p21-dependent Inhibition of Colon Cancer Cell Growth by Mevastatin Is Independent of Inhibition of G1 Cyclin-dependent Kinases*

Received for publication, July 5, 2003, and in revised form, August 11, 2003
Published, JBC Papers in Press, August 19, 2003, DOI 10.1074/jbc.M307194200

Chinweike Ukomadu‡ and Anindya Dutta§¶
From the §Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia 22908 and the ¶Division of Gastroenterology, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Mevastatin arrested HCT116 colon cancer cells at the G1/S transition and increased cellular levels of p211MIP/WMAP1. p21-deficient colon cancer cells continued to proliferate in the presence of mevastatin. Although p21 was necessary for the G1/S block, the G1 cyclin-dependent kinases (Cdks) cyclin E-Cdk2 and cyclin D-Cdk4 remained active. Despite the activity of the G1 Cdks the retinoblastoma protein was hypophosphorylated due to unknown mechanisms that were dependent on the p21 protein. The resulting decrease in cyclin A mRNA and protein led to a decrease in the activity of cyclin A-Cdk2. Therefore, although p21 was required for the G1/S arrest of HCT116 colon cancer cells by mevastatin, its mode of action was more complicated than the simple formation of a physical complex with cyclin-Cdk2. This mechanism of inhibition is different from that seen in prostate cancer cells (Ukomadu, C., and Dutta, A. (2003) J. Biol. Chem. 278, 4840–4846) where the activating phosphorylation of cyclin E-Cdk2 is suppressed and p21 is not required, suggesting the existence of cell line-specific differences in the mechanism by which statins arrest the cell cycle.

The inhibitors of the rate-limiting enzyme in cholesterol synthesis, hydroxymethylglutaryl-coenzyme A reductase, have been used for more than 2 decades as first line agents for management of hypercholesterolemia (1). In recent years it has become obvious that these drugs, collectively known as statins, affect additional biological processes. They have been used as anti-inflammatory molecules with potential therapeutic efficacy in the treatment of multiple sclerosis and experimental encephalomyelitis (2, 3) and Alzheimer’s disease (4). These drugs have also been shown to have growth-inhibitory effects in a number of normal and cancer cell lines (5, 6) and have been studied as potential cancer chemotherapeutic agents (7). Because they block a proximal step in cholesterol synthesis, a logical explanation for the growth-altering effects was inhibition of isoprenylation of key regulatory proteins (Ras, Rap, etc.) with their subsequent defective subcellular localization. However, experiments done with lovastatin showed that inhibition of Cdk21 through a Ras-independent pathway accounted for the growth-inhibitory effects (8). Recent studies have focused on how statins inhibit Cdk2 activity. Because growth inhibition has been accompanied by an induction of cyclin-dependent kinase inhibitors p21 and/or p27 (9), the growth-deterrent effects are believed to result from the induction of these proteins and subsequent inhibition of Cdk2. While some experiments have suggested that the statins elevate cyclin-dependent kinase inhibitor by acting as proteasome inhibitors (10) others have suggested that statins act by transcriptional activation of the cyclin-dependent kinase inhibitor (11). In a recent study on PC3 cells, mevastatin inhibited Cdk2 activity by preventing its activating phosphorylation despite depletion of p21 by siRNA transfection (12), suggesting that alternative targets exist for the antiproliferative effects of these drugs. Although it is clear that this group of drugs has potent antiproliferative effects, there has not been a careful search for lineage-specific effects in the molecular mechanism of inhibition of cell proliferation.

The studies reported here evaluated the molecular mechanism of inhibition of colon cancer cell proliferation by mevastatin. Mevastatin, an analog of lovastatin, led to a G1 block and lack of proliferation in HCT116 cells. We have conclusive genetic evidence that p21, but not the related cyclin-dependent kinase inhibitors p27 or p57, is responsible for this effect in HCT116 cells. Although p21 was absolutely required for the inhibitory effect of mevastatin, the inhibition of the kinase activity cannot be accounted for simply by the increased association of p21 with cyclin-Cdk2. In HCT116 cells suppressed by mevastatin, cyclin D-Cdk4 and cyclin E-Cdk2 activities were present in vitro and in vivo. Surprisingly retinoblastoma protein (Rb) was underphosphorylated in vivo despite the activity of cyclin D- and E-associated kinases. These results confirm the requirement for p21 in HCT116 cells but suggest that hypophosphorylation of Rb is effected by p21 without direct inhibition of G1 Cdks through complex formation. These results also underscore the different mode of action of mevastatin in the HCT116 colon cancer cells compared with that in the previously studied PC3 prostate cancer cells.

EXPERIMENTAL PROCEDURES

Materials, Cell Culture, and Flow Cytometry—PC3 cells were from the ATCC (Manassas, VA). The cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. HCT116 cells (wild type and p21-deficient) were the kind gift of Dr. Bert Vogelstein (The Johns Hopkins University). Cells were grown

* This work was supported by National Institutes of Health Grant R01 CA89406 (to A. D.) and by a Howard Hughes Medical Institute postdoctoral fellowship and a Foundation of Digestive Health and Nutrition research scholar’s award (to C. U.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 434-924-1227; Fax: 434-924-5069; E-mail: ad8q@virginia.edu.

‡ The abbreviations used are: Cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; siRNA, small interfering RNA; STAT, signal transducers and activators of transcription; CBP, cAMP-response element-binding protein (CREB)-binding protein; C/EBP, CCAAT/enhancer-binding protein.
as described previously (13, 14). All experiments described below were performed with cells harvested 48 h after mevastatin treatment except where otherwise stated.

Mevastatin (compactin) and roscovitine were from Sigma and were dissolved in dimethyl sulfoxide (Me$_2$SO). Unless indicated 10 µM mevastatin and 50 µM roscovitine were added to subconfluent cells. Control cells were treated with equivalent amount of Me$_2$SO. Cells for flow cytometry were processed at respective times as described previously (15). Antibodies to Cdk2, cyclin E, cyclin A, p57, p21, and p27 were obtained from Santa Cruz Biotechnology. Additional antibodies to Cdk2 and cyclin E were obtained from Neomarkers. Antibody to γ-tubulin was from Sigma. Anti-Rb antibody was the kind gift of Dr. E. Harlow (Harvard University). Anti-phospho-Rb antibody (serine 807/811) was from Cell Signaling Technologies (Beverly, MA). Radioactive isotopes [γ-32P]ATP, [3H]thymidine, and [35S]methionine were from PerkinElmer Life Sciences.

**Immunoblots, Immunoprecipitations, Kinase Assays, and [35S]Methionine Labeling**—These experiments were performed as described previously (12).

**Immunofluorescence**—Cells grown on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature. Cells were permeabilized in 0.5% Triton X-100 in phosphate-buffered saline for 20 min and then blocked in 3% bovine serum albumin solution for 1 h. Primary antibody to γ-tubulin was added at a 1:1000 dilution and incubated overnight at room temperature. After three washes in 3% bovine serum albumin, rhodamine-conjugated secondary antibody was added and incubated at 37 °C for 1 h. Cells were washed and processed for microscopy.

**siRNA Transfections**—Two p21 oligonucleotides (5'-AAGCTCTACCTTCCCCAGGGGCC (p21-1) and 5'-AACATACTGCGCTGGACTGTTTT (p21-2)) were synthesized. Both were equally effective in reducing p21 levels. p21-2 oligonucleotide was used for the experiments reported here. A siRNA to the firefly luciferase (GL2) was used in control experiments (16). Cells were transfected and processed as described previously (12).

**RESULTS**

**p21 Is Necessary for the Cell Cycle Inhibition Noted with Mevastatin Treatment**—Previous experiments have documented that p21 and/or p27 (5, 6, 11) levels are increased following treatment with statins. Interestingly depletion of p21 in PC3 cells by siRNA transfection did not prevent mevastatin-induced inhibition (12). We evaluated the role of p21 in the statin-induced inhibition of HCT116 colon cancer cell proliferation. For these experiments, we used HCT116 cells that are either wild type for p21 (p21+/+) or deficient in p21 (p21−/−) (13, 14). Cells were treated with 10 µM mevastatin and subjected to fluorescence-activated cell sorting (Table I) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay (Fig. 1, A and B). By 36 h after treatment with mevastatin, a prominent G$_1$ block was present in p21+/+ cells but not in p21−/− HCT116 cells (Table I). Furthermore the proliferation of p21-containing cells was inhibited by mevastatin, while that of p21-deficient cells was unaffected (Fig. 1, A and B). Parallel tritiated thymidine experiments also showed that thymidine incorporation was inhibited only in the p21+/+ cells (not shown). These findings suggest that, in the colon cancer cell line HCT116, p21 is required for mevastatin-induced inhibition of cell proliferation.

**Cdk2 Is Not Inhibited by Mevastatin in p21-deficient HCT116 Cells**—We next evaluated whether Cdk2 kinase is inhibited in a p21-dependent manner. Fig. 2A shows an immunoblot of lysates from p21+/+ and p21−/− HCT116 cells. As expected, the level of Cdk2 remained constant in both cell lines with or without mevastatin treatment. p21 was induced in p21+/+ cells in the presence of mevastatin and was absent in the p21-deficient cells. Immunoprecipitation with antibodies to Cdk2 (Fig. 2B) showed a very minor increase in associated p21 along with a dramatic inhibition of kinase activity. Although both p27 and p57 were present in p21−/− cells they did not substitute for the role of p21 in the inhibition of Cdk2 or cell proliferation. At face value, these results might be interpreted as showing that the induction of p21 and association with Cdk2 is responsible for the inhibition of Cdk2 and G$_1$/S block. As will become obvious with further exploration, this is not the case.

Since siRNA against p21 did not abolish the antiproliferative effect of mevastatin in the PC3 prostate cancer cells, the apparent requirement of p21 for the same activity in HCT116 cells could be due to secondary changes in the HCT116 p21−/− cells (12). We therefore decided to confirm the p21 requirement for the effect of mevastatin in HCT116 cells by acute depletion of p21 using RNA interference. Fig. 2C (middle panel) shows
that the transfected oligonucleotide could decrease p21 effectively in HCT116 cells even in the presence of mevastatin. In contrast a control siRNA did not reduce p21 levels or inhibit the mevastatin-induced enhancement of p21. In addition, p21 depletion did not lead to enhancement of p21 levels (bottom panel). Quantitative Western blots suggested a 5–10-fold decrease in p21 levels in mevastatin-treated, p21 siRNA-transfected cells compared with mevastatin-treated, control siRNA-transfected cells. Immunoprecipitation of transfected cell lysates with anti-Cdk2 antibody also showed a significant decrease in associated p21 (Fig. 2D, middle panel, compare lanes 2 and 4). Kinase assays against histone H1 showed that this decrease in p21 leads to the persistence of kinase activity in the presence of mevastatin (Fig. 2D, bottom panel, compare lanes 2 and 4). Therefore, contrary to the results in PC3 cells, p21 is indeed required in HCT116 cells for the antiproliferative effect of mevastatin.

The Inhibitory Effect of Mevastatin in HCT116 Cells Is Due to Enhancement of p21 mRNA Levels Not Stabilization of p21 Protein—Two mechanisms have been proposed for the increase in p21 protein levels in cultured cells following treatment with statins. In PC3 cells, mRNA levels are elevated following mevastatin. 2 In addition p21 promoter deletion experiments performed in PC3-M cells have identified a segment of the promoter responsible for the lovastatin-induced enhancement of p21 mRNA (11). Experiments in MOA-MB-157 cells, however, have suggested that p21 levels are enhanced because lovastatin acts as an inhibitor of the proteasome leading to greater protein stability (10). We performed experiments to evaluate which of these mechanisms accounted for the elevated levels of p21 in HCT116 cells following mevastatin treatment. As shown in Fig. 3A, treatment of p21+/+ cells with mevastatin led to an increase in p21 mRNA levels. No p21 was detected in p21-deficient cells with or without mevastatin. Fig. 3B shows pulse-chase experiments of [35S]methionine-labeled p21 in the presence and absence of mevastatin. There was no detectable change in the half-life of p21 with mevastatin treatment suggesting that the mechanism of p21 enhancement in HCT116 cells is by increasing the p21 mRNA level and is not due to stabilization of the p21 protein.

Mevastatin Treatment Leads to a p21-dependent Decrease in Cyclin A Protein and mRNA Levels—Since mevastatin blocked the cells in G1 with decreased Cdk2 kinase activity, we separately evaluated the activities of cyclin A- and E-associated Cdk2. Fig. 4A shows immunoblots of whole cell lysates at specific times following mevastatin treatment. In both cell lines (H1101 p21+/+ and p21−/−) the levels of cyclin E remained relatively constant (top panel). In contrast, the amount of cyclin A declined within 48 h in the p21-sufficient cells but not in the p21-deficient HCT116 cells (bottom panel). This would suggest either a p21-dependent effect on the cyclin A protein or cyclin A mRNA. In Northern blots performed on lysates 48 h after mevastatin addition, the level of cyclin E mRNA remained constant, while there was a suppression of cyclin A mRNA in HCT116 p21+/+ cells (Fig. 4B). In p21−/− HCT116 cells we observed continued expression of cyclin A mRNA. Therefore, cyclin A transcription is not directly suppressed by mevastatin, but a p21-dependent activity is required for the suppression.

C. Ukomadu and A. Dutta, unpublished.
Because cyclin A transcription is dependent on E2F (17) we wondered whether the inhibition of cyclin E-Cdk2 and the subsequent underphosphorylation of Rb explains the decrease in cyclin A mRNA and protein levels in mevastatin-treated p21+/− cells.

Distinct Effects of Mevastatin on in Vitro Kinase Activities of Cyclin E-Cdk2 from PC3 and HCT116 Cells—Treatment of HCT116 cells with mevastatin (Fig. 5A) inhibited cyclin A-associated kinase activity but not cyclin E-associated kinase activity. As expected there was no decrease in cyclin E- or A-associated kinase activities in p21+/− cells following mevastatin treatment. Thus the inhibition of in vitro kinase in the Cdk2 immunoprecipitates from p21+/− HCT116 cells (Fig. 2B) is accounted for mostly by the lack of cyclin A and is not due to inhibition of cyclin E-Cdk2 kinase.

Interestingly in PC3 cells both cyclin E- and cyclin A-associated Cdk2 activity was inhibited by mevastatin (12). Fig. 5B further illustrates this discrepancy between cyclin E-Cdk2 from mevastatin-treated PC3 and HCT116 cells. While there was complete loss of kinase activity in PC3 cells by 36 h after mevastatin treatment, greater than 70% of the cyclin E-Cdk2 activity remained in HCT116 cells. Fig. 5C quantitates this difference in inhibition of cyclin E-Cdk2 from PC3 or HCT116 cells following treatment with mevastatin.

This difference in the histone H1 kinase activity of Cdk2 immunoprecipitates (Fig. 2B) versus cyclin E immunoprecipitates (Fig. 5, A–C) from mevastatin-treated HCT116 cells is intriguing. In contrast to complete loss of activity in the Cdk2 immunoprecipitates, significant activity was retained in the cyclin E immunoprecipitate. This difference was confirmed with antibodies to Cdk2 and cyclin E from different sources. We suggest that the anti-Cdk2 antibodies preferentially immunoprecipitate free Cdk2 that is not complexed with any cyclin. Since free Cdk2 was enriched in mevastatin-treated cells because of suppression of cyclin A transcripts, it appears that all the kinase activity is lost upon mevastatin treatment in Fig. 2B. The cyclin E immunoprecipitation in Fig. 5 selectively isolated only Cdk2 complexed with cyclin E and eliminates free Cdk2 and thus accurately portrays the residual activity of the cyclin E-Cdk2 complex upon mevastatin treatment.

Mevastatin Treatment Leads to Underphosphorylation of Rb in Vivo Despite Retained in Vitro Cyclin E-Cdk2 Activity—We next focused on why cyclin E-Cdk2 activity in vitro was normal but the cell cycle was blocked. One possibility was that cyclin E-Cdk2 activity on a relevant substrate in vivo was inhibited despite the in vitro activity against histone H1. The state of phosphorylation of Rb was examined following mevastatin treatment (Fig. 6A). In the top panel equal amounts of lysates from mevastatin-treated and untreated cells were analyzed by immunoblots using an antibody that recognizes hyper- and hypophosphorylated forms of Rb. In p21+/− HCT116 cells, the slowly migrating band corresponding to the hyperphosphorylated Rb was lost with mevastatin treatment. In p21+/− cells Rb remained hyperphosphorylated corresponding to a failure of mevastatin to block the cell cycle. To further illustrate this, we performed parallel immunoblots with an antibody that specifically recognizes the hyperphosphorylated form of Rb. Fig. 6A (lower panel) confirms that hyperphosphorylation of Rb was inhibited by mevastatin treatment of the p21+/− HCT116 cell line. Thus mevastatin effectively prevents Rb phosphorylation in vivo, although cyclin E-Cdk2 is active in vitro.
The hypophosphorylation of Rb in mevastatin-treated HCT116 cells could be secondary to inhibition of cyclin D-Cdk4 kinase activity. As shown in Fig. 6B, following mevastatin treatment there was no change in complex formation between cyclin D1 and Cdk4 (middle and right panels), no increase in association of p21 or p27 with cyclin D1 (middle panel), and no decrease in Cdk4 kinase activity (right panel). Therefore the hypophosphorylation of Rb in vivo cannot be due to loss of Cdk4 kinase activity.

The observations above may be explained by two possible hypotheses. 1) Cyclin E activity measured in vitro is not representative of the activity in vivo, or 2) cyclin E is active in vivo, and Rb hypophosphorylation occurred via a cyclin E-independent mechanism.

A disparity in the activity of cyclin E-Cdk2 in vitro and in vivo could be explained by a number of technical factors. A differential activity of cyclin E-Cdk2 against H1 and Rb was ruled out because we see no difference in the in vitro activity of cyclin E-Cdk2 on histone H1 or a fusion protein containing the carboxyl-terminal fragment of Rb. A low affinity inhibitor of the enzyme might be lost during extraction and immunoprecipitation of the kinase. [35S]-Methionine labeling in vivo followed by immunoprecipitation of cyclin E under lower stringencies of extraction and washing did not, however, reveal any novel proteins co-immunoprecipitated from mevastatin-treated cells. Differential cellular localization of cyclin-Cdk complexes was also investigated, but we saw no differences in localization of p21, Cdk4, cyclin D1, cyclin E, and Cdk2 with prominent nuclear staining of all factors 48 h after mevastatin treatment (not shown). Failure to extract the total representative pool of cyclin E-Cdk2 might also account for the disparity. To address this cell fractionation experiments were performed (Fig. 6C). Phosphorylated Rb levels decreased with mevastatin treatment showing the successful action of mevastatin (panels 1 and 2). Hypophosphorylated Rb was enriched in the pellet fraction consistent with previous fractionation results (18). MCM10, a DNA replication initiation factor, remained in the nuclease-resistant fraction (P) providing a control for the integrity of the fractionation (panel 3). The decrease in MCM10 with mevastatin treatment is expected as the protein is loaded on to chromatin in the S phase (19). Cyclin E and Cdk2 were extracted totally in the same soluble fraction before or after mevastatin treatment ruling out the possibility that we leave behind a large pool of the kinase during our extraction. p21 was present almost exclusively in the same fraction as the cyclin E-Cdk2, so we are not fractionating the kinase from its inhibitor during the extraction process (Fig. 6C, panels 4–6, lanes 1 and 4). Therefore the disparity between the in vitro and in vivo kinase activity of cyclin E-Cdk2 cannot be accounted for by technical artifacts introduced during cell lysis.

The second possibility is that cyclin E-Cdk2 is active in vivo implying that another cellular factor accounts for the hypophosphorylation of Rb following mevastatin treatment. Centrosome duplication is dependent on the activity of cyclin E-Cdk2 (20, 21). If cyclin E-Cdk2 is globally inhibited in vivo, centrosome duplication will be suppressed in the mevastatin-treated HCT116 cells. Fig. 7A shows immunofluorescence pictures after staining with an antibody to γ-tubulin to detect centrosomes. The left and middle panels are representative of the majority of the cells seen in untreated HCT116 and PC3 cells as well as in mevastatin-treated HCT116 cells. However, in mevastatin-treated PC3 cells (right panel), the majority of the cells contained an unduplicated centrosome. Quantitative analysis of centrosome duplication in PC3 cells treated with roscovitine, an inhibitor of cyclin-dependent kinases, showed a decrease in the number of duplicated chromosomes (Fig. 7B, left). Treatment of PC3 cells with mevastatin also showed a similar decrease in the number of cells with duplicated chromosomes consistent with significant inhibition of cyclin E-Cdk2 activity in these cells. However, mevastatin treatment of HCT116 cells showed that the number of cells with two or more centrosomes was not decreased by mevastatin treatment. These experiments suggest that cyclin E-Cdk2 is active as a kinase in HCT116 cells but that another p21-dependent mechanism accounts for the underphosphorylation of Rb that results in the cyclin A decrease and cell cycle block.

Lastly we examined the kinetics of Rb dephosphorylation in HCT116 cells following mevastatin treatment (Fig. 7C). A significant increase in the hypophosphorylated form of Rb was detectable within 24 h (panel 1, compare lanes 1 and 2) with more profound reduction by 36 h following mevastatin treatment (panel 2, compare lanes 1 and 3). By 48 h there was no detectable phospho-Rb (panels 1 and 2, lanes 4 and 5). In contrast, the levels of cyclin A remained relatively unchanged during the first 36 h before it declined (panel 3). This suggests
that the decrease in cyclin A levels is subsequent to the dephosphorylation of Rb. We examined the cyclin E-Cdk2 activity using the same lysates (panel 5) and noted a persistence of a significant fraction of the kinase activity. After an initial decrease of 30% of the kinase activity (quantitated by band excision and scintillation counting) in the first 24 h (compare lanes 1 and 2), the kinase activity remained fairly constant during the duration of the experiment. This disparity between the lack of Rb hyperphosphorylation and persistent cyclin E-Cdk2 activity suggests the existence of another mechanism for the dephosphorylation of Rb in mevastatin-treated cells.

**DISCUSSION**

Like its analog lovastatin, mevastatin inhibited cell proliferation. In HCT116 cells, this antiproliferative effect paralleled the increase in cellular levels of p21, leading to the assumption that the increased association of p21 with Cdk2 is sufficient to account for the kinase inhibition and cell cycle block. The mechanism for the enhancement of p21 protein levels following mevastatin treatment appears to be secondary to increased mRNA levels of p21 following mevastatin treatment. The pivotal role of p21 in the effect of mevastatin is illustrated by the failure of the statin to suppress growth of p21-deficient HCT116 cells. In addition p21-deficient HCT116 cells contained active Cdk2 kinases and hyperphosphorylated Rb implying that the cell cycle is not blocked. While previous studies have documented the increased levels of p27 upon lovastatin treatment in breast, HeLa, HL 60, fibroblast, and pulmonary arterial smooth muscle cells (5, 22, 23) our experiments showed that only p21 was induced in HCT116 cells. The failure of mevastatin to inhibit growth of p21−/− HCT116 cells constitutes the first genetic proof that p21 is an important mediator of the observed antiproliferative effects in some cells.

Despite the requirement of p21, a number of observations rule out a simplistic model where enhanced p21 interaction with G1 cyclin-Cdk accounts for the antiproliferative effect. 1) Cyclin E-Cdk2 and cyclin D1-Cdk4 were complexed to p21 but remained relatively active in mevastatin-treated cells. 2) Although p27 and p57 were present in p21-deficient cells there was no compensatory increase of these Cdk inhibitors upon mevastatin treatment. 3) The in vitro activities of cyclin E-Cdk2 derived from PC3 or HCT116 cells were differently affected following mevastatin treatment, although similar amounts of p21 were associated with the kinase in the two cases (as reported here and in Ref. 12). 4) Although Rb phosphorylation was suppressed in HCT116 cell lines the centrosome duplication suggests that cyclin E-Cdk2 remains active in vivo. Therefore, other factors must contribute to the suppression of Rb phosphorylation in vivo and enhance the cell cycle block.

How can p21 inhibit the cell cycle without inactivating the G1 Cdkks by direct association? Results are beginning to emerge in the literature suggesting that p21 has other targets in the cell (for a review, see Ref. 24). For example, p21 protects cells from apoptosis by association with and inhibition of stress-activated protein kinase, apoptosis signal-regulating kinase-1, and the Caspase 3 enzyme. p21 also affects cell adhesion and motility by interacting with integrins and with Rho kinases. Several studies (26–30) also suggest that p21 directly regulates
the activity of transcription factors like NF-κB, STAT3, Myc, C/EBP, and E2F. In addition, by interacting with and inhibiting the CBP/p300 histone acetylases, p21 can have indirect effects on chromatin structure and gene regulation. Any of these mechanisms might be responsible for the p21-dependent inhibition of Rb phosphorylation and G1 arrest induced by mevastatin without an inhibition of G1 Cdk.

We have begun to dissect the complexities involved in the inhibition of Cdk2 activity by mevastatin. Although the inhibition was accompanied by an induction of p21 and was dependent on p21 it would be erroneous to conclude that the inhibition is due to the direct action of p21 on the Cdk2 protein. In a popular model of regulation of Cdk in G1 and S, it is believed that cyclin D-Cdk4 initiates the G1/S transition by

**Fig. 7. Centrosome duplication assays.** A, mevastatin-treated and untreated cells were stained with γ-tubulin and then with rhodamine-conjugated secondary antibody. The figure shows some representative views of centrosomes (arrows) seen in mevastatin-treated and untreated cells. B, a bar graph depicting the percentage of cells with duplicated centrosomes in roscovitine- or mevastatin-treated and untreated cells. Hatched bars represent untreated cells, while black bars represent either roscovitine-treated PC3 cells (left, PC3(Ros)), mevastatin-treated PC3 cells (middle, PC3(MEV)), or mevastatin-treated HCT116 cells (right). An average of 200 cells was counted per experiment. The graph represents mean ± S.D. from three separate experiments. C, time course of Rb dephosphorylation. 25 μg of cellular lysate for each time point was used for Western blots. UNT is lysate from untreated cells. Top panel, immunoblot with total Rb antibody shows a predominance of the phosphorylated Rb in untreated cells (upper arrow) with increasing amounts of hypophosphorylated forms detectable at 24 h (lower arrow). Second panel, immunoblot with phospho-Rb antibody showing a significant decrease in phosphorylated Rb most obvious at 36 h. Third panel, time course of cyclin A abundance showing loss of cyclin A protein by 48 h. Fourth panel, Cdk2 content as loading control. Fifth panel, cyclin E-Cdk2 activity of lysates. 250 μg of lysate was immunoprecipitated for kinase assay on histone H1. Note that cyclin E activity persists after an initial decrease of 30%. cyc, cyclin; Rbp, phosphorylated Rb; MEV, mevastatin.
phosphorylating Rb and releasing some E2F transcription factor (Fig. 8). The E2F promotes transcription of cyclin E, leading to activation of cyclin E-Cdk2, which further phosphorylates Rb and releases more E2F providing a positive feedback loop (Fig. 8, dashed arrows). Eventually a critical mass of free E2F is available to promote transcription from the cyclin A promoter (and that of other DNA replication proteins) leading to the activation of the S phase Cdk cyclin A-Cdk2 and progression of the cell into S phase. Cyclin E-Cdk2 kinase activity was seen in mevastatin-treated cells despite the hypophosphorylation of Rb suggesting that at least 70–80% of this kinase can be active without full activation of the positive feedback loop. In contrast, the failure to activate cyclin A promoter and form an active cyclin A-Cdk2 kinase can be attributed to the hypophosphorylated Rb and failure of the positive feedback loop. It is also now clear that the bulk of Cdk2 inhibition seen upon mevastatin treatment is due to the disappearance of cyclin A transcripts and not due to a direct action of p21 on Cdk2.

It is instructive to compare the data presented here with the effect of mevastatin in another cell line, the PC3 prostate cancer cell line (Table II) (12). In mevastatin-treated PC3 cells, Rb was hypophosphorylated because of inhibition of cyclin E-Cdk2 due to the loss of activating phosphorylation of Cdk2 on threonine 160. This inhibition was not dependent on p21 as p21-deficient cells continued to be inhibited by mevastatin. In contrast, in the HCT116 cells mevastatin had a minimal effect on the kinase activity of cyclin E-Cdk2 but decreased the phosphorylation of Rb in vivo. Since the centrosome duplication assays suggested that cyclin E-Cdk2 activity is maintained in vivo, the un inhibited kinase activity of cyclin E-Cdk2 is not an in vitro artifact. Since the hypophosphorylation of Rb was dependent on the presence of p21 in these cells, one of the atypical targets of p21 could be responsible for the elevation of a factor that prevents the phosphorylation of Rb. This factor could act to sequester the Rb or could be an enzyme whose activity is necessary before Rb is phosphorylated by Cdkks in vivo. Alternatively the atypical targets may activate a Rb-specific phosphatase.

The variation in the mechanism of action of statins on two different cancer cell lines was unexpected, particularly because the final effect was the same: cell cycle block at the G1/S transition with hypophosphorylated Rb. These observations strongly suggest that the mechanism of action of statins against cancers should be tested separately for each cancer instead of making generalized conclusions from a few cell lines. An intriguing commonality between the action of mevastatin on PC3 and HCT116 cells is worth discussing. In PC3 cells the substrate Cdk2 was not phosphorylated on Thr-160 despite the in vitro activity of the Cdk2-activating kinase, cyclin H-Cdk7. In HCT116 cells the substrate Rb was not hyperphosphorylated despite the activity of the relevant kinases, cyclin D1-Cdk4 and cyclin E-Cdk2. Although we have not yet been successful in detecting such an activity, it is possible that the explanation may lie in substrate-specific phosphatases that are activated by mevastatin in the two cell lines.

In summary, we provide the first genetic evidence for the dependence on p21 for the antiproliferative effects of statins. We show that, in the colon cancer cell line HCT116, cell cycle block was due to hypophosphorylation of Rb and a decrease in cyclin A levels. Because p21 is a potent inhibitor of Cdkks in vitro, induction of p21 occurring concurrently with inhibition of Cdk2 in cells has led to the assumption that the p21 inhibits the kinase by association with the enzyme. Our studies suggested that p21 has diverse regulatory effects in vivo separate from the simple association and direct inhibition of Cdkks. Future studies will be undertaken to gain an understanding of the mechanisms responsible for the p21-dependent hypophosphorylation of Rb in mevastatin-treated HCT116 cells.

Acknowledgment—We thank the members of the Dutta laboratory for valuable discussions and help.

REFERENCES
1. Retterstol, K., Staugaard, M., Gorbitz, C., and Ose, L. (1996) Am. J. Cardiol. 78, 1369–1374
2. Greenwood, J., Walters, C. E., Pryce, G., Kanuga, N., Beraud, E., Baker, D., and Adamson, P. (2003) FASEB J. 17, 905–907
3. Yousef, S., Stute, O., Patarroyo, J. C., Ruiz, P. J., Radoevich, J. L., Hur, E. M., Bravo, M., Mitchell, D. J., Sobel, R. A., Steinman, L., and Zamvil, S. S. (2002) Nature 420, 78–84
4. Vega, G. L., Weiner, M. F., Lipton, A. M., Von Bergmann, K., Latjohann, D., Moore, C., and Svetlik, D. (2003) Arch. Neurol. 60, 510–515
5. Rao, S., Lowe, M., Herlitzck, T. W., and Keyomarsi, K. (1998) Oncogene 17, 2393–2402
6. Keyomarsi, K., Sandoval, L., Band, V., and Pardee, A. B. (1991) Cancer Res. 51, 3602–3609
7. Kim, W. S., Kim, M. M., Choi, H. J., Yoon, S. S., Lee, M. H., Park, K., Park, C. H., and Kang, W. K. (2001) Investig. New Drugs 19, 81–83
8. DeCleue, J. E., Vass, W. C., Papageorge, A. G., Lowy, D. R., and Williams, B. M. (1991) Cancer Res. 51, 712–717
9. El-Kabes, S., Gray-Sablin, J., Herlitzck, T. W., and Keyomarsi, K. (1999) Exp. Cell Res. 252, 211–223
10. Rao, S., Porter, D. C., Chen, X., Herlitzck, T., Lowe, M., and Keyomarsi, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7797–7802
11. Lee, S. J., Ha, M. J., Lee, J., Nguyen, P., Choi, Y. H., Pirnia, F., Kang, W. K., Wang, X. F., Kim, S. J., and Trepel, J. B. (1998) J. Biol. Chem. 273, 10618–10623
12. Ukomadu, C., and Dutta, A. (2003) J. Biol. Chem. 278, 4840–4846
13. Waldman, T., Kinzler, K. W., and Vogelstein, B. (1995) Cancer Res. 55, 5187–5190
14. Waldman, T., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1996) Nature 381, 713–716
15. Yamaguchi, R., and Dutta, A. (2000) Exp. Cell Res. 261, 271–283
16. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* **411**, 494–498
17. Schulze, A., Zerfass, K., Spitzkovsky, D., Middendorp, S., Berges, J., Helin, K., Jansen-Durr, P., and Henglein, B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11264–11268
18. Mittnacht, S., and Weinberg, R. A. (1991) *Cell* **65**, 381–393
19. Izumi, M., Yatagai, F., and Hanaoka, F. (2001) *J. Biol. Chem.* **276**, 48526–48531
20. Okuda, M., Horn, H. F., Tarapore, P., Tokuyama, Y., Smulian, A. G., Chan, P. K., Knudsen, E. S., Hofmann, I. A., Snyder, J. D., Bove, K. E., and Fukasawa, K. (2000) *Cell* **103**, 127–140
21. Angus, S. P., Fribourg, A. F., Markey, M. P., Williams, S. L., Horn, H. F., DeGregori, J., Kowalik, T. F., Fukasawa, K., and Knudsen, E. S. (2002) *Exp. Cell Res.* **276**, 201–213
22. Hengst, L., and Reed, S. I. (1996) *Science* **271**, 1861–1864
23. Fouty, B. W., and Rodman, D. M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 501–509
24. Coqueret, O. (2003) *Trends Cell Biol.* **13**, 65–70
25. Henglein, B., Chenivesse, X., Wang, J., Eck, D., and Brechot, C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5490–5494
26. Delavaine, L., and La Thangue, N. B. (1999) *Oncogene* **18**, 5381–5392
27. Snowden, A. W., Anderson, L. A., Webster, G. A., and Perkins, N. D. (2000) *Mol. Cell. Biol.* **20**, 2676–2686
28. Coqueret, O., and Gascan, H. (2000) *J. Biol. Chem.* **275**, 18794–18800
29. Kitaura, H., Shinshii, M., Uchikoshi, Y., Ono, T., Tsurimoto, T., Yoshikawa, H., Iguchi-Ariga, S. M. M., and Ariga, H. (2000) *J. Biol. Chem.* **275**, 10477–10483
30. Harris, T. E., Albrecht, J. H., Nakanishi, M., and Darlington, G. J. (2001) *J. Biol. Chem.* **276**, 29200–29209

**p21 and Mevastatin**