Rapamycin (Sirolimus) Inhibits Proliferating Cell Nuclear Antigen Expression and Blocks Cell Cycle in the G_1 Phase in Human Keratinocyte Stem Cells

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Abstract

Because the immunosuppressant rapamycin (sirolimus) blocks T cell proliferation in G_1 phase, it has been proposed as a potential treatment for psoriasis, a skin disease characterized by T cell activation and keratinocyte stem cell hyperproliferation. To determine another potentially important mechanism through which rapamycin can act as an antipsoriatic agent, we tested its direct effect on keratinocyte stem cell proliferation in vitro as well as in vivo. In vivo cell cycle quiescent (G_0 phase) stem cell keratinocytes in primary culture sequentially express de novo cyclin D1 and proliferating cell nuclear antigen (PCNA), prior to S phase entry, and upregulate β1 integrin. Rapamycin inhibited the growth of keratinocytes that were leaving quiescence as well as those already in cell cycle without affecting cell viability. Although β1 integrin^{bright} expression was not affected, the number of β1 integrin^{bright} cells entering S/G_2/M was significantly lowered by rapamycin. Cells treated with rapamycin exhibited decreased PCNA expression while cyclin D1 expression, which precedes PCNA expression in the cell cycle, was not affected. We found similar effects on stem cell keratinocytes in patients with psoriasis treated systemically with rapamycin. Because PCNA is required for cell cycle progression from G_1 to S phase, our data indicate that inhibition of PCNA protein synthesis may be an important regulatory element in the ability of rapamycin to exert a G_1 block. (*J. Clin. Invest.* 1997. 99:2094–2099.) Key words: keratinocytes • PCNA • psoriasis

Introduction

In this study, we examined the effect of rapamycin (sirolimus) on in vitro primary epidermal cell cultures. In primary culture, keratinocyte stem cells acquire high levels of β1 integrin, exit G_0, and enter the proliferative phase of the cell cycle (1, 2). To identify the point in cell cycle at which rapamycin acts, we used flow cytometric analysis using antibodies to mark stem cell activation (β1 integrin), G_1 points in cell cycle (proliferating cell nuclear antigen (PCNA)[1] and cyclin D1), as well as DNA content to mark S and G_2/M progression from G_0/G_1.

PCNA is first expressed in mid-G_1 (3). PCNA expression peaks in S phase but continues to be weakly expressed in G_2 and M phases of the cell cycle (3). Not only is it involved in DNA replication (4, 5) and repair (6), but it also plays a role in cell cycle regulation (7, 8). D-type cyclins are important for progression from the G_0/G_1 to S phases of cell cycle (9–11). They are cell-type specific; cyclin D2 is thought to be the major D-type cyclin expressed in keratinocyte cells (12). However, we have recently shown that keratinocytes express cyclin D1 when they traverse from cell cycle quiescence (G_0) into cell cycle prior to G_1 PCNA expression (13).

In vivo epidermal growth is regulated by the degree to which stem cells in basal epidermis remain in the quiescent (G_0) or proliferative (G_1/S/G_2/M) phase of cell cycle. This process is aberrant in psoriasis. Epidermal hyperproliferation in psoriasis is characterized by a markedly increased percentage of normally quiescent basal (stem) keratinocytes in the proliferative phases of cell cycle (14). T lymphocytes infiltrating psoriatic lesions may play a role in the induction and maintenance of keratinocyte hyperproliferation in the disease (15). Cyclosporine and FK-506 (tacrolimus), macrocyclic immunosuppressants that block T cell activation, have been used as treatments for psoriasis but their use is often times limited because of side effects such as nephrotoxicity and hypertension.

Rapamycin is a macrocyclic immunosuppressant with demonstrated ability to block T cell proliferation via a G_1 cell cycle blockade (16). Rapamycin does not block the cyclosporine- and FK-506–sensitive calcineurin phosphatase pathway that is critical for T cell receptor-mediated signal transduction, but it does inhibit the S6 kinase pathway used not only by T cells but also by other cell types for cell cycle progression (17–19). Whereas cyclosporine and FK-506 exert minimal effects on keratinocyte proliferation at dosages achievable in vivo (20), rapamycin, because of its distinct cell cycle signaling target of action, may inhibit keratinocyte growth in vitro (21). If rapamycin indeed blocks keratinocyte proliferation as well as T cell activation, it could be a treatment with rapid effects in psoriasis.

Data presented here indicate that rapamycin does not affect stem cell β1 integrin activation. Rather, rapamycin acts af-

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1. *Abbreviations used in this paper:* EtOH, ethanol; K1/K10, keratin 1/keratin 10; PCNA, proliferating cell nuclear antigen; PI, propidium iodide.

ter cyclin D1 induction but prior to PCNA expression and DNA synthesis in keratinocytes. These results indicate a specific G_1 block in keratinocyte stem cells by rapamycin, which was verified in skin of patients with psoriasis who were receiving systemic rapamycin. Our data show that this G_1 block is a result of rapamycin's inhibition of PCNA synthesis. This stresses the importance of PCNA in the regulation of cell progression from G_1 into S phase.

Methods

Human subjects. Keratome biopsies were taken from the buttock area of normal volunteers. As part of a phase I pharmacokinetic clinical trial, patients with psoriasis received 5 mg/m^2 per d oral rapamycin for 1 wk. Keratome biopsies were also taken from these patients before treatment (day 0), 2 d into treatment, and on the last day of treatment (day 7). Patients had not used systemic immunosuppressives for at least 4 wk and systemic retinoids, corticosteroids, or phototherapy for at least 3 wk before entry into the study. All procedures were approved by the Institutional Review Board of the University of Michigan Medical Center, and informed consent was obtained from each subject.

Initiation of primary cultures. Epidermal cell suspensions were prepared from keratomes of normal subjects as described previously (14). Cells were plated at a concentration of 1×10^6 cells/ml into 6-well culture plates (2 ml/well) or into 25 cm^2 culture flasks (5 ml/flask) and cultured in keratinocyte basal media (KBM) (Keratinocyte-SFM media without bovine pituitary extract and EGF; GIBCO Laboratories, Grand Island, NY) supplemented with 1% FBS. Cultures were treated with 20 nM rapamycin (Wyeth-Ayerst, Philadelphia, PA) dissolved in absolute ethanol (EtOH) or with absolute EtOH alone as negative control 2 h or 24 h after initiation of culture (short-term culture) or 72 h after initiation of culture (long-term culture). After 72 h of additional culturing following rapamycin or EtOH treatment, the adherent cells were treated with trypsin (0.025% trypsin + 0.01% EDTA) and collected. Cell viability was assessed with trypan blue (GIBCO Laboratories) to determine rapamycin cytotoxicity. Cells were then fixed in 70% EtOH and kept at −20°C at least overnight.

Colony formation assay. Epidermal cell cultures were prepared as described above. After culturing, cells were fixed in 1% formalin for 15 min on the culture plates at 4°C and stained with a solution of 50% Nile blue and 50% rhodamine B (both from Sigma Chemical Co., St. Louis, MO).

Flow cytometric assay of keratinocyte subset proliferation. The EtOH-fixed cells were stained with the following mAbs: anti–β1 integrin (4B4; Coulter Immunology, Hialeah, FL), anti–PCNA (PC10; Boehringer Mannheim Biochemicals, Indianapolis, IN), anti–cyclin D1 (PharMingen, San Diego, CA), anti–keratin 1/keratin 10 (K1/K10) (ICN Pharmaceuticals Inc., Costa Mesa, CA), and isotype controls (purified mouse IgG1 and IgG2a; Sigma Chemical Co.); then stained with the secondary antibodies goat anti–mouse IgG1 conjugated to FITC and IgG2a conjugated to PE (both from Boehringer Mannheim Biochemicals) and resuspended in propidium iodide (PI) with RNase A, both from Sigma Chemical Co., as in the method of Bata-Csorgo et al. (1) and Gong et al. (22). Samples were stored at 4°C and analyzed by flow cytometry within 24 h, described in detail elsewhere (14). Data were analyzed using Coulter Elite (Coulter Cytometry) and Modfit (Verity Software House, Inc., Topsham, ME) software.

Statistical analysis. Paired two-tailed Student's *t* test was used to compare rapamycin-treated and control cultures.

Results

Rapamycin inhibits establishment of keratinocyte primary cultures. Normal fresh ex vivo epidermal cells were plated in KBM + 1% FBS. In order to capture potential rapamycin effects on cells leaving quiescence as well as cells already in cell cycle, we treated cultures with rapamycin 1 and 3 d after plating. At both time points, cell growth was inhibited by rapamycin treatment as demonstrated by reduced colony numbers and size ($n = 8$). Fig. 1 shows a representative experiment. At a concentration of 20 nM, rapamycin did not affect cell viability as determined by trypan blue staining of harvested cells (data not shown).

Rapamycin does not affect β1 integrin upregulation. To determine whether reduced culture establishment (by morphology) was because of an inhibition of the ability of stem cell keratinocytes to upregulate their β1 integrin expression, and thereby affect adhesion/signaling, we analyzed 3-d-old cultured keratinocytes that were exposed to rapamycin 2 or 24 h after plating using a flow cytometric assay described by Bata-Csorgo et al. (1). Keratinocyte stem cells will express high levels of β1 integrin (β1 integrin^{bright} population) by about 72 h of culture but at 48 h after plating, a β1 integrin^{bright} population is usually not yet evident.

Cultures stained with anti-β1 integrin FITC demonstrated that rapamycin treatment had no effect on the percentage of β1 integrin^{bright} cells appearing in the culture by 72 h, as compared with the EtOH-treated control culture in four of four experiments. The average difference in β1 integrin^{bright} cells between the treated and untreated cultures was 4±1% (Table I, compare columns 2 and 3).

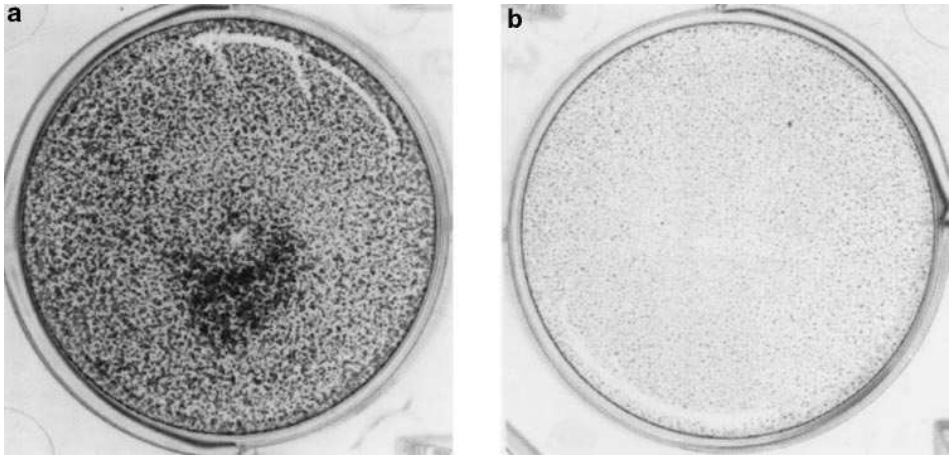

Figure 1. Keratinocyte proliferation is inhibited by rapamycin. Normal epidermal cells were plated in KBM + 1% FBS and treated with control EtOH (*a*) or 20 nM rapamycin (*b*) 72 h into culture. Adherent cells were fixed with 1% formalin and stained with Nile Blue and rhodamine B 72 h after rapamycin or EtOH addition.

*Table I. Effect of Rapamycin on Cell Cycle of β1 Integrin^{bright} Cells**

	% β1 integrin^{bright}		% S/G2/M	
Experiment	Rapamycin	Medium	Rapamycin	Medium
1	43.6	37.1	18.5	33.8
2	14.5	11.2	17.2	30.1
3	38.2	35.8	5.8	17.1
4	53.3	57.8	24.2	52.5

*Normal adult epidermal cells were cultured in KBM + 1% FBS and treated with 20 nM rapamycin in EtOH or an equal volume of EtOH as negative control 2 h after plating. Adherent cells were harvested 72 h later, fixed in 70% EtOH and co-stained with anti-β1 integrin and PI for cell cycle analysis.

PCNA expression is inhibited by rapamycin. PCNA expression can be used as a marker of cell entry into G_1. The PCNA mAb (clone PC10) used in this study labels both free PCNA and PCNA associated with replicating DNA. However, Bravo and McDonald-Bravo (4) have shown that only PCNA associated with DNA replication sites is detected after methanol fixation. This same study as well as others (5, 6) also show that, after Triton extraction, only the bound PCNA remains in cells. Therefore, as a result of fixation and extraction, the PCNA mAb is labeling only the PCNA associated with DNA replication sites. After 48 h in culture few cells, if any, express PCNA. By 72 h, however, proliferating cells (which are primarily stem cells and thus, β1 integrin^{bright}) are expressing PCNA. In all short-term culture experiments (n = 4) rapamycin inhibited PCNA expression. The PCNA expression of the rapamycin-treated culture is only slightly higher than the nonspecific binding of the isotype control while the EtOH-treated culture has higher PCNA expression (Fig. 2). Both a PCNA^{high} and a PCNA^{low} population are present in the EtOH-treated control culture (Fig. 2, solid thin line). By contrast, the rapamycin-treated culture contains only the PCNA^{low} population (Fig. 2, dashed line). The reduction in PCNA expression is almost a log less, indicating a significant, but not complete, inhibition of PCNA expression. There was an average 48±6% reduction in the PCNA mean channel fluorescence of the rapamycin-treated cultures relative to the PCNA mean channel fluorescence of the EtOH control cultures.

Cyclin D1 expression is not affected by rapamycin. Cells express D-type cyclins in G_1 and the association of these cyclins with their cyclin-dependent kinases and PCNA suggests that they may be crucial for G_1 restriction point (start) regulation. Because adult keratinocytes express cyclin D1 and not cyclin D2 in their transition from G_0 to G_1/S while already cycling keratinocytes express cyclin D2 (13), short-term primary cultures treated with rapamycin 2 h after plating were fixed and stained with anti-cyclin D1. Relative to isotype control (Fig. 3, solid dark line), the antibody-stained cells in both rapamycin-treated and EtOH-treated cultures exhibited the same cyclin D1 expression, as indicated by essentially overlapping histograms of cell count versus fluorescence intensity (Fig. 3, dashed and solid thin lines). The difference in cyclin D1 mean channel fluorescence was only 5±5% (n = 2). Both the rapamycin-treated and the EtOH-treated cultures contained positive cells stained with anti-cyclin D1 in equal numbers as indicated by similar numbers of cells with increased fluorescence intensity relative to the isotype control staining.

Stem cell proliferation is inhibited by rapamycin. Although PCNA expression was markedly reduced, it was not completely inhibited by rapamycin treatment. In the EtOH-treated control culture, there is a population with intermediate expression of PCNA (PCNA^{low} population) which corresponds to the level of PCNA expression of the majority of rapamycin-treated cells still expressing PCNA (Fig. 2). To determine if this degree of PCNA reduction was associated with blocked progression into actual DNA synthesis (S/G_2/M), short-term cultures were treated with rapamycin or EtOH and the adherent cells collected and fixed with 70% EtOH. The cells were then co-stained with anti-β1 integrin and the DNA stain PI. The relative size of the β1 integrin^{bright} cell population (percent β1 integrin^{bright} cells of all harvested epidermal cells) and the percent S/G_2/M phase cells among the β1 integrin^{bright} cells for each experiment are shown in Table I. Although the appearance of β1 integrin^{bright} cells was not affected by rapamycin treatment (Table I, compare columns 2 and 3, P = 0.46), rapamycin did indeed suppress the number of β1 integrin^{bright} cells undergoing DNA synthesis, based on cellular PI content (n = 4,

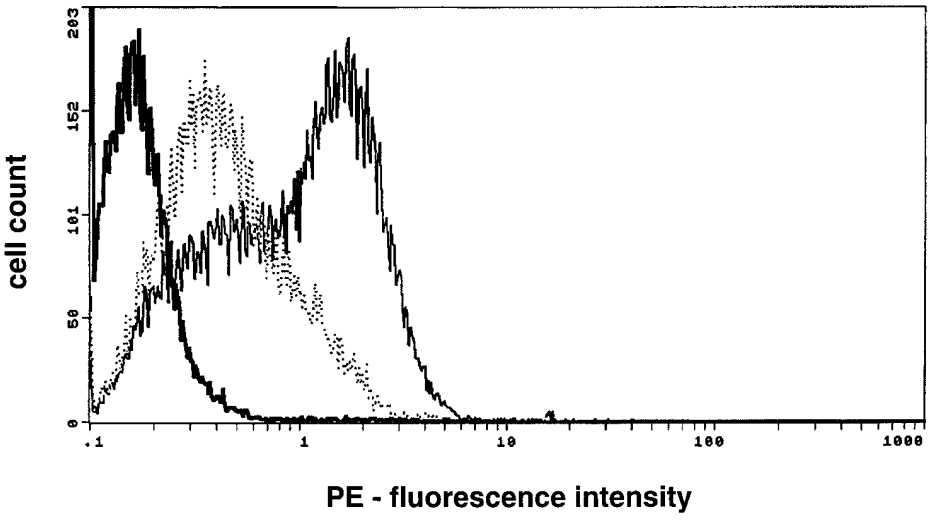

Figure 2. PCNA expression of rapamycin-treated and EtOH-treated short-term keratinocyte cultures. Normal adult epidermal cells were plated in KBM + 1% FBS and treated with rapamycin or control EtOH 2 h after plating. 72 h after plating, adherent cells were harvested and fixed in 70% EtOH, then stained with anti-PCNA PE. The one-parameter histogram shows PCNA expression (*PE*) of the epidermal cells along the *x*-axis. Nonspecific fluorescence was detected by isotype staining (*solid dark line*). The culture treated with EtOH (*solid thin line*) had a distinct PCNA$^+$ population while the culture treated with rapamycin (*dashed line*) was only slightly brighter than the isotype control.

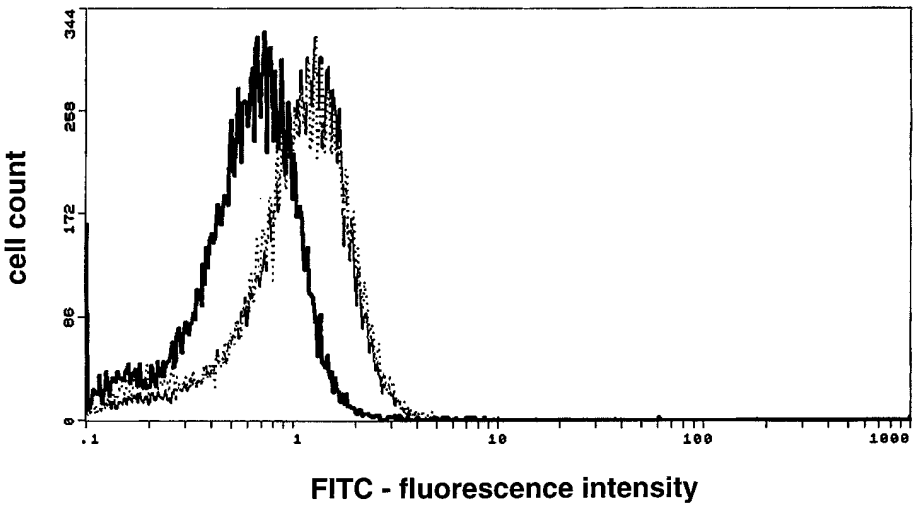

Figure 3. Cyclin D1 expression of rapamycin-treated and EtOH-treated short-term keratinocyte cultures. Normal epidermal cells from the same representative experiment as in Fig. 2 were stained with anti-cyclin D1 FITC. The one-parameter histogram shows cyclin D1 expression (*FITC*) of the epidermal cells along the *x*-axis. Nonspecific fluorescence was detected by isotype staining (*solid dark line*). The rapamycin-treated culture (*dashed line*) and the EtOH-treated culture (*solid thin line*) have almost identical cyclin D1 fluorescence.

52±5% decrease in $S/G_2/M$ phase cells) (Table I, compare columns 4 and 5, $P = 0.02$). Thus, the decreased PCNA expression because of rapamycin treatment is associated with a block in the ability of stem cells entering G_1 (β1 integrin^{bright} cyclin D1$^+$) to transit into S phase. Other studies have suggested that cells will not enter S phase without a critical level of PCNA (8). Although the rapamycin-treated cells express an intermediate level of PCNA, these cells may lack this critical PCNA level and therefore be unable to enter $S/G_2/M$, as our data have shown.

Rapamycin inhibits epidermal stem cell PCNA expression and proliferation in vivo. To determine if these effects documented in primary ex vivo cultures are achievable in vivo, keratomes from patients with psoriasis who received rapamycin (5 mg/m^2 per d) were examined for epidermal keratinocyte stem cell PCNA expression and cell cycle inhibition. Keratomes were taken at three time points: before treatment (day 0), two days into treatment, and on the final day of treatment (day 7). Epidermal cells were fixed with 70% EtOH and stained with anti-K1/K10, anti-PCNA, and PI (to quantify cellular DNA content). Over time, the PCNA mean channel fluorescence of the epidermal stem cell population (K1/K10$^-$ cells) decreases with time ($n = 2$) (Fig. 4). These results indicate that the PCNA expression in individual stem cells was inhibited by rapamycin treatment in vivo. The cell cycle analysis of the K1/K10$^-$ population demonstrates a similar trend. In both subjects, the percent of cells in $S/G_2/M$ decreased over time (Fig. 5). Our data show that rapamycin inhibits PCNA expression and stem cell proliferation not only in the in vitro keratinocyte cultures but in keratinocytes from patients with psoriasis as well.

Discussion

We have shown that rapamycin inhibits normal human stem cell keratinocyte proliferation by blocking cell cycle progression in G_1 phase. Numerous studies have shown a similar effect on T cells. Thus, rapamycin may be a novel treatment for psoriasis, a skin disease with characteristic T cell infiltration and keratinocyte hyperproliferation.

The specific point in G_1 at which keratinocyte stem cells were blocked was localized to be prior to PCNA expression but after cyclin D1 induction. Cyclin D1 expression precedes expression of PCNA as quiescent (G_0 phase) stem cell keratinocytes progress into cell cycle (13). β1 integrin upregulation, another characteristic change that keratinocytes undergo in the early in vitro culture as they progress from G_0 into G_1 (1, 2), was not affected by rapamycin. Both cyclin D1 and PCNA expression require de novo protein synthesis in these cells, unlike β1 integrin^{bright} expression which is probably regulated through

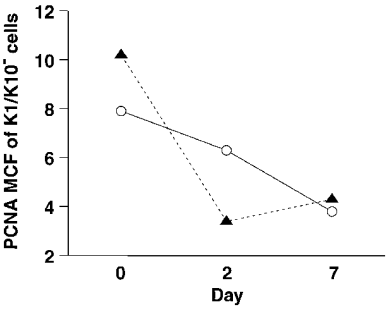

Figure 4. PCNA mean channel fluorescence (MCF) of K1/K10$^-$ cells in vivo. Epidermal cells from psoriatic patients treated with 5 mg/m^2/d rapamycin ($n = 2$) were taken at three time points during rapamycin treatment and stained with anti-K1/K10 and anti-PCNA. The line graph shows PCNA MCF of K1/K10$^-$ keratinocytes at days 0, 2, and 7. Over time, the PCNA MCF of the K1/K10$^-$ population (stem cells) decreased with rapamycin treatment in both subjects. Open circle, patient 1; closed triangle, patient 2.

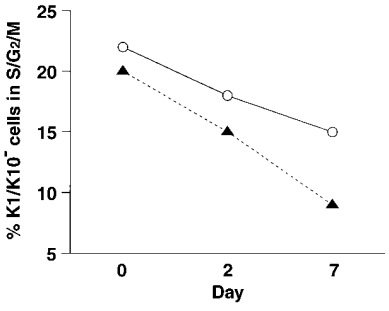

Figure 5. Effect of rapamycin on cell cycle of K1/K10$^-$ cells in vivo. Epidermal cells from the same psoriatic patients analyzed in Fig. 4 were stained with anti-K1/K10 and PI to quantify cellular DNA content. The line graph shows the percent of the K1/K10$^-$ population (stem cells) in $S/G_2/M$ at days 0, 2, and 7. In both subjects (open circle, patient 1 and closed triangle, patient 2), the percentage of stem cells proliferating steadily decreases over the period of rapamycin treatment.

changes in the glycosylation state of integrin subunits rather than new synthesis of integrin subunits (23).

The gene for the mid-G_1 PCNA protein is regulated by the E2F transcription factor (24). It has been shown that, other than regulating DNA synthesis, PCNA may be important in cell cycle progression. New synthesis of PCNA is required before cells can enter S phase (25). Studies using antisense oligonucleotides to PCNA or its mRNA have demonstrated that these forms of treatment prevent cell entry into S phase (7, 8). The level of PCNA expression is highest at the G_1/S transition, when cells are progressing in the cell cycle (25, 26).

Because PCNA expression is critical for cell cycle progression into S phase, the suppression of PCNA protein production may be the critical restricting mechanism of rapamycin's ability to block keratinocyte proliferation.

Previous studies have shown that rapamycin inhibits $p34^{cdc2}$ kinase activity (27, 28). $p34^{cdc2}$ has been implicated in regulation of the G_1/S phase transition in mammalian cells. $p34^{cdc2}$ may be involved in phosphorylation of the retinoblastoma gene product, $p110^{Rb}$. $p110^{Rb}$ links the cell cycle with gene transcription by controlling the expression of genes necessary for progressing through cell cycle (29–31).

Rapamycin has been found to inhibit $p110^{Rb}$ phosphorylation (32, 33). In its hypophosphorylated form, $p110^{Rb}$ has been found to complex with the cellular transcription factor E2F (34, 35). Thus, rapamycin-induced hypophosphorylation of $p110^{Rb}$, because of the drug's inhibition of $p34^{cdc2}$ kinase activity, allows it to remain bound to E2F. In this complexed form, E2F is inactive (35–37) and, as such, cannot transcribe the PCNA gene. Through this pathway, rapamycin may prevent expression of E2F-regulated proteins critical for G_1 progression, such as PCNA.

Other studies have shown that rapamycin blocks phosphorylation of a specific ribosomal protein, S6, by inhibiting p70 S6 kinase activation (38–40). The actual function of phosphorylated S6 is unclear but it has been correlated with growth and increased protein synthesis in rat thymocytes (41).

Terada et al. (42) have shown that rapamycin inhibits upregulation of ribosomal protein mRNA and protein in mitogen-activated primary T lymphocytes. Their study found that rapamycin did not affect the levels of nonribosomal proteins but did delay the synthesis of late G_1 and S phase proteins (42). Protein synthesis is required throughout G_1 in order for cells to progress into S phase (43).

Our work is the first to demonstrate that rapamycin inhibits the synthesis of PCNA, a cell cycle regulatory protein necessary for cells to traverse from G_1 into S phase. The inhibition of PCNA synthesis by rapamycin can serve as an important tool in the investigation of cell cycle regulation. Our results also show that the epidermis, by harboring cell cycle quiescent (G_0 phase) stem cells which uniquely establish in vitro keratinocyte colonies provides a useful system for investigating cell cycle regulation.

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References

1. Bata-Csorgo, Zs., C. Hammerberg, J.J. Voorhees, and K.D. Cooper. 1995. Kinetics and regulation of human keratinocyte stem cell growth in short-term primary ex vivo culture. Cooperative growth factors from psoriatic lesional T lymphocytes stimulate proliferation among psoriatic uninvolved, but not normal, stem keratinocytes. *J. Clin. Invest.* 95:317–327.

2. Jones, P.H., S. Harper, and F.M. Watt. 1995. Stem cell patterning and fate in human epidermis. *Cell.* 80:83–93.

3. Celis, J.E., and A. Celis. 1985. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. *Proc. Natl. Acad. Sci. USA.* 82:3262–3266.

4. Bravo, R., and H. Macdonald-Bravo. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell Biol.* 105:1549–1554.

5. Celis, J.E., P. Madsen, A. Celis, H.V. Nielsen, and B. Gesser. 1987. Cyclin (PCNA, auxiliary protein of DNA polymerase δ) is a central component of the pathway(s) leading to DNA replication and cell division. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 220:1–7.

6. Celis, J.E., and P. Madsen. 1986. Increased nuclear cyclin/PCNA antigen staining of non S-phase transformed human amnion cells engaged in nucleotide excision DNA repair. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 209:277–283.

7. Jaskulski, D., J.K. DeRiel, W.E. Mercer, B. Calabretta, and R. Baserga. 1988. Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. *Science (Wash. DC).* 240:1544–1546.

8. Liu, Y.-C., R.L. Marraccino, P.C. Keng, R.A. Bambara, E.M. Lord, W.-G. Chou, and S.B. Zain. 1989. Requirement for proliferating cell nuclear antigen expression during stages of the Chinese hamster ovary cell cycle. *Biochemistry.* 28:2967–2974.

9. Sherr, C.J. 1993. Mammalian G_1 cyclins. *Cell.* 73:1059–1065.

10. Dowdy, S.F., P.W. Hinds, K. Louie, S.I. Reed, A. Arnold, and R.A. Weinberg. 1993. Physical interaction of the retinoblastoma protein with human D cyclins. *Cell.* 73:499–511.

11. Pines, J. 1995. Cyclins and cyclin-dependent kinases: theme and variations. *Adv. Cancer Res.* 66:181–212.

12. Tam, S.W., A.M. Theodoras, J.W. Shay, G.F. Draetta, and M. Pagano. 1994. Differential expression and regulation of cyclin D1 protein in normal and tumor human cells: association with cdk4 is required for cyclin D1 function in G1 progression. *Oncogene.* 9:2663–2674.

13. Bata-Csorgo, Zs., K.D. Cooper, K. Kang, J.J. Voorhees, and C. Hammerberg. 1996. Differential expression suggesting different functions of D-type cyclins in normal human keratinocytes. *J. Investig. Dermatol.* 106:828. (Abstr.)

14. Bata-Csorgo, Zs., C. Hammerberg, J.J. Voorhees, and K.D. Cooper. 1993. Flow cytometric identification of proliferative subpopulations within normal human epidermis and the localization of the primary hyperproliferative population in psoriasis. *J. Exp. Med.* 178:1271–1281.

15. Valdimarsson, H., B.S. Baker, I. Jonsdottir, and L. Fry. 1986. Psoriasis: a disease of abnormal keratinocyte proliferation induced by T lymphocytes. *Immunol. Today.* 7:256–259.

16. Dumont, F.J., M.J. Staruch, S.L. Koprak, M.R. Melino, and N.H. Sigal. 1990. Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J. Immunol.* 144:251–258.

17. Liu, J., J.D. Farmer, Jr., W.S. Lane, J. Friedman, I. Weissman, and S.L. Schreiber. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell.* 66:807–815.

18. Liu, J., M.W. Albers, T.J. Wandless, S. Luan, D.G. Alberg, P.J. Belshaw, P. Cohen, C. MacKintosh, C.B. Klee, and S.L. Schreiber. 1992. Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. *Biochemistry.* 31:3896–3901.

19. Dumont, F.J., M.R. Melino, M.J. Staruch, S.L. Koprak, P.A. Fischer, and N.H. Sigal. 1990. The immunosuppressive macrolides FK-506 and rapamycin act as reciprocal antagonists in murine T cells. *J. Immunol.* 144:1418–1424.

20. Taylor, R.S., K.D. Cooper, J.T. Headington, V.C. Ho, C.N. Ellis, and J.J. Voorhees. 1989. Cyclosporine therapy for severe atopic dermatitis. *J. Am. Acad. Dermatol.* 21:580–583.

21. Duncan, J.I. 1994. Differential inhibition of cutaneous T-cell-mediated reactions and epidermal cell proliferation by cyclosporin A, FK-506, and rapamycin. *J. Investig. Dermatol.* 102:84–88.

22. Gong, J., F. Traganos, and Z. Darzynkiewicz. 1993. Simultaneous analysis of cell cycle kinetics at two different DNA ploidy levels based on DNA content and cyclin B measurements. *Cancer Res.* 53:5096–5099.

23. Kim, L.T., S. Ishihara, C.-C. Lee, S.K. Akiyama, K.M. Yamada, and F. Grinnell. 1992. Altered glycosylation and cell surface expression of β1 integrin receptors during keratinocyte activation. *J. Cell Sci.* 103:743–754.

24. Lee, H.-H., W.-H. Chiang, S.-H. Chiang, Y.-C. Liu, J. Hwang, and S.-Y. Ng. 1995. Regulation of cyclin D1, DNA topoisomerase I, and proliferating cell nuclear antigen promoters during the cell cycle. *Gene Expr.* 4:95–109.

25. Baptist, M., J.E. Dumont, and P.P. Roger. 1993. Demonstration of cell cycle kinetics in thyroid primary culture by immunostaining of proliferating cell nuclear antigen: differences in cyclic AMP-dependent and -independent mitogenic stimulations. *J. Cell Sci.* 105:69–80.

26. Gazitt, Y., G.W. Erdos, and R.J. Cohen. 1993. Ultrastructural localiza-

tion and fluctuation in the level of the proliferating cell nuclear antigen and myc oncoproteins in synchronized neuroblastoma cells. *Cancer Res.* 53:1899–1905.

27. Howe, P.H., G. Draetta, and E.B. Leof. 1991. Transforming growth factor β1 inhibition of $p34^{cdc2}$ phosphorylation and histone H1 kinase activity is associated with G1/S-phase growth arrest. *Mol. Cell. Biol.* 11:1185–1194.

28. Morice, W.G., G.J. Brunn, G. Wiederrecht, J.J. Siekierka, and R.T. Abraham. 1993. Rapamycin-induced inhibition of $p34^{cdc2}$ kinase activation is associated with G_1/S-phase growth arrest in T lymphocytes. *J. Biol. Chem.* 268: 3734–3738.

29. Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell.* 81:323–330.

30. Furukawa, Y., H. Piwnica-Worms, T.J. Ernst, Y. Kanakura, and J.D. Griffin. 1990. *cdc2* gene expression at the G_1 to S transition in human T lymphocytes. *Science (Wash. DC).* 250:805–808.

31. Lees, J.A., K.J. Buchkovich, D.R. Marshak, C.W. Anderson, and E. Harlow. 1991. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:4279–4290.

32. Marx, S.O., T. Jayaraman, L.O. Go, and A.R. Marks. 1995. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. *Circ. Res.* 76:412–417.

33. Terada, N., R.A. Franklin, J.J. Lucas, J. Blenis, and E.W. Gelfand. 1993. Failure of rapamycin to block proliferation once resting cells have entered the cell cycle despite inactivation of p70 S6 kinase. *J. Biol. Chem.* 268:12062–12068.

34. Nevins, J.R. 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science (Wash. DC).* 258:424–429.

35. Chellappan, S.P., S. Hiebert, M. Mudryj, J.M. Horowitz, and J.R. Nevins. 1991. The E2F transcription factor is a cellular target for the RB protein. *Cell.* 65:1053–1061.

36. Hiebert, S.W., S.P. Chellappan, J.M. Horowitz, and J.R. Nevins. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes Dev.* 6:177–185.

37. Bagchi, S., R. Weinmann, and P. Raychaudhuri. 1991. The retinoblastoma protein copurifies with E2F-I, and E1A-regulated inhibitor of the transcription factor E2F. *Cell.* 65:1063–1072.

38. Price, D.J., J.R. Grove, V. Calvo, J. Avruch, and B.E. Bierer. 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science (Wash. DC).* 257:973–977.

39. Terada, N., J.J. Lucas, A. Szepesi, R.A. Franklin, K. Takase, and E.W. Gelfand. 1992. Rapamycin inhibits the phosphorylation of p70 S6 kinase in IL-2 and mitogen-activated human T cells. *Biochem. Biophys. Res. Commun.* 186: 1315–1321.

40. Calvo, V., M. Wood, C. Gjertson, T. Vik, and B.E. Bierer. 1994. Activation of 70-kDa S6 kinase, induced by the cytokines interleukin-3 and erythropoietin and inhibited by rapamycin, is not an absolute requirement for cell proliferation. *Eur. J. Immunol.* 24:2664–2671.

41. Wettenhall, R.E.H., and G.J. Howlett. 1979. Phosphorylation of a specific ribosomal protein during stimulation of thymocytes by concanavalin A and prostaglandin E_1. *J. Biol. Chem.* 254:9317–9323.

42. Terada, N., K. Takase, P. Papst, A.C. Nairn, and E.W. Gelfand. 1995. Rapamycin inhibits ribosomal protein synthesis and induces G1 prolongation in mitogen-activated T lymphocytes. *J. Immunol.* 155:3418–3426.

43. Brooks, R.F. 1977. Continuous protein synthesis is required to maintain the probability of entry into S phase. *Cell.* 12:311–317.