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Effect of Cyclin E Overexpression on Lovastatin-Induced G1 Arrest and RhoA Inactivation in NIH3T3 Cells

Paramita M. Ghosh,[1] Marissa L. Moyer,[1] Glen E. Mott,[1] and Jeffrey I. Kreisberg[1,2]*

[1]Department of Pathology, University of Texas Health Science Center, San Antonio, Texas 78284
[2]South Texas Veterans Health Care System, Audie Murphy Branch, San Antonio, Texas 78284

Abstract The HMG-CoA reductase inhibitor, lovastatin, blocks targeting of the Rho and Ras families of small GTPases to their active sites by inhibiting protein prenylation. Control NIH3T3 cells, and those overexpressing human cyclin E protein were treated with lovastatin for 24 h to determine the effects of cyclin E overexpression on lovastatin-induced growth arrest and cell rounding. Lovastatin treatment (10 μM) of control 3T3 cells resulted in growth arrest at G1 accompanied by actin stress fiber disassembly, cell rounding, and decreased active RhoA from the membranous protein fraction. By contrast, in NIH3T3 cells overexpressing cyclin E, lovastatin did not cause loss of RhoA from the membrane (active) protein fraction, actin stress fiber disassembly, cell rounding or growth arrest within 24 h. Analysis of cell cycle proteins showed that 24 h of lovastatin treatment in the control cells caused an elevation in the levels of the cyclin-dependent kinase inhibitor $p27^{kip1}$, inhibition of both cyclin E- and cyclin A-dependent kinase activity, and decreased levels of hyperphosphorylated retinoblastoma protein (pRb). By contrast, lovastatin treatment of the cyclin E overexpressors did not suppress either cyclin E- or cyclin A-dependent kinase activity, nor did it alter the level of maximally phosphorylated pRb, despite increased levels of $p27^{kip1}$. However, by 72 h, the cyclin E overexpressors rounded up but remained attached to the substratum, indicating a delayed response to lovastatin. In contrast with lovastatin, inactivation of membrane-bound Rho proteins (i.e., GTP-bound RhoA, RhoB, RhoC) with botulinum C3 transferase caused cell rounding and G1 growth arrest in both cell types but did not inhibit cyclin E-dependent histone kinase activity in the cyclin E overexpressors. In addition, 24 h of cycloheximide treatment caused depletion of RhoA from the membrane (active) fraction in neo cells, but in the cells overexpressing cyclin E, RhoA remained in the active (membrane-associated) fraction. Our observations suggest that (1) RhoA activation occurs downstream of cyclin E-dependent kinase activation, and (2) overexpression of cyclin E decreased the turnover rate of active RhoA. J. Cell. Biochem. 74:532–543, 1999.

Key words: $p27^{kip1}$; C3 transferase; morphology; cell cycle; actin stress fibers

Cell cycle progression is accomplished by the sequential activation of cyclin-dependent kinases (cdks). The key regulatory elements in cdk activation are the cyclin subunits, which form complexes with cdks [Graña and Reddy, 1995]. Ectopic expression of cyclins D or E accelerated the G1 phase of the cell cycle, indicating that they were rate-limiting [Ohtsubo and Roberts, 1993; Quelle et al., 1993; Resnitzky et al., 1994]. The important cyclin-cdk complexes required in mammalian cells for cell-cycle progression from G1 to S phase, past the restriction point, are cyclins D1–3/cdk4 or 6, cyclin E/cdk2, and cyclin A/cdk2 [Won et al., 1992; Koff et al., 1991, 1992; Girard et al., 1991; Zindy et al., 1991; Bates et al., 1994]. Both cyclin D- and cyclin E-dependent kinases must phosphorylate the retinoblastoma protein, pRb, to release and activate transcription factors such as E2F [Lundberg and Weinberg, 1998; Kato et al., 1993; Buchkovich et al., 1989; Chen et al., 1989; Mihara et al., 1989]. E2F transcriptionally activates genes, such as cyclin A, which maintains pRb phosphorylation through S phase [Chelappan et al., 1991; Devoto et al., 1992; Resnitzky et al., 1995; Nevins et al., 1997]. The enzymatic activity of cyclin/cdk complexes is also modulated by cdk inhibitors, including $p27^{kip1}$, $p21^{cip1}$, and $p16^{ink4}$ [for review, see Sherr and Roberts, 1995; Peeper et al., 1994]. For example, $p27^{kip1}$ is expressed at high levels in

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*Correspondence to: Jeffrey I. Kreisberg, Department of Pathology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284.
E-mail: kreisberg@uthscsa.edu

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quiescent cells and in cells in G1 arrest after treatment with transforming growth factor-TGF-β, cyclic adenosine monophosphate (cAMP), or lovastatin [Hengst and Reed, 1996; Hengst et al., 1994; Kato et al., 1994; Ravitz et al., 1996; Winston et al., 1996]. Both cyclin D/cdk4 and cyclin E/cdk2 compete for binding to inhibitor proteins such as $p27^{kip1}$ [Sherr and Roberts, 1995]. After growth factor stimulation, cyclin D1 transcription is stimulated, which results in cdk4/cdk6 activation [Zhu et al., 1996]. Elevated levels of cyclin D1 protein bind to and deplete inhibitory proteins, such as $p27^{kip1}$, which results in activation of cyclin E/cdk2 and maximum phosphorylation of pRb [Lundberg and Weinberg, 1998]. E2F is released after pRb phosphorylation, cyclin A/cdk2 is activated, and DNA synthesis is initiated [Chelappan et al., 1991; Girard et al., 1991].

Cells also require an intact cytoskeleton to progress through the restriction point [Bohmer et al., 1996]. Treatment of cells with cytochalasin D to disrupt actin microfilaments results in G1 arrest despite treatment with growth factors. The development of actin stress fibers may provide a mechanical signal by increasing cell tension, affecting expression of genes necessary for cell cycle progression [Wilson et al., 1995; Maniotis et al., 1997]. Stimulation of cell growth by the small GTPase Ras also results in changes in the actin cytoskeleton [Prendergast and Gibbs, 1993]. Microinjection experiments demonstrated that several proteins of the Ras family (e.g., Ras, Rac, and Rho) act in concert and function in a cascade in which activation of Rac by Ras causes membrane ruffling, which in turn stimulates Rho-mediated actin stress fiber assembly [Nobes and Hall, 1995; Chant and Stowers, 1995; Bar-Sagi and Feramisco, 1986; Rodriguez-Viciana et al., 1997]. Microinjection of Rho proteins directly into cells also stimulated cell proliferation [Olson et al., 1995].

Small GTPases such as Rho and Ras act as molecular switches, active when GTP-bound and inactive when GDP-bound [Bourne et al., 1990]. The small GTPases are post-translationally modified by isoprenylation. The Ras proteins (e.g., H-Ras, N-Ras, and K-Ras) are farnesylated, while the Rho family of proteins (e.g., Rho, Rac, cdc42) are geranylgeranylated [Grunler et al., 1994]. Separate classes of signaling proteins regulate GTPase activity by influencing the transition between the active and inactive states. Only isoprenylated small GTPases can be bound by the regulatory proteins [Hori et al., 1991]. Isoprenoid production is inhibited by the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor, lovastatin [Grunler et al., 1994]. At high concentrations, lovastatin arrests cell growth in the G1 phase of the cell cycle by increasing the levels of $p27^{kip1}$ [Hengst et al., 1994; Hengst and Reed, 1996]. Lovastatin treatment also results in cell rounding and loss of adhesion due to disassembly of actin stress fibers [Fenton et al., 1992]. Geranylgeranylpyrophosphate (GGPP) is a metabolite of mevalonic acid whose production is inhibited by lovastatin [Grunler et al., 1994]. The addition of GGPP to lovastatin-treated cells causes protein geranylgeranylation and overcomes the cell growth inhibitory properties of lovastatin [Hirai et al., 1997; Noguchi et al., 1998]. In addition, geranylgeranylation restores Rho localization to its active site in the membrane, which results in actin stress fiber assembly and cell spreading [Ghosh et al., 1997].

In this report, we present data suggesting that activation of RhoA occurs downstream of cyclin E-dependent kinase activation. Overexpression of cyclin E resulted in increased cell growth and actin stress fiber assembly due to increased active (membranous) RhoA. Despite an elevation in $p27^{kip1}$ levels after lovastatin treatment in NIH3T3 cells overexpressing cyclin E, RhoA remained activated (i.e., membrane-bound), pRb remained hyperphosphorylated, and G1/S progression remained unaltered. Our results suggest that activation of RhoA in cyclin E overexpressing NIH3T3 cells resulted from alterations to the turnover rate of membrane-bound RhoA.

MATERIALS AND METHODS

Cell Culture

Control NIH3T3 cells stably transfected with the vector containing the neomycin-resistant gene only and NIH3T3 cells stably overexpressing human cyclin E or cyclin D1 were kindly provided by Dr. Richard K. Assoian (Department of Pharmacology, University of Pennsylvania, Philadelphia, PA)[(Zhu et al., 1996]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 1% Fungi-Bact Solution (Irvine Scientific, Santa Ana, CA), and 400 μg/ml G418 Sulfate (Mediatech, Herndon, VA).

Pharmocological Treatment

Lovastatin was a gift from Merck Research Laboratories (Rahway, NJ) and was prepared as described elsewhere [Ghosh et al., 1997]. C3 transferase exoenzyme was prepared and transfected into cells with lipofectin (Gibco) as described previously [Chong et al., 1994; Kreisberg et al., 1997]. Cycloheximide (Sigma Chemical Co., St. Louis, MO) was dissolved in water at a stock concentration of 10 mg/ml and was used at a final concentration of 10 μg/ml.

Fluorescence Microscopy

TRAMP cells were grown on coverslips under required conditions. After the experiment, the coverslips were fixed for 1 h in a modified Zamboni fixative (800 mg paraformaldehyde dissolved in 3 ml hot saturated picric acid by dropwise addition of 1 N NaOH, cooled, 180 mg NaCl added, and the volume adjusted to 20 ml with 50 mM Na phosphate buffer, pH 7.4). The fixed cells were permeabilized with 0.4% Triton X-100 in PBS for 2 min at room temperature, and incubated 20 min in 2 U/ml rhodamine phalloidin (Molecular Probes, Eugene, OR). Observations were made under standard epifluorescence optics (Zeiss, Thornwood, NJ).

Growth Curves

Cell growth was assessed over a period of 5 days. Cells were plated in duplicate in 35-mm dishes at a concentration of 0.5×10^4 cells/dish, trypsinized and counted using a hemocytometer. Each point on the growth curve represents the mean of two dishes, each dish being counted three times.

Flow Cytometry

NIH3T3 cells were grown under desired conditions in 100-mm dishes at 500,000 cells/dish. For flow cytometry, the cells were trypsinized, pipetted off the surface in 2 ml of growth medium, and spun down in a tabletop centrifuge. The medium was aspirated, the pellet washed once in phosphate-buffered saline (PBS) and the cells resuspended in 500 μl 70% ethanol. After incubation for 30 min at −20°C, the cells were repelleted, and washed twice in 1% BSA/PBS. For nuclear staining with propidium iodide, the cells were suspended in staining medium (150 μl PBS, 50 μl of 1 mg/ml RNase A [Sigma], and 100 μl of 100 μg/ml propidium iodide [Sigma] and incubated overnight at 4°C. Flow cytometry was conducted on FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were illuminated with 200 mW of 488 nm light produced by an argon-ion laser. Fluorescence was read through a 630/22 nm bandpass filter. Data were collected on 20,000 viable cells as determined by forward and right angle light scatter and stored as frequency histograms, and then analyzed using MODFIT (Verity software, Topsham, ME).

Western Blotting

NIH3T3 cells were grown on 100-mm dishes at 500,000 cells/dish. Whole cell extracts were prepared by washing the cells twice in PBS, and lysing in 250 μl RIPA buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 10 mM EGTA, 20 mM β-glycerophosphate, 150 mM NaCl, 0.1% Nonidet P-40, 50 μg/ml PMSF, 10 μg/ml SBTI, 2 μg/ml leupeptin, 1 μg/ml aprotinin). For Triton X-114 phasing, cells were washed twice in ice-cold PBS, and scraped into 3 ml lysing buffer as described elsewhere [Ghosh et al., 1997]. Proteins were quantitated using a BCA assay (Pierce, Rockford, IL) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed at 45 mA for about 4 h, using vertical slab units (Hoeffer, San Fransisco, CA). The gels were electroblotted overnight at 200 mA using a Trans-Blot Cell (BioRad, Hercules, CA) onto 0.2-μm PVDF membrane (BioRad Laboratories, Hercules, CA). The blots were stained with antibodies (Santa Cruz Laboratories, Santa Cruz, CA), which were detected by enhanced chemiluminescence (Pierce) after incubation with a horseradish peroxidase (HPO)-labeled secondary antibody.

Immunoprecipitations and Kinase Assays

Cells were grown on 100-mm dishes at 500,000 cells/dish under required conditions. For immunoprecipitation, 250 μg of protein obtained from whole cell lysates in RIPA buffer was precleared with 25 μl of 50% protein A-sepharose beads in 400 μl of RIPA containing 1 μg/ml BSA for 1 h. The supernatants were incubated with the appropriate antibody (1 μg/

sample) overnight. Next, 20 µl of protein A-sepharose was added for 1 h, and the immunocomplexes washed three times with RIPA. Samples were separated by SDS-PAGE, and proteins detected by Western blotting. Cdk2 histone kinase assays were performed as per Zhu et al. [1996]. 50 mg of total protein was immunoprecipitated as before with the appropriate antibody, the samples washed twice in RIPA, and twice in 1× kinase buffer (20 mM Tris, pH 7.5, 4 mM $MgCl_2$). The beads were resuspended in reaction mix (4 µl of 5× kinase buffer, 25 µM ATP, 1 mM DTT, 2 µg Histone H1, 5 mCi γ^{32}P-ATP (3,000 Ci/mmol), water to 20 µl). The mixture was incubated at 37°C for 30 min, at the end of which, 4× Laemmli sample buffer was added to 1× concentration, and samples run on 12% SDS-PAGE. Gels were dried and exposed to X-ray film at −80°C. It was determined that the histone kinase assays were within the linear range.

RESULTS

Overexpression of Cyclin E in NIH3T3 Cells Delays Lovastatin-Induced Cell Rounding and Actin Stress Fiber Disassembly

NIH3T3 cells transfected with the neomycin-resistant vector only (to be referred to as neo cells) were elongated and contained actin stress fibers (Fig. 1A). Overexpression of human cyclin E in these cells caused an apparent increase in the amount of actin stress fibers, resulting in a flatter cell morphology (Fig. 1B). This suggested that cyclin E affected the assembly of the actin cytoskeleton. The Rho family of small GTPases regulate the assembly of the actin cytoskeleton [Hall, 1998]. These proteins

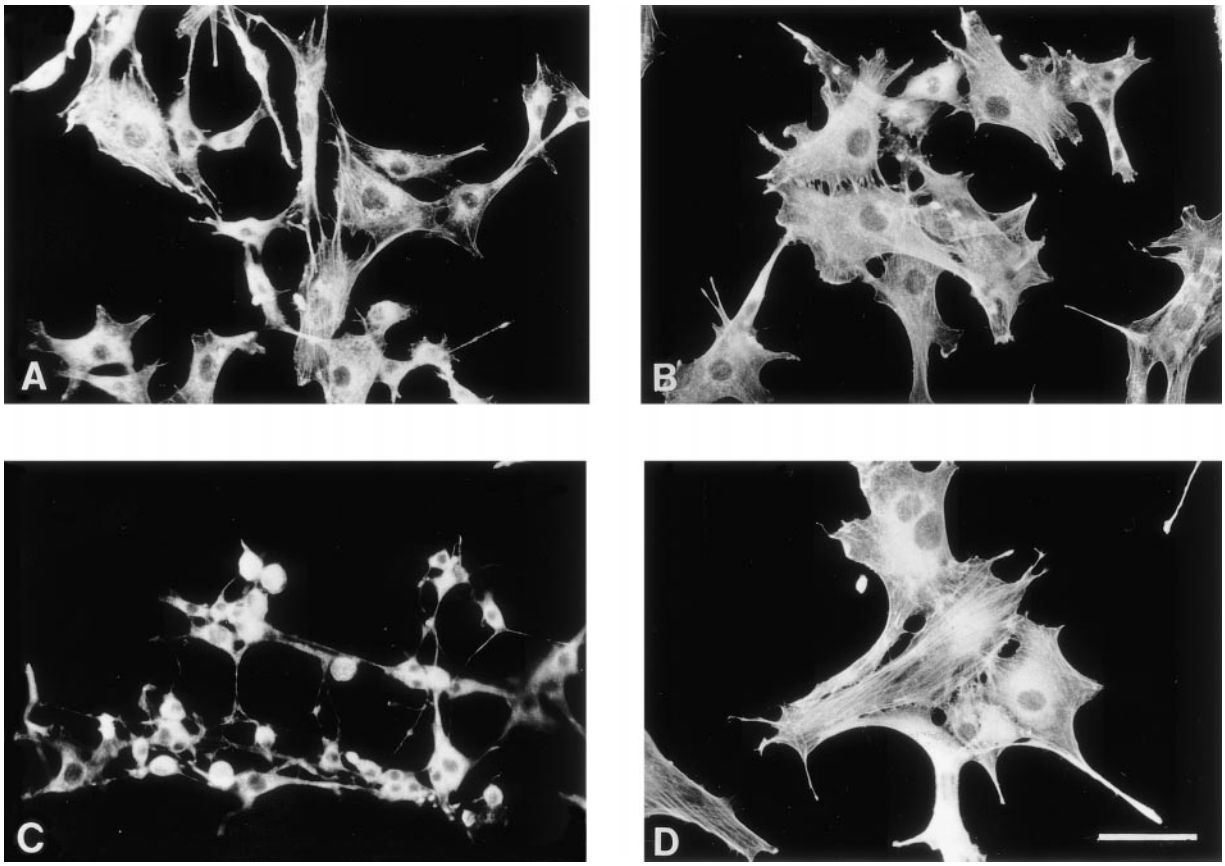

Fig. 1. Fluorescence photomicrographs showing the effect of 24 h of treatment with 10 µM lovastatin on cell morphology and actin stress fiber assembly as detected by rhodamine phalloidin staining. **A:** Untreated neo cells were elongated and displayed actin stress fibers. **B:** NIH3T3 cells overexpressing cyclin E displayed abundant actin stress fibers and appeared to be flatter than neo cells. **C:** Lovastatin-treated neo cells were round and displayed actin filament disassembly. **D:** The cyclin E overexpressors did not round up after 24 h of treatment with 10 µM lovastatin. Scale bar = 50 µm.

require isoprenylation for activation [Fenton et al., 1992], a process that is prevented by the inhibition of isoprenoid synthesis by lovastatin [Grunler et al., 1994]. To study the mechanism by which cyclin E affects actin cytoskeleton assembly, and to determine whether this process is mediated by small GTPases, we treated both the neo cells and the cyclin E overexpressors with lovastatin. In neo cells, 24 h of lovastatin treatment (10 μM) resulted in cell rounding and actin stress fiber disassembly (Fig. 1C). By contrast, the cyclin E overexpressors remained flat and well spread, and actin stress fibers remained intact in the presence of lovastatin (Fig. 1D). Overexpression of cyclin D1, on the other hand, did not protect NIH3T3 cells from the effects of lovastatin (not shown). By 72 h of lovastatin treatment, the neo cells had detached from the culture dish and were apoptotic as evidenced by staining with the DNA binding fluorochome Hoechst 33825 [Oberhammer et al., 1993] (not shown). On the other hand, after 72 h of lovastatin treatment, many of the cyclin E overexpressors were round, and actin stress fibers were disassembled, but the cells remained attached. By 5 days of lovastatin treatment, some of the cyclin E overexpressing cells had detached from the substratum and undergone apoptosis (not shown). Thus, the effects of lovastatin on the cyclin E overexpressors were delayed in comparison to its effects on the neo cells.

Overexpression of Cyclin E Delayed Growth Arrest Induced by Lovastatin

Growth curves of the two cell types grown in the presence or absence of lovastatin are shown in Figure 2. Similar to previous reports [Zhu et al., 1996; Ohtsubo and Roberts, 1993], the neo cells grew at a slower rate than 3T3 cells overexpressing cyclin E. On lovastatin treatment, the rate of growth was dramatically decreased in neo cells, but the cyclin E overexpressors were largely unaffected (Fig. 2). Cells were subjected to flow cytometric analysis to determine whether this was due to a growth arrest in the G1 phase of the cell cycle (Table I). After 48 h of lovastatin treatment, although both types of cells displayed fewer cycling cells, 60% more cyclin E overexpressing cells were in S-phase compared with neo cells (Table I). After 72 h of lovastatin treatment, the cyclin E overexpressing cells were growth arrested in G1 (not shown).

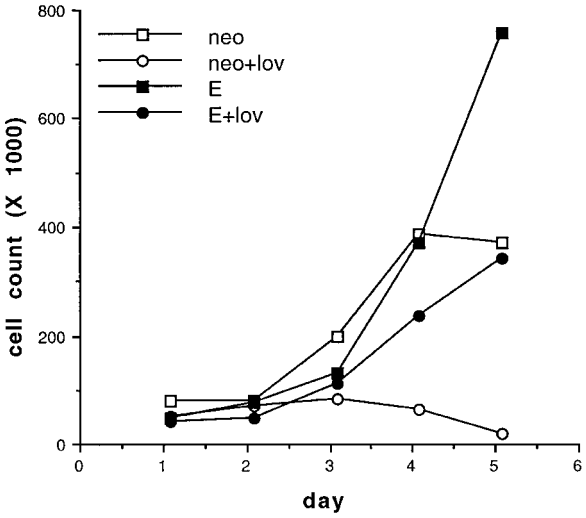

Fig. 2. Effect of lovastatin on cell proliferation. Neo cells and the cyclin E overexpressing cells were plated in duplicate in 35-mm dishes at a concentration of 0.5×10^4 cells/cm^2 either untreated or in the presence of 10 μM lovastatin. At the end of 1–5 days, the number of attached cells was counted using a hemocytometer under a light microscope. Untreated neo cells exhibited an initial growth rate comparable to that of the cyclin E overexpressors, but while the latter kept on growing even after 5 days, the neo cells did not. neo cells in lovastatin alone displayed decreased cell numbers after 72 h, but the cyclin E overexpressors did not show an indication of slowing down until day 5, although some of these cells had rounded up by day 3. Each time point is the mean ±S.D. of two dishes. Repetition of this experiment several times yielded similar results.

TABLE I. Flow Cytometric Analysis of Various Clones of NIH3T3 Cells With or Without 10 μM Lovastatin in the Incubation Medium for 48 h Showing Cells in Each Phase of the Cell Cycle

Conditions	% of cells in G0/G1	% of cells in S-phase	% of cells in G2/M
neo	70.16	18.8	11.04
neo + lovastatin	87.66	5.87	6.47
E	62.29	20.90	16.81
E + lovastatin	73.99	9.31	16.7

Overexpression of Cyclin E Delayed Loss of Membrane-Associated (Active) RhoA After Lovastatin Treatment

Actin stress fiber assembly is regulated by the small GTPase RhoA [Ridley and Hall, 1992]. To determine whether the increase in actin stress fibers observed in the cyclin E overexpressors resulted from an increase in active RhoA, we compared the amount of membrane-associated RhoA versus cytosolic RhoA in the two cell types with or without lovastatin treatment.

Membrane-associated RhoA is activated, i.e., GTP-bound [Takai et al., 1995; Lang et al., 1996]. Overexpression of cyclin E caused an increase in total cellular RhoA (Fig. 3). The increase in actin stress fiber assembly in NIH3T3 cells overexpressing cyclin E was most likely the result of increased expression of membrane-associated (GTP-bound) RhoA in these cells (Fig. 3, cf. C_M with A_M). Also, the difference in cellular responsiveness to lovastatin treatment corresponded to differences in RhoA activation. That is, in neo cells, 24 h of lovastatin treatment resulted in a reduction of membrane-associated RhoA while cytosolic RhoA increased

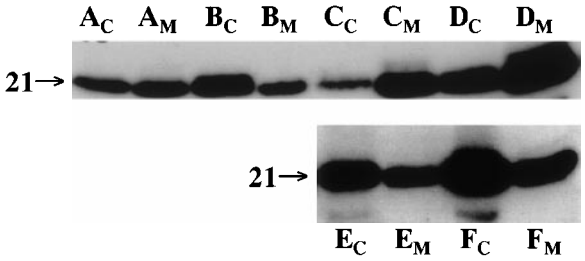

Fig. 3. Western blot analysis of the effect of lovastatin on RhoA (p21) localization in neo and cyclin E overexpressing cells. Cells were incubated with lovastatin (10 μM) for 24 or 72 h, harvested and extracted with Triton X114 into cytosolic (C) and membranous (M) cellular fractions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed and proteins electroblotted and probed with an antibody to RhoA. At 24 h after plating (top), untreated neo cells **(A)** displayed immunoreactive RhoA in the membrane fraction (A_M). Treatment with lovastatin **(B)** caused RhoA to accumulate in the cytosolic fraction (B_C). In untreated cyclin E overexpressors **(C)**, RhoA is found almost exclusively in the membrane cellular fraction (C_M), and after lovastatin addition **(D)**, nearly all RhoA remained in the membrane (D_M). However, after 72 h of lovastatin treatment (bottom), RhoA accumulated in the cytosol (F_C) in these cells as well. 72 h of culture without (E_C, E_M) and with lovastatin (F_C, F_M).

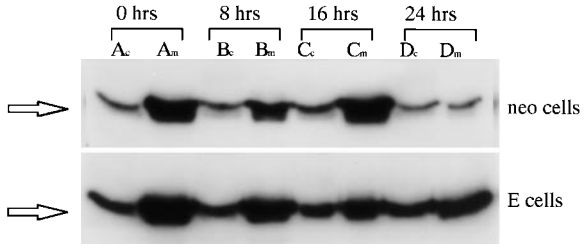

Fig. 4. Western blot estimating the turnover rate of active (membrane-bound) RhoA in neo and cyclin E overexpressing cells. The cells were plated onto 10-cm dishes and incubated with 10 μg/ml cycloheximide for 8–24 h. Neo cells (top) displayed a decrease in active RhoA after treatment for 24 h (Dm), but in the cells overexpressing cyclin E (bottom) RhoA remained membrane-bound (Dm).

(Fig. 3, cf. B_M with B_C). On the other hand, in cells overexpressing cyclin E, RhoA remained in the membrane-associated fraction despite the presence of lovastatin (Fig. 3, D_M). In contrast with RhoA, the small GTPase Ras remained in the membrane at all times in both types of cells (not shown). After 72 h of lovastatin treatment, when the cyclin E overexpressors were round and actin stress fibers were disassembled, RhoA was depleted from the membrane (active) fraction and accumulated in the cytosolic (inactive) fraction (Fig. 3, F_C and F_M), similar to neo cells.

Overexpression of Cyclin E Decreased the Turnover Rate of Membrane-Bound RhoA

The results shown in Figure 3 suggested that the half-life of active (membrane-associated) RhoA increased with overexpression of cyclin E in NIH3T3 cells. To confirm this, neo cells and those overexpressing cyclin E were treated with 10 μg/ml cycloheximide, to prevent RhoA synthesis, leaving the degradative pathway intact. In the presence of cycloheximide for 24 h, there was nearly complete degradation of RhoA from the membrane (active) fraction in the neo cells (Fig. 4, top, D_m), but the cyclin E overexpressors retained RhoA in the membrane (active) fraction (Fig. 4, bottom, D_m). These data suggested that cyclin E overexpression increased the expression of active RhoA by decreasing its turnover rate.

Lovastatin Treatment Elevated $p27^{kip1}$ Levels in Both Neo Cells and Cyclin E Overexpressors but Failed to Inhibit Hyperphosphorylation of pRb and Cyclin E- and Cyclin A-Associated Histone Kinase Activities in Cells Overexpressing Cyclin E

Transition through the restriction point before entry into S phase is dependent on hyperphosphorylation of pRb [Weinberg, 1995]. Both cyclin A- and cyclin E- dependent kinase activity is essential for the G1/S transition, making them major regulatory components of the onset of DNA replication [Lundberg and Weinberg, 1998]. Lovastatin treatment has been shown to induce cell cycle arrest at G1 by increasing the expression of $p27^{kip1}$ [Hengst et al., 1994; Hengst and Reed, 1996]. This results in inactivation of cyclin/cdk complexes and pRb hypophosphorylation. In the absence of lovastatin, $p27^{kip1}$ levels were higher in the neo cells compared with the cyclin E overexpressors, however, 24 h of lovastatin treatment increased $p27^{kip1}$ levels in

both cell types (Fig. 5). The levels of another cdk inhibitor, $p21^{cip1}$ were not altered after cyclin E overexpression (not shown). Cyclin E-associated histone kinase activity was elevated 3× in untreated cyclin E overexpressors compared with neo cells, similar to previous reports [Ohtsubo and Roberts, 1993; Zhu et al., 1996] and remained more than 2× elevated after lovastatin treatment at a level comparable to untreated neo cells (Fig. 6A). Cyclin A-associated histone kinase activity also remained elevated after lovastatin-treatment in cyclin E overexpressing cells, whereas in the neo cells, 24 h of lovastatin treatment resulted in a 70% reduction in kinase activity (Fig. 6B). Similarly, the levels of hyperphosphorylated pRb were increased in the cells expressing human cyclin E, compared with neo cells. After 24 h of lovastatin treatment, the neo cells displayed a decrease in hyperphosphorylated pRb, and a simultaneous increase in hypophosphorylated pRb (Fig. 7). Reflecting the increase in $p27^{kip1}$ on lovastatin treatment, the cyclin E overexpressors also displayed an increase in hypophosphorylated pRb; however, the level of hyperphosphorylated pRb did not decrease.

Inactivation of RhoA With C3 Transferase Exoenzyme Caused Both Neo Cells and NIH3T3 Cells Overexpressing Cyclin E to Round Up and Growth Arrest in G1 Phase of the Cell Cycle

Treatment with the toxin C3 from *Clostridium botulinum* inactivates Rho proteins (RhoA, RhoB, RhoC, but not Rac or Cdc42) by ADP ribosylation at Asn 41 [Just et al., 1995]. Unlike lovastatin, which affects prenylation, and, therefore, activation of only newly synthesized Rho proteins, C3 can inhibit prenylated Rho, including membrane-bound (active) RhoA. To inhibit previously prenylated and membrane-bound Rho, we treated both neo cells and the cyclin E overexpressors with C3 (0.9 μg/ml); 24 h of treatment caused cell rounding, actin stress fiber disassembly, and growth arrest in the G1 phase of the cell cycle in both neo cells and the cyclin E overexpressors (Fig. 8; Table II), demonstrating that the function of active RhoA in cell spreading, actin stress fiber assembly, and cell cycle progression cannot be bypassed by cyclin E overexpression. Cyclin A-associated histone kinase activity was depressed in both types of cells after C3 treatment (Fig. 9A), but cyclin E-associated histone kinase activity was largely unchanged in the cyclin E overexpressors (Fig. 9B).

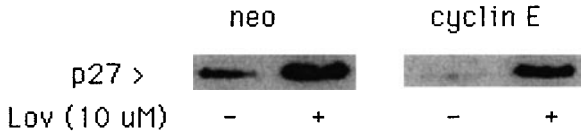

Fig. 5. Western blot showing the amount of total $p27^{kip1}$ present in neo cells and in the cyclin E overexpressors. Note that the cyclin E overexpressors contained less $p27^{kip1}$ than the neo cells. In both cases, the total amount of $p27^{kip1}$ increased after lovastatin treatment.

DISCUSSION

Our results show that NIH3T3 cells overexpressing cyclin E displayed accelerated growth and an increase in actin stress fibers, which resulted in a greater degree of cell spreading than control 3T3 cells. The increase in actin stress fibers correlated with an increase in active, membrane-associated RhoA in the cyclin E overexpressing cells. Both control (neo) cells and the cyclin E overexpressors displayed an increase in $p27^{kip1}$ levels in response to lovastatin treatment; however, only neo cells, and not the cyclin E overexpressors, demonstrated RhoA inactivation, actin stress fiber disassembly, cell rounding and G1 growth arrest after 24 h. In contrast with lovastatin, inactivation of Rho proteins (RhoA, RhoB, and RhoC) with the C3 transferase exoenzyme caused cell rounding, actin stress fiber disassembly and G1 growth arrest in both types of cells. Prolonged treatment with lovastatin (72 h), however, caused the cyclin E overexpressors to round up and RhoA to diminish from the membrane accompanied by growth arrest similar to that seen in the neo cells after 24 h of exposure to lovastatin. This effect was shown to be due to the increased half-life of RhoA in NIH3T3 cells overexpressing cyclin E. Thus, (1) active Rho was necessary for cell spreading and proliferation in both neo cells and the cyclin E overexpressors, but (2) the effect of lovastatin, which prevented prenylation, and therefore, targeting of newly synthesized Rho to its active site in the membrane, was delayed in the cyclin E overexpressors, as compared with the control cells. This delay can be attributed to the continued presence of membrane-bound RhoA in the cyclin E overexpressing cells, caused by a de-

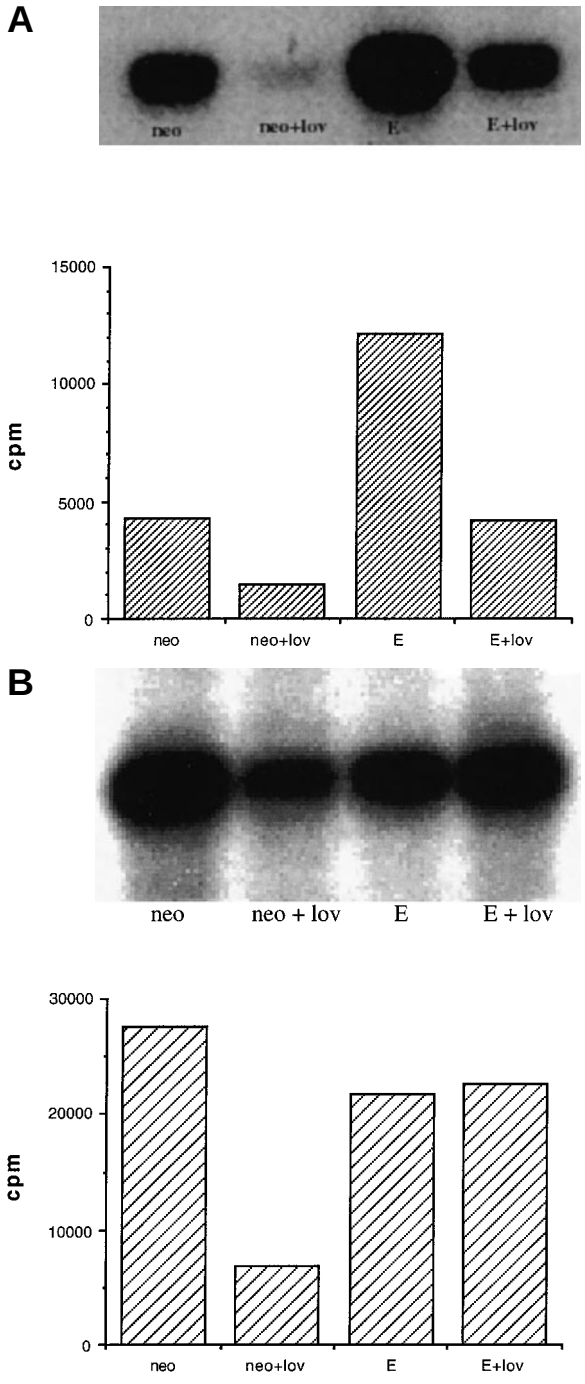

Fig. 6. **A:** Autoradiogram demonstrating increased cyclin E-associated histone kinase activity in the cyclin E overexpressors after 24 h of lovastatin treatment ,as compared with neo cells treated with lovastatin for the same period of time. Cell lysates were collected in RIPA buffer and immunoprecipitated with an antibody to cyclin E. cdk2 activity was assayed by histone phosphorylation. ^{32}P-histone was analyzed on a β-scanner. **B:** Cyclin A-associated histone kinase activity was also assayed after immunoprecipitation with an antibody to cyclin A. As shown, cyclin A-associated histone kinase activity was preserved after 24 h of lovastatin treatment in cyclin E overexpressing cells, but not in the neo cells. The histone kinase assays were within the linear range.

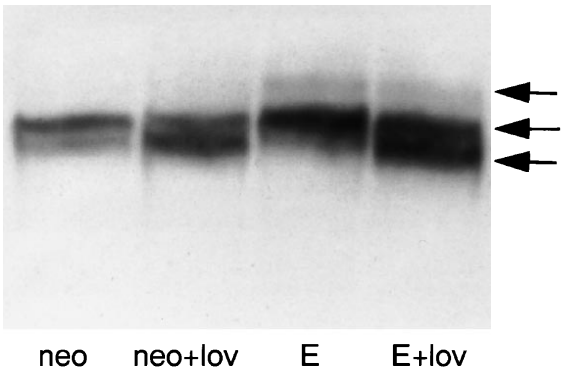

Fig. 7. Western blot demonstrating pRb phosphorylation. The level of pRb phosphorylation affects the migration of the protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Hypophosphorylated or partially phosphorylated pRb migrates more rapidly than does maximally phosphorylated pRb. After lovastatin treatment, neo cells (neo+lov) demonstrated an increase in hypophosphorylated pRb (lowest arrow). In the cyclin E overexpressors (E), there is an increase in maximally phosphorylated pRb (uppermost arrow) which remains unaltered after lovastatin treatment (E+lov).

creased turnover rate of active RhoA in these cells, as compared with the neo cells.

The increase in membrane-bound, and total cellular, RhoA in 3T3 cells overexpressing cyclin E, as well as the increase in the half life of RhoA on cyclin E overexpression, suggests that RhoA acts downstream of cyclin E-associated cdk2 activation. The importance of RhoA in the induction of cyclin E expression, cdk2 activation and G1/S transition in rat astrocytes has been reported [Tanaka et al., 1998]. Recently, Hu et al. [1999] have shown in Chinese hamster embryo fibroblasts, that RhoA regulates cyclin E/cdk2 activity, which is required for $p27^{kip1}$ degradation. Our data shows for the first time that the amount of membrane-bound (active) RhoA increased in response to overexpression of cyclin E. These results imply that cyclin E-dependent kinases regulate events upstream of RhoA activation. In addition to activation of cyclin-dependent kinases, cells require an assembled cytoskeleton to undergo cell cycle progression [Bohmer et al., 1996]. This includes the development of actin stress fibers by activated RhoA. Formation of actin stress fibers and cell spreading is required for the maximal expression of cyclin D1 [Bohmer et al., 1996]. This is followed by cyclin E expression. Phosphorylation of pRb by cyclin D1/cdk4 or 6 and cyclin E/cdk2 releases the E2F transcription factors [Lundberg and Weinberg, 1998; Chelappan et al., 1991]. According to Ohtani et al.

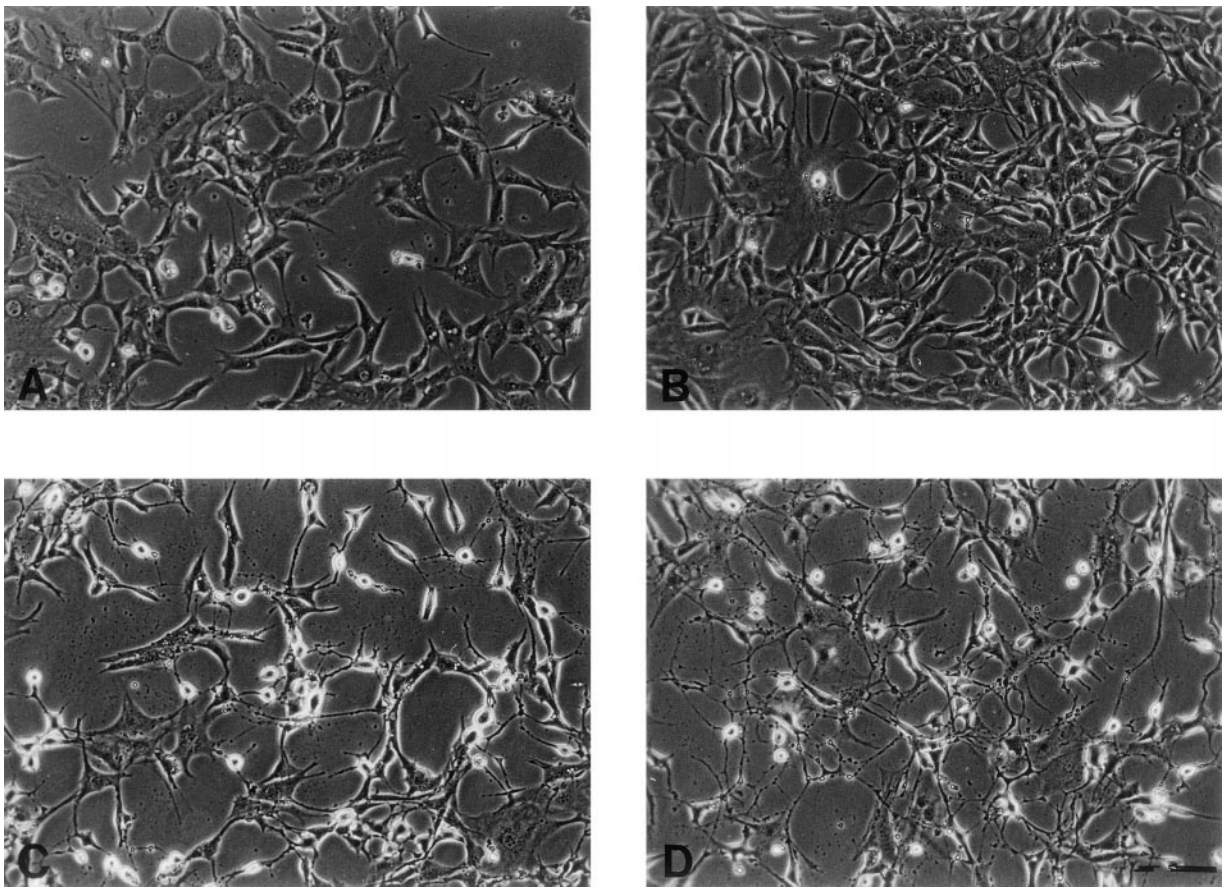

Fig. 8. Effect of C3 transferase exoenzyme on NIH3T3 cell spreading. **A:** neo cells. **B:** Cyclin E overexpressors. After treatment with C3 transferase (0.9 mg/ml), both the neo cells **(C)** and the cyclin E overexpressors **(D)** round up, indicating that Rho inactivation results in cell rounding.

TABLE II. Flow Cytometric Analysis of Various Clones of NIH3T3 Cells With or Without 0.9 ng/ml C3 Toxin in the Incubation Medium for 24 h

Conditions	% of cells in G0/G1 phase	% of cells in S-phase	% of cells in G2/M phase
neo	53.79	29.41	16.80
neo + C3	64.34	15.28	20.38
E	39.60	38.70	21.70
E + C3	53.99	19.29	26.72

[1995], E2F gene products activate the cyclin D1 promoter. Since cyclin D1 expression requires cytoskeletal integrity [Bohmer et al., 1996], a parallel pathway would exist for the stimulation of actin stress fiber assembly, which would also be affected by cyclin E overexpression.

Inactivation of RhoA by C3 treatment caused cell cycle arrest in G1 in both neo cells and the cyclin E overexpressors, indicative of a role for RhoA in cell cycle progression in NIH3T3 cells. In support of this, cyclin A-associated histone kinase activity was reduced in both types of cells. However, cyclin E-asociated histone kinase activity, which was increased in NIH3T3 cells overexpressing cyclin E compared with neo cells, was not inhibited after C3 treatment in either type of cell. This again suggests that RhoA activation is downstream of cyclin E/cdk2 activation.

The increase in cyclin E-associated kinase activity in the cyclin E overexpressors is to be expected. By contrast, there was no change in the response of $p27^{kip1}$ in the two cell lines to lovastatin. Several recent papers conclude that the importance of RhoA in cell cycle progression is due to its ability to decrease the levels of the cyclin-dependent kinase inhibitors $p27^{kip1}$ and $p21^{cip1}$ [Hirai et al., 1997; Weber et al., 1997; Olson et al., 1998]. Hirai et al. [1997] showed in rat thyroid endothelial cells, that Rho proteins

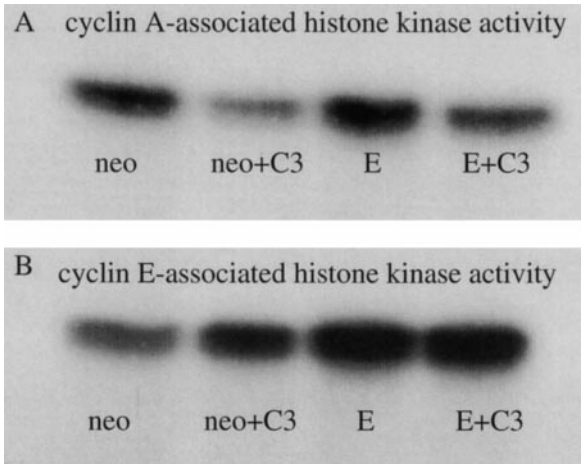

Fig. 9. **A:** Autoradiogram demonstrating decreased cyclin A-associated histone kinase activity in the neo cells and the cyclin E overexpressors after treatment with C3 transferase exoenzyme (0.9 μg/ml) overnight, as compared with untreated cells. As in Figure 6, the cyclin E overexpressing cells had more kinase activity than the neo cells. **B:** Cyclin E-associated histone kinase activity in neo cells and cyclin E overexpressors after C3 treatment. There was no change in the kinase activity after C3 treatment in either cell type, suggesting that RhoA activation occurs downstream of cyclin E-associated kinase activity. The cyclin E overexpressors had more kinase activity than the neo cells.

were responsible for $p27^{kip1}$ degradation. Our data, on the other hand, showed that lovastatin treatment of cyclin E overexpressing cells elevated $p27^{kip1}$ levels despite the continued presence of active (membrane-bound) RhoA. Thus, in our system, RhoA activation does not cause $p27^{kip1}$ degradation. Along these lines, the same group [Tanaka et al., 1998] recently showed in rat astrocytes, that $p27^{kip1}$ levels did not change after treatment with the HMG-CoA reductase inhibitor, pravastatin, although RhoA was translocated from the membrane to the cytosol. It was also suggested that the influence of RhoA on cell cycle progression was mediated by $p21^{cip1}$ [Olson et al., 1998]. However, we did not observe a direct relationship between $p21^{cip1}$ expression and RhoA expression. Again, lovastatin treatment did not appear to affect membrane-bound (active) Ras in either type of NIH3T3 cell. This is consistent with a recent report by Noguchi et al. [1998], who determined that activation of RhoA and not Ras was necessary for G1-to-S transition in rat thyroid cells.

In conclusion, our results show an intimate association between the cell cycle machinery and the cellular localization of the small GTPase RhoA. We suggest that (1) because RhoA activation is affected by cyclin E-dependent kinase activity, RhoA activation is downstream of cyclin E in the signaling pathway; and (2) $p27^{kip1}$ accumulation after lovastatin treatment is not dependent on RhoA inactivation by lovastatin.

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