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Lovastatin arrests CHO cells between the origin decision point and the restriction point

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Abstract Asynchronously growing Chinese hamster ovary (CHO) cells treated with the pro-drug, β-lactone ring form of lovastatin were arrested in G_1-phase. Subsequent removal of lovastatin resulted in the synchronous entry of cells into S-phase regardless of the presence of mevalonic acid. Lovastatin-arrested cells contained hypophosphorylated retinoblastoma protein (Rb) and required serum mitogens to enter S-phase after lovastatin removal, indicating that cell-cycle arrest is prior to the restriction point (R-point). However, in contrast to quiescent cells, intact nuclei prepared from lovastatin-arrested cells were competent for DNA replication when introduced into *Xenopus* egg extracts. Initiation of replication by *Xenopus* egg cytosol took place specifically within the dihydrofolate reductase (DHFR) origin locus, demonstrating that cells were arrested after the origin decision point (ODP). We conclude that the β-lactone ring form of lovastatin is an effective reagent with which to synchronize CHO cells between the ODP and R-point, without resulting in the withdrawal of cells from the cell-cycle into a quiescent state.

Key words: Cell-cycle; Initiation of DNA replication; Lovastatin; *Xenopus* egg extract

1. Introduction

Lovastatin inhibits 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, preventing the reduction of HMG-CoA to mevalonate, the rate limiting step in cholesterol biosynthesis [1,2]. Lovastatin has also been shown to synchronize mammalian cells in G_1-phase [3–6] but the mechanism of cell-cycle arrest has remained a mystery. Cell-cycle arrest was originally shown to be reversed by the addition of mevalonate [3–6], suggesting that the mevalonate pathway plays an important role in G_1-phase progression. In order to be effective in the inhibition of HMG-CoA reductase activity, lovastatin must first be converted from its pro-drug, β-lactone ring form to its dihydroxy open ring form [3–5,7,8]. However, a recent report found that the pro-drug form of lovastatin, which does not inhibit HMG-CoA reductase, inhibits the proteasome and is considerably more active in arresting mammalian cells in G_1-phase than after conversion to the open form [8].

The stage of G_1-phase at which lovastatin exerts its effect is also not clear. Some studies have suggested that cells are arrested in early G_1-phase, prior to the restriction point (R-point) [3], while others have concluded that lovastatin arrested cells in late G_1-phase [9]. Our previous studies have characterized several G_1-phase hallmarks in Chinese hamster ovary (CHO) cells that take place as cells exit mitosis after synchronization by mitotic shake-off (Fig. 1). In particular, we have defined a novel point when the specific sequences at which replication will initiate within the CHO DHFR locus are established [10]. This point, termed the origin decision point (ODP), was originally defined by introducing nuclei from cells synchronized at different times during G_1-phase into a cell-free replication system derived from *Xenopus* eggs. Replication initiates at dispersed sites with nuclei isolated from cells synchronized prior to the ODP, whereas replication initiates at the same specific origin sites utilized in vivo with nuclei isolated after the ODP [10]. The ODP was subsequently verified in vivo, in the absence of the *Xenopus* egg extract system, demonstrating that it is a bona fide novel G_1-phase event [11]. The ODP is independent of mitogen stimulation, and takes place several hours prior to phosphorylation of the retinoblastoma tumor suppressor protein (Rb) and the restriction point (R-point), the point at which entry into S-phase becomes independent of serum mitogens [11,12]. Hence, our studies demonstrate the existence of at least three distinct stages of G_1-phase (Fig. 1): (I) the pre-ODP stage when cells have not yet selected where replication will initiate, (II) the post-ODP stage prior to the R-point, when origins have been selected but cells may still withdraw from the cell-cycle into a state of quiescence, and (III) the period after the R-point during which cells prepare for DNA synthesis. Here, we demonstrate that the pro-drug form of lovastatin can effectively synchronize CHO cells during the post-ODP, pre-R-point stage of G_1-phase, without causing a withdrawal of cells from the cell-cycle into a quiescent state.

2. Materials and methods

2.1. Cell culture and synchrony

CHOC 400 cells, a derivative of CHO cells in which the DHFR gene has been amplified to approximately 1000 copies per cell by stepwise selection in methotrexate [13], were cultured as described [12]. Lovastatin (Sigma, M2147) was dissolved directly in 60% ethanol and stored at −20°C, preserving its β-lactone ring structure. We did not have success in synchronizing greater than 80% of CHOC 400

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cells in G_1-phase with the activated, open ring form of lovastatin, which was converted from the pro-drug form as described [4]. Asynchronous cultures of CHOC 400 cells were treated with 5 μM lovastatin pro-drug. Within 24 h cells began to round up in culture even though 10% still remained in S-phase as measured by BrdU incorporation. By 48 h of treatment, approximately 40% of cells detached from the tissue culture dish. 32 h incubation was found to be optimal for synchrony without significant cell detachment. These observations are consistent with those of Rao et al. [8], who showed that 5 μM of lovastatin pro-drug was sufficient for G_1-phase synchrony of a human tumor cell line but that 10 μM resulted in significant apoptosis. To release synchronized cells, lovastatin medium was removed, cells were washed three times with DMEM medium and fresh medium was added. In some experiments, fresh medium was supplemented at this point with mevalonic acid (Sigma, M4667) at the indicated concentrations (Fig. 2). Mitotic synchrony was achieved as described [14].

2.2. Flow cytometry and immunohistochemistry

For flow cytometry, cells were trypsinized and fixed with 70% ethanol. Cells were then resuspended with phosphate buffered saline (PBS) containing 1 mg/ml RNase A and incubated at 37°C for 30 min. 1×10^6 cells were stained with 20 μg/ml propidium iodide and analyzed for DNA content with a Becton Dickinson FACSII flow cytometer. In parallel experiments, aliquots of cells were labeled with 33 μg/ml of BrdU for 30 min, and then stained with anti-BrdU antibodies as described [15].

2.3. Immunoblotting

Cells were washed twice with PBS, and lysed directly on the dish in loading buffer (50 mM Tris (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue). Cell lysates were scraped into Eppendorf tubes, boiled in a water bath for 5 min and stored at −20°C. Protein concentration was determined by acid precipitation of proteins, followed by the Bio-Rad protein assay, according to Bio-Rad instructions. Approximately 50 μg of the cell lysates were subjected to 6.5% SDS–PAGE gel. Proteins were transferred to an Immobilon-P membrane, incubated with anti-Rb (C-15; Santa Cruz Biotechnology) and detected by ECL as described [12].

2.4. Preparation of intact nuclei and in vitro replication in Xenopus egg extracts

Cells were permeabilized with digitonin on ice for 5 min, and the percentage of intact nuclei were measured by fluorescence microscopy as described [14]. *Xenopus* egg extracts were prepared as described [14]. Intact nuclei were incubated with *Xenopus* egg extracts (17 000 nuclei per μl extract) in the presence or absence of 3 mM 6-dimethylaminopurine (6-DMAP) at 21°C, as described [14]. The fraction of DNA that replicated was measured by acid precipitation as described [14].

2.5. The early labeled fragment hybridization (ELFH) assay for mapping replication origin sites

The ELFH assay has been described in detail [14], with subsequent modifications [16]. Briefly, intact nuclei were incubated with *Xenopus* egg extracts (17 000 nuclei per μl extract) in the presence of aphidicolin (100 μg/ml) at 21°C for 2 h. Nuclei were then washed free of extract and aphidicolin and nascent DNA synthesized close to origin sites was pulse labeled in vitro with [α-^{32}P]dATP. Radiolabeled DNA was then isolated, sheared to small fragments and hybridized to a panel of probes spanning 120 kb of the DHFR locus, which were immobilized on nylon membranes. The relative amounts of hybridization of nascent DNA to each of these probes was then normalized for probe size, dATP content and hybridization efficiency as described [14], and then plotted vs. map position to give a curve describing the relative frequency of initiation at different sites throughout the DHFR locus.

3. Results and discussion

3.1. The pro-drug form of lovastatin arrests CHO cells in G_1-phase, prior to the R-point

Despite several attempts, we were not successful in synchronizing greater than 80% of CHOC 400 cells in G_1-phase with the activated, open ring form of lovastatin (not shown). Since

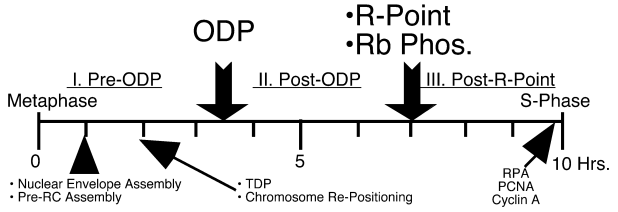

Fig. 1. G_1-phase hallmarks in CHOC 400 cells. The assembly of a nuclear envelope [10,21], including a complete nuclear membrane and lamina takes place within approximately 45 min after metaphase. Coincident with nuclear envelope formation is the association of Mcm proteins with chromatin [22], a hallmark of pre-replication complex (pre-RC) assembly and the transition to replication competence (often referred to as replication 'licensing'). Over the next 60–90 min, DNA sequences are gradually re-positioned within the nucleus and, by 2 h after mitosis, the program for the order in which chromosomal segments will be replicated is established [21] (timing decision point; TDP). Between 3 and 4 h after metaphase cells pass through the origin decision point (ODP), when the specific sequences at which replication will initiate within the CHO DHFR locus are established [10]. Phosphorylation of the retinoblastoma tumor suppressor protein (Rb) takes place 6–8 h after metaphase, coincident with the R-point [12]. Within 2 h after passage through the R-point, cyclin A protein accumulates [12], DNA synthesis proteins RPA and PCNA associate with replication origins [22], and DNA synthesis begins (approximately 50% of cells enter S-phase at 10 h after metaphase; [10–12]).

it was recently reported that the pro-drug form of lovastatin may be more effective at synchronizing mammalian cells in G_1-phase [8], we treated CHOC 400 cells with the pro-drug form of lovastatin, which does not inhibit HMG-CoA reductase [8]. After 32 h exposure to lovastatin, less than 5% remained in S-phase (as measured by BrdU incorporation) and flow cytometry analysis indicated that greater than 90% were in G_1/G_0-phase (Fig. 2). To determine whether mevalonic acid was necessary to release cells from lovastatin arrest, lovastatin synchronized cells were transferred to fresh medium containing various concentrations of mevalonic acid (Fig. 2). Results revealed that the presence of mevalonic acid had no influence on progression of the cell-cycle after release from lovastatin. Together, these data are consistent with those reported by Rao et al. [8], suggesting that lovastatin does not inhibit the cell-cycle through an effect on HMG-CoA reductase but through an independent mechanism dependent on the pro-drug form of lovastatin. Previous results reporting cell synchrony with the open ring form of lovastatin were likely due to the fact that preparations of lovastatin after chemical conversion to the open form are still significantly contaminated with the β-lactone pro-drug [8].

Having demonstrated efficient synchrony of CHOC 400 cells with the lovastatin pro-drug, we first wanted to determine whether cell-cycle arrest was prior to the R-point. We have previously shown that the R-point is coincident with phosphorylation of the retinoblastoma (Rb) tumor suppressor protein in cycling cells (Fig. 1 and [12]). Hence, extracts prepared from lovastatin-arrested cells were analyzed by immunoblotting with an anti-Rb antibody. Results revealed that, after 24 h of lovastatin treatment (when 10% of cells still could be detected in S-phase, as BrdU-positive cells), both phosphorylated and dephosphorylated forms of Rb were detectable whereas after 32 h, only the unphosphorylated form of Rb was detected (Fig. 3A). After release from lovastatin arrest, Rb phosphorylation was not observed for 6–9 h. Con-

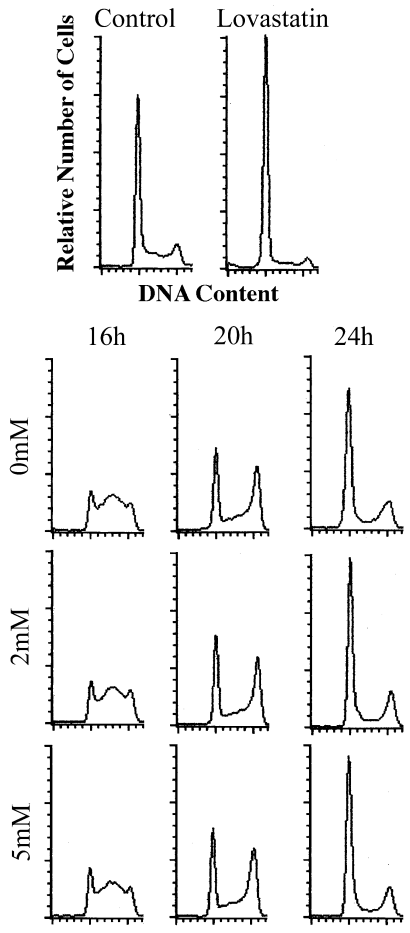

Fig. 2. CHO cells are arrested in a G_1/G_0-phase by the pro-drug of lovastatin. Asynchronous cultures of CHOC 400 cells were incubated in the absence (control) and presence (lovastatin) of 5 μM pro-drug form of lovastatin for 32 h. Aliquots of lovastatin-arrested cells were released for 16, 20 or 24 h in the absence or presence of the indicated concentration of mevalonate. Cells were then harvested and DNA content was analyzed by flow cytometry.

sistent with our previous results [12], 50% of cells entered S-phase 2–3 h after Rb phosphorylation (Fig. 3B).

Since the R-point is defined as a transition from serum dependent to serum independent entry into S-phase, we examined whether the entry into S-phase after release from lovastatin arrest required serum mitogens. Cells were released from a lovastatin arrest and shifted to serum-free medium at various times thereafter. All cultures were collected at 15 h after release, pulse labeled with BrdU, and the percentage of cells that had entered S-phase was evaluated (Fig. 3C). Results revealed that lovastatin-arrested cells required serum for entry into S-phase and gradually passed through a transition to serum independent entry into S-phase 6–9 h after release from the lovastatin block. This transition coincides with the time of Rb phosphorylation under these conditions. We conclude from these experiments that the lovastatin arrest point is upstream of the R-point.

3.2. Cells arrested by lovastatin are replication competent

Cells deprived of growth factors or exposed to inhibitors of protein synthesis during G_1-phase exit the cell-cycle and enter a state of quiescence termed G_0 [1]. Since G_0 and G_1 cells both harbor unreplicated genomes, the experiments described in Figs. 2 and 3 cannot distinguish whether cells arrested by lovastatin have exited the cell-cycle and entered G_0, or whether they have remained in G_1-phase. Recent studies have demonstrated that intact nuclei from quiescent cells are not competent for DNA replication when introduced into *Xenopus* egg extracts [12,17,18], whereas nuclei collected during G_1-phase initiate DNA replication efficiently in these same extracts [10,12]. To determine whether nuclei from lovastatin-arrested cells are replication competent, CHOC 400 cells were treated with the pro-drug form of lovastatin for 32 h. Aliquots of these cells were released in fresh medium for 2 or 6 h or remained arrested. Intact nuclei were then prepared from each of these samples, introduced into a *Xenopus* egg extract, and the efficiency of DNA replication was evaluated. Results (Fig. 4) revealed that the efficiency of DNA replication with nuclei from cells in the lovastatin-arrested state (0 h) was indistinguishable from that with nuclei isolated from cells released for 2 or 6 h. Both the efficiency of DNA replication and the length of the lag period before the onset of in vitro DNA synthesis was similar to that observed in our previous studies with nuclei from cells synchronized in G_1-phase after mitotic selection [10,12,14]. By contrast, DNA synthesis was almost undetectable within nuclei from these same cells after they were arrested in G_0 by serum or isoleucine deprivation [12].

Several characteristics of DNA synthesis with nuclei from

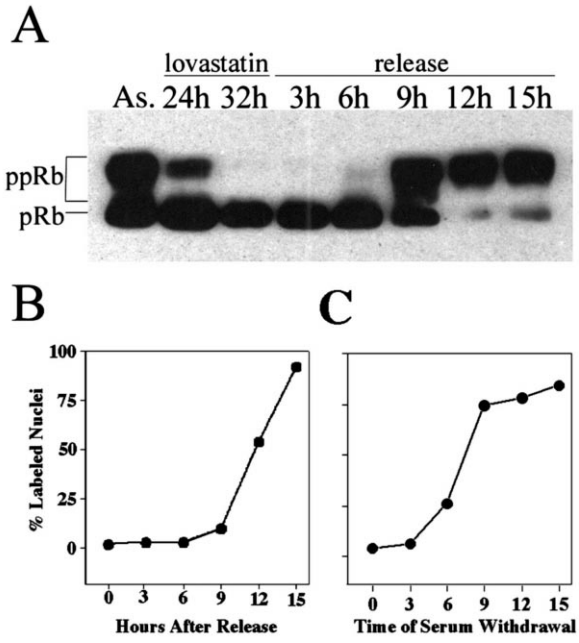

Fig. 3. CHO cells are arrested by lovastatin with hypophosphorylated Rb. A: Cell lysates were prepared from cells treated with lovastatin for 24 or 32 h and from cells treated for 32 h and released for 3, 6, 9, 12, or 15 h and subjected to immunoblotting with an anti-Rb antibody. Cell lysates from asynchronous cells (As.) were also analyzed as a control. The unphosphorylated (pRb) and phosphorylated (ppRb) forms of Rb are indicated. B: Cells from A treated with lovastatin for 32 h and released were pulse labeled with BrdU for 30 min, fixed and stained with an anti-BrdU antibody. The percentage of labeled nuclei at each time point was scored. C: Lovastatin-arrested cells were released into complete medium. At the indicated time, cells were washed in serum-free medium and further incubated in serum-free medium. At 15 h after release, all cultures were pulse labeled with BrdU and the percentage of cells that had entered S-phase was evaluated as in B.

lovastatin-arrested cells were consistent with the de novo initiation of DNA replication, rather than DNA repair synthesis or the elongation of pre-primed templates within contaminating S-phase cells. First, pulse labeling lovastatin-arrested cells in culture with BrdU prior to nuclear isolation revealed that less than 5% of these cells were synthesizing DNA at the time of nuclear isolation, whereas >99% incorporated BrdUTP in vitro, demonstrating that the DNA synthesis observed is dependent upon the in vitro replication system. Second, in vitro DNA synthesis did not begin until after a 10–30 min lag period, which is required for the assembly of initiation complexes within G_1-phase nuclei, whereas DNA synthesis within S-phase nuclei begins immediately upon contact with the extract [14,15]. Finally, in vitro DNA synthesis was sensitive to 6-DMAP (see Fig. 4), a protein kinase inhibitor that prevents the initiation of DNA replication but not elongation or repair synthesis [14,15,19]. Taken together, these results demonstrate that lovastatin arrest does not result in the loss of replication competence, strongly suggesting that lovastatin does not cause cells to enter into a quiescent state similar to the G_0 state of serum-arrested cells. Further insight into the nature of the lovastatin-arrested state will require the examination of pre-replication complex proteins, cyclins, cyclin dependent kinases (cdks), and cdk inhibitors.

3.3. Lovastatin arrests CHO cells in the post-ODP stage of G_1-phase

Fig. 3B shows that cells do not enter S-phase until 11–12 h after release from lovastatin arrest. This length of time is at least as great as the entire length of G_1-phase (Fig. 1). This could indicate that cells require a recovery period after lovastatin treatment, or it could mean that cells are arrested very early in G_1-phase. Since we have determined several early G_1-phase hallmarks in CHOC 400 cells (Fig. 1), we were in a good position to distinguish between these two possibilities. We first examined whether lovastatin-arrested cells are in the pre-ODP or post-ODP stages of G_1-phase by mapping the sites of initiation within the DHFR locus when nuclei are introduced into *Xenopus* egg extracts [14]. Nuclei from lovastatin-arrested cells and cells released from lovastatin arrest for 6 h were introduced into a *Xenopus* egg extract containing aphidicolin. Aphidicolin allows the initiation of replication and the formation of short primers but prevents the processive elongation of replication forks, resulting in the trapping of nascent DNA strands close to their sites of initiation [14,15]. These nascent strands were then radioactively labeled and hybridized to a panel of 18 probes distributed over a 120 kb region within the DHFR locus [14,15]. Initiation sites within the DHFR locus were then revealed by plotting the relative amounts of label at each map position (Fig. 5). As a control for cells in the pre-ODP stages of G_1-phase, cells were synchronized in mitosis and released into G_1-phase for 2 h. As shown in Fig. 5, initiation of DNA replication took place within the DHFR origin locus with nuclei from lovastatin-arrested cells as well as cells released from lovastatin arrest for 6 h, whereas initiation sites were distributed throughout the DHFR locus in nuclei from pre-ODP control cells. We conclude that the lovastatin pro-drug arrests CHO cells in the post-ODP stage of G_1-phase.

In normal cycling cells, phosphorylation of Rb takes place 3–4 h after the ODP and entry into S-phase takes place 6–7 h

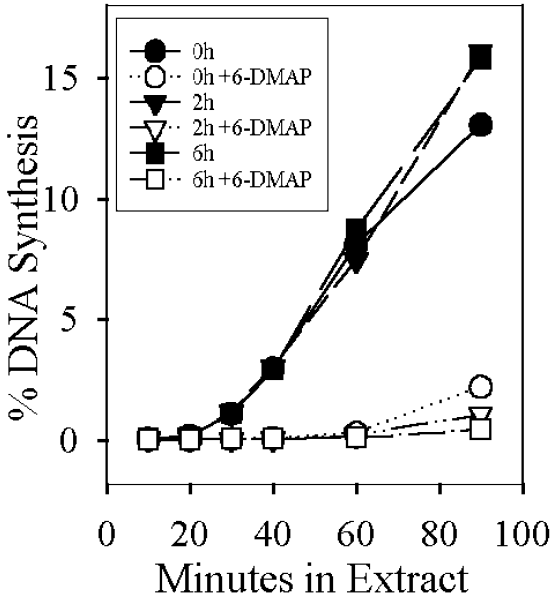

Fig. 4. Lovastatin-arrested cells are in a replication competent state. Intact nuclei were prepared from lovastatin-arrested cells and from cells released from lovastatin arrest for 6 h. Nuclei were then introduced into a *Xenopus* egg extract supplemented with [α-^{32}P]dATP in either the absence (closed symbols) or presence (open symbols) of 3 mM 6-DMAP. The percentage of input DNA replicated was determined at the indicated times by acid precipitation [14].

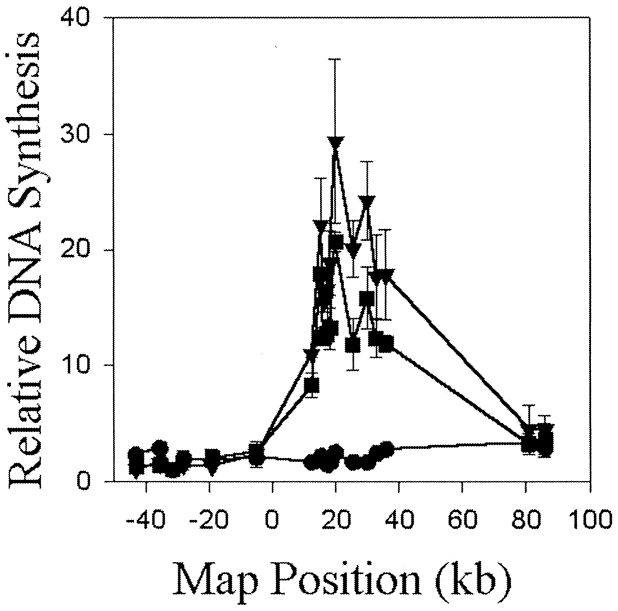

Fig. 5. Lovastatin arrests CHO cells in the post-ODP stage of G_1-phase. Intact nuclei were prepared from lovastatin-arrested cells (squares) and from cells released from lovastatin arrest for 6 h (triangles). Nuclei were then introduced into a *Xenopus* egg extract and the sites of initiation were evaluated using the ELFH assay (Section 2). The relative amounts of hybridization of the earliest labeled nascent DNA to each probe from the DHFR locus are plotted against the map position of each probe. As a control, intact pre-ODP nuclei (circles) were prepared from cells synchronized in mitosis and released into G_1-phase for 2 h, as described [10,12,14]. Shown are the mean values from three independent experiments and the standard deviation of the mean.

after the ODP (Fig. 1). Clearly, lovastatin-arrested cells, which take 11–12 h to enter S-phase after release (Fig. 3B) yet are arrested in the post-ODP stage of G_1-phase (Fig. 5), must require several hours to recover from the effects of the drug. Since lovastatin arrest does not result in the entry of cells into a quiescent state (Fig. 4), what might be the basis of this recovery period? Other investigators have shown that lovastatin induces the disassembly of actin stress fibers within human mesangial cells within 24 to 48 h [20]. We observed that CHOC 400 cells treated with lovastatin are rounder in shape than untreated cells and re-flatten after release. We have also shown that CHOC 400 cells become arrested in the post-ODP stages of G_1-phase when they are prevented from attaching to a solid substrate (S.M. Keezer and D.M. Gilbert, unpublished observations). Hence, one possibility is that lovastatin-arrested cells need time to reform actin stress fibers and return to a normal morphology before they can passage through the R-point and enter S-phase.

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