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Rapamycin inhibits E2F-dependent expression of minichromosome maintenance proteins in vascular smooth muscle cells

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Abstract

Rapamycin inhibits vascular smooth muscle cell (VSMC) proliferation and rapamycin-eluting stents represent a novel therapeutic strategy for preventing postangioplasty restenosis. The precise molecular mechanism, for rapamycin-mediated inhibition of VSMC cell cycle progression and DNA replication remain to be elucidated. Minichromosome maintenance proteins (MCM) are essential regulators of DNA replication and the objective of this study was to examine the effect of rapamycin on their expression in rat aortic VSMC. Rapamycin substantially inhibited mitogen-induced MCM6 and MCM7 mRNA and protein expression in a dose-dependent fashion. Transient transfection experiments revealed that rapamycin inhibited MCM6 and MCM7 promoter activity, implicating a transcriptional mechanism. MCM6 and MCM7 transcriptional activation is regulated by E2F and activity of a luciferase reporter plasmid driven by four E2F elements was also significantly inhibited by rapamycin. The inhibitory effect of rapamycin on MCM6 and MCM7 was reversed by overexpression of E2F, indicating that their downregulation by rapamycin involves an E2F-dependent mechanism. These observations suggest that rapamycin inhibits MCM6 and MCM7 expression by blocking their E2F-dependent transactivation which may contribute importantly to the inhibition of VSMC DNA synthesis by rapamycin.

Keywords: Rapamycin; Restenosis; Vascular smooth muscle cells; Minichromosome maintenance proteins; DNA replication; E2F

Proliferation of vascular smooth muscle cells (VSMC) constitutes a key event during the pathogenesis of restenosis after percutaneous coronary revascularization [1–3]. Although the inception of coronary stenting has significantly reduced the rate of restenosis, in-stent restenosis continues to be with an estimated restenosis rate of about 20% still a major public-health burden [4,5]. Evidence is accumulating from experimental [6–9] and clinical [10,11] studies that local delivery of the macrolide immunosuppressant rapamycin from coated stents may be highly efficacious in preventing postangioplasty restenosis. Several cellular and molecular mechanisms have been implicated in the antiproliferative activity of rapamycin. Rapamycin has been demonstrated to inhibit VSMC proliferation [12–14] and platelet-derived growth factor-induced migration of VSMC [13], which are two crucial events during the pathogenesis of neointima formation. The proposed mechanism by which rapamycin exerts its antiproliferative efficacy involves an inhibition of the $G_1 \rightarrow S$ phase transition by binding to the cytosolic receptor FKBP 12 [15], prevention of $p27^{kip1}$ degradation [8,13], and inhibition of retinoblastoma protein (Rb) phosphorylation [8,12].

Cell cycle progression requires increased Rb phosphorylation to release the S phase transcription factor E2F [16–18]. Minichromosome maintenance (MCM)

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proteins constitute a signature component of eukaryotic DNA replication and guarantee that DNA replication occurs only once per mitotic cycle (reviewed by [19,20]). The six members of the MCM protein family, MCM2–MCM7, are recruited as heterohexameric complexes onto replication origins during the G1 phase of the cell cycle, resulting in the formation of a prereplicative complex (pre-RC). Assembly of the pre-RC begins with the binding of a six-subunit origin recognition complex (ORC) to specific origin sites in the genome determining where replication will occur [21,22]. In early G1, the ORC recruits Cdc6 protein which mediates the loading of the MCM complex to chromatin, resulting in chromatin being 'licensed' for replication in the subsequent S phase [23,24]. Phosphorylation of the MCM complex by the Cdc7–Dbf4 kinase (DDK) during S phase and activation of S phase-promoting cyclin-dependent kinases (CDKs) induce a conformational change in the MCM complex and promote the association of Cdc45 with the MCMs at origins [25,26]. Upon formation of the Cdc45–MCM complex the duplex DNA unwinding is initiated and various replication proteins, including DNA polymerases, are recruited onto unwound DNA for the initiation of DNA synthesis [27,28].

Despite recent advances in the understanding of cell cycle regulation, studies investigating the effect of rapamycin on genes regulating VSMC DNA replication have not been performed. The objective of this study was to examine the effect of rapamycin on MCM expression during the VSMC cell cycle. We report here that the mitogen-induced expression of MCM6 and MCM7, two important regulators of DNA replication, is attenuated at a transcriptional level by rapamycin. The mechanism by which rapamycin inhibits MCM expression likely involves an inhibition of E2F-dependent transactivation during the $G_1 \rightarrow S$ transition. Local delivery of rapamycin to VSMC after coronary stent implantation, therefore, should block MCM expression, inhibit DNA synthesis, and prevent intimal hyperplasia which should protect against the development of restenosis.

Materials and methods

Materials. Rapamycin was commercially obtained from LC Laboratories. DMEM, L-glutamine, OPTI-MEM, TRIzol reagent, and LipofectAMINE 2000 were obtained from Life Technologies. PerfectHyb Plus hybridization buffer was purchased from Sigma. Fetal bovine serum was purchased from Irvine Scientific. Hybond enhanced chemiluminescence nitrocellulose membranes, nylon N^+ membranes, Rediprime II random prime labeling system, horseradish peroxidase-linked anti-mouse antibody, and enhanced chemiluminescence Western blotting detection reagents were obtained from Amersham Pharmacia Biotech. The goat polyclonal antibody against MCM6 (sc-9843), the mouse monoclonal antibody against MCM7 (sc-9966), and the mouse–anti-goat IgG antibody (sc-2354) were from Santa Cruz Biotechnology. The E2F-1 IgG (05-379) was purchased from Upstate and the phospho-Rb Ser 807/811 antibody (No. 9308) was from Cell Signaling Technology. [α-^{32}P]dCTP was commercially obtained from ICN. The Dual Luciferase Reporter Assay System and pRL-CMV were purchased from Promega. cDNA for MCM6 and the human MCM6 promoter pHSMCM6-Luc(-754) driven by a luciferase reporter were provided by Dr. Hiroshi Nojima (Department of Molecular Genetics, Osaka University, Japan) [29]. The human MCM7 promoter pHsMCM7-Luc(-558) luciferase reporter plasmid and MCM7 cDNA were previously described [30]. The pE2F-Luc luciferase reporter plasmid containing four E2F enhancer elements was obtained from Clontech. The E2F expression vector pCMV-E2F was obtained from Dr. Axel H. Schönthal [31]. Adenovirus encoding human E2F-1, driven by the cytomegalovirus (CMV) immediate-early promoter (Adx-E2F), was provided by Dr. Robb MacLellan (Department of Cardiology, University of California, Los Angeles, CA) [32]. Rapamycin was commercially obtained from LC Laboratories.

Cell culture. Rat aortic smooth muscle cells (RASMC) were prepared from the thoracic aorta of 2- to 3-month-old Sprague–Dawley rats by using the explant technique. Cells at passage three to eight were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Cells were grown to 60–70% confluency and made quiescent by serum starvation (0.4% FBS) for at least 24 h. Rapamycin or vehicle (DMSO) was added 30 min prior to the addition of 10% FBS. For all data shown, each individual experiment was performed using an independent preparation of RASMC.

Western immunoblotting. Cells were harvested and sonicated in solubilization buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA, 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM sodium vanadate; 10 μg/ml each aprotinin and leupeptin; and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were cleared by centrifugation and protein concentrations were determined by Lowry assay. Cell lysates containing equal amount of protein were resolved by SDS–polyacrylamide gel electrophoresis. Protein was transferred electrophoretically to a nitrocellulose membrane. After blocking in 20 mM Tris–HCl (pH 7.6) containing 150 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dried milk, blots were incubated with specific antibodies for pRb ser807/811, MCM6, MCM7, or E2F-1. Immunoreactive bands were visualized by incubation with peroxidase-conjugated anti-mouse IgG antibody, anti-rabbit IgG antibody, or anti-goat IgG antibody. The antigen–antibody complex was detected using an enhanced chemiluminescence detection system. Quantification of the Western blots was performed by densitometry.

RNA isolation and Northern blotting. RNA was isolated using TRIzol reagent as described by the manufacturer. Fifteen micrograms of total RNA was denatured in formamide and formaldehyde and electrophoresed through 1% formaldehyde-containing agarose gels. After electrophoresis, RNA was transferred to nylon membrane by capillary blotting and UV cross-linked. Hybridization was performed using PerfectHyb Plus hybridization buffer as directed. Probes for MCM6 and MCM7 used in the hybridization were radiolabeled with [α-^{32}P]dCTP using Rediprime II random prime labeling system. Blots were co-probed for CHOB, a constitutively expressed housekeeping gene encoding a ribosomal protein, to assess equal loading of samples. All experiments were repeated at least three times with different cell preparations.

Transient transfections. For analysis of MCM6, MCM7, and E2F transcriptional activation RASMC were transfected with 500 ng/ml of the pHSMCM6-Luc(-754), pHsMCM7-Luc(-558), or pE2F-Luc reporter plasmid using LipofectAMINE 2000. To investigate the effect of rapamycin on the MCM7 promoter in cells overexpressing E2F, RASMC were co-transfected with 500 ng/ml pHsMCM7-Luc(-558) and the E2F expression vector pCMV-E2F. Twenty-four hours after transfection, cells were starved in DMEM containing 0.4% FBS for 24 h and then pretreated with the indicated concentrations of rapamycin for 30 min prior to the addition of 10% FBS. Luciferase activity was assayed after 24 h using a Dual Luciferase Reporter Assay System (Promega). Transfection efficiency was adjusted by normalizing to

Renilla luciferase activities generated by co-transfection with 5 ng/ml pRL-CMV (Promega). All experiments were repeated at least three times with different cell preparations.

Adenoviral vector and infection. RASMC were infected with 100 PFU/cell adenovirus encoding human E2F-1, driven by the CMV immediate-early promoter (Adx-E2F) in DMEM containing 0.4% FBS for 24 h. After further starvation for 24 h, cells were pretreated with rapamycin for 30 min and 10% FBS was added for 12 h. Recombinant type 5 adenovirus expressing green fluorescent protein (GFP) gene was used as a control vector (Adx-GFP) in all experiments. All experiments were repeated at least three times with different cell preparations.

Statistics. Data were expressed as means ± standard error of the mean (SEM). Statistical significance was determined using Student's *t* test. Values of $p < 0.05$ were considered to be statistically significant.

Results

Rapamycin inhibits Ser807/811 phosphorylation of the retinoblastoma protein

The Rb protein product is a nuclear phosphoprotein that arrests cells during G1 by indirectly repressing transcription of genes required for $G_1 \rightarrow S$ phase transition. Cell cycle progression requires increased phosphorylation of the retinoblastoma protein to release the S phase transcription factor, E2F, which regulates expression of genes encoding the enzymatic machinery for DNA synthesis. We, therefore, investigated the effect of rapamycin on Rb phosphorylation at specific phosphorylation sites, such as Ser807/811, which mediate CDK-dependent regulation of Rb function and are important for cells to exit G1 and enter S phase [33]. Compared to quiescent RASMC, phosphorylation of Ser807/811 increased significantly after 24 h stimulation with 10% FBS (Fig. 1). Pretreatment with rapamycin substantially inhibited mitogen-induced phosphorylation of Ser807/811 in a dose-dependent fashion ($89.9 \pm 7.8\%$ inhibition (100 nM rapamycin) vs. 10% FBS, $n = 3$, $p < 0.05$). In combination, these findings suggest that Rb phosphorylation is a major target for rapamycin, which inhibits VSMC proliferation, at least in part, by inhibition of Rb phosphorylation at specific phosphorylation sites, such as Ser807/811.

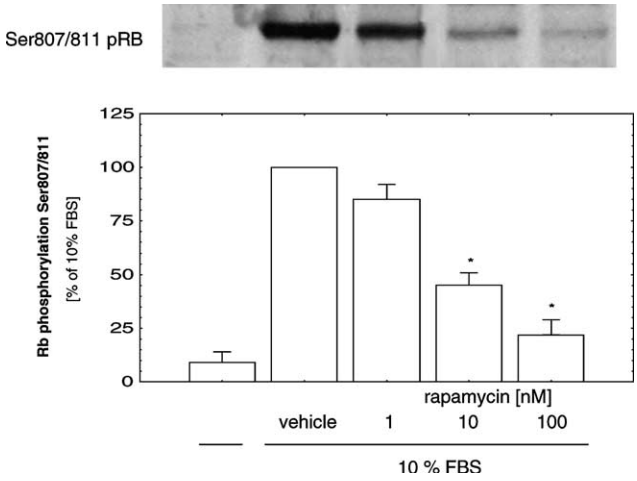

Fig. 1. Rapamycin inhibits Rb phosphorylation in RASMC. Quiescent RASMC (—) were stimulated with 10% FBS for 24 h. Cells were preincubated with the indicated concentrations of rapamycin or vehicle (DMSO) 30 min prior to addition of 10% FBS. Whole cell proteins (75 μg) were assayed by Western immunoblotting using an anti-phospho-Rb Ser 807/811 antibody. Results were expressed as percent (means ± standard error of the mean) of the mitogenic induction of Rb protein phosphorylation at Ser 807/811. The autoradiogram is representative of three separate experiments and data are expressed as means ± standard error of the mean (*$p < 0.05$ vs. 10% FBS).

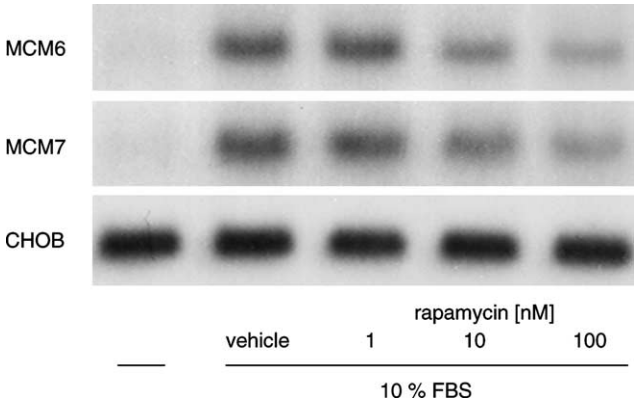

Fig. 2. Rapamycin inhibits growth-induced MCM6 and MCM7 mRNA expression in RASMC. Quiescent RASMC (—) were preincubated with rapamycin or vehicle (DMSO) 30 min prior to growth stimulation with 10% FBS. Twelve hours after stimulation, cells were harvested and total RNA was analyzed for MCM6 and MCM7 mRNA expression by Northern blotting. Co-hybridization for CHOB, a constitutively expressed housekeeping gene encoding a ribosomal protein, was employed to assess equal loading of samples. The autoradiogram is representative of three separate experiments.

Rapamycin inhibits MCM6 and MCM7 mRNA and protein expression in RASMC

MCM are essential for the initiation and completion of the S phase during the cell cycle [19,20,34]. To further examine whether rapamycin affects S phase gene expression, quiescent RASMC were pretreated with rapamycin for 30 min, stimulated with 10% FBS to re-enter the cell cycle, and analyzed for MCM6 and MCM7 expression. Northern blot analysis revealed that MCM6 and MCM7 mRNA levels are low in quiescent cells, consistent with these cells being growth-arrested and not replicating DNA (Fig. 2). Stimulation with FBS resulted in a marked increase of MCM6 and MCM7 mRNA expression after 12 h (3.4 ± 0.4, 3.8 ± 0.6-fold induction vs. quiescent cells). This induction of MCM6 and MCM7 mRNA was substantially attenuated by rapamycin ($68.2 \pm 5.6\%$, $71.2 \pm 6.9\%$ inhibition vs. 10% FBS after 12 h treatment with 100 nM rapamycin, $n = 3$, $p < 0.05$). The inhibition of MCM6 and MCM7 mRNA expression observed after administration of rapamycin

was dose-dependent. At the concentrations used, no cytotoxic effects were observed, as evidenced by the lack of cell detachment and a failure to take up the vital dye trypan blue.

Western blot analysis revealed that the inhibition of the mitogenic induction of MCM6 and MCM7 mRNA by rapamycin correlated with a significant dose-dependent attenuation of MCM6 and MCM7 protein expression 24 h after growth stimulation ($61.1 \pm 8.3\%$, $52.7 \pm 6.0\%$ inhibition vs. 10% FBS after 24 h treatment with 100 nM rapamycin, $n = 3$, $p < 0.05$, Fig. 3). Under the same experimental conditions we also analyzed the inhibitory effect of rapamycin on the mitogenic induction of MCM6 and MCM7 expression by platelet-derived growth factor (PDGF-BB, 20 ng/ml) and insulin (1 μM). Similar to the findings on FBS-stimulated RASMC, rapamycin exhibited a comparable inhibition on PDGF + insulin induced MCM6 and MCM7 mRNA and protein expression. Taken together, these findings indicate that the inhibition of MCM6 and MCM7 protein expression by rapamycin results from an inhibition of MCM6 and MCM7 mRNA expression. Reduced MCM6 and MCM7 mRNA levels by rapamycin could reflect either decreased transcription and/or mRNA stability.

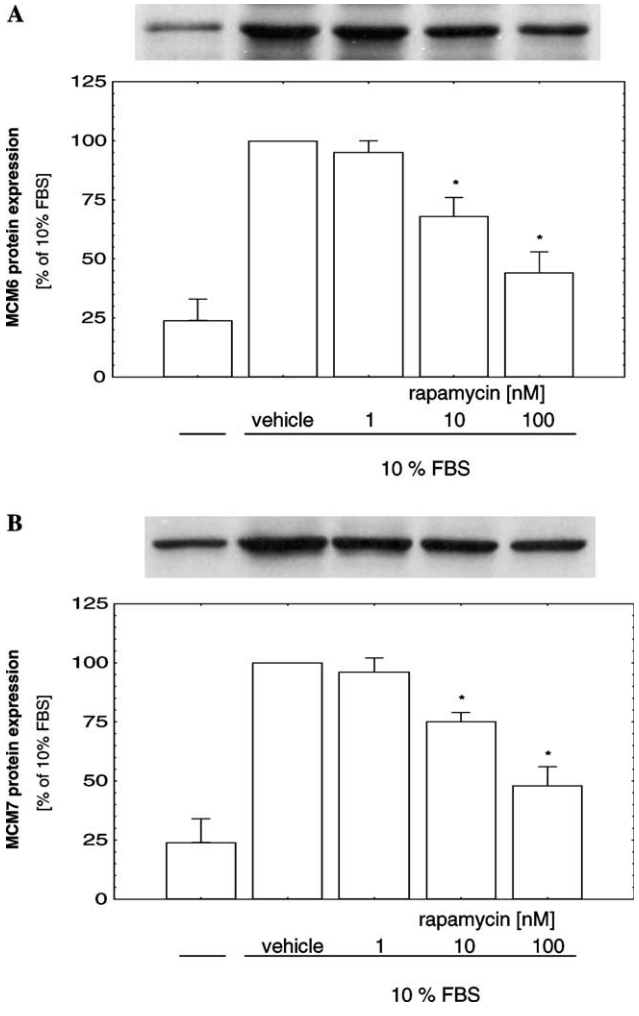

Fig. 3. Rapamycin inhibits growth-induced MCM6 and MCM7 protein expression in RASMC. RASMC were growth-arrested in G_0/G_1 by incubation in 0.4% FBS for at least 24 h (—). Cells were then preincubated with rapamycin (1–100 nM) or vehicle (DMSO) 30 min prior to growth stimulation with 10% FBS. Twenty-four hours after stimulation whole cell proteins (40 μg) were subjected to immunoblotting using specific MCM6 (A) and MCM7 (B) antibodies. Each autoradiogram is representative of three separate experiments and data are expressed as means ± standard error of the mean (*$p < 0.05$ vs. 10% FBS).

Rapamycin inhibits MCM6 and MCM7 promoter activity

To further understand the mechanism involved in the inhibition of MCM expression by rapamycin, we next examined the effect of rapamycin on MCM6 and MCM7 transcription. RASMC were transiently transfected with human MCM6 and MCM7 promoter fragments driving expression of the luciferase reporter gene. The MCM6 promoter fragment employed contains a 789 bp DNA segment (−754 to +35) and the MCM7 promoter fragment consists of a 505 bp DNA segment (558 to −54). MCM6 and MCM7 promoter activity was significantly induced after mitogenic stimulation with 10% FBS (Fig. 4, 3.2 ± 0.6, 6.3 ± 1.4-fold induction vs. quiescent cells, $n = 3$, $p < 0.05$). Rapamycin substantially inhibited mitogen-induced transcriptional activation of MCM6 and MCM7 in a dose-dependent fashion ($79.3 \pm 7.4\%$, $74.4 \pm 7.1\%$ inhibition vs. 10% FBS after 24 h treatment with 100 nM rapamycin, $n = 3$, $p < 0.05$). These data demonstrate that rapamycin inhibits MCM6

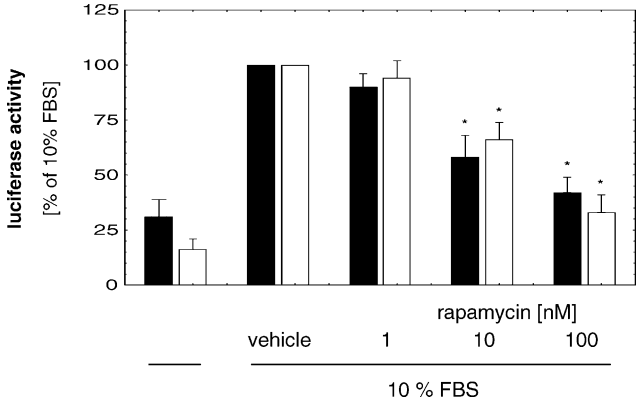

Fig. 4. Rapamycin attenuates MCM6 and MCM7 transcriptional activation. RASMC were transiently transfected with pHsMCM6-Luc (−754) (black bars) and pHsMCM7-Luc(-558) (white bars) promoter fragments driving expression of luciferase reporter genes and serum-starved for 24 h (—). Thirty minutes before growth stimulation with 10% FBS cells were pretreated with the indicated concentrations of rapamycin or vehicle (DMSO). Twenty-four hours after stimulation luciferase activity was assayed. Transfection efficiency was adjusted by normalizing firefly luciferase activities to *Renilla* luciferase activities generated by co-transfection with pRL-CMV. All experiments were repeated at least three times with different cell preparations. Data are expressed as means ± standard error of the mean (*$p < 0.05$).

and MCM7 expression, at least in part, at the transcriptional level.

Rapamycin inhibits mitogen-induced E2F-dependent transactivation

The promoter regions of MCM6 contain five E2F sites, while three E2F sites have been identified in the MCM7 promoter [29,30]. Growth-stimulated Rb phosphorylation results in the release of E2F, which in turn activates the S phase genes, including MCM6 and MCM7. Data in Fig. 5 show the effect of rapamycin on the activity of a luciferase reporter plasmid driven by multiple E2F binding sites. FBS-induced E2F transcriptional activity was markedly inhibited by treatment with rapamycin (1–100 nM) ($82.1 \pm 6.9\%$ inhibition after 24 h, $n = 3$, $p < 0.05$). These data demonstrate that the downregulation of MCM6 and MCM7 transcription by rapamycin occurs, at least in part, through an inhibition of their E2F-dependent transactivation.

Overexpression of E2F-1 reverses the inhibitory effect of rapamycin on MCM6 and MCM7

To corroborate that E2F is the critical target for rapamycin-mediated inhibition of MCM expression, we employed an adenoviral expression vector to overexpress E2F independent of Rb phosphorylation. In Adx-GFP-infected cells, stimulation with FBS resulted in a marked increase of MCM7 mRNA (Fig. 6A) and protein (Fig. 6B) which was potently blocked by 100 nM rapamycin. Western blot analyses of total cell lysates from VSMC treated with rapamycin or rapamycin plus 10% FBS showed no effect of rapamycin on the expression of E2F-1 (Fig. 6B). Infection of RASMC with Adx-E2F resulted in a strong overexpression of

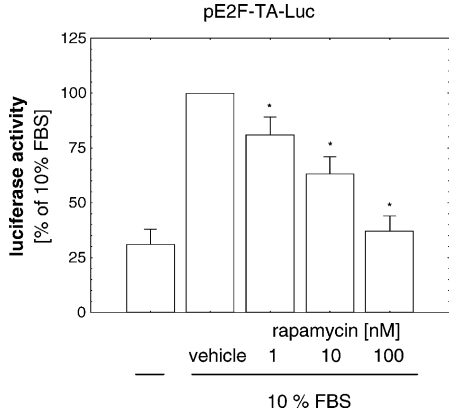

Fig. 5. Rapamycin inhibits growth-induced E2F transcriptional activation. RASMC were transiently transfected with a luciferase reporter plasmid driven by multiple E2F binding sites driving expression of luciferase reporter gene (pE2F-Luc). Transfected cells were serum-starved for 24 h. Thirty minutes before stimulation with 10% FBS cells were pretreated with rapamycin or vehicle (DMSO). Twenty-four hours after stimulation luciferase activity was assayed. Transfection efficiency was adjusted by normalizing firefly luciferase activities to *Renilla* luciferase activities generated by co-transfection with pRL-CMV. All experiments were repeated at least three times with different cell preparations. Data are expressed as means ± standard error of the mean (*$p < 0.05$).

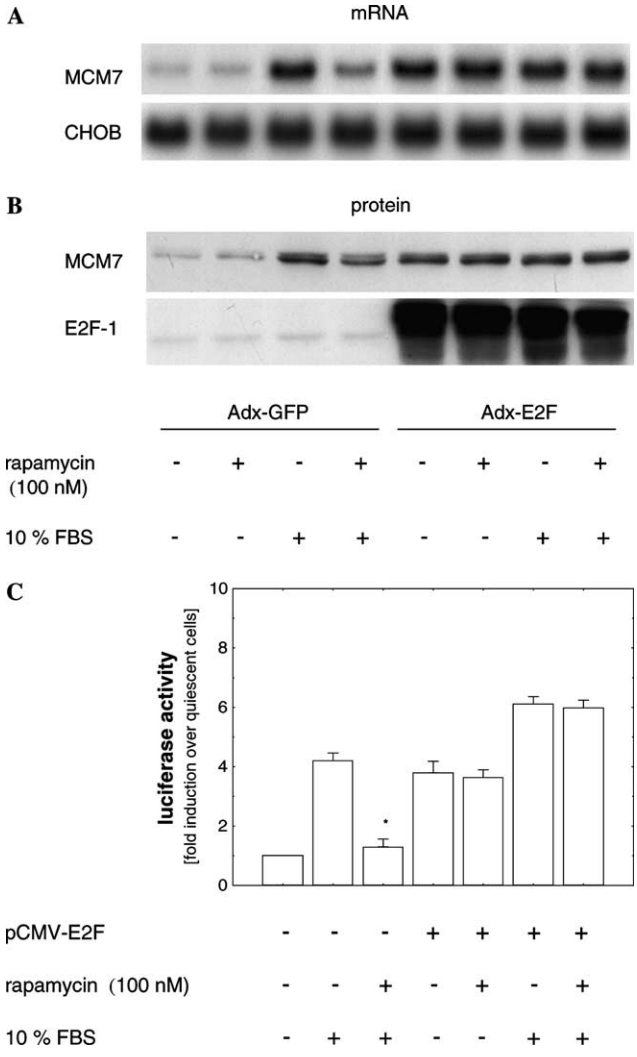

Fig. 6. The inhibitory effect of rapamycin on MCM7 expression is reversed by overexpression of E2F. Quiescent RASMC were infected with 100 PFU/cell adenovirus overexpressing human E2F-1, driven by the CMV immediate-early promoter (Adx-E2F) for 24 h. Infection of RASMC with recombinant type 5 adenovirus expressing green fluorescent protein (Adx-GFP) served as control. After infection, cells were pretreated with rapamycin (100 nM) 30 min prior to stimulation with 10% FBS. Twelve hours after stimulation, cells were harvested and total RNA were analyzed for MCM7 mRNA expression by Northern blotting (A). Twenty-four hours after stimulation whole cell proteins (40 μg) were analyzed by immunoblotting using a specific MCM7 antibody (B). Overexpression of human E2F-1 was monitored by immunoblotting with a specific human E2F-1 antibody. Each autoradiogram is representative of three separate experiments. (C) RASMC were transfected with the MCM7 promoter pHsMCM7-Luc(-558) alone or co-transfected with an E2F-expression vector (pCMV-E2F) and stimulated as described in Fig. 4. Experiments were repeated three times with different cell preparations. Data are expressed as means ± standard error of the mean (*$p < 0.05$).

E2F-1 protein which induced MCM7 expression (mRNA and protein) in quiescent cells. Rapamycin had no effect on MCM7 expression in quiescent cells infected with Adx-E2F. In RASMC ectopically overexpressing E2F, rapamycin was ineffective in suppressing serum-induced MCM7 mRNA and protein expression. Comparable results were obtained for MCM6 (data not shown).

To further investigate whether overexpression of E2F also reverses the inhibitory effect of rapamycin on MCM7 transcription, RASMC were co-transfected with the MCM7 promoter and an expression vector overexpressing E2F (Fig. 6C). Consistent with our observation that adenovirus-mediated overexpression of E2F induces MCM7 mRNA expression in quiescent cells, the MCM7 promoter activity was induced 3.8 ± 0.35-fold by overexpessing E2F ($n = 3$, $p < 0.05$). Stimulation with FBS resulted in a slightly further increase of the MCM7 promoter activity (1.52 ± 0.27-fold induction over quiescent cells co-transfected with pCMV-E2F, $n = 3$, $p < 0.05$). However, rapamycin exhibited no effect on the E2F-induced or mitogen-induced MCM7 promoter activity in cells that were co-transfected with the E2F expression vector. In combination, these data strongly suggest that the inhibitory effect of rapamycin on mitogen-induced MCM6 and MCM7 expression is mediated by an inhibition of E2F-dependent transactivation through prevention of E2F release from Rb.

Discussion

Several lines of evidence have suggested that rapamycin inhibits VSMC proliferation by targeting important cell cycle regulators controlling Rb phosphorylation and $G_1 \rightarrow S$ phase transition during the VSMC cell cycle [12–14]. The details of rapamycin effects on cascades downstream of Rb phosphorylation that lead to DNA replication have been poorly investigated. Therefore, the objective of the present study was to elucidate whether an inhibition of Rb phosphorylation and E2F-dependent transactivation of target genes translate into an effective inhibition of downstream S phase gene expression in VSMC. The present study provides evidence for an important role of rapamycin in inhibiting MCM gene expression, which are essential components for the initiation of DNA replication during the S phase of the cell cycle. The present results show that rapamycin inhibits expression of MCM6 and MCM7 in VSMC at a transcriptional level through an inhibition of their E2F-dependent transactivation. Retention of E2F by hypophosphorylated Rb appears to be the principal molecular mechanism for the antiproliferative action of rapamycin in VSMC.

Initial studies by Gregory et al. [6] demonstrated that intraperitoneal injection of rapamycin resulted in a dose-dependent inhibition of neointimal proliferation caused by either chronic alloimmune or mechanical injury in a rat model. Subsequent studies by Marx et al. [12] and Poon et al. [13] reported that rapamycin inhibits proliferation of VSMC through an inhibition of $G_1 \rightarrow S$ phase progression associated with an inhibition of cyclin-dependent kinase 2, cyclin D1, and Rb phosphorylation. These early findings were followed by further in vitro studies of Braun-Dullaeus et al. [14] demonstrating that rapamycin inhibits several G1 cyclins and cyclin-dependent kinases as well as the cyclin-dependent kinase inhibitor $p21^{cip1}$ in coronary artery smooth muscle cells. Using balloon angioplasty of porcine coronary arteries, Gallo et al. [8] showed that rapamycin inhibits neointima formation in vivo which was associated with increased levels of $p27^{kip1}$ and an inhibition of Rb phosphorylation. Consistent with these observations, we find that rapamycin inhibited Rb phosphorylation in RASMC, providing further evidence that Rb is an important target for the antiproliferative activity of rapamycin.

MCM are required for both the initiation and elongation stages of DNA replication [19,20,34]. The concept that MCM6 and MCM7 may play an important regulatory role during VSMC proliferation arises from several recent findings of novel MCM/protein and MCM/chromatin interactions. MCM6 and MCM7 together with MCM4 form a subcomplex of MCM that have DNA helicase, DNA binding, and DNA ATPase activities, suggesting that these MCM are required for strand unwinding during chromosome replication [35–37]. Since MCM have been identified as essential components of DNA replication and the question whether rapamycin affects the expression of regulatory S phase genes has not been addressed, we examined the effect of rapamycin on mitogen-induced expression of MCM6 and MCM7 in VSMC. We demonstrate that rapamycin potently attenuates mitogen-induced expression of MCM6 and MCM7 in RASMC at a transcriptional level. The inhibitory effect of rapamycin on MCM6 and MCM7 expression was abrogated after overexpression of E2F independent of Rb phosphorylation. This result is consistent with rapamycin inhibiting MCM6 and MCM7 expression mainly by targeting the action of the S phase transcription factor E2F.

Phosphorylation of Rb results in a conformational change that releases sequestered E2F, enabling it to transactivate target genes encoding the enzymatic machinery for DNA synthesis [16–18]. E2F transcriptional activity is a rate limiting step for mitosis [17,18] and any influence of rapamycin on this process would have an impact on VSMC $G_1 \rightarrow S$ progression. In T cells rapamycin abrogates the activity of $p70^{s6k}$, the 40S ribosomal protein S6 kinase, which is able to regulate E2F transcriptional activity [38]. The MCM6 and MCM7 promoters contain functional E2F binding sites [29,30],

suggesting that E2F is responsible for the coordinate expression of MCMs during progression through the cell cycle. Therefore, rapamycin could inhibit mitogen-induced MCM transcription by blocking their E2F-dependent transactivation. One mechanism by which rapamycin could abrogate mitogen-induced E2F activity is to downregulate cellular levels of E2Fs. However, Western blot analyses of total cell lysates from VSMC treated with rapamycin alone or in combination with the mitogen revealed no effect on the expression of E2F-1. To monitor E2F transcriptional activity in VSMC, we employed a reporter plasmid driven by four E2F binding sites upstream of a luciferase gene. Consistent with a central role for E2F in regulating MCM expression, we find that rapamycin substantially inhibits the transcriptional activation of this reporter plasmid. Conversely, overexpression of E2F prevents the inhibition of MCM7 promoter activity by rapamycin. These results indicate that rapamycin inhibits MCM6 and MCM7 by blocking their E2F-dependent transactivation in VSMC. Although a large body of evidence indicates that Rb indirectly controls the rate of cell proliferation via its interaction with a constellation of transcription factors, including E2F, Elf-1, Sp-1/Sp-3, and C/EBP (reviewed by [39]), Rb and MCM7 have been also reported to form complexes in vitro and in vivo resulting in a direct inhibition of DNA replication [40]. Therefore, additional studies investigating the molecular pathways by which antiproliferative agents inhibit DNA replication are indeed warranted.

In summary, the present study provides data linking the inhibition of Rb phosphorylation by rapamycin to downstream DNA replication by preventing mitogen-induced MCM6 and MCM7 expression. We show that rapamycin inhibits MCM6 and MCM7 expression at a transcriptional level, suggesting that rapamycin inhibits expression of genes that are essential for DNA replication during the S phase of the cell cycle. Overexpression of E2F reverses the inhibitory effects of rapamycin on MCM6 and MCM7 expression and transcriptional activation of a luciferase reporter plasmid driven by multiple E2F elements was substantially inhibited by rapamycin. The effects of rapamycin on E2F-dependent transactivation reveal a molecular mechanism for the inhibitory effect on MCM6 and MCM7 expression and imply that prevention of E2F-dependent transactivation of target genes by blocking Rb phosphorylation is the underlying mechanism for the antiproliferative effects of rapamycin on VSMC.

Acknowledgments

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