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Lovastatin mediated G1 arrest in normal and tumor breast cells is through inhibition of CDK2 activity and redistribution of p21 and p27, independent of p53

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Previously, we reported that lovastatin, a potent inhibitor of the enzyme HMG CoA reductase also acts as an antimitogenic agent by arresting cells in the G1 phase of the cell cycle resulting in cell cycle-independent alteration of cyclin dependent kinase inhibitors (CKIs). In the present study we have investigated the nature of the CKIs (p21 and p27) alterations resulting in G1 arrest in both normal and tumor breast cell lines by lovastatin. We show that even though lovastatin treatment causes G1 arrest in a wide variety of normal and tumor breast cells irrespective of their p53 or pRb status, the p21 and p27 protein levels are not increased in all cell lines treated suggesting that the increase in p21 and p27 protein expression per se is not necessary for lovastatin mediated G1 arrest. However, the binding of p21 and p27 to CDK2 increases significantly following treatment of cells with lovastatin leading to inhibition of CDK2 activity and a subsequent arrest of cells in G1. The increased CKI binding to CDK2 is achieved by the redistribution of both p21 and p27 from CDK4 to CDK2 complexes subsequent to decreases in CDK4 and cyclin D3 expression following lovastatin treatment. Lastly, we show that lovastatin treatment of 76N-E6 breast cell line with an altered p53 pathway also results in G1 arrest and similar redistribution of CKIs from CDK4 to CDK2 as observed in other breast cell lines examined. These observations suggest that lovastatin induced G1 arrest of breast cell lines is through a p53 independent pathway and is mediated by decreased CDK2 activity through redistribution of CKIs from CDK4 to CDK2.

Keywords: lovastatin; cell cycle; cyclins; CKIs; CDKs; adaptor molecule

Introduction

The mammalian cell cycle, defined as a sequence of events between two cell divisions, is positively regulated by cyclins and cyclin dependent kinases (CDKs) which associate to form hetrodimeric complexes (Sherr, 1994, 1996; Elledge *et al*., 1996; Nasmyth, 1996). Mitogenic stimuli results in the phosphorylation and thereby activation of cyclin-CDK complexes by CDK activating kinase, CAK (Fisher and Morgan, 1994; Makela *et al*., 1994; Harper and Elledge, 1998). The activated cyclin/CDK complexes in turn sequentially phosphorylate substrates such as the retinoblastoma protein (pRb) throughout the cell cycle (Ewen *et al*., 1993a; Matsushime *et al*., 1994; Sherr, 1996). Phosphorylation of pRb which is necessary for the progression through G1 is regulated primarily by cyclin D/CDK4/CDK6 complexes while the cyclin E/CDK2 complex regulates the passage of cells from late G1 to S phase and further contribute to pRb hyper-phosphorylation (Sherr, 1994, 1996; Bartek *et al*., 1997). The hypophosphorylated pRb serves as a tumor suppressor by interacting with and inhibiting cellular proteins such as E2F-DP heterodimeric transcription factors which activate many genes required for DNA replication pivotal for G1/S transition (Weinberg, 1995; Bartek *et al*., 1996, 1997; Ikeda *et al*., 1996). The complete hyperphosphorylation of pRb by cyclin E/CDK2 complexes and consequent activation of E2F-DP transcription complex are thought to play a major role in overcoming of the restriction point (Pardee, 1989; Planas-Silva and Weinberg, 1997b).

Progression through the cell cycle and the restriction point is also negatively regulated through the association with CDK inhibitors, CKIs (Elledge and Harper, 1994; Sherr and Roberts, 1995; Harper and Elledge, 1996; Harper, 1997). There are two families of structurally distinct CKIs, the CIP/KIP family which inhibit a broad range of CDKs by selectively binding and inhibiting the fully associated cyclin/CDK complexes and the INK family which bind specifically to CDK4 and/or CDK6 and inhibit complex formation with cyclin D (Elledge *et al*., 1996; Sherr, 1996; Harper, 1997). The CKI p21 (CIP1/WAF1), the first mammalian CKI to be identified was simultaneously characterized by several laboratories as the major p53 inducible gene (WAF1) (El-Deiry *et al*., 1993) as a CDK inhibitor protein (CIP1, p21 and p20CAP1) (Gu *et al*., 1993; Harper *et al*., 1993; Xiong *et al*., 1993) as a protein highly expressed in senescent fibroblasts (SDI) (Noda *et al*., 1994), and as a melanoma differentiation associated gene (mda 6) (Jiang and Fisher, 1993). p27 (KIP1), similar in amino acid sequence and inhibitory specificity to p21, was identified as a protein associated with inactive cyclin E/CDK2 complexes in TGF-β1 treated and contact inhibited cells (Polyak *et al*., 1994a,b), as a protein that interacts with cyclin D1/CDK4 complexes (Toyoshima and Hunter, 1994), and in cells arrested in G1 (Hengst *et al*., 1994). Although p21 is induced by p53 in response to DNA damage resulting in CDK inhibition and G1 growth arrest (Dulic *et al*., 1994), it can also be induced by p53

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independent mechanisms; by serum, PDGF and EGF in embryonic fibroblasts from p53 knockout mice (Michieli *et al*., 1994), by serum starvation in p53 mutant human breast carcinoma cells (Sheikh *et al*., 1994), by EGF in squamous carcinoma cells (Jakus and Yeudall, 1996), by TGF-β1 in p53 mutant cells (Elbendary *et al*., 1994; Datto *et al*., 1995; Reynisdottir *et al*., 1995) and by lovastatin in breast cancer cells (Gray-Bablin *et al*., 1997). p27 is similarly induced by lovastatin (Hengst *et al*., 1994; Hengst and Reed, 1996; Gray-Bablin *et al*., 1997), by TGF-β1 (Polyak *et al*., 1994a), following cell to cell contact inhibition, by rapamycin and by agents that induce cAMP mediated growth arrest (Kato *et al*., 1994; Nourse *et al*., 1994; Polyak *et al*., 1994b; Toyoshima and Hunter, 1994). Hence, p21 and p27 may function similarly to inhibit CDK activity and proliferation in response to different environmental stimuli. Furthermore, removal by degradation/inactivation of p27 may be necessary for proliferation (Pagano *et al*., 1995).

We and others have previously reported that treatment of mammary epithelial cells or HeLa cells by lovastatin results in the induction of p21 and p27 in mammary cells (Gray-Bablin *et al*., 1997) and p27 in HeLa cells (Hengst *et al*., 1994; Hengst and Reed, 1996). Lovastatin is an inhibitor of HMG COA reductase which is the rate limiting enzyme of the cholesterol biosynthesis pathway (Alberts *et al*., 1980). Though lovastatin has been primarily prescribed for patients with high cholesterol levels (Rettersol *et al*., 1996) it has also been used as an effective agent in cell synchronization for both tumor and normal cells (Keyomarsi *et al*., 1991; Keyomarsi, 1996). The inhibition of the cholesterol biosynthesis pathway by lovastatin not only blocks mevalonate synthesis (the product of HMG COA reductase) but also prevents the farnesylation and geranylgeranylation (intermediate products of the cholesterol pathway) of several signal transduction proteins such as Ras, Rap and many G proteins, thereby preventing their proper intracellular localization and function (Goldstein and Brown, 1990; Maltese, 1990). The induction of p21 and p27 by lovastatin in breast cancer cells seems to be independent of the Ras pathway (Keyomarsi *et al*., 1991). Furthermore, the lovastatin mediated CKI induction is also through cell cycle independent mechanisms, distinct from other G1 arresting agents/conditions such as serum starvation or double thymidine block (Gray-Bablin *et al*., 1997).

In this study, we have investigated the nature of induction of p21 and p27 by lovastatin in both normal and tumor-derived mammary epithelial cells. We show that lovastatin is capable of inducing G1 arrest in both normal and tumor breast cells. Furthermore, we provide evidence that it is not the increase in protein levels of the p21 and p27 *per se*, but the increase in the binding of both p21 and p27 to CDK2 complexes and the resulting decrease in activity of cyclin/CDK2 complexes that is essential for lovastatin mediated G1 arrest of normal and tumor cells. We also show that the increased binding of CKIs to CDK2 correlates with decreased CDK4 and cyclin D3 levels and the subsequent release of p21 and p27 from the cyclin D/CDK4 complex, providing evidence for the redistribution of both p21 and p27 under physiological conditions *in vivo*. Lastly, we show that binding of CKIs to CDK2 or the switching of partners from CDK4 to CDK2 following lovastatin treatment is through a p53 independent pathway.

Results

Lovastatin synchronizes a broad range of tumor and normal breast cancer cells

In order to determine whether lovastatin would have a differential affect of G1 mediated arrest of normal versus tumor breast cells, three normal and six tumor cell lines were treated with lovastatin at the same dose of duration (i.e. 40 μM for 36 h) (Table 1). The normal cells examined were 76N and 70N (both mortal) and MCF-10A (immortalized). The tumor cells used were classified on their ability to express tumor suppressor genes p53, pRb and estrogen receptor (ER). The ER positive tumor cells are MCF-7, ZR75T, and T47D and the ER negative tumor cells are MDA-MB-157, MDA-MB-231 and Hs578T. In the ER positive group all 3 tumor cell lines are also wild type for pRb and p53, except for T47D, which has a p53 mutant phenotype. In the ER negative group all three tumor cell lines are negative for p53 and pRb except for MDA-MB-157 which has a wild type pRb. However, the pRb in these cells is expressed at very low levels (see Figure 1) and is inactive because of high levels of p16 and overexpression of cyclin E (Keyomarsi *et al*., 1995). p16 has been shown to transcriptionally down regulate pRb expression (Fang *et al*., 1998) while the overexpression of cyclin E results in constitutive hyperphosphorylation of pRb rendering it inactive as a tumor-suppressor (Gray-Bablin *et al*., 1996). In the case of Hs578T, it too is pRb negative as published

Table 1 Cell cycle profiles of lovastatin treated normal and tumor (RB and p53 positive and negative) breast cells

Cell lines	*Lovastatin,* 40 μM h	*ER*	*p53*	*pRb*	*%G1*	*%S*	*%G2*
76N	0	−	+	+	56.6	12.1	31.3
	36				72	1.2	27
MCF-10A	0	−	+	+	65	12	23
	36				90	1.6	8.4
70N	0	−	+	+	60	13	27
	36				78	1.2	21
MCF-7	0	+	+	+	77	11	12
	36				82	4	14
ZR75T	0	+	+	+	67	19	14
	36				85	6	9
T47D	0	+	−	+	73	13	14
	36				86	4	10
Hs578T	0	−	−	−	62	16	20
	36				83	5	12
MDA-MB-157	0	−	−	±	55	12	32
	36				60	4.3	36
MDA-MB-231	0	−	−	−	54	22	24
	36				80	7	13

Estrogen Receptor (ER), p53 and pRb status of cell lines were determined previously (Gray-Bablin *et al*., 1996, and references within). + indicates wild-type, − indicates mutant or deleted, ± indicates wild-type but functionally inactive (see text). Percentages of cells in different phases of the cell cycle for each cell line following lovastatin treatment were obtained from flow cytometric measurements of DNA contents from three separate experiments and the average of the values are indicated

previously (Gray-Bablin *et al*., 1996). In this cell line the pRb protein, although expressed at very low levels (Figure 1), is highly susceptible to degradation (data not shown). Therefore, Hs578T, MDA-MB-231 and MDA-MB-157 cell lines are considered functionally pRb negative as we previously described (Gray-Bablin *et al*., 1996).

These studies revealed that lovastatin treatment for 36 h resulted in G1 arrest in both tumor and normal cell lines irrespective of their p53, pRb, or ER status (Table 1). However, the percent S phase decrease may be dependent on the expression of the aforementioned proteins. For example, treatment of normal breast epithelial cells with lovastatin resulted in the largest S phase decrease (89% drop from untreated cells) while the S phase in p53/pRb/ER positive or negative cells dropped by 68% from the untreated controls. Furthermore, no changes in cell cycle distribution were observed after 36 h in untreated control cultures (data not shown). These observations suggest that although lovastatin is capable of arresting both normal and tumor cells in G1, the degree of synchronization is more profound in normal cells. Next, we examined the pattern of expression of key cell cycle regulators in these three classes of cell lines following treatment of cells with lovastatin (Figure 1).

Lovastatin treatment causes a decrease in the levels of CDK4 and cyclin D3 in both normal and tumor cells

To determine which key cell cycle regulators were required for lovastatin mediated G1 arrest, we examined the expression of several positive and negative cell cycle proteins in both normal and tumor cells. A subset of the cell lines from Table 1 consisting of two normal cell lines, 76N and MCF-10A, two tumor cell lines which are p53, pRb and ER positive, MCF-7, ZR75T and two tumor cell lines which are p53, pRb and ER negative, Hs578T and MDA-MB-157 cell lines were analysed. The 76N and MCF-10A cells were chosen as they represented normal cells obtained from two different lineages, 76N cell lines are normal mortal cells obtained from reduction mammoplasty while MCF-10A cell lines were immortalized from normal breast epithelial cell strain, MCF-10, after cultivation in medium containing low calcium concentrations (Soule *et al*., 1990).

All cells within each category were treated with 40 μM lovastatin for 36 h and at the indicated times following treatment, cells were harvested and subjected to Western blot analysis with antibodies to p27, p21, p16, pRb, p53, CDK2, CDK4, cyclin D1 and cyclin D3 (Figure 1). These analyses revealed that the total protein levels of p21 and p27 were induced significantly only in the cells with pRb/p53/ER negative status suggesting that lovastatin causes an induction of these CKI's through a p53 independent mechanism. In p53 and pRb positive cells (MCF-7 and ZR75T) the basal levels of p21 and p27 were very high and no subsequent increase in these CKI's were seen when examining total protein levels. Normal cells on the other hand show an increase in p27 but a decrease in p21 following lovastatin treatment. The p27 levels accumulate by sixfold in MCF-10A cells and 2.5-fold in 76N cells reproducibly. INK CKI, p16 was expressed only in MDA-MB-157 cells and its levels did not change following lovastatin treatment.

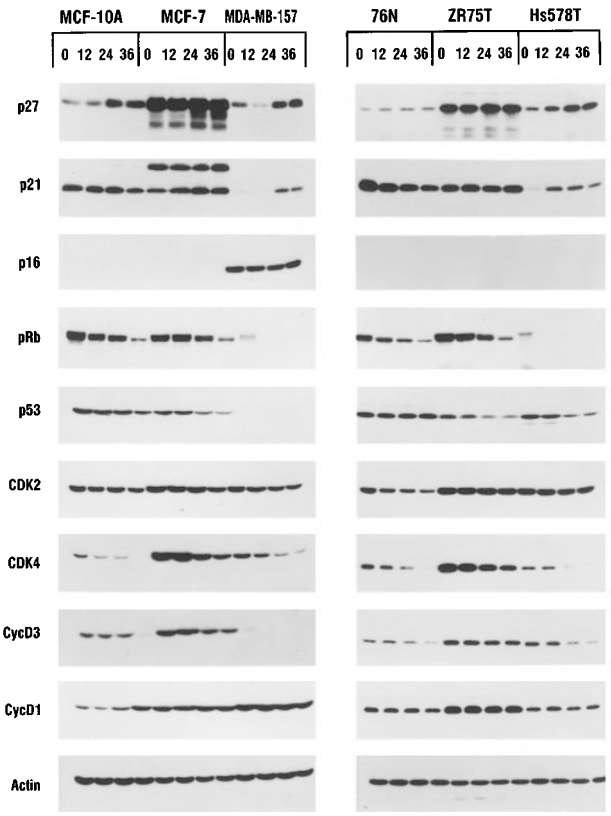

Figure 1 Expression of positive and negative cell cycle regulators in normal and tumor breast cells following lovastatin treatment. All cells were cultured in medium containing 40 μM lovastatin. At the indicated times following treatment cells were harvested, cell lysates prepared and subjected to Western blot analysis: 50 μg of protein extract from each condition was analysed by Western blot analysis with the indicated antibodies or actin used for equal loading. The blots were developed by chemiluminescence reagents. The same blots were sequentially hybridized with different antibodies (see Materials and methods). The blots were stripped between the antibodies in 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), and 2% SDS for 10 min at 55°C

The levels of p53 and pRb tumor suppressor protein decreased in both normal and ER/p53/pRb positive tumor cell lines. The simultaneous decrease in p53 and p21 in normal cells suggest that in normal cells p21 expression may be strongly influenced by p53. A similar decrease in p21 is not noticed in p53 positive tumor cell lines (MCF-7 and ZR75T) even though p53 levels decreased, suggesting that the p53-p21 pathway in these tumor cell lines is not as tightly controlled as seen in normal. Analysis of cyclins and CDKs revealed that following lovastatin treatment CDK4 and cyclin D3 protein levels decreased in all cell lines while cyclin D1 and CDK2 levels remained relatively unchanged. Collectively these results reveal that the only cell cycle regulators which consistently decrease in response to lovastatin are CDK4 and cyclin D3. Furthermore, the increases of p21 and p27 at the protein levels were only apparent in ER/p53/pRb negative cell tumor cell lines and were not universally observed in lovastatin induced G1 arrest which occurred in all cells regardless of their p53 or pRb status (Table 1). Lastly, the arrest of MDA-MB 157 and Hs578T cells which are p53 and pRb negative implies that the activity of the tumor

suppressors is not required for lovastatin mediated G1 arrest. This analysis raised the question if and how p21 and p27 could play a role in lovastatin mediated G1 arrest and how the decrease in the CDK4 and cyclin D3 in response to lovastatin could contribute to this arrest?

Inactive cyclin CDK2 complexes in lovastatin treated cells are due to increased p21 and p27 binding

A likely explanation for lovastatin mediated G1 arrest in the different cell types is that lovastatin treatment of cells results in the inhibition of CDK2 activity which is necessary for cells to overcome the restriction point in the G1 phase of the cell cycle. In order to examine the kinase activity associated with CDK2 in normal and tumor cells, we measured the phosphorylation of histone H1 in immunoprecipitates prepared from lovastatin treated cells using an antibody to CDK2 (Figure 2a). This analysis revealed that treatment of all cells by lovastatin resulted in a rapid decrease of CDK2 activity and by 36 h, the time when G1 arrest fully manifests itself in cells, the level of CDK2 activity reaches its nadir in all cells examined. These data suggest that lovastatin mediated G1 arrest results in lowered CDK2 activity in all cells regardless their p53, pRb, normal or tumor status.

To determine if the decreased activity of the CDK2 is due to its association with CKIs, a two step experiment consisting of an immunoprecipitation with anti-CDK2 antibody followed by Western blot analysis with p21 or p27 was performed (Figure 2b). These analyses revealed that the decreased CDK2 activity observed (Figure 2a) was concomitant with increased binding of p27 to CDK2 in all normal and tumor cell lines treated with lovastatin. Furthermore, all tumor cell lines treated with lovastatin exhibited increased binding of p21 to CDK2 as well. In normal cells however, not only were the p21 total protein levels decreased by lovastatin treatment (Figure 1), but the binding of p21 to CDK2 also decreased (Figure 2b), despite complete inhibition of the CDK2 activity (Figure 2a). Collectively these results suggest that the G1 arrest induced by lovastatin is concomitant with decreased activity of CDK2 mediated by increased binding of p27 (in all cells) and p21 (only in tumor cells) to CDK2. Interestingly, MCF-7 and ZR75T which exhibited no increases in p21 or p27 expression (Figure 1) revealed a clear increase in binding of both these CKIs to CDK2 (Figure 2b). These observations raise the question that if the total levels of p21 and p27 are not induced by lovastatin (Figure 1) what accounts for the increased binding of these inhibitors to CDK2 in MCF-7 and ZR75T cells (Figure 2b)?

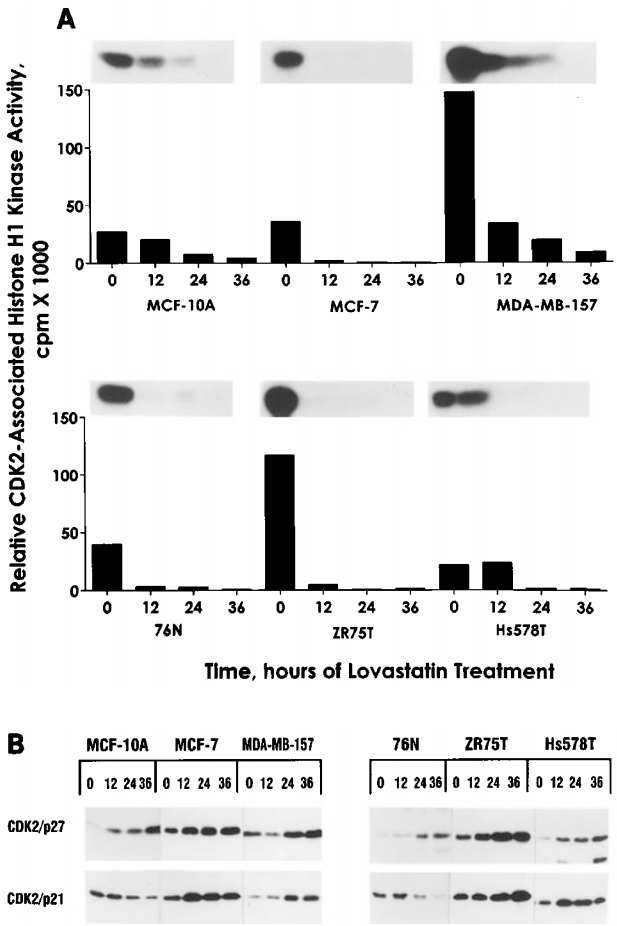

Figure 2 Lovastatin treatment reduces CDK2 activity by increased p21 and p27 binding. All cells were cultured in medium containing 40 μM lovastatin. At the indicated times following treatment cells were harvested, cell lysates prepared and subjected to (**a**) Histone H1 kinase analysis or (**b**) Immune-complex formation. For kinase activity, equal amounts of protein (300 μg) from cell lysates were prepared from each cell line at the indicated times following lovastatin treatment and immunoprecipitated with anti-CDK2 antibody (polyclonal) coupled to protein A beads using histone H1 as substrate. For each cell line we show the resulting autoradiogram of the histone H1 SDS–PAGE and the quantitation of the histone H1 associated kinase activities by scintillation counting. For immunoprecipitation followed by Western blot analysis, equal amounts of protein (300 μg) from cell lysate prepared from each cell lines were immunoprecipitated with anti-CDK2 (polyclonal) coupled to protein A beads and the immunoprecipitates were subjected to Western blot analysis with the indicated antibodies

Lovastatin treatment causes the CKIs to switch from binding to CDK4 to CDK2

It has been proposed that cyclin D/CDK4 complexes play a critical role in titrating p21 and p27 by binding to them, resulting in decreased amounts of p21 and p27 which would otherwise bind to and inhibit cyclin/CDK2 complex activity (LaBaer *et al*., 1997). At low concentrations, the binding of p21 and p27 to CDK4 will not inhibit cyclin D/CDK4 activity but rather promote efficient binding of cyclin D with CDK4, and as such function as adaptor molecules (LaBaer *et al*., 1997). Since lovastatin causes the synchronization of cells apparently by increasing binding of p21 (tumor cells) and p27 (normal and tumor cells) to CDK2 complexes, it can be hypothesized that this increased binding of CKI's to CDK2 may be due to the switching of the CKI's from CDK4 to CDK2, mediated by lovastatin. To test this hypothesis and to examine this adaptor molecule theory we examined the association of p21 and p27 to CDK4 following lovastatin treatment (Figure 3). Our results clearly demonstrate that in untreated normal and ER/p53/pRb positive tumor cells, p21 and p27 bind to CDK4, and upon treatment with lovastatin, both p21 and p27 are released from CDK4 in a time dependent fashion (Figure 3) which corresponds to their binding (i.e.

switching partners) to CDK2 (Figure 2b). The apparent increase in p27 levels observed in 76N cells following lovastatin treatment seems to be the primary event leading to CDK2 inactivation; primary to redistribution of p27 from CDK4 to CDK2. However, in MCF-7 and ZR75T cells, where no detectable increase in the levels of p21 and p27 following lovastatin treatment is observed, the switching of these CKIs from CDK4 to CDK2 occurs concomitantly with decrease in CDK2 activity, suggesting that in these ER/p53/pRb positive tumor cells the p21 and p27 switch from CDK4 to CDK2 is the primary event leading to CDK2 inactivation. In both sets of cell lines we show that lovastatin creates a signal for these CKIs to switch partners from CDK4 to CDK2 and such switching of partners is concurrent with G1 arrest. The signal that initiates the switching may be the decrease in CDK4 and cyclin D3 protein levels observed in all cells examined following treatment with lovastatin (Figure 1).

The p53/pRb/ER negative tumor cell lines reveal a different pattern of CKI/CDK4 binding than either the normal cells or the p53/pRb/ER positive tumor cell lines (Figure 3). MDA-MB-157 cells which have very low levels of p21 and p27 show no binding of these proteins to CDK4. In fact CDK4 is bound to p16 which is expressed in high quantities in this cell line and can titrate and thus prevent any CDK4 complex formation with p21 or p27. In the Hs578T cell line which do not express detectable p16 levels, CDK4 is not titrated and binds only to p27 in untreated cells and treatment of cells with lovastatin decreases the binding of p27 to CDK4. Collectively, these observations support the validity of the adaptor molecule theory where p21 and p27 switch partners from CDK4 to CDK2 specifically for ER/p53/pRb positive cells which have endogenously high levels of p21 and p27. Furthermore, the decreased binding of the CKIs to CDK4 also suggest that lovastatin affects the cellular pathways that provide the switching signal for CKIs to redistribute from cyclin/CDK4 complex to cyclin/CDK2 complexes.

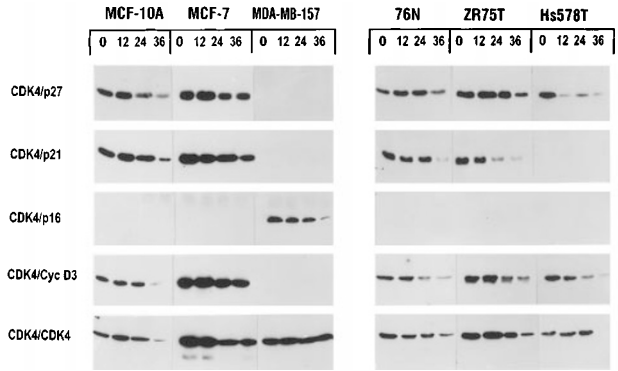

Figure 3 Redistribution of p21 and p27 from CDK4 to CDK2 following lovastatin treatment. All cells were cultured in medium containing 40 μM lovastatin. At the indicated times following treatment cells were harvested, cell lysates prepared and subjected to Immune-complex formation. Equal amounts of protein (300 μg) from cell lysate prepared from each cell line was immunoprecipitated with anti-CDK4 (polyclonal) coupled to protein A beads and the immunoprecipitates were subjected to Western blot analysis with the indicated antibodies

CDK2 is completely sequestered by p21 and p27 following lovastatin treatment

To determine whether the increased binding of p21 and p27 to CDK2 is sufficient to inactivate CDK2, we examined the proportion of CDK2 bound to p21 and p27 following lovastatin treatment. For these experiments MCF-7 and ZR75T cell lines were chosen as the basal levels p21 and p27 are very high in these cell lines (Figure 1) and treatment with lovastatin results in increased binding of both CKIs to CDK2 (Figure 2). To evaluate the proportion of CDK2 in complex with p21 and p27 we immunodepleted cell extracts with anti-p21 and anti-p27 antibodies. These extracts were prepared from both cell lines before and after lovastatin treatment. Following immunodepletion, the final supernatant was subjected to Western blot analysis with antibodies to p21, p27, CDK2, CDK4 and actin (Figure 4). These results revealed that upon immunodepletion of cells of p21 and p27, there is little or no CDK2 present following lovastatin treatment. CDK4 levels were also not detectable following p21 and p27 immunodepletion in lovastatin treated cells (Figure 4) partly due to the decrease in total CDK4

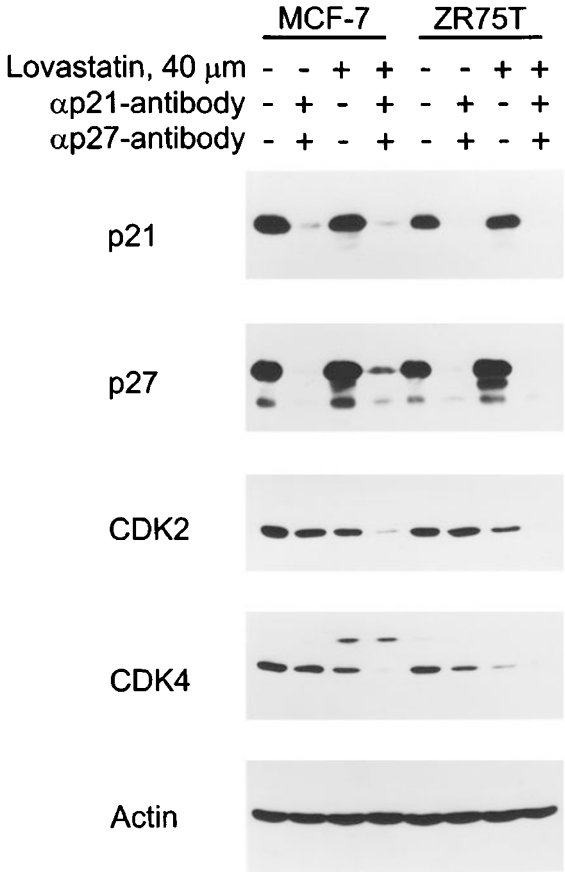

Figure 4 Immunodepletion of p21 and p27 in lovastatin treated cells also depletes the cells of CDK2. Cells were cultured in medium 40 μM lovastatin for 0 and 36 h. Following treatment cells were harvested, cell extracts prepared and immunodepleted with antibodies against p21 and p27. Following three rounds of immunodepletion with anti-p21 and anti-p27 coupled to protein A beads, or protein A beads alone, the remaining supernatant (50 μg/lane) were subjected to Western blot analysis with the indicated antibodies

levels following treatment (Figure 1). Hence, upon lovastatin treatment, CDK4 levels decrease, p21 and p27 levels do not change yet their binding to CDK2 increase, and CDK2 activity greatly declines. Furthermore lovastatin results in sequestering of CDK2 by these CKIs suggesting that p21 and p27 alone will inhibit CDK2 activity. These observations also suggest that the amounts of p21 and p27 released from CDK4 complexes in lovastatin treated cells is sufficient to block the CDK2 activity in these cells.

Lovastatin mediated G1 arrest is through a p53 independent pathway

To directly examine the role of p53 in the G1 arrest induced by lovastatin in breast epithelial cells, we investigated perturbation of the cell cycle by lovastatin in 76N cells transformed by the human papilloma virus E6 (76N-E6) (Band *et al*., 1991). Initially, we examined the expression of key cell cycle regulatory proteins in 76N-E6 as compared to 76N parental cell line (Figure 5b, right panel). This analysis showed that aside from lack of expression of p53 and p21 in 76N-E6 cell line, the other cell cycle regulatory proteins are similarly expressed between 76N-E6 and the parental 76N cells. Next, to examine the effects of lovastatin on 76N-E6 cells we treated them with 40 μM lovastatin for 36 h. Such treatment resulted in a G1 arrest of 76N-E6 cells despite the absence of p53 (Figure 5a). At the indicated times following treatment, cells were harvested and subjected to Western blot analysis with antibodies to key cell cycle regulators (Figure 5b). These analyses reveal that as expected the cells did not express any p53 due to its rapid degradation by the transfected E6 oncogene. However, both p27 and p21 levels were induced following lovastatin treatment (Figure 5b) which is unlike what was observed in the 76N parental p53 wild-type cells (Figure 1) or HeLa cells where only p27 was shown to be induced by lovastatin (Hengst *et al*., 1994; Hengst and Reed, 1996). These results suggest that the elimination of p53 in 76N-E6 cells results in induction of p21 by lovastatin through a p53 independent mechanism and that p53 expression is not critical for lovastatin mediated G1 arrest.

All the other cell cycle regulatory proteins examined in 76N-E6 cells revealed a similar pattern of alteration following lovastatin treatment as observed in normal 76N cells (Figure 1); i.e. the expression of hyperphosphorylated pRb, CDK4 and cyclin D3 decreased rapidly and significantly in response to lovastatin while the levels of CDK2 and cyclin D1 remain relatively unchanged (Figure 5b). Similarly, CDK2 activity also decreased profoundly in 76N-E6 cells following lovastatin treatment and G1 arrest (Figure 5c). We also examined the association of p21 and p27 with CDK2 following lovastatin treatment and found that the binding of both p21 and p27 to CDK2 increased significantly during the course of lovastatin treatment, suggesting that p21 and p27 are indeed acting as inhibitors of CDK2 leading to G1 arrest in 76N-E6 cells independent of p53 (Figure 5d). Lastly, we examined the association of p21 and p27 with CDK4 following lovastatin treatment and found that in untreated 76N-E6 cells both p21 and p27 bind to CDK4 only to redistribute from CDK4 to CDK2 upon treatment with lovastatin (Figure 5e). These results

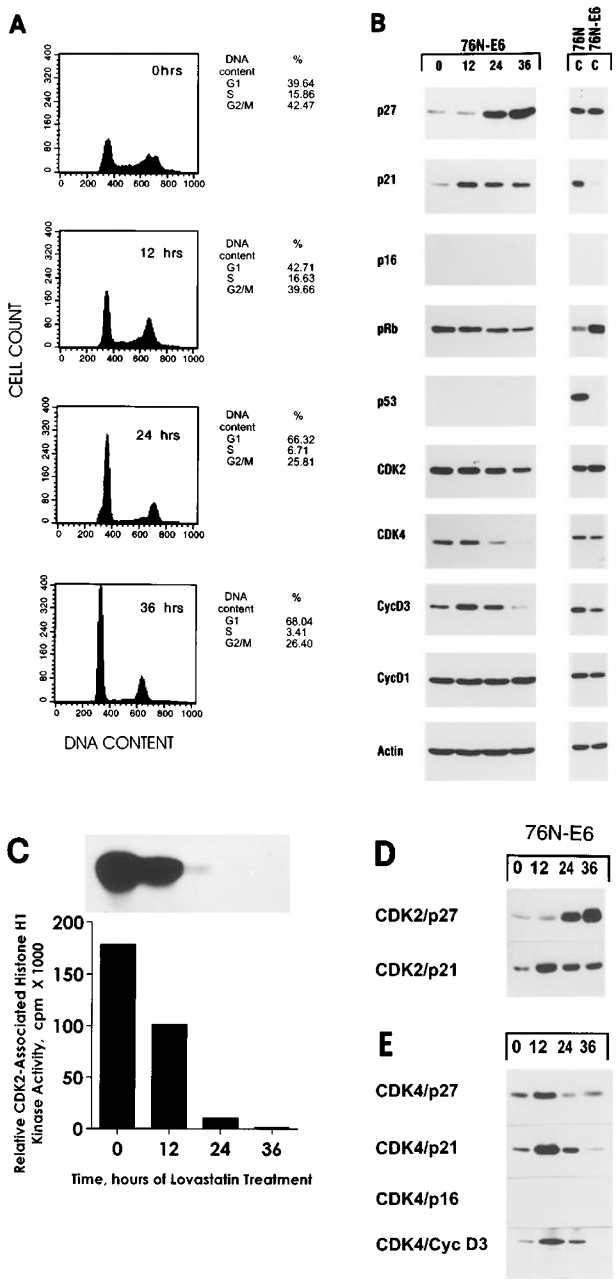

Figure 5 Induction of CKIs in 76N-E6 cells following lovastatin mediated GI arrest. 76N-E6 cells treated with 40 μM lovastatin. At the indicated times following treatment cells were harvested and subjected to (**a**) Flow cytometry. Percentages of cells in different phases of the cell cycle for 76N-E6 were determined from flow cytometric measurements of DNA content. (**b**) Western blot analysis. Fifty μg of protein extracts from each condition was analysed by Western blot analysis with the indicated antibodies or actin used for equal loading and the blots were developed using the chemiluminescence reagents. The right panel shows Western blot analysis using cell extracts from untreated 76N parental and 76N-E6 cell lines on the same blot with identical exposure times. (**c**) Histone H1 kinase analysis. Equal amounts of protein (300 μg) from cell lysates were prepared at the indicated times following lovastatin treatment and immunoprecipitated with anti-CDK2 antibody (polyclonal) coupled to protein A beads using histone H1 as substrate. The autoradiogram of the histone H1 SDS–PAGE and the quantitation of the histone H1 associated kinase activities by scintillation counting are presented. (**d**) CDK2 Immune-complex formation and (**e**) CDK4 Immuno-complex formation. For immunoprecipitation followed by Western blot analysis, equal amounts of protein (300 μg) from cell lysates prepared from lovastatin treated cells were immunoprecipitated with anti-CDK2 (polyclonal) (**d**) or anti-CDK4 (polyclonal) (**e**) coupled to protein A beads and the immunoprecipitates were subjected to Western blot analysis with the indicated antibodies

suggest that the lovastatin mediated G1 arrest in 76N-E6 cells, the concomitant decrease in CDK2 activity, the increased expression of p21 and p27, the increased association of these CKIs to CDK2, and lastly the redistribution of CKIs from CDK4 to CDK2 are all independent of p53.

Discussion

The outcome of CKI induction in most cells is the cessation of cell proliferation, differentiation or even cell death. In tumor cells the regulation of the CKIs is altered leading to either lack of function, or expression. Hence, if the CKIs could be induced consistently in tumor cells, and their induction lead to G1 arrest the goal of controlling the proliferation of cancer cells could be achieved. As shown in this study we observe that lovastatin can cease cell proliferation in a wide variety of normal and tumor breast cells, independent of their p53, pRb or ER status. We also provide evidence that it is not the increase in the levels of the CKIs per se but the increased binding of p21 and p27 to CDK2 and the consequent reduction of CDK2 activity that is actually responsible for the G1 arrest caused by lovastatin in both normal and tumor breast cell lines. The increased binding of CKIs to CDK2 is through a p53 independent pathway. Our results also reveal that lovastatin like other growth arresting agents such as TGF-β (Reynisdottir and Massague, 1997) use the switching of CKI from CDK4 complex to CDK2 as a method to initiate growth arrest.

Redistribution of both p21 and p27 from CDK4 complexes to CDK2 complexes following lovastatin treatment

Recently a new functional role has been assigned to p21 and p27, that of facilitating cyclin D/CDK4 assembly *in vitro* or acting as adaptor molecules (Poon *et al*., 1995; LaBaer *et al*., 1997; Planas-Silva and Weinberg, 1997a; Prall *et al*., 1997). Adaptor molecules facilitate the association or complex formation between proteins without hindering in the function of the complex. Both *in vitro* studies using purified p21, p27 or p57 (KIP2) and *in vivo* studies using cells transiently transfected with p21, CDK4 and cyclin D1, showed an abundance of assembled CDK4/cyclin D1 complex which increased directly with increasing inhibitor levels. In fact, the addition of p21, p27 and p57 were able to promote assembly of the cyclin CDK4 complexes by 35–80-fold without inhibiting the activity of the complex (LaBaer *et al*., 1997). Hence, at low concentrations p21 and p27 can act as adaptor molecules, facilitate the binding of cyclin D/CDK4 complexes, promote the phosphorylation of pRb and progression through G1, while at higher concentrations, the CKIs can switch partners bind to CDK2 and inhibit cell cycle progression. Similarly, other studies examining the effect of estrogen on the cell cycle of ER positive MCF-7 cells revealed that the addition of β-estradiol or estrogen were able to rescue synchronized cells and induce progression through the cell cycle by increasing the level of cyclin D and increased binding of p21 and p27 to the cyclin D/CDK4 complexes from the cyclin E/CDK2 complexes (Planas-Silva and Weinberg, 1997a; Prall *et al*., 1997). This redistribution of p21 caused a marked induction of the cyclin E/CDK2 kinase activity. Similarly, studies with the growth inhibitor TGF-β also showed redistribution of the cyclin kinase inhibitors, i.e. CKIs switch from CDK4 to CDK2 in response to growth inhibitory activity of TGF-β. In addition TGF-β resulted in the reduced synthesis of CDK4 and cyclin D leading to the subsequent dissociation of cyclin D/CDK4 complexes and redistribution of p27 from cyclin D/CDK4 complex to cyclin E/CDK2 leading to a G1 arrest (Ewen *et al*., 1993b). Subsequent studies have suggested that the redistribution of p27 following TGF-β treatment was due to the increased binding of p15 to cyclin D/CDK4 complexes in Mink Lung cells (Reynisdottir *et al*., 1995; Reynisdottir and Massague, 1997). It therefore has been proposed that cyclin D/CDK4 complexes apart from phosphorylating pRb, titer the p21 and p27 in the cells and hence prevent the binding of the CKIs to cyclin/CDK2 complexes.

In the present study we also observe that treatment of cells with lovastatin induce a cascade of events leading to cessation of cell proliferation through the redistribution of the CKIs from cyclin D/CDK4 complexes to cyclin E/CDK2 complexes. We observed that lovastatin treatment of all cells examined, normal or tumor caused a reduced synthesis of both cyclin D3 and CDK4 but not cyclin D1. A similar decrease in CDK4 and cyclin D3 but not D1 was seen in retinoic acid arrested MCF-7 cell (Zhu *et al*., 1997) and glucocorticoids arrested U2OS and SAOS2 cells (Rogatsky *et al*., 1997) suggesting that the rapid reduction in cyclin D3 or CDK4 levels may be the key signal for redistribution of the CKIs. In the present study we observed that the CKI binding to CDK4 complexes reduced rapidly and significantly following lovastatin treatment (Figure 4). This reduction was seen in both normal and ER/p53/pRb positive tumor cell lines. Hence, the redistribution of both p21 and p27 from CDK4 complexes to CDK2 complexes, apparently signaled by a decrease in the expression of CDK4 and cyclin D3, leads to G1 arrest caused by lovastatin treatment. This switching also explains the increased binding of the CKI's to CDK2 complexes that is observed in ER/p53/pRb positive cells (Figure 2) without the corresponding increase in CKI levels, as these cell lines have a high basal levels of these CKIs (Figure 1). The high levels of both CKIs could be attributed to increase cyclin D/CDK4 activity required to phosphorylate pRb in these cells and thereby giving these tumor cells a growth advantage. We also show that when cell extracts prepared from lovastatin treated cultures of ER/p53/pRb positive tumor cell lines (i.e. MCF-7 and ZR75T) are immunodepleted of both p21 and p27, there is little to no CDK2 or CDK4 left in the final supernatant. These results suggest that CDK2 is completely sequestered by p21 and p27 following lovastatin treatment in the absence of any CKI accumulation. Furthermore, the amounts of p21 and p27 released from CDK4 complexes which then associate with CDK2 complexes in lovastatin treated cells are sufficient to block the CDK2 activity in these cells.

Lovastatin mediated G1 arrest is p53 independent

In this study we analysed the effect of lovastatin on a broad range of breast cells, normal and tumor with

different p53 status. We found that whether a cell is p53 wild-type or mutant the effect of lovastatin was the same in all cells and resulted in G1 arrest suggesting that the p53 pathway is not involved in the G1 arrest induced by lovastatin. We observed this p53 independence under three different conditions. First, the protein levels and CDK2 binding of p21 and p27 in p53/Rb negative cells, (MDA-MB 157 and Hs578T) increased dramatically following lovastatin treatment, resulting in the G1 arrest of these cells. MDA-MB-157 is null for p53 while Hs578T harbors a mutant p53. Hence, the increase in p21 levels following lovastatin treatment in these cells is independent of p53. Secondly, MCF-7 and ZR75T cells which are p53 and pRb positive tumor cells showed a decrease in p53 levels following treatment with lovastatin (Figure 1) even though the binding of p21 and p27 to CDK2 increased resulting in inhibition of CDK2 activity and subsequent G1 arrest (Figure 2). In these cells the increased binding of p21 to CDK2 was independent of p53, due to decrease in p53 levels in response to lovastatin (Figure 1). Finally, and most directly, we show that 76N-E6 cells, stably transformed by the human papilloma virus E6 which renders p53 inactive (Band *et al*., 1990; 1991), not only were G1 arrested by lovastatin treatment but also revealed an induction of p21 and p27 expression followed by increased binding of p21 (and p27) to CDK2. The above results using three different cell types which are either p53/pRb wild-type, or p53/pRb mutant, or harbor an inactive p53 (i.e. 76N-E6) clearly reveal that the p21 induction and subsequent G1 arrest mediated by lovastatin is p53 independent.

Lovastatin is a widely used drug for patients suffering from hypercholesterolemia (Rettersol *et al*., 1996). Investigators attempted to use lovastatin as an agent for treatment of cancer because it inhibits the cholesterol biosynthesis pathway and tumor cells have an increased level of cholesterol synthesis (Bernstein and Ross, 1993). However, these studies and recent clinical trials were inconclusive in providing a role for lovastatin as an anti-cancer agent (Thibault *et al*., 1996). On the other hand, it is reasonable to evaluate the chemo-preventative effect of this drug since data from a large clinical trial of lovastatin for reducing serum cholesterol produced the unexpected finding of a 33% decrease in cancer incidence (Stein *et al*., 1993). Furthermore, lovastatin has also been shown to inhibit metastasis of highly metastatic B16F10 mouse melanoma in nude mice (Jani *et al*., 1993). Lastly, in the present study we show that lovastatin treatment of cells leading to G1 arrest is through the induction of p21 and p27 and subsequent inhibition of CDK2 activity. Collectively the above studies suggest that lovastatin may have chemo-preventative properties by inducing the inhibitory activity of the negative regulators of the cell cycle. It therefore is quite pertinent to investigate the direct mechanism by which lovastatin activates the CKIs in the cells, whether by inducing their expression in otherwise CKI negative cells, or mediating their redistribution to cyclin/CDK complexes which inhibit progression through the cell cycle. The universality and the p53 independent action of lovastatin in cessation of cell proliferation, also make it a very attractive agent for use as a potential chemo-preventative agent.

Materials and methods

Materials, cell lines and culture conditions

Lovastatin was kindly provided by William Henkler (Merck, Sharp and Dohme Research Pharmaceuticals, Rathway, NJ, USA). Serum was purchased from Hyclone Laboratories (Logan, Utah, USA) and cell culture medium from Life Technologies, Inc. (Grand Island, NY, USA). All other chemicals used were reagent grade. Before addition to cultures, lovastatin was converted from its inactive lactone prodrug form to its active dihydroxy-open acid as described previously (Keyomarsi *et al*., 1991; Keyomarsi, 1996). The culture conditions for 76N, 70N normal cell strains, MCF-10A immortalized cell line, and MCF-7, ZR75T, MDA-MB-157, Hs578T, T47D, and MDA-MB-231 breast cancer cell lines were described previously (Keyomarsi and Pardee, 1993; Keyomarsi *et al*., 1995). 76N-E6 cell line (a gift from Dr V Band, Tufts Medical Institute, Boston, MA, USA) were immortalized and cultured as described previously (Band *et al*., 1990, 1991). All cells were cultured and treated at 37°C in a humidified incubator containing 6.5% CO_2 and maintained free of mycoplasma as determined by Hoechst staining (Hessling *et al*., 1980).

Synchronization and flow cytometry

Synchronization by lovastatin treatment was performed as described previously (Keyomarsi *et al*., 1991). Briefly medium was removed 24–36 h after the initial plating, replaced with fresh medium plus 40 μM lovastatin for 0–36 h. Cells were harvested at the indicated times and flow cytometry analysis was performed. For Fluorescence-Activated Cell Sorter (FACS) analysis 10^6 cells were centrifuged at 1000 *g* for 5 min, fixed by the gradual addition of ice cold 70% ethanol (30 min at 4°C) and washed with phosphate buffered saline. Cells were then treated with RNAse (10 ug/ml) for 30 min at 37°C, washed once with phosphate buffered saline and resuspended and stained in 1 ml of 69 μM propidium iodide in 38 mM sodium citrate for 30 min at room temperature. The cell cycle phase distribution was determined by analytical DNA Flow cytometry as described previously (Keyomarsi *et al*., 1995).

Western blot and immune complex kinase analysis

Cell lysates were prepared and subjected to Western blot analysis as previously described (Keyomarsi *et al*., 1995). Briefly, 50 μg of protein from each condition was electrophoresed in each lane of either a 7% sodium dodecyl sulfate-polyacrylamide gel (SDS–PAGE) (pRb), 10% SDS–PAGE (p53, cyclin A, cyclin D1, cyclin D3), 13% SDS–PAGE (p21, p27, CDK2, CDK4), or a 15% SDS–PAGE (p16), and transferred to Immobilon P overnight at 4°C at 35 mV constant volts. The blots were blocked overnight at 4°C in Blotto (5% nonfat dry milk in 20 mM Tris, 137 mM NaCl, 0.25% Tween, pH 7.6). After six, 10 min washes in TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween, pH 7.6), the blots were incubated in primary antibodies for 3 h. Primary antibodies used were pRb monoclonal antibody (PharMingen, San Diego, CA, USA), at a dilution of 1:100, monoclonal antibody to p16 (a gift from Jim DeCaprio, Dana Farber Cancer Institute) at a dilution of 1:20, CDK2, CDK4 and p27, monoclonal antibodies (Transduction Laboratories, Lexington, KY, USA) each at a dilution of 1:100, p21 and p53 monoclonal antibodies (Oncogene Research Products/Calbiochem, San Diego, CA, USA) at a dilution of 1:100 cyclin D1 monoclonal antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA) at a dilution of 1:100, and actin

monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN, USA) at 0.63 μg/ml in Blotto. Following primary antibody incubation, the blots were washed and incubated with goat anti-mouse horseradish peroxidase conjugate at a dilution of 1:5000 in Blotto for 1 h and finally washed and developed with the Renaissance chemiluminescence system as directed by the manufacturers (NEN Life Sciences Products, Boston, MA, USA).

For immunoprecipitations followed by Western blot analysis 300 μg of cell extracts were used per immunoprecipitation with polyclonal antibody to CDK2 [CDK2 antibody was generated by immunizing rabbits with multiple antigenic peptide (MAP peptides) (Posnett *et al*., 1988) consisting of the 30 amino acids of the N terminal region of the CDK2 protein. MAP peptides consist of branched lysines, with each lysine directly attached to the peptide. Rabbits were primed and boosted subcutaneously, with 2 mg of MAP peptide without a carrier protein and emulsified in complete or incomplete (four boosts) adjuvant, respectively. The rabbits were boosted in 3 week intervals and the titer and the specificity of the serum was monitored by ELISA (Enzyme-linked immunosorbent assay) following each boost.] or CDK4 (a gift from Dr M Pagano, New York University Medical Center, NY, New York) (Tam *et al*., 1994) in lysis buffer containing 50 mM Tris buffer pH 7.5, 250 mM NaCl, 0.1% NP-40, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 0.5 mM PMSF, 50 mM NaF, 0.5 mM Sodium Ortho-Vanadate. The protein/antibody mixture was incubated with protein A Sepharose for 1 h and the immunoprecipitates were then washed twice with lysis buffer and four times with kinase buffer (50 mM Tris HCL pH 7.5, 250 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 0.1 mg/ml BSA). The immunoprecipitates were then electrophoresed on 10% (cyclin D3) 13% (p21, p27, CDK4 and CDK2) and 15% gels (p16) transferred to Immobolin P, blocked and incubated with the indicated antibodies at dilutions described above. For Histone H1 kinase assay the immunoprecipitates were incubated with kinase assay buffer containing 60 μM cold ATP and 5 μCi of [^{32}P]ATP in a final volume of 50 μl at 37°C for 30 min. The products of the reaction were then ana-lysed on a 13% SDS–PAGE gel. The gel was then stained, destained, dried and exposed to X-ray film. For quantitation, the protein bands corresponding to histone H1 were excised and radioactivity was measured by scintillation counting.

For immunodepletion, three sequential immunodepletions were carried out with 500 μg of each cell extract using either anti-p21 and anti-p27 polyclonal antibodies (Santa Cruz Biochemicals, Santa Cruz, CA, USA) bound to protein A beads or protein A beads alone. Fifty μg aliquots of the remaining supernatant were subjected to Western blot analysis as described above to analyse the presence of remaining proteins.

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