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Inhibition of the 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Pathway Induces p53-independent Transcriptional Regulation of p21$^{WAF1/CIP1}$ in Human Prostate Carcinoma Cells*

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Progression through the cell cycle is controlled by the induction of cyclins and the activation of cognate cyclin-dependent kinases. The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor lovastatin induces growth arrest and cell death in certain cancer cell types. We have pursued the mechanism of growth arrest in PC-3-M cells, a p53-null human prostate carcinoma cell line. Lovastatin treatment increased protein and mRNA levels of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$, increased binding of p21 with Cdk2, markedly inhibited cyclin E- and Cdk2-associated phosphorylation of histone H1 or GST-retinoblastoma protein, enhanced binding of the retinoblastoma protein to the transcription factor E2F-1 *in vivo*, and induced the activation of a p21 promoter reporter construct. By using p21 promoter deletion constructs, the lovastatin-responsive element was mapped to a region between −93 and −64 relative to the transcription start site. Promoter mutation analysis indicated that the lovastatin-responsive site coincided with the previously identified transforming growth factor-β-responsive element. These data indicate that in human prostate carcinoma cells an inhibitor of the HMG-CoA reductase pathway can circumvent the loss of wild-type p53 function and induce critical downstream regulatory events leading to transcriptional activation of p21.

Cell cycle progression is controlled by the orderly activation of cyclin-dependent kinases (Cdks)[1] (1–4). Cdk activation is regulated at multiple levels including the induction and degradation of cyclin protein, Cdk phosphorylation by CAK, the cyclin-activating kinase, and the synthesis of Cdk-inhibitory proteins (5). Cdk inhibitors can negatively regulate cell cycle progression in response to a variety of antiproliferative signals including DNA damage, differentiation, contact inhibition, and senescence (6–8). p21$^{WAF1/CIP1}$, the first Cdk inhibitor to be identified, is required for p53-induced growth arrest (9). The p21 protein negatively regulates cell cycle progression by inhibiting Cdk catalytic activity and by inhibiting proliferating cell nuclear antigen, which is required for DNA synthesis (10). By inhibiting the activity of G_1 cyclin-Cdk complexes, p21 blocks phosphorylation of the retinoblastoma protein (pRB). This leaves pRB in its active, hypophosphorylated form, in which it is able to induce G_1 arrest through negative regulation of the heterodimeric transcription factor E2F/DP (11–13).

We have found that human prostate carcinoma cells are very sensitive to the induction of growth arrest and cell death by the HMG-CoA reductase inhibitor lovastatin (14).[2] Lovastatin blocks the rate-limiting step in the cholesterol-synthesis pathway, the formation of mevalonic acid from HMG-CoA (15–17). The study presented here investigates the mechanism by which lovastatin induces cell cycle arrest in the androgen-independent prostate carcinoma line PC-3-M, which is lacking both p53 alleles (18). The data show that lovastatin treatment caused a marked shift of pRB to its underphosphorylated form and enhanced the *in vivo* binding of pRB and E2F-1. In addition, lovastatin inhibited Cdk activity, and this inhibition was associated with elevated protein and RNA expression of the Cdk inhibitor p21, similar to the induction of p21 by lovastatin in human breast cells (19). We investigated the mechanism of lovastatin-induced p21 expression using p21 promoter-reporter assays. Lovastatin treatment induced transcriptional activation of the p21 promoter as indicated by increased activity of a p21 promoter-luciferase reporter construct, and this transcriptional response did not require the p53 response element. All effects of lovastatin were completely reversed by addition of mevalonic acid. These data demonstrate a link between the mevalonate pathway and activation of the promoter of the critical cell cycle-regulatory protein p21. To begin to understand the mechanism of lovastatin-induced transcription, promoter deletion and mutation studies were performed. This analysis demonstrated that lovastatin responsiveness is mediated by a promoter-proximal region that coincides with the recently identified transforming growth factor-β (TGF-β)-responsive element.

MATERIALS AND METHODS

Cells—The human prostate carcinoma cell line PC-3-M was a gift of Dr. James Kozlowski, Department of Urology, Northwestern University Medical School (20). Cells were cultured as described previously (21).

Antibodies—Polyclonal antibodies to cyclin E, Cdk2, Cdk4, and monoclonal anti-E2F-1 (clone 20) were obtained from Santa Cruz Biotechnology. Monoclonal anti-pRB antibody (clone G3–245) and monoclonal anti-p21 antibody were purchased from PharMingen.

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[1] The abbreviations used are: Cdk, cyclin-dependent kinase; pRB, retinoblastoma protein; GST, glutathione *S*-transferase; TGF-β, transforming growth factor-β; TβRE, transforming growth factor-β-responsive element; kb, kilobase pair; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

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TABLE I
Effect of lovastatin on cell cycle distribution in PC-3-M cells

Exponentially growing PC-3-M cells were treated at 0 time with lovastatin (10 μM), and flow cytometric cell cycle analysis was performed comparing untreated controls with cells treated with lovastatin for 24 and 48 h.

Time	G_1	S	G_2/M
h			
0	20	49	31
24	76	7	17
48	91	4	5

Cell Cycle Analysis—Cells were fixed in 50% ethanol and incubated with RNase A and the DNA intercalating dye propidium iodide, and cell cycle phase analysis was performed by flow cytometry using a Becton Dickinson FACStar flow cytometer and Becton Dickinson Cell Fit software.

Immunoprecipitation and Western Blot Analysis—Immunoprecipitations were performed as described (22). Cell lysates were incubated with anti-Cdk2 polyclonal antibody, anti-E2F-1, or normal rabbit serum. The immunocomplex was collected on protein A-Sepharose (Sigma) and analyzed by SDS-polyacrylamide gel electrophoresis using enhanced chemiluminescence detection (Amersham Pharmacia Biotech). Western blot analysis was performed as described (23).

Northern Blot Analysis—Total RNA was isolated from cells lysed in guanidinium thiocyanate solution, and Northern blot analysis was carried out as described previously (24) using ^{32}P-labeled (ICN) cDNA probes and 30 μg of total RNA per lane. The human p21 DNA probe was a gift of Dr. David Givol.

Immune Complex Kinase Assay—Immune complex kinase assays were performed as described (22). Briefly, cell lysates were incubated with primary antibody, and immune complexes were collected on protein A-Sepharose beads and resuspended in kinase assay mixture containing [γ-^{32}P]ATP (ICN) and GST-RB or histone H1 (Life Technologies, Inc.) as substrate. The GST-RB fusion protein containing amino acids 379–928 of human pRB (25) was a gift of Dr. Frederic Kaye, NCI, National Institutes of Health. The proteins were separated on 10% SDS-polyacrylamide gels, and bands were detected by autoradiography.

p21 Promoter-Luciferase Constructs—Drs. Bert Vogelstein and Todd Waldman, The Johns Hopkins Oncology Center, provided the full-length 2.4-kb human p21 promoter-chloramphenicol acetyltransferase constructs as well as p21 promoter deletion constructs, in which 300 base pairs were progressively removed from the 3′ terminus. Three constructs were tested as follows: a 2.4-kb p21 promoter fragment containing the p21 transcription initiation start site at its 3′ end and a p53 response element at the 5′ end, a 2.1-kb fragment consisting of the 2.4-kb fragment from which 300 base pairs, including the p53 response element, had been deleted from the 5′ end, and a 0.3-kb fragment consisting of the 300 base pairs most proximal to the transcription start site (9). The original chloramphenicol acetyltransferase constructs were digested with *Hin*dIII and *Xho*I and subcloned into the *Hin*dIII-*Xho*I sites of the luciferase reporter vector pGL2-basic (Promega Corp.), to create p21 promoter-luciferase constructs. The deletion constructs p21P 93-S (0 to −93), p21P Sma Δ1 (0 to −63) p21P Sma Δ2 (full-length p21 promoter from which the region −64 to −110 was deleted) and p21P (0 to −100), and the p21P 93-S mutants were created as described previously (26).

Luciferase Assay—PC-3-M cells were co-transfected with the p21 promoter-luciferase reporter constructs and with the β-galactosidase reporter vector pCMVβ (Promega) using LipofectAMINE (Life Technologies, Inc.), as recommended by the manufacturer. Following transfection the cells were incubated for 6 h; the medium was exchanged, and the cells were incubated for an additional 36 h in the presence or absence of 10 μM lovastatin. The cells were then lysed, and luciferase activity in the lysates was assayed using a Dynatech ML1000 luminometer (Dynatech Laboratories). Luciferase activity was normalized to β-galactosidase activity which was assayed using the β-galactosidase Enzyme Assay System (Promega).

RESULTS

Lovastatin Induces G_1 Arrest and Cell Death—24 h after 10 μM lovastatin addition there was an increase in the percent of PC-3-M cells in G_1. By 48 h following lovastatin addition greater than 90% of the PC-3-M cells were arrested in G_1 (Table I). Cell viability was consistently greater than 75% in cells treated for 48 h, after which there was a marked loss of viability, culminating in cell death (data not shown).

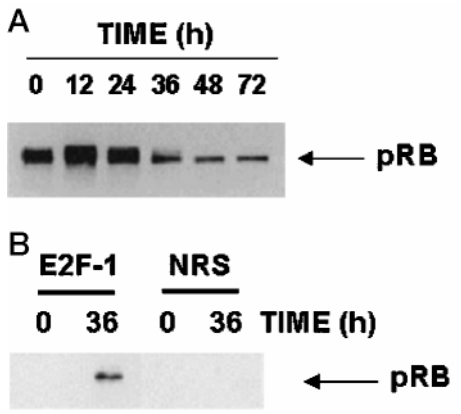

FIG. 1. **Hypophosphorylation of pRB and enhanced association of pRB and E2F-1 in PC-3-M cells after exposure to lovastatin.** *A*, pRB was detected by Western blot analysis in cell lysates using 30 μg of protein from untreated cells (0 h) and in cells 12, 24, 36, 48, and 72 h after exposure to 10 μM lovastatin. *B*, total cell lysates (2 mg of protein) from control cells and cells incubated with 10 μM lovastatin for 36 h were immunoprecipitated with anti-E2F-1 antibody or normal rabbit serum (*NRS*). Immune complexes were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with anti-pRB antibody. Proteins were detected by enhanced chemiluminescence.

Loss of Phosphorylated pRB in Cells Exposed to Lovastatin—In cells treated with lovastatin pRB appeared as a single band on Western blot analysis, migrating in the position characteristic of unphosphorylated or hypophosphorylated pRB (27) (Fig. 1*A*). The position of pRB after lovastatin coincided with the position of pRB detected with an antibody specific for the un- or hypophosphorylated form (Pharmingen clone G99–549, data not shown).

Lovastatin Enhances Association of pRB and E2F-1—Association of pRB with the transcription factor E2F-1 was almost undetectable by co-immunoprecipitation analysis of the untreated log phase cells (Fig. 1*B*). However, 36 h after addition of lovastatin there was a strong increase in the association of pRB and E2F-1.

Levels of Cdk2, Cdk4, and Cyclin E Protein Are Unchanged following Lovastatin Treatment—The G_1/S transition is regulated by D-type cyclins which bind to and activate Cdk4 and Cdk6 and cyclin E, which activates Cdk2. Because lovastatin arrests PC-3-M cells in G_1, we tested whether lovastatin decreased protein levels of Cdk4, Cdk2, or cyclin E, and as demonstrated in Fig. 2*A*, there was little detectectable change in the level of these proteins, even after 48 h of exposure to lovastatin. It should be noted, however, that the actual effects of lovastatin on these proteins may be larger than is apparent in the Western blot because of the nonlinearity of the chemiluminescence detection system.

Lovastatin Inhibits Cdk2 and Cyclin E-associated Kinase Activity—Cdk2- and cyclin E-associated kinase activity were reduced to undetectable levels after 36 h treatment with lovastatin (Fig. 2*B*). These results indicated that the inhibition of Cdk2- and cyclin E-associated kinase activity might involve binding of a Cdk inhibitor protein to the Cdk2-cyclin E complex.

Lovastatin Induces p21 Protein and Association of p21 with Cdk2—Western blot analysis showed that lovastatin induced a marked increase in the level of the Cdk inhibitor p21 (Fig. 3*A*). We next asked whether the p21 protein induced by lovastatin treatment was associated with Cdk2 *in vivo*. As shown in Fig. 3*B*, p21 was detected in anti-Cdk2 immunoprecipitates from lovastatin-treated cells and not in immunoprecipitates from

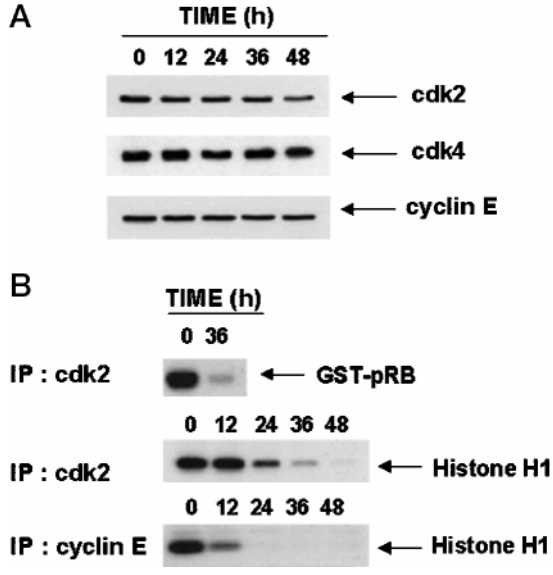

FIG. 2. **Effect of lovastatin on levels of Cdk2, Cdk4, and cyclin E protein and Cdk2-dependent and cyclin E-dependent kinase activity.** *A*, PC-3-M cells were treated with 10 μM lovastatin for the times indicated. Total cell lysates were prepared, and Western blot analysis was performed using anti-Cdk2, anti-Cdk4, or anti-cyclin E antibody and enhanced chemiluminescence detection. *B*, effect of lovastatin on Cdk2 kinase and cyclin E-associated kinase activity. PC-3-M cells were treated with lovastatin, and cell lysates were immunoprecipitated (*IP*) with anti-Cdk2 or cyclin E antibody, and kinase activity was assayed using GST-RB or histone H1 as substrate.

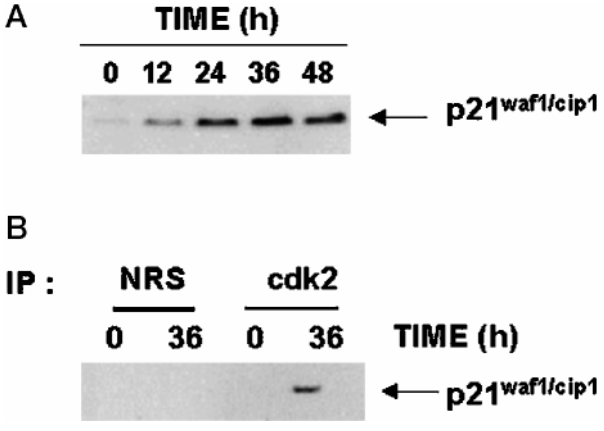

FIG. 3. **Lovastatin induces the cyclin-dependent kinase inhibitor p21 and association of p21 with Cdk2.** *A*, cells were incubated with 10 μM lovastatin for the time indicated, and total cell lysates were subjected to Western blot analysis using anti-p21 antibody and enhanced chemiluminescence detection. *B*, cells were treated with 10 μM lovastatin for 36 h, and cell lysates (2 mg protein) were immunoprecipitated (*IP*) with anti-Cdk2 antibody, separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with anti-p21 antibody followed by enhanced chemiluminescence detection.

untreated cells. Thus lovastatin induced the association of p21 with Cdk2, suggesting that lovastatin-induced p21 blocks Cdk2 kinase activity by direct binding to the cyclin-Cdk2 complex.

Lovastatin Induces p21 mRNA—To examine the mechanism by which lovastatin induces p21 we performed Northern blot analysis of the effect of lovastatin on the level of p21 mRNA. The 2.4-kb p21 transcript was increased in PC-3-M cells as early as 12 h after lovastatin treatment (Fig. 4). The kinetics of induction were consistent with the time course of inhibition of Cdk activity. The increase in p21 mRNA induced by lovastatin was blocked by actinomycin D (data not shown), suggesting that lovastatin may be regulating p21 mRNA levels through an effect on p21 transcription.

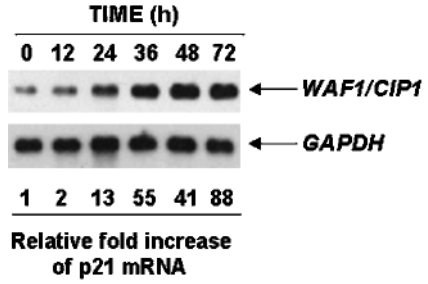

FIG. 4. **Lovastatin induces expression of p21 mRNA.** Cells were incubated with 10 μM lovastatin for the times indicated. Total RNA was prepared, and p21 mRNA was detected by Northern blot analysis. The same blot was probed with a cDNA to glyceraldehyde-3′-phosphate dehydrogenase (*GAPDH*) to demonstrate comparable levels of mRNA per lane. The fold increase in p21 mRNA level was determined by integrating the signal using NIH Image version 1.6 software.

p53 acts at the transcriptional level to increase p21 expression in response to DNA damage (9). In PC-3-M cells the p53 gene has been reported to be truncated and p53 protein absent (18). As a control we performed Western and Northern blot analysis for p53 and found no evidence of p53 expression in control or lovastatin-treated cells (data not shown). These data demonstrate that lovastatin is regulating the level of p21 mRNA through a p53-independent mechanism.

Transcriptional Activation of the p21 Promoter by Lovastatin—To test for transcriptional regulation of p21 by lovastatin p21 promoter-luciferase reporter constructs were transfected into PC-3-M cells, and luciferase activity was measured in the presence and absence of lovastatin. The full-length 2.4-kb promoter construct containing a p53 response element was activated 3–4-fold by lovastatin (Fig. 5*A*). Activation was not seen with a control vector consisting of the pGL2-basic luciferase reporter vector alone, and activation of the p21 promoter by lovastatin was completely blocked by incubation of the cells with 5 mM mevalonic acid, demonstrating that the ability of lovastatin to activate the p21 promoter is mediated by a component of the HMG-CoA reductase pathway (data not shown).

A 2.1-kb construct lacking the p53 response element was activated by lovastatin to the same extent as the full-length promoter (Fig. 5*A*). Constructs consisting of 300 base pairs and 100 base pairs proximal to the transcription start site were fully activated by lovastatin when compared with the 2.4-kb promoter. Thus, the lovastatin-responsive site is localized to the promoter proximal region between 0 and −100 base pairs. Three deletion constructs were employed to further map this region of the p21 promoter. A construct consisting of 0 to −93 base pairs was fully responsive to lovastatin (Fig. 5*B*). In contrast, a construct consisting of the region from 0 to −63 was non-responsive to lovastatin. A deletion construct consisting of the full-length promoter minus the region −64 to −110 was also non-responsive to lovastatin. These data localize the region responsive to lovastatin to the sequences between −64 and −93 base pairs.

The region between −64 and −93 contains two Sp1 binding sites. The second of these sites, between −78 and −84, has been identified as both the TGF-β-responsive element (TβRE) and the butyrate-responsive element (28). To test whether the lovastatin-responsive element coincides with this Sp1 binding site, we tested two constructs of the 0 to −93 region in which the Sp1 site was mutated. Mutant 1, containing 10 consecutive mutated base pairs, did not respond to lovastatin (Fig. 6). Mutation of bases −78 and −79 also resulted in loss of lovastatin responsiveness. These data localize the lovastatin-responsive region and suggest that Sp1 may play a critical role in the activation of p21 transcription in response to lovastatin.

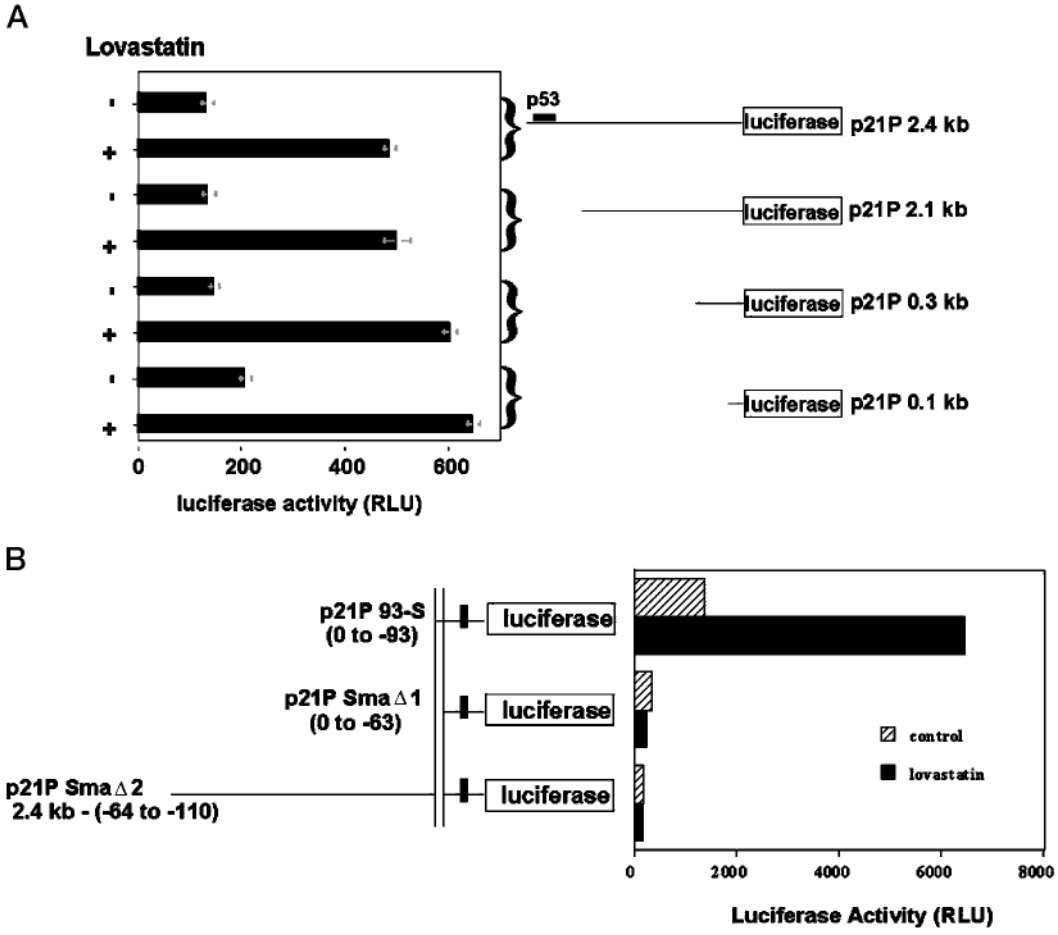

FIG. 5. **Deletion analysis of the activation of the p21 promoter by lovastatin.** PC-3-M cells were transiently transfected with p21 promoter luciferase constructs (10 μg) and incubated for 6 h; the medium was changed; the cells were incubated for an additional 36 h in the presence or absence of 10 μM lovastatin, and luciferase activity was measured. *A*, transfection of the full-length 2.4-kilobase promoter construct and three progressive deletion constructs. The results are expressed as the mean ± S.D. of data from three separate samples and are representative of five separate experiments. *B*, mapping of the lovastatin-responsive region using two transcription start site-proximal constructs and a full-length promoter construct with an interstitial deletion. *RLU*, relative luciferase units.

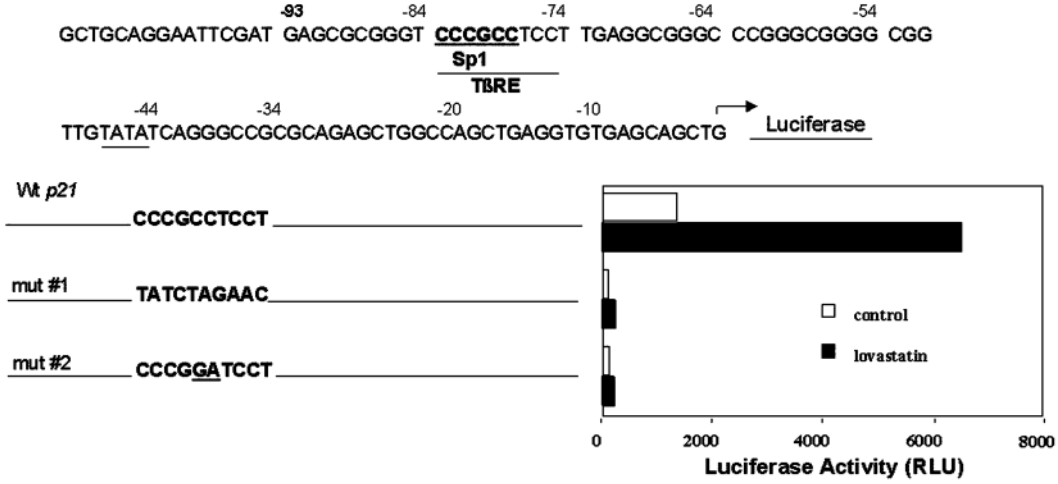

FIG. 6. **Mutation analysis of the activation of the p21 promoter by lovastatin.** PC-3-M cells were transfected with the wild-type p21 promoter-reporter deletion construct p21P 93-S or with mutant p21P 93-S constructs and lovastatin-induced luciferase activity was measured. *RLU*, relative luciferase units.

DISCUSSION

The hormone refractory prostate cancer cell line studied here represents the most advanced and least treatable form of adult carcinoma (29, 30). Progressive malignant tranformation in epithelial tumors has been associated with a variety of genetic changes (31). Recent data suggest that mutations in cell cycle genes are the most common genetic change in tumor cells (32). Although inactivation or deletion of the RB gene itself is limited to a subset of human tumors (33), it has been suggested that continued molecular characterization may reveal that virtually all tumors harbor a mutation in one of the genes responsible for regulating G_1 progression (32) and that the final

common target of the genetic changes in G_1 regulatory proteins is the functional inactivation of pRB (13).

Passage from G_1 to S phase is regulated by D-type cyclins and their cognate kinases Cdk4 and Cdk6, which act by phosphorylating and inactivating pRB prior to the restriction point at which cells commit to DNA synthesis (34), and by cyclin E-Cdk2, which acts later in G_1 (35). Genetic changes associated with G_1 cyclins and their Cdks in human cancers include overexpression of cyclin D1, overexpression or mutation of Cdk4, loss of expression of the cyclin D-directed Cdk inhibitor p16, mutation or deletion of p53 and RB, and overexpression of cyclin E (36–39). The data presented here demonstrate that in the human androgen-independent prostate carcinoma cell line PC-3-M, the mevalonate pathway inhibitor lovastatin induces G_1 arrest, blocks pRB phosphorylation, and increases *in vivo* binding of pRB to E2F-1, consistent with restoration of the growth-regulatory activity of pRB. Furthermore, lovastatin blocks cyclin E-Cdk2 kinase activity, in association with enhanced expression of the Cdk inhibitor p21.

Lovastatin has been shown previously to increase the protein level of the Cdk inhibitor $p27^{KIP1}$ in HeLa cells, HL60 cells, and normal fibroblasts (40, 41). There was no effect on $p27^{KIP1}$ in the PC-3-M cells (data not shown), and thus G_1 arrest appears to be independent of $p27^{KIP1}$ in the prostate cancer cells studied.

Inhibition of the mevalonate pathway has many ramifications including loss of the protein isoprenylation modifications farnesylation and geranylgeranylation, loss of sterol synthesis, and potential perturbation of *N*-linked glycosylation through inhibition of dolichol synthesis (42). To begin to map the target of lovastatin action within the mevalonate pathway, we incubated PC-3-M cells with the squalene synthase inhibitor zaragozic acid (43). We found that zaragozic acid treatment had no effect on p21 levels or cell cycle distribution (data not shown). Because lovastatin effects on the cell cycle were completely reversible by mevalonate and not mimicked by zaragozic acid, the critical point in the mevalonate pathway appears to be between HMG-CoA reductase and squalene synthase. One possible mechanism of lovastatin action is through Ras inactivation due to inhibition of Ras isoprenylation. Evidence suggesting that Ras may not be the mediator of the lovastatin response was presented by De Clue *et al.* (44) who showed that lovastatin arrests the growth of Ras-transformed cells even when the transforming Ras protein utilizes a myristate-dependent rather than farnesyl-dependent membrane-targeting moiety, and thus is not dependent on the mevalonate pathway. These data do not, however, eliminate Ras inactivation as the mechanism of lovastatin-induced G_1 arrest, and further experimental approaches, including studies with specific farnesyltransferase inhibitors, will be required.

Although lovastatin itself may not be the optimal HMG-CoA reductase inhibitor for cancer treatment, it has been shown to induce growth arrest and cell death in tumor cells *in vitro* and in athymic mice *in vivo* (44–47). A phase I study of lovastatin was completed recently at the National Cancer Institute, in which it was demonstrated that doses as high as 25 times the standard anti-hypercholesterolemic dose were well tolerated (48). More precise identification of the target of lovastatin action would aid in developing new anticancer drugs that act through perturbation of the HMG-CoA reductase pathway.

In addition to p53, recent studies have demonstrated activation of the p21 promoter by TGF-β (49, 50), vitamin D_3 (51), phorbol ester, okadaic acid (52–54), STAT1 (55), and butyrate (28). Activation by vitamin D_3 and STAT1 occurs more than 500 base pairs upstream of the lovastatin-responsive region (51, 55). In the U937 myeloid leukemia cell line, it has been demonstrated that activation of p21 transcription by phorbol esters and okadaic acid is mediated by the promoter region between −154 and +16 and that Sp1 plays a critical role in this response. In K562, SW480, and HepG2 cells the activation of p21 transcription by phorbol ester was mapped to the region between −95 and −121, and AP2 was shown to act as a positive regulator of the phorbol ester response. The TβRE is located between −71 and −86 relative to the p21 transcription start site (49, 50). We found that the lovastatin response region maps between −64 and −93. Furthermore, mutation of the TβRE abrogated lovastatin-induced p21 promoter activation. Thus the lovastatin response site corresponds with the TβRE. One possible mechanism for the induction of p21 by lovastatin in PC-3-M cells would be induction of TGF-β by lovastatin and activation of p21 transcription by TGF-β. It is likely, however, that lovastatin effects on p21 are unrelated to TGF-β because PC-3-M cells have an attenuated response to TGF-β, and direct addition of TGF-β1 to PC-3-M cells did not activate the p21 promoter reporter construct (data not shown). Recently it was reported that butyrate activates the p21 promoter and that this response maps to the TβRE (28). Sp1 is required for p21 promoter activation in response to both TGF-β and butyrate (28, 50). We have shown that mutation of an Sp1 binding site also blocks lovastatin activation of the p21 promoter. Several studies have shown that Sp1 is a critical factor in regulating transcriptional responses to pRB. Sp1 binds to the pRB-responsive element, and Sp1 binding is required and sufficient for promoter activation in response to pRB (56–58). Although the overall level of pRB protein decreases in PC-3-M cells in response to lovastatin, that decrease is predominantly or entirely in the hyperphosphorylated form (Fig. 1*A* and data not shown), and there is a substantial increase in active pRB as assessed by the markedly enhanced E2F-pRB binding in the *in vivo* coimmunoprecipitation experiment (Fig. 1*B*). These data suggest that both pRB and Sp1 may play a role in lovastatin-induced activation of the p21 promoter.

The data presented here demonstrate that lovastatin induces p21 transcription in the absence of wild-type p53. The most frequently mutated tumor suppressor in human cancer is p53. Therefore, p53-independent induction of p21 presents an attractive target for anticancer drug development (59). The data presented here suggest that perturbation of the mevalonate pathway may offer a new approach to transcriptional control of this critical negative growth-regulatory gene.

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