x 10⁴ cells were plated in quadruplicates with 20% FCS-DMEM medium for 44 h at which point 20 µl of rapamycin or FK520 were added to the cultures giving final concentrations of 0.001, 0.1, 1.00, and 1000 ng/ml. After 68 h, 0.5 µCi of [3H]thymidine was added for 4 h. Cellular proliferation was determined by measuring [3H]thymidine uptake. The results are for a representative experiment. Rapamycin at 1 ng/ml also inhibited BC3H1 cell proliferation (see Fig. 2).
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**FIG. 2. Time course for rapamycin-induced inhibition of BC3H1 proliferation.** BC3H1 cells were cultured with either no drug (control), rapamycin (1 ng/ml), or FK520 (1 ng/ml), and [3H]thymidine incorporation was determined as described in the legend to Fig 1. [3H]thymidine was added for the final 4 h at days 1, 2, and 3. The results are expressed as mean counts/min; error bars represent standard deviation.

**FIG. 3. FK520 competes with rapamycin for FKBP12.** BC3H1 cells were cultured with either 0.5 ng/ml rapamycin alone or 0.5 ng/ml rapamycin and increasing concentrations of FK520 as indicated. Cells were pulsed with [3H]thymidine and harvested after 72 h. Control cells were cultured in identical conditions but received no drugs. The results are for a representative experiment. Increasing concentrations of FK520 blocked the antiproliferative effects of rapamycin, indicating that these effects are mediated by binding to FKBP12.

**FIG. 4. Rapamycin induces α-actin expression.** BC3H1 cells (~30% confluence) were cultured in DMEM medium containing 20% FCS. Rapamycin or FK520 (100 ng/ml) was added, and control cultures received no drugs. Each time point represents data from three experiments. α-Actin mRNA levels were determined by hybridizing Northern blots to an α-actin cDNA. The same Northern blots were stripped and probed with a cDNA encoding glyceraldehyde-phosphate dehydrogenase. Quantification of the signals on Northern hybridizations was with a PhosphorImager. α-Actin mRNA levels were normalized to glyceraldehyde-phosphate dehydrogenase levels.

**FIG. 5. Rapamycin decreases p34cdc2 kinase activity.** BC3H1 cells were cultured with either 190 ng/ml rapamycin or medium alone (control). After 1 h incubation at 37°C, FCS was added to the cultures to a final concentration to 20%. The 0 time point represents cell lysates made just before the addition of serum. 100 μg of protein from each time point was used for immunoprecipitation with a C-terminal-specific polyclonal anti-p34cdc2 antibody, followed by a kinase assay using histone H1 as a substrate. 15 μl of the reaction mixture was run on 12% SDS-polyacrylamide gels. The inset shows an immunoblot indicating p34cdc2 protein levels. Fifty μg of protein from the same cell lysates was size-fractionated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Immunoblotting was carried out using a C-terminus-specific polyclonal anti-p34cdc2 antibody as described under "Experimental Procedures." Only the relevant portion of the gel is shown. The levels of p34cdc2 protein as determined by immunoblotting (inset) were relatively constant, despite treatment with rapamycin or FK520, whereas the p34cdc2 kinase activity expressed as picomoles of 32P transferred per min are decreased at 0.5 h in the rapamycin-treated samples compared with controls. No such decrease in p34cdc2 kinase activity was seen with FK520.

**TABLE 1**

| Cell cycle analysis |
|---------------------|
| Cells were precultured for 24 h in media containing drug vehicle only, 100 ng/ml rapamycin, or 100 ng/ml FK520. Cells were then stained with propidium iodide, and cell cycle distributions were analyzed using flow cytometry. Aliquots (200 μl) were removed from each sample and pulsed with 2 μCi of [3H]thymidine. | |
| | G1 | S | G2 |
| Control | 48.5 | 7.4 | 33.9 |
| Rapamycin | 69.2 | 3.9 | 20.6 |
| FK520 | 55.1 | 7.8 | 25.9 |

which induces α-actin but deinduces β-actin (26).

**Rapamycin Inhibits cdc2 Kinase Activity—To explain the G1 arrest caused by rapamycin-FKBP12, we examined whether cell cycle proteins or kinases associated with G1 to S transition were affected. Recent studies have shown that p34cdc2, a 34-kDa protein kinase implicated in G1 to S and G2 through M transitions in yeast, may have similar functions in mammals. In proliferating cells, p34cdc2 levels are constant throughout the cell cycle. However, the catalytic activity of p34cdc2 is modulated by phosphorylation and dephosphorylation and by cell cycle-associated proteins known as cyclins. To examine the phosphorylation of p34cdc2, BC3H1 cell lysates were immunoprecipitated with a p34cdc2 C-terminus-specific antiserum. p34cdc2 kinase activity was determined using histone H1 as a substrate. Rapamycin-FKBP12 decreased p34cdc2 kinase activity within 30 min after addition of drug (100 ng ml−1) to proliferating cells (Fig. 5). FK520 did not inhibit p34cdc2 kinase activity (not shown). The decrease in p34cdc2 kinase activity was not due to a decrease in p34cdc2 protein levels which remained relatively constant as shown in Fig. 5, an immunoblot demonstrating the levels of p34cdc2 protein in the same samples assayed for kinase activity. The decrease in p34cdc2 kinase activity was associated with a decrease in cyclin A levels (Fig. 6). Cyclin A levels were determined in the same samples used for
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Fig. 6. Rapamycin inhibits cyclin A expression. Protein lysates from the same experiment described in Fig. 5 (BC3H1 cells) were cultured with either 100 ng/ml rapamycin or medium alone) were used to determine cyclin A levels. Fifty μg of cell lysates were size-fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and stained with a rabbit anti-human cyclin A polyclonal antibody as described under “Experimental Procedures.” Immunoblots were scanned and densitometry performed using Image 1.36 software. The inset shows the cyclin A bands from the immunoblot.

p34<sup>cdc2</sup> kinase activity assays with a polyclonal anti-human cyclin A that cross-reacts with murine cyclin A.

**DISCUSSION**

We showed previously that FKBP12 is physically associated with the calcium release channel/ryanodine receptor in skeletal muscle and that it was expressed at high levels in all types of muscle which we examined (20). To understand the cellular role of FKBP12 in muscle, we took advantage of recent observations that rapamycin-FKBP12 inhibits cellular proliferation in non-muscle cells (5, 6, 10, 11). Our studies show that rapamycin is a potent inhibitor of proliferation in muscle cells (BC3H1) and that the effects are similar to those seen in non-muscle cells. In contrast, FK520, an FK506 analog, had no effect on the proliferation (Fig. 1). To establish that the rapamycin effect was via FKBP12, increasing concentrations of FK520 were used to compete with rapamycin. A 100-fold excess of FK520 was required to reverse the proliferative inhibition of rapamycin (Fig. 1). These findings were similar to those reported for rapamycin-FKBP12 in non-muscle cells (5, 6).

FK506-FKBP12 complexes inhibit the serine-threonine phosphatase activity of calcineurin (3), whereas rapamycin down-regulates the phosphorylation of p70 S6 kinase (6, 10, 11). More recently it was reported that the target for rapamycin is the phosphatidylinositol 3-kinase homologue TOR2 (27) and that inhibition of TOR2 activity caused G<sub>1</sub> arrest. Earlier studies had suggested that p34<sup>cdc2</sup> might have a role in the G<sub>1</sub> to S transition in mammals but supporting data were lacking. Since rapamycin accumulated cells in G<sub>1</sub> phase, we speculated that p34<sup>cdc2</sup> could be the target for rapamycin. While our experiments were in progress, it was reported that rapamycin inhibits p34<sup>cdc2</sup> kinase activity in T lymphocytes (11). To understand the mechanism underlying the inhibition of p34<sup>cdc2</sup> kinase activity in muscle, we examined the levels of cyclins known or thought to be associated with p34<sup>cdc2</sup> and possibly required for kinase activation. Cyclin A levels were decreased only in the rapamycin-treated cells, and the time course for this decrease matched that seen for the decrease in p34<sup>cdc2</sup> kinase (Fig. 6). Cyclin A has not been identified as a marker for G<sub>1</sub> arrest, and its decrease could simply reflect arrest of the cell cycle after rapamycin treatment which may occur before cyclin A levels rise. Cyclin D2 and D3 mRNAs and were unchanged, although protein levels were not examined. Our finding that the G<sub>1</sub> arrest is associated with a decrease in p34<sup>cdc2</sup> activity supports a role for p34<sup>cdc2</sup> in the G<sub>1</sub> to S transition as well as in the G<sub>2</sub> to M transition in mammals.

We also found that the inhibition of proliferation was associated with induction of myogenic differentiation. This indicates that rapamycin-FKBP12 not only causes cell cycle arrest but also induces the myogenic differentiation pathway. Moreover, the nature of this induction is unique because in addition to activating the muscle-specific gene encoding α-actin, the non-muscle form, β-actin, also remained activated. Both fibroblast growth factor and transforming growth factor β have been shown to inhibit myogenic differentiation (28). In BC3H1 cells fibroblast growth factor specifically inhibits the expression of the ryanodine receptor and the dihydropyridine receptor (calcium channels involved in skeletal muscle excitation-contraction coupling) probably via direct inhibition of muscle-specific transcription factors (17). The findings in the present study suggest that the mechanism of rapamycin activation of myogenic differentiation may involve signaling pathways which differ from those activated by growth factor withdrawal. Moreover, since rapamycin was able to induce differentiation in subconfluent culture and in the presence of 20% FCS, it is apparent that the effects of rapamycin-FKBP12 dominate over those of growth factor withdrawal.

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