Phosphorylation of cdk2 on threonine 160 is essential for kinase activity. Mevastatin, an inhibitor of cholesterol synthesis, inhibits cell growth through inhibition of cdk2 and this has been suggested to be due to enhancement of p21 levels. In a prostate cancer cell line, PC3, mevastatin treatment led to elevated levels of p21 and caused a small increase in the p21 associated with cdk2. However, this increase in the associated p21 appeared out of proportion with the resulting dramatic inhibition of kinase activity. Using RNA interference we show that mevastatin inhibits cdk2 activity despite lack of induction of p21, p27, and p57. Instead the kinase was inhibited due to a decrease in activating phosphorylation. Phosphorylation of cdk2 from mevastatin-treated cells with exogenous cyclin-dependent kinase (cdk)-activating enzymes restored its functional activity. The only known mammalian cyclin B-cdk7mat1 complex (cdk2-activating kinase, Cak), was not inhibited by mevastatin, suggesting either that a different CAK is responsible for cdk2 phosphorylation in vivo or that the regulation is at the level of substrate accessibility or of cdk2 dephosphorylation. These results suggest that mevastatin inhibits cdk2 activity in PC3 cells through the inhibition of Thr-160 phosphorylation of cdk2, providing a novel example of regulation of cdk2 at this level.

The basic cell cycle machinery is composed of regulatory cyclin subunits complexed to cyclin-dependent kinase (cdk) subunits (1). When active, the cyclin-cdk complex phosphorylates specific substrates in a cell cycle-dependent manner. Cyclin E-cdk2 is active in mid G1 close to the restriction point, cyclin A-cdk2 from the beginning of S to M, whereas cyclin B-cdk1 is active at the G2-M transition (2). After binding of the cyclin to the cdk, the resulting heterodimer remains inactive because of the presence of inhibitory phosphorylation and/or lack of activating phosphorylation. cdk2 contains two sites of inhibitory phosphorylations on threonine 14 and on tyrosine 15. It also contains a site for activating phosphorylation on threonine 160 (3). The activation of cyclin E or A-cdk2 requires the dephosphorylation of Thr-14 and Tyr-15 and the phosphorylation of Thr-160. The former is accomplished through the action of the dual specificity phosphatase CDC25 (4) and the latter through the action of the Cdk-activating enzyme; CAK (5). Inhibitory proteins, which associate with and inactivate the kinase, further regulate cyclin-cdk2 (6). p21 and its close relatives p27 and p57 belong to this class of cdk inhibitors (CKIs).

Regulation of cdk in response to different stimuli has until now been described at the level of CKI induction or changes in the inhibitory phosphorylation on Thr-14 and Tyr-15. Following gamma radiation or exposure to other DNA-damaging agents, p21 is induced in a p53-dependent manner to inhibit cdk2 and block the cell cycle at the G1-S transition (7). Intra-S-phase checkpoints activated by DNA damage and mitotic checkpoints induced by DNA damage or incomplete DNA replication lead to inhibitory phosphorylation of cdk2 (8) on Thr-14 and Tyr-15. In contrast, regulation of a cyclin-cdk complex at the level of Thr-160 phosphorylation appears to be rare.

Because of the pivotal role of cyclin E or A-cdk2 in cell cycle progression, molecules that inhibit their activities are likely to have antineoplastic properties. Inhibitors of the rate-limiting enzyme in cholesterol synthesis, hydroxymethyl-glutaryl-CoA reductase, have growth deterrent effects in a number of normal and cancer cell lines (8, 9). Collectively known as "statins," these drugs are used for treatment of hypercholesterolemia (10). One explanation for their growth-altering effects is that the lack of isoprenylation of key regulatory proteins like Ras, Rap, and others leads to defective subcellular localization of these key proteins. Lovastatin, however, inhibits cdk2 through a ras-independent pathway (11). Growth inhibition was accompanied by an induction of CKIs, p21, and/or p27 (9), due to the inhibition of proteasomes by statins (12). The increased association of p21 with cyclin-cdk2 that accompanies the cdk2 kinase inhibition is believed to account for the cell cycle block. However, definitive evidence that p21 and/or p27 are necessary for growth inhibition by statins has been lacking, and alternate pathways by which statins could inhibit cdkks have not been explored.

The studies reported here evaluate the molecular mechanism of inhibition of cell proliferation by mevastatin. Mevastatin, an analogue of lovastatin, leads to a profound G1 block in a prostate (PC3) cancer cell line. Although p21 levels are elevated and its association with cdk2 increased, it is unlikely that the inhibition of kinase activity and cellular proliferation are caused by p21. Instead a decrease in the activating phosphorylation of cdk2 likely accounts for the antiproliferative effects of this drug.

MATERIALS AND METHODS

Materials, Cell Culture, and Flow Cytometry—PC3 cells from the ATCC (Manassas, VA) were grown in RPMI medium supplemented with 10% fetal bovine serum and antibiotics.

Mevastatin (compactin, Sigma) was dissolved in Me2SO, and 10 μM was added to subconfluent cells. TGF-β1 (Invitrogen) was dissolved in deionized water and used at 80 μM. Cells for flow cytometry were processed as previously described (13). Antibodies to cdk2, cyclin E, cyclin A, p57, p21, p27, and phosphotyrosine were from Santa Cruz
Biology. Anti-Rb antibody was the kind gift of Dr. E. Harlow (Harvard University). Anti-phospho-Rb antibody was from Cell Signaling Technologies (Beverly, MA). Duplex oligoribonucleotides were from Dharamco (Lafayette, CO). Radioactive isotopes, [$\gamma^{-32}$P]ATP, [$\gamma^{-33}$P]orthophosphate, and [$\beta^{-35}$S]methionine were from PerkinElmer Life Sciences. Recombinant cyclin Hcdk2 was the kind gift of Dr. Robert Fisher (Sloan-Kettering). The expression plasmid PGEX-cdk1p was the kind gift of Dr Mark Solomon (Yale University).

Immunoblotting—Cells were treated with lysin buffer (50 mM, Tris-HCl pH 7.4, 0.2% Nonidet P40, 150 mM NaCl, 1 mM EDTA, and protease inhibitors). Lysates were clarified by centrifugation, and protein concentration was determined by the Bradford assay method. 30 μg of total cellular protein was loaded per lane and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and immunoblots were performed as previously described (14). 10–8 cm 12% SDS-polyacrylamide gels were used to resolve samples except in experiments for the resolution of the two forms, where a 13–15 cm 11% SDS-polyacrylamide gel was used.

Immunoprecipitations and Kinase Assays—Cells were treated with lysin buffer supplemented with 50 mM NaF, 1 mM Na$_3$VO$_4$. Equal amounts of lysates (200–1500 μg of protein) were immunoprecipitated with anti-cdk2, cyclin E, or cyclin A antibodies. The pellet was washed three times with lysin buffer and twice in kinase wash buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, 5 mM MnCl$_2$, 5 mM dithiothreitol, 10 μM ATP, 0.5 μM of [$\gamma^{-32}$P]ATP, 2 μg of histone H1). The mixture was incubated at 30 °C for 30 min, and the reaction was stopped by the addition of equal volume of 2× sample buffer. For cdk2 activation, kinase assays were carried out as previously described (15, 16). For phosphatase assays, cells were solubilized in lysin buffer devoid of EDTA and phosphatase inhibitors (unless otherwise indicated). Substrates were incubated with cell lysates in the presence of either 10 mM MgCl$_2$, or 10 mM CaCl$_2$, for 30 min at 30 °C, and the reaction was stop by addition of sample buffer.

[35S]Methionine and [32P] Labeling—Subconfluent cells were grown with or without mevastatin for 42 h. For [$\beta^{-35}$S]methionine labeling, the medium was removed, and the cells were washed 2× in phosphate-buffered saline and incubated in methinione free medium with or without mevastatin for 1 h. Cells were labeled for 4 h in methionine-free medium, supplemented with 5% dialyzed fetal calf serum, 25 μCi/ml [$\beta^{-35}$S]methionine (specific activity, 1175 Ci/mmol) with or without mevastatin. Proteins were extracted with lysis buffer as above, and protein concentration was determined by the Bradford assay. For [$\gamma^{-32}$P] labeling of cellular proteins, cells were incubated in phosphate free medium for 2 h and then in medium containing 350 μCi/ml [$\gamma^{-32}$P]orthophosphate for 3 h prior to harvesting.

RNAi Transfections—A 21 oligonucleotide corresponding to nucleotides 1938–1960 (p21–2) was synthesized. A control RNAi to cyclin H (194–206) was also synthesized. This RNAi had no effect on cellular levels of cyclin H and was used as a control. Cells were transfected twice with 60 μmol oligonucleotide at 0 and 24 h. 4 h after the second transfection, cells were washed and fresh media were supplemented with either mevastatin or Me$_2$SO. Cells were harvested 48 h later.

RESULTS

Mevastatin Treatment Leads to a G$_1$ Block and Decrease in Kinase Activity of cdk2—As has been reported with lovastatin treatment of PC3-M cells (17), mevastatin treatment leads to a significant decrease in the S phase population with a concurrent increase in G$_1$ noted by 24 h, and a more pronounced G$_1$ block by 36 h (Fig. 1A). This inhibition of cell growth is paralleled by a dose-dependent inhibition by mevastatin on the activity of cdk2 (not shown). Immunoblot analysis of cell lysates after 36 h of mevastatin treatment shows that, although the levels of the cell cycle-regulated proteins cdk2, p27, and p57 remained unchanged, the amount of p21 was increased (Fig. 1B). Immunoprecipitation of cell lysates with antibodies to cdk2 confirm that, although the amount of cdk2 remained relatively unchanged, there was a small but perceptible increase in p21 associated with cdk2 (Fig. 1C). This rather modest increase in p21 was accompanied by a dramatic inhibition of kinase activity of cdk2 following mevastatin treatment. These experiments confirm previous observations that the increase in cellular p21 and the subsequent inhibition of cdk2 parallel growth inhibition by statins.

p21 Is Induced in PC3 Cells following TGF-β1 Treatment but without Concurrent Inhibition of cdk2 in Vitro—Although mevastatin treatment clearly led to induction of p21, the modest (2–fold) increase in the amount of p21 associated with the inhibited cdk2 made us question whether this association was the only mechanism of kinase inhibition. In support of the existence of other mechanisms, treatment of PC3 cells with TGF-β1 induces p21 (Fig. 2A) and increased association of p21 with cdk2 (Fig. 2B), but no inhibition of kinase activity (Fig. 2B) or induction of a G$_1$-S block (data not shown). The increase in p21 association with cdk2 is more profound in TGF-β1-treated than with mevastatin-treated cells (compare with Fig. 1C). This observation suggests that a simple association of p21 with cdk2 does not explain the entire antiproliferative effect of mevastatin. Because it is possible that a factor in TGF-β1-treated cells abrogates the inhibition of cdk2 by p21, we pre-treated cells with TGF-β1 for 12 h prior to the addition of mevastatin. The addition of mevastatin to TGF-β1-treated cells inhibited cdk2 activity (Fig. 2C) and stopped cell proliferation (data not shown). Therefore, the kinase activity of p21 associated cdk2 in TGF-β1-treated cells was not caused by the induction of a novel p21 resistance factor by this cytokine.

Fig. 1. Cell cycle block by mevastatin. A, mevastatin leads to a G$_1$ block in PC3 cells. Percentage of cells in G$_1$ (black bars), S (hatched bars), and G$_2$/M (white bars) at indicated times following addition of Me$_2$SO (Control) or mevastatin (MEV). Experiments are the average of three separate experiments. Arrow bars represent mean ± S.D. B, immuno blot of lysates of PC3 cells in the absence (–) and presence (+) of mevastatin. 36 h after addition of mevastatin, cells were lysed and 30 μg of total protein was loaded per lane. Although levels of cdk2, p27, and p57 remained relatively unchanged, a significant increase in p21 level was noted. C, Cdk2 complex from PC3 cells. 200 μg of total cellular protein from PC3 cells was immunoprecipitated with an antibody to cdk2 and immunoblotted with antibodies to cdk2 and p21. cdk2 levels remained unchanged (top), whereas a small increment in associated p21 was noted (middle). The bottom panel shows the dramatic reduction in the activity of cdk2 from mevastatin-treated cells.
p21-depleted cdk2 from Mevastatin-Treated Cells Is Inhibited—Because the p21-containing complexes of cyclin-cdk2 from TGF-β1-treated cells is active, we wondered if p21 solely accounted for the inhibition of cdk2 in mevastatin-treated cells. We prepared lysates from mevastatin-treated and untreated cells and subjected equal amounts of the lysate to sequential immunoprecipitations with anti-p21 antibody until the supernatant was depleted of p21. Fig. 3 A shows no detectable p21 in the supernatant of the fifth immunoprecipitate of mevastatin-treated and untreated lysates (lanes 4 and 6). The p21-depleted supernatants were then immunoprecipitated with antibody to cdk2. The cdk2 immunoprecipitated from the depleted supernatants was devoid of p21 (Fig. 3A, lanes 7 and 8). In Fig. 3B, anti-cdk2 immunoblot of total lysates (top, lanes 1 and 2) show that cdk2 content is similar. Cdk2 immunoblots of p21 immunoprecipitates (lanes 3–6) show that only a small amount of cdk2 is associated with p21 (upper band best seen in lane 5). Parallel cdk2 immunodepletion experiments show that most of the total p21 is non-cdk2 bound (not shown). Thus, it is not surprising that cdk2 immunoprecipitates of p21-depleted lysates (top, lanes 7 and 8) show that similar amounts of the protein are present in mevastatin-treated and untreated cells. However, kinase assays against histone H1 show that the cdk2 from mevastatin-treated cells is still inhibited (bottom). This suggests that enhancement of p21 levels and its increased association with cdk2 may not be the only mechanism of cdk2 inhibition by mevastatin.

Mevastatin Blocks Cell Proliferation in the Absence of p21—The above observations led us to question whether mevastatin can block p21-deficient PC3 cells. We used RNA interference (RNAi) to deplete PC3 cells of p21. Fig. 4A shows that p21 siRNA-transfected cells treated with mevastatin have lower levels of p21 when compared with untreated cells (lanes 1 and 4). The decrease of p21 was not accompanied by a compensatory increase of p27 or p57 levels. In addition a control oligonucleotide duplex did not prevent the mevastatin-induced elevation of p21 (Fig. 4A, lanes 5 and 6) confirming the specificity of the synthetic oligonucleotide for p21. Fig. 4B shows an example of a quantitative Western blot of mevastatin-treated cells with or without transfection of p21 siRNA. Although p21 is detectable in 5 μg of lysate from untransfected cells, it is barely detectable in 50 μg of oligonucleotide-transfected cell lysate. An average of multiple experiments suggests that RNAi decreases p21 level by 10- to 16-fold in mevastatin-treated cells. Immunoprecipitations with antibodies to cdk2 show no associated p21 in oligonucleotide-transfected cells (Fig. 4C), confirming that the cdk2 complex is devoid of p21. Despite the lack of p21, the Rb protein is hypophosphorylated (Fig. 4D, top two panels, lanes 3 and 4) and cdk2 kinase activity blocked (bottom two panels, lanes 3 and 4) upon mevastatin treatment. The kinase inhibition and hypophosphorylation of Rb is equivalent to that in mevastatin-treated but untransfected (lane 2) or control oligonucleotide-transfected cells (lane 6). FACS analysis of p21 siRNA-transfected cells shows a decrease in the S phase population upon mevastatin treatment that is similar to that of untransfected and control RNAi-transfected cells (Fig. 5E). These results show that inhibition of cdk2 is not dependent on the ability of mevastatin to elevate p21 protein levels in PC3 cells.
The Cyclin E/cdk2 Complex Is Intact but Inhibited in Mevastatin-treated PC3 Cells

We next evaluated changes in cyclins A and E, the regulatory cyclins of cdk2, upon mevastatin treatment. Following mevastatin treatment, the level of cyclin E protein remained relatively constant, but the amount of cyclin A declined within 48 h (Fig. 5A) in parallel with a decline in cyclin A mRNA (data not shown). Immunoprecipitation with antibodies to cyclin A and E and subsequent histone H1 kinase assays show that cyclin A/cdk2 was inhibited (Fig. 5B, top panel). Interestingly, cyclin E/cdk2 was also inhibited (middle panel), despite the presence of an intact cyclin E/cdk2 complex. The discrepancy in the kinase activity (bottom) of cyclin E/cdk2 from TGF-β1 and mevastatin-treated cells was not caused by differences in the cdk2:p21 ratio.

The Cyclin E/cdk2 Complex Is Intact but Inhibited in Mevastatin-treated PC3 Cells—We next evaluated changes in cyclins A and E, the regulatory cyclins of cdk2, upon mevastatin treatment. Following mevastatin treatment, the level of cyclin E protein remained relatively constant, but the amount of cyclin A declined within 48 h (Fig. 5A) in parallel with a decline in cyclin A mRNA (data not shown). Immunoprecipitation with antibodies to cyclin A and E and subsequent histone H1 kinase assays show that cyclin A/cdk2 was inhibited (Fig. 5B, top panel). Interestingly, cyclin E/cdk2 was also inhibited (middle panel), despite the presence of an intact cyclin E/cdk2 complex.
Mevastatin and cdk2 Activation

Fig. 6. Reduced phosphorylation of cdk2 upon mevastatin treatment of PC3 cells. A, Western blot of cdk2 immunoprecipitates with antityrosine phosphate antibody (lower panel) shows no change in phosphotyrosine content. The upper panel is a cdk2 immunoblot as a loading control. B, immunoprecipitation of lysates from [32P]orthophosphate- or [35S]methionine-labeled PC3 cells with anti-cdk2 antibody. Proteins were transferred to nitrocellulose and processed for autoradiography (top) and immunoblotted with anti-cdk2 antibody (bottom). Phosphate incorporation into cdk2 was decreased with mevastatin treatment. The immunoblot confirms equal loading of the three lanes. C, immunoprecipitation of cdk2 from [32P]orthophosphate or [35S]methionine-labeled cells. The band shown is the 32-kDa cdk2 protein. Note the decreased incorporation of radiolabeled phosphate into cdk2 derived from mevastatin. D, immunoblot of cell lysates and cyclin E immunoprecipitates from untreated, TGF-β1, and mevastatin-treated PC3 cells. Samples were analyzed on a SDS-polyacrylamide gel under conditions that resolve the two forms of cdk2. Samples were electrophoresed an additional 30 min after the dye front had exited the gel (for better resolution of the two forms of cdk2). Western blot was performed with anti-cdk2 antibody. The relative amount of the slower migrating band (asterisk) corresponds to cdk2 not phosphorylated on Thr-160. E, H1 kinase assay of cyclin Ecdk2 from untreated (UNT), TGF-β1, and mevastatin-treated PC3 cells were treated with either GST or GST-Cak1 in the presence of 1 mM cold ATP and [γ-32P]ATP. Lanes 1 and 2 contained no lysate from PC3 cells and confirm that Cak1 has no intrinsic kinase activity against histone H1. Lanes 3 and 4 were analyzed on a SDS-PAGE into a fast migrating band containing the activating phosphate on Thr-14 and Tyr-15 phosphorylation (Fig. 6A). Because this finding could be explained on the basis of a decreased pool of cyclin A associated with cyclin Ecdk2 from PC3 cells were incubated as in vitro activation by GST-Cak1. Immunoprecipitates of cyclin Ecdk2 from untreated (UNT), TGF-β1, and mevastatin-treated PC3 cells were treated with either GST or GST-Cak1 in the presence of 1 mM cold ATP for 30 min at 30°C. The pellet was washed, and histone H1 and [γ-32P]ATP were then added. cdk2 kinase activity inhibited with mevastatin treatment (lane 5) was restored by GST-Cak1 (lane 6). Lanes 1 and 2 contained no lysate from PC3 cells and confirm that Cak1 has no intrinsic kinase activity against histone H1. F, H1 kinase assay of cyclin Ecdk2 following in vitro activation by recombinant cyclin Hcdk7. Immunoprecipitates of cyclin Ecdk2 from PC3 cells were incubated as in D with or without recombinant cyclin Hcdk7. The pellet was washed, and histone H1 and [γ-32P]ATP were then added. Cyclin Hcdk7 restores the cdk2 kinase activity (compare lanes 5 and 6). Lanes 1 and 2 show mock immunoprecipitations devoid of lysate and confirm that the enhanced kinase activity against histone H1 is not secondary to the presence of cdk2.

Because the cyclin D1-cdk4 kinase activity was unchanged (data not shown), the mevastatin-induced cell cycle block appears to be a result of the inhibition of cyclin E-cdk2. Previous studies have shown that cyclin A transcription is dependent on E2F (18), so that inhibition of cyclin E-cdk2 and underphosphorylation of Rb could explain the decrease in cyclin A mRNA, protein levels, and lack of cyclin A-associated kinase activity.

Immunoprecipitation of mevastatin and TGF-β1-treated cell lysates with cyclin E antibody and immunoblots of the precipitates with anti-cdk2 and p21 antibodies were performed to examine the amount of p21 associated with cyclin E-cdk2 in the two lysates (Fig. 5C). In support of the hypothesis that p21 is not responsible for the inhibition of cdk2, the ratio of cdk2 to p21 in each complex was not different despite the dramatic difference in kinase activity (Fig. 5C, bottom panel).

Decrease in Activating Phosphorylation May Account for Inhibition of cdk2 in PC3 Cells—The inhibition of cyclin E-cdk2 kinase activity was seen even when p21 induction was prevented by RNAi (Fig. 4D, bottom panel). Because cyclin E-cdk2 was inhibited despite an intact complex and dependent on p21, we examined whether there were changes in the regulatory phosphorylation of cdk2. Immunoprecipitates of cdk2 probed with antiphosphotyrosine antibody showed no increase in the content of phosphotyrosine, a marker of inhibition through Thr-14 and Tyr-15 phosphorylation (Fig. 6A). However upon metabolic labeling of cells with [32P]orthophosphate, phosphorylation of cdk2 was suppressed following mevastatin treatment but not in untreated or TGF-β1-treated cells (Fig. 6B). Because this finding could be explained on the basis of a decreased pool of cyclin A-cdk2 complexes, we repeated these experiments by immunoprecipitation with an antibody to cyclin E. Fig. 6C shows that there is a decrease in the phosphorylation of cyclin E-associated cdk2 from mevastatin-treated cells. It has been previously reported that cdk2 can be resolved on SDS-PAGE into a fast migrating band containing the activating phosphate on Thr-160 and a slower migrating band devoid of this phosphate (19). Western blot of cell lysates with anti-cdk2 antibody showed a decrease in the faster migrating phosphorylated band in mevastatin-treated PC3 cells (Fig. 6C, upper panel). To selectively examine the cyclin E-cdk2 population, the mobility of cdk2 associated with cyclin E was examined (Fig. 6D, lower panel). Consistent with our previous results, the de-phosphorylated form of cdk2 (slower migrating, labeled with an asterisk) was increased upon mevastatin treatment. Because
cause of the similar content of p21 in the cyclin E-cdc2 from TGF-β1 and mevastatin-treated cells (Fig. 6C), the difference in phosphorylation could not be ascribed to an unequal amount of p21 in the kinase complex.

The activating phosphorylation of cdkks is under the control of the cdk-activating kinase, Cak. In yeast, a monomeric protein Cak1p (15) is required for activation of cdkks. In mammalian cells a trimeric enzyme consisting of Cdk7, cyclin H, and MAT1 is felt to be the putative Cak (20, 21). We evaluated the ability of recombinant yeast Cak to activate the inhibited cyclin E-cdc2 from mevastatin-treated cells (Fig. 6E). As previously reported (15), GST-Cak1 by itself has no intrinsic histone H1 kinase activity (lane 2). Preincubation of cyclin E-cdc2 from mevastatin-treated cells with GST-Cak1, however, induced the histone H1 kinase activity of the former (compare lanes 5 and 6). Fig. 6F shows a similar experiment performed with recombinant cyclin H-cdc2, the putative mammalian Cak. In accordance with the observation with yeast Cak, cyclin H-cdc2 activates the inhibited cdk2 from mevastatin-treated cells (compare lanes 5 and 6). Cdk7 does not phosphorylate histone H1 (lane 2). These observations support the notion that the inhibition of cyclin Ecdc2 upon mevastatin treatment of PC3 cells is due to a decrease in activating phosphorylation on Thr-160.

The decrease in activating phosphorylation on Thr-160 could be a result of 1) decrease in level or activity of the trimeric cyclin H-cdc2-mat1 complex (Cak), 2) decrease in level or activity of a novel Cak, or 3) increase of a phosphatase targeted to phospho-Thr-160. Immunoblots to evaluate the levels of cdk7 and cyclin H showed no changes in their respective cellular levels following mevastatin treatment (data not shown). Immunoprecipitation of the complex with cdk7 antibodies followed by analysis of its ability to phosphorylate bacterially produced GST-cdk2 showed no decrease in the Cak activity following mevastatin treatment (Fig. 7A). We have been unable to see any change in cdk2 phosphatase activity in mevastatin-treated lysates. For example, in Fig. 7B, cyclin-cdk2 was immunoprecipitated from untreated cells and incubated with increasing amounts of mevastatin-treated lysate supplemented with 10 mM MgCl₂. After incubation, the pellets were washed and the ability of the kinase to phosphorylate histone H1 was determined. If cdk2 was dephosphorylated on Thr-160, there would be a decrease in kinase activity. No decrease in the activity of cdk2 was seen. In Fig. 7C, GST-cdk2 was phosphorylated in vitro with yeast Cak1p. The phosphorylated product was then used as the substrate for phosphatase assays. Cell lysate was incubated with phosphorylated GST-cdk2. Lane 1 shows the control with no added lysate, lanes 2 and 3 show reactions containing untreated lysates, and lanes 4 and 5 are reactions containing lysates from mevastatin-treated cells. Lanes 3 and 5 represent cell lysates with or without phosphatase inhibitors (Phase −). There is no perceptible difference in the phosphatase activity of lysates from mevastatin-treated and untreated cells. We have performed similar reactions, with supplemental calcium, with and without ATP and have been unable to see enhanced cdk2 phosphatase activity in mevastatin-treated cells. We are therefore left with the possibility that a novel Cak is responsible for the phosphorylation of cyclin E-cdk2 in vivo, and this is suppressed upon mevastatin treatment. Alternatively, the cyclin E-cdk2 is sequestered from cyclin H-cdc2 Cak in mevastatin-treated cells.

**DISCUSSION**

Like its analog, lovastatin, mevastatin inhibits cell growth. In PC3 cells, this antiproliferative effect parallels an 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 p21 and p27 levels are enhanced in lovastatin-treated cells via the inhibition of the proteasomes (12). Although there is no doubt that p21 levels are elevated in mevastatin-treated cells, our studies suggest that p21 may not always be the active inhibitor of the cdk2 kinase. In fact, a model where p21 interacts with the cyclin-cdc2 to inhibit its activity cannot explain the observations we report here, for the following reasons. 1) The p21 associated with cdk2 appears out of proportion with the dramatic reduction of kinase activity noted with mevastatin treatment. TGF-β1 enhances the levels of p21 and increases its association with cdk2 but does not inhibit the kinase activity. Thus a mere 2-fold increase in the association of p21 with the kinase complex upon treatment with mevastatin is unlikely to be enough to inhibit the cdk2. 2) Elimination of p21 from PC3 cells by RNA interference does not prevent the mevastatin-induced cell cycle block and cdk2 inhibition, suggesting that p21 is dispensable for these effects of mevastatin. 3) Lastly, in experiments to be reported elsewhere we find that, consistent with the above observations, overexpression of p21 in PC3 cells does not result in cell cycle arrest, despite elevated levels and association of p21 with cyclin E-cdk2. This implies that the p21 protein in PC3 cells is inactive in the inhibition of the cdk2. The inactivity of p21 in these cells is thus the primary reason for unmasking a second pathway by which mevastatin inhibits cdk2.

In PC3 cells a decrease in activating phosphorylation of cdk2 is the critical factor that suppresses cyclin E-cdk2 kinase. This might be secondary to decreased levels or activity of the cdk2-
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activating kinase (Cak). There is some debate regarding the identity of the physiologic Cak in mammalian cells. Although the trimeric complex of cyclin H, cdk7, and mat1 (20, 21) was long felt to assume this role, a number of lines of experimental evidence suggest that there might well be a second Cak in mammalian cells. First, in yeast the physiologic cak1p is a monomer that has no significant homology to any of the members of the trimeric mammalian Cak. Second, the trimeric Cak complex is a constituent of the basal transcriptional unit TFII-H, whereas the yeast cak1p plays no role in transcription (22). Lastly, recent experimental evidence suggests that a second Cak activity can be identified in chromatographic fractions of HeLa cell lysates (23) and that an immunologically similar protein can be identified in HepG2 cells with antibodies to the yeast Cak1p (24).

We have not yet detected enhanced cdk2 phosphatase activity in cell extracts following mevastatin treatment. Furthermore, the constitutively active phosphatase (protein phosphatase type 2C) that has been identified in mammalian cells targets Thr-160 of monomeric cdk2 for dephosphorylation. Because the decreased phosphorylation of cdk2 from mevastatin-treated cells is seen on cyclin E-complexed cdk2, it is unlikely that enhanced protein phosphatase type 2C activity is responsible. As a result, we favor the possibility that a novel mammalian Cak is suppressed or that cdk2 is sequestered from cyclin H/cdk7/mat1 Cak in mevastatin-treated PC3 cells.

The failure to phosphorylate Rb upon mevastatin treatment leads to decreased cyclin A transcription, decreased cyclin A complexed with cdk2, and loss of cyclin A/cdk2 kinase activity, further contributing to the cell cycle block. Therefore, the decrease in cyclin A kinase activity occurs through two different mechanisms: 1) a primary decrease in Thr-160 phosphorylation of cyclin E/cdk2 and 2) a secondary decrease of cyclin A mRNA and protein.

In summary, we provide evidence for a novel pathway by which mevastatin inhibits cyclin-dependent kinases and cell cycle progression in PC3 cells. Treatment of hepatoma-derived HepG2 cells with TGF-β was reported to decrease the activating phosphorylation on cyclin E/cdk2 without any increase in p21 or other cdk inhibitors (24). Our results provide a second example where the activating phosphorylation on cdk2 is regulated independently of changes in associated cyclin or cdk inhibitors. Because p21 is a potent inhibitor of cdk2 in vitro, there are many examples where the induction of p21 occurring concurrently with inhibition of cdk2 has led to the assumption that the p21 inhibits the kinase by simple and direct association in the affected cells. Our studies suggest that, in some of these cells, p21 may be dispensable for the inhibition of the kinase and that additional inhibitory pathways may be in use.

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